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The effects of exercise on intracellular Ca^{2+} homeostasis, Ca^{2+}-regulating gene expression and mitogen-induced cell proliferation of murine splenic lymphocytes

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I declare that this thesis is my original work and other sources of information have been properly quoted. This work has not been previously presented to obtain any other degree from any other university. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the “Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice”.

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List of abbreviation

$[Ca^{2+}]i$	Intracellular Ca^{2+} concentration
ARC	Audio Return Channel
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AP-1	Activating protein-1
AUC	Area under curve
CIF	Calcium influx factor
CaM	Calmodulin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CRAC	Calcium release–activated calcium
Con A	Concanavalin A
Ct	Cycle threshold
DMSO	Dimethyl sulfoxide
DMEM	Modification of eagle's Medium
DAG	Diacylglycerol
DHP	Dihydropyridine
EGTA	Ethylene glycol bis (β-aminoethyl)-ether N, N, N', N' tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
IP3	Inositol triphosphate
IP3R	Inositol 1,4,5-trisphosphate receptor
IL-2	Interleukin 2
MDA	Malondialdehyde
mg	Microgram
mM	Millimole
NFAT	Nuclear factor of activated T-cells
nM	Nanomol
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T-cells
PMCA	Plasma membrane Ca^{2+} ATPase
PLC-γ	Phospholipase C-γ
PBS	Phosphate-buffered saline

PM	Plasma membrane
PKC	Protein kinase C
PIP2	Phosphatidylinositol 4,5-bisphosphate
PHA	Phytohemagglutinin
P_{Ca}/P_{Na}	P is permeability
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RyRs	Ryanodine receptors
ROS	Reactive oxygen species
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
STIM	Stromal interaction molecule
SOCE	Store-operated calcium entry
TRP	The transient receptor potential channel
TRPV	Transient Receptor Potential Vanilloid
TRPM	A family of transient receptor potential ion channels
Th	T helper cells
TRPC	A family of transient receptor potential cation channels
TRPN	A member of the transient receptor potential channel family of ion channels
TRPA	A family of transient receptor potential ion channels
TRPP	Transient Receptor Potential Polycystic
TRPML	Transient receptor potential cation channel, mucolipin subfamily
TREK-1	TWIK1-related K + channel
μM	Micromole
VDCCs	Voltage-dependent calcium channels
v/v	Volume to Volume
$\dot{V}_{O2max}$	Maximal oxygen uptake
4-αPDD	4α-phorbol 12,13-didecanoate

Summary

Moderate intensity exercise improves immune functions, whereas excessive exercise has been shown to impair the immune response. The effect of exercise on immunity is mediated via numerous factors, but the exact molecular basis still isn't clear. Intracellular Ca^{2+} is a final focus of cellular signaling transduction, and many Ca^{2+}-regulating factors control intracellular Ca^{2+} transients. Thus, this research focused on the change in intracellular Ca^{2+} concentration and sought to investigate whether or not exercise could affect intracellular Ca^{2+} homeostasis, Ca^{2+}-regulating gene expression and mitogens-induced cell proliferation in murine splenic lymphocytes in order to uncover the potential mechanism by which exercise influences immune functions. In this study, lymphocytes were isolated from spleens. Intracellular Ca^{2+} was determined from Fura-2(AM)-loaded cell suspensions by using a fluorescence spectrometer. The combination of flow cytometry and CFSE-labeling techniques was used for the determination of cell proliferation. The expressions of Ca^{2+}-regulating genes were determined by qPCR analysis. Compared with the control group, basal $[Ca^{2+}]i$ was significantly elevated ($P<0.001$, $n=62$) and chronic voluntary exercise significantly elevated PHA-induced $[Ca^{2+}]i$ in Ca^{2+} buffer ($P<0.05$, $n=5$); either in Ca^{2+} containing buffer or in Ca^{2+} free PBS solution, Con A or OKT3-induced change of $[Ca^{2+}]i$ was significantly higher in the chronic exercise group than in the control group ($P<0.05$,$n=5$); CD3+ T cells from the chronic exercise group showed higher mitogen-induced cell proliferation levels than from the control group ($P<0.05$,$n=5$). However, the expression of Ca^{2+}-regulating genes, STIM1, ORAI1,ORAI2, Cav1.2, Cav2.3,IP3R2,TRPV4,TRPM1,TRPM5,TRPC1,MCU, P2X7,and P2Y14 were significantly downregulated ($P<0.05$,$n=5$). Acute exercise elevated basal $[Ca^{2+}]i$ and Con A or OKT-induced $[Ca^{2+}]i$ and Mn^{2+} influx, and reduced mitogens-induced cell proliferation in splenic lymphocytes at the 3rd hour after exercise ($P<0.05$,$n=5$). Compared with the non-exercise group, PMCA, SERCA, P2X7, and TRPC1 genes expressions were significantly downregulated ($P<0.05$,$n=5$), IP3R2 expression was significantly upregulated at the 3rd hour after exercise ($P<0.01$,$n=5$). This study suggested that chronic voluntary exercise enhanced the sensitivity of mitogens or OKT3-evoked transmembrane Ca^{2+} influx in murine splenic lymphocytes, i.e. high intracellular Ca^{2+} transients with low Ca^{2+}-regulating gene expression; this enhanced Ca^{2+} was followed by enhanced cellular functions; the downregulation of Ca^{2+} homeostasis-related factors expression might be served as a self-protective mechanism against elevated

intracellular Ca^{2+} signals. Secondly, a single bout of endurance exercise with high intensity might cause “delayed” intracellular Ca^{2+} upburst and impairment of cellular function in murine splenic lymphocytes. The enhanced calcium aren't turned into an enhanced proliferation could mean exercise-induced the production of free radical serve as an obstruction mechanism of intracellular signal transduction.

1 Introduction

1.1 The physiological nature of exercise and strenuous exercise-induced local inflammatory response

Physical activity has been defined as bodily movement produced by skeletal muscles, which results in energy expenditure (Pate et al. 1995).The types of exercise include a single bout of acute exercise with high-intensity, and chronic regular exercise with moderate-intensity, according to exercise intensity, duration and frequency. Exercise can enhance marked transient physiological changes in blood flow, pH, O_2 consumption, substrate depletion, energy expenditure, metabolic responses, osmotic pressure, ATP concentration, heat dissipation, the release of acute phase proteins dehydration state, body temperature, activity of sympathetic nervous system, stress hormones levels, production of free radical and cytokines. So exercise can be categorized as a subset of complex “stressor”, which results in a unique perturbation and disruption of homeostasis in organism. These exercise-related factors have the important immunoregulatory roles. Many clinical physical stressors induce a pattern of hormonal and immunological responses that have similarities to that of exercise (Pedersen & Hoffman-Goetz 2000). The interactions between strenuous exercise and the immune system constitute a model of stress response (Hoffman-Goetz & Pedersen 1994).Exercise represents an excellent, controllable, quantifiable and reproducible quantifiable model with which to study the effects of stress on immunity (Hoffman-Goetz & Pedersen 1994; Pedersen et al. 1994; Pedersen & Hoffman-Goetz 2000).

Physical exercise has been considered by some as a muscle-damaging exercise (Shephard 1999).It is generally accepted that strenuous exercise causes a local inflammatory response in muscles. Exercise, especially if which includes strenuous eccentric muscle contraction action, has been considered as a local muscle-damaging activity (Shephard 1999) due to the important alterations in some biochemical parameters which are surrogate markers of skeletal muscle damage or injury(Gravina et al. 2012) incurring the release of various substances such as intracellular cytokines and chemokines, ultimately resulting in a local inflammatory response, that may include migration and infiltration of leukocytes into the tissue. The immune system plays a critical role in regulating the inflammatory process. To

counteract the unwanted effects of an inflammatory process, the immune system produces anti-inflammatory factors that, in response to excessive inflammation, persist for long periods and then may result in immune suppression(Dinarello 1997). The immune function doesn't recover from exercise and some functions can become chronically depressed. Exercise-induced immunosuppression is characterized by a suppression of NK cell activity, lymphocyte proliferation. Short-term suppression of the immune system is not dangerous; moreover exercise-induced immune-suppression could have a "protective" role against overtraining. In my opinion, changes of immune functions that exercise induces correspond with the model of super-compensation, the appropriate immunosuppression that exercise induces could be a necessary condition to improve immune functions. However, chronic suppression leaves the body vulnerable to infection and disease.

1.2 The fundamental definition of relationship between exercise and the immune functions

1.2.1 The immunoregulatory role of exercise

Exercise can modulate immunity and induce numerous changes in immunological parameters, such as immune cell count, helper-to-suppressor T cell ratio, and proliferative response to a mitogen. Paradoxically, it appears that exercise is a double-edged sword (i.e. it has both a positive and a negative effect on the immune function or health). The general hypothesis is that regular moderate exercise can exert a positive effect on the immune system and improve immune cell metabolism adaptations and consequently immune function which may reflect an increased ability to counteract immune challenges and contribute to the immune response, and reduce the number of infections and improve human health and longevity. The frequent and regular exercise boost the immune system that is associated with a number of human health benefits, and it is a non-pharmacological treatment modality for the diseases (Pedersen & Saltin 2006). The recommendation about exercise from the Center for Disease Control and Prevention and the American College of Sports Medicine recommends that all adults should exercise most, preferably all, days of the week for at least 30 minutes or more of moderate intensity (Pate et al. 1995).

Conversely, excessive amounts of high-intensity exercise (e.g. marathon running) might promote immunodepression and transient immune disturbance, and it have

harmful effects on health that is associated with the increased risk of infection. Immune function decreases due to strenuous exercise-induced alterations in the pro/anti-inflammatory cytokine balance, decreased in circulating secretary immunoglobulin concentrations, cytolytic activity of NK cells, ratio of CD4 to CD8 cells and proliferative ability of lymphocytes， lower circulating numbers of immune cells, impaired antibody synthesis, mitogen-stimulated inflammatory cytokine production. Strenuous exercise might impair cell-mediated immunity to lead to a state of inflammation; such a response might explain partly the increased risk of infection in athletes. Furthermore, strenuous exercise is harder to perform when the immune system is being challenged. The magnitude and direction of exercise-induced immune alteration and adaptation depend on the type and volume of exercise workload (including duration, and intensity, frequency, and chronicity of exercise), subject fitness and training state as well as time after the last exercise session, therefore outcomes are highly variable.

1.2.2 The effects of acute exercise on the immune functions

In experimental models, an acute bout of exercise is stressor inducing changes of immune functions during and after the challenge. There was a study shown that a single bout of exercise exerts an array of effects on immune parameters and causes acute change of many components in the immune system, although only in a limited time frame and the characteristics of the loading (Radak et al. 2001). Exercise has a biphasic effect on immune function. Exercise causes an increase in circulating NK cells, NK cytolytic activity during brief and prolonged exercise. However, after exercise, the concentration of NK cytolytic activity declines below pre-exercise values. The percentage of NK cells was suppressed below pre-exercise values only after intense long duration (>60 min) exercise, but is not suppressed following moderate exercise(Pedersen & Hoffman-Goetz 2000) or unchanged compared with pre-exercise values (Mackinnon 1989). Various immune cell functions temporarily impaired following acute bouts of intense exercise may last between 3h to 72 h (Nieman 2000). It has also been reported that NK cell activity was not lower on a per NK cell basis after moderate exercise; in fact, NK cell activity on a “per cell” basis was elevated after exercise (Nieman et al. 1993b). Although prolonged exercise appears to produce post exercise suppression of immune functions, a consistent post exercise pattern has not emerged. Attention has been given to determining whether the exercise-induced change in NK activity is simply due to numerical redistribution of NK cells or whether it reflects a true diminution of NK cytotoxicity on a per-cell basis.

There was a study that shows that the lowest NK cell activity can measured at 2~4 h after the strenuous and prolonged endurance exercise bouts (Pedersen et al. 1990),the changes reflect mainly an altered NK cell distribution, with little change in per cell cytotoxicity (Hoffman-Goetz & Pedersen 1994; Brenner et al. 1996; Shephard & Shek 1999). However, it also was suggested that the increased ratio of low cytotoxic (CD56bright) to high cytotoxic (CD56dim) NK-cells was responsible for the exercise-induced reduction in total NK-cell cytotoxicity (Suzui et al. 2004).

Strenuous bouts of intense training and competitions are known to affect immunological functions in elite athletes (Gleeson 2006). Both T cell and B cell functions appear to be sensitive to increases in training load in well-trained athletes undertaking a period of intensified training(Gleeson et al. 2012). However, intense exercise to exhaustion has been shown to decrease in vitro responses to T and B-cell mitogens, T-helper to-suppressor cell ratio and cytokine responses (Kohut et al. 2001), decrease in circulating numbers of T1 cells ,T1 cell cytokine production, T cell proliferative responses, and B cell immunoglobulin synthesis. Steensberg et al. (2001), reported that 2.5 h of treadmill running (75% of maximalVO_2) caused a significant decrease in the percentage of circulating Th cells producing T1 cytokines immediately and 2 h after exercise. The prolonged practice of elite competitive sport is associated with immune disturbances. It remains unclear whether the immune response to viral infection is inhibited by intensive exercise.

1.2.3 The effects of chronic exercise on the immune functions

The scientific evidence shows that chronic aerobic exercise training can enhance NK cell cytotoxicity on a per cell basis (Woods et al. 1999). But not all, cross-sectional studies have shown an enhanced NKCA in endurance athletes when compared with non-athletes(Nieman et al. 1995a; Nieman et al. 2000). Peripheral blood NK cell number and percentage are generally normal in athletes, although NKCA may be higher at rest in athletes compared with nonathletes(Nieman et al. 1995a).Trained rodents also demonstrate a greater NKCA(Jonsdottir et al. 1997; Hoffman-Goetz 1998). Several prospective studies using moderate endurance training regimens of 8~15 weeks duration have reported no significant elevation in NKCA relative to sedentary controls(Nieman et al. 1993a; Nieman et al. 1998; Nieman & Pedersen 1999). Watson et al. (1986) reported that 15 week of training at 75–85% maximal O_2 uptake decreased NK cell cytolytic activity without altering NK cell counts. But Long-term, high intensity exercise can suppress NKAC and decrease the circulating

NK cell number.

Regular exercise training with moderate intensity studies suggest improvements in mitogen-induced CD4+T-lymphocyte proliferation(Shinkai et al. 1995), lower numbers of senescent T-cells(Spielmann et al. 2011), enhanced T cell mediated immunity, counts of T cells, B cells, and immunoglobulins (Pahlavani et al. 1988; Ferry et al. 1992; Elphick et al. 2003a; Elphick et al. 2003b), enhanced vaccination antibody responses (Kohut et al. 2004; Woods et al. 2009), increased expression of certain anti-oxidative enzymes, lowered inflammatory response to bacterial challenge in human or animal models compared with the typical sedentary individuals. But these results are not universal (Ferry et al. 1991; Lin et al. 1993).There was a study showed that chronic voluntary wheel running increased the number of antibody-producing cells in response to vaccination and reduced the clearance rate of radio labeled IgG (Suzuki & Tagami 2005). Moraska & Fleshner (2001) have provided data showing that 4 weeks of voluntary wheel running reduced stress-induced immunosuppression. Cross-sectional studies of in vitro immune function quite consistently suggest enhanced T-lymphocyte proliferation(Nieman et al. 1993a; Shinkai et al. 1995; Di Pietro et al. 1996; Gueldner et al. 1997) among highly trained athletes vs. untrained subjects. Single sessions of submaximal exercise transiently reduced lymphocyte function in men and that this effect occurred irrespective of the subjects' fitness level (MacNeil et al. 1991).Although elite athletes are not clinically immune deficient, their chronic exposure to stress related to regular intensive training could lead to development of an immune depression(Gleeson 2006). This may affect not only cell-mediated immunity and inflammation-by decreasing Th-1cell cytokine production(Gleeson 2007), but also innate immune functions. The following is the review in which intracellular Ca2+homeostasis-regulating in lymphocytes is emphasized.

1.3 The important functional role of intracellular Ca^{2+} and the regulation of intracellular Ca^{2+} homeostasis

Cytoplasmic Ca^{2+}, one of the most ancient and energetically inexpensive cations, is a highly versatile and final common focus in the cellular signaling transduction network. The increase or decrease in intracellular Ca^{2+} concentration, i.e. $[Ca^{2+}]i$, can act as a switch to turn numerous biological responses "on" or "off". Ca^{2+} as the most ubiquitous second messenger administers a wide range of cellular functions and fates, including gene expression(Negulescu et al. 1994; Feske et al. 2001),

cross-talk between different enzyme systems (Scharenberg & Kinet 1998), transcription factor activation (Dolmetsch et al. 1997), free radical metabolism (Castilho et al. 1995), cell motility (Randriamampita & Trautmann 2004), contraction, secretion, synaptic transmission, fertilization, nuclear pore regulation and transcription (Fill & Copello 2002), protein phosphorylation (Yang et al. 2006), production of various cytokines (Inada et al. 2006), cell proliferation(Choudhry et al. 1994) and differentiation (Khaidukov & Litvinov 2005), apoptosis (Phaneuf & Leeuwenburgh 2001) and necrosis(Oshimi et al. 1996).

$[Ca^{2+}]i$ is a sensitive marker of cellular homeostasis. In resting cells, $[Ca^{2+}]i$ lies between 50~200nM, whereas Ca^{2+} concentration in extracellular space or in reticular system within cell is between 1~5 mM (Krebs 1998). Stress may affect the intracellular Ca^{2+} dynamics. In cells, a localized and transient increase in $[Ca^{2+}]i$ is not sufficient to activate transcription by Ca^{2+} storage release, which requires a more sustained Ca^{2+} influx across plasma membrane through Ca^{2+} channels. Sustained increase in $[Ca^{2+}]i$ is required for biologic responses in cell. During T cell activation, relatively subtle, often oscillatory, fluctuations in intracellular Ca^{2+} concentration have an essential role in activating a Ca^{2+}-sensitive transcription factor-nuclear factor of activated T cells (NFAT) that is responsible for directing the chronic expression of cytokine genes (Timmerman et al. 1996).And the cell replication rate in vitro has been found to be positively correlated with Ca^{2+} concentration in culture medium (Swierenga et al. 1978). Increased Ca^{2+} fluxes after receptor crosslinking could be correlated with an increased propensity towards apoptosis (Cyster & Goodnow 1995). One can define a range of $[Ca^{2+}]i$ values (400~900nM) which appear optimal for T cell proliferation; lower $[Ca^{2+}]i$ values are suboptimal, higher values are cytotoxic (Donnadieu et al. 1995). Extremely high $[Ca^{2+}]i$ that disrupts Ca^{2+} homeostasis or potentially activates some Ca^{2+}-sensitive messagers can be detrimental to T cells. Studies using in vitro and in vivo imaging of T cells have shown that an increase in intracellular Ca^{2+} concentration results in reduced mobility and rounding of otherwise polymorphic T cells(Negulescu et al. 1996; Delon et al. 1998), whereas inhibition of the Ca^{2+} increase prevents these processes. The decrease in intracellular Ca^{2+} concentration also has been linked to lymphocyte proliferative change. The decrease in intracellular Ca^{2+} concentration of unstimulated lymphocytes may indicate impaired cellular function and an increased risk of infection.

The cellular surface has large numbers of ion channels that regulate the timing

intracellular Ca^{2+} transients, including: (1) the Ca^{2+} channels of plasma membrane, which are responsible for Ca^{2+} entry into cells from extracellular space. The Ca^{2+} channels of plasma membrane are divided into four kinds of membrane(Mooren & Kinne 1998): store-operated Ca^{2+} channels gated by depletion of intracellular Ca^{2+} stores, such as 'calcium release-activated calcium' CRAC channels, the depletion of Ca^{2+} stores is able to gate the entry of extracellular Ca^{2+}; voltage-gated Ca^{2+} channels that mediate Ca^{2+} entry into cells in response to membrane depolarization; receptor-operated Ca^{2+} channels gated by ligand/receptor interaction, some of which are TRP family members, inositol-1,4,5-triphosphate receptors (InsP3R) Ca^{2+} channels, P2X receptor; and stretch- activated Ca^{2+} channels gated by physical parameters, e.g., temperature, and mechanical forces, which are often members of the TRP (transient receptor potential) family, such as TRPV1~4, TRPM8; (2)the K^+ channels of plasma membrane, including voltage- gated and Ca^{2+}-activated K^+ channels, which provides the electrical driving force for Ca^{2+} entry into cells from extracellular space(Rink & Deutsch 1983; Partiseti et al. 1992; Lewis & Cahalan 1995); (5)the non-selective cation channels of plasma membrane;(3) the Ca^{2+} channels of intracellular stores, including IP3Rs and ryanodine receptors (RyRs), responsible for Ca^{2+} release from internal stores; (4)the Na^+ channels on plasma membrane, including TRPM4; (6)SERCA (endoplasmic reticulum Ca^{2+} -ATPase) pump and PMCA (plasma membrane Ca^{2+} ATPase) pump, which return Ca^{2+} to intracellular stores and extrude it from cells, respectively;(7) Buffering by intracellular Ca^{2+} stores, e.g., endoplasmic reticulum, mitochondria, embosoms, lysosome-related organelles, Golgi apparatus, nucleus, and mitochondria; (8) intracellular Ca^{2+} binding proteins(Mooren & Kinne 1998).

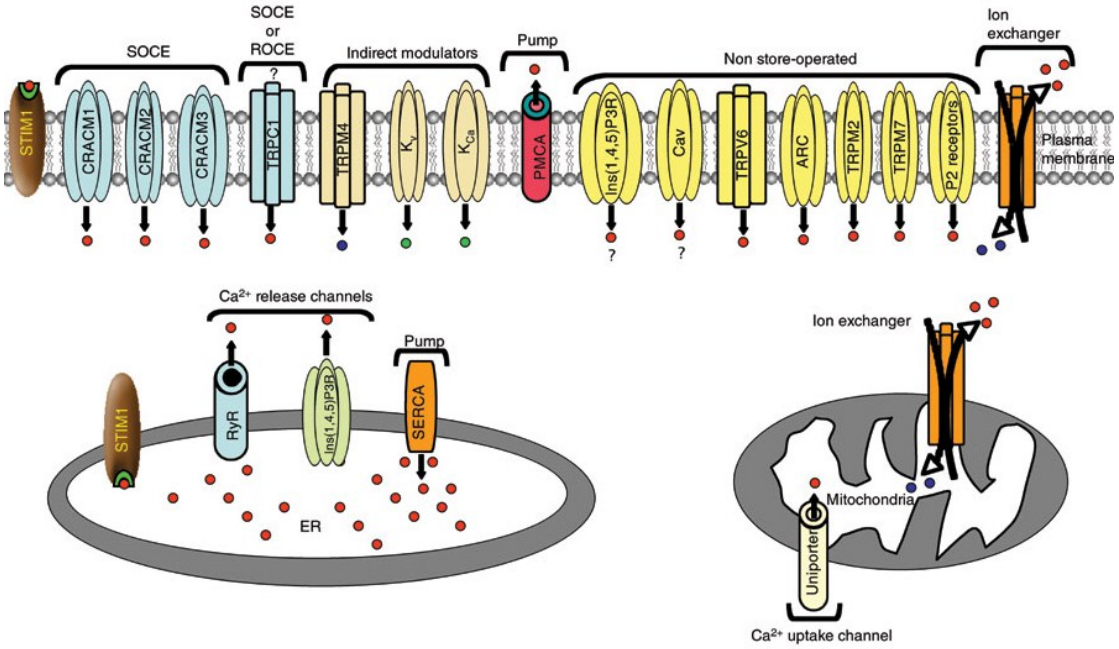

Figure 1-*The departments which regulate intracellular Ca^{2+} homeostasis. Red dots, Ca^{2+}; blue dots, Na^{+}; green dots, K^{+}; ?, controversial route. ROCE, receptor-operated Ca^{2+} entry; K_v, voltage-gated K^{+} channel; K_{Ca}, Ca^{2+}-activated K^{+} channel; PMCA, plasma membrane Ca^{2+} ATPase; Ins(1,4,5)P_3R, Ins(1,4,5)P_3 receptor; TRPV6, transient receptor potential, vanilloid, member 6; ARC, arachidonate-regulated, Ca^{2+}-selective; P2 receptors, purinergic receptors; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; ER, endoplasmic reticulum. From: Vig M, Kinet JP. Calcium signaling in immune cells. Nat Immunol. 2009;10 (1):21-7.*

1.4 The departments to regulate intracellular Ca^{2+} transients

1.4.1 The departments in endoplasmic reticulum

1.4.1.1 Ryanodine receptors

Ryanodine receptors that are large homotetrameric, acted as sentinels can be gated by allosteric coupling to voltage-gated Ca^{2+} channels (in the case of RyR1) and by Ca^{2+} (all isoforms) to regulate endoplasmic reticulum Ca^{2+} appropriate release into cytoplasm. All three isoforms (RyR1, RyR2,and RyR3) can be activated by cyclic ADP ribose (cADPr). Also, RyRs are structurally and functionally related to another intracellular Ca^{2+} release channel, inositol 1,4,5-trisphosphate receptors(Mikoshiba 1993; Dawson 1997; Taylor et al. 1999), and RyRs exist in a macromolecular organization complexed with numerous accessory proteins(Marks et al. 2002).

1.4.1.2 Ins (1,4,5) P3 receptors

Ins (1,4,5)P3 receptors are large tetrameric Ca^{2+} channels. InsP3R-1, -2,and -3, which are all expressed in T-cells are activated by InsP3, and serve as Ca^{2+} release channels by which Ca^{2+} is transported from the stores into the cytosol. There was a study showed that $InsP_3R$-3 was involved in the generation of monophasic single Ca^{2+} transients, whereas InsP3R-1 and InsP3R-2 were involved in the generation of Ca^{2+} oscillations with differing frequencies(Miyakawa et al. 1999). Lymphocyte activation as well as apoptosis is strictly dependent on the mobilization of the $InsP_3$-sensitive calcium pool (Jayaraman et al. 1995; Khan et al. 1996). In both T and B cells, $InsP_3R3$ is up-regulated in cells undergoing apoptosis (Mikoshiba 1997). The inhibition of downstream events may be achieved simply by reducing the overall levels of InsP3Rs, rather than the specific levels of one particular isotype(Sugawara et al. 1997). If T-cells were deficient in IP3R-1 those cells were resistant to apoptosis induced by dexamethasone, T-cell receptor stimulation, ionizing radiation and Fas molecules (Jayaraman & Marks 1997). Ca^{2+} release from the endoplasmic reticulum into the cytosol through $InsP_3$ receptor Ca^{2+} channels is essential for lymphocytes function.

1.4.1.3 SERCA enzymes

One mechanism regulating the endoplasmic reticulum Ca^{2+} release is the SERCA enzyme activity, which mediates the reuptake of Ca^{2+} from the cytoplasm into the endoplasmic reticulum-associated calcium storage organelles and generate a strong calcium concentration gradient between the cytosol (low nanomolar) and the endoplasmic reticulum lumen (high micromolar) (Wuytack et al. 2002). SERCA enzyme activity is involved in the control of cell proliferation (Cheng et al. 1996). Precisely regulated SERCA activity is essential for normal cell function and survival. SERCA enzymes are encoded by three genes (SERCA1, 2, and 3). The expression of SERCA isoenzymes is tissue-specific and developmentally regulated(Lacabaratz et al. 1996).SERCA2b expression is universal. In T lymphocytes, SERCA2b is co-expressed with SERCA3(Launay et al. 1997).The calcium affinity of SERCA3 is significantly inferior to that of SERCA2b(Chandrasekera et al. 2009). The co-expression of SERCA2b and SERCA3 within the same cell is thus involved in the fine regulation of the calcium uptake characteristics of the endoplasmic reticulum. The SERCA inhibitors (thapsigargin) trigger the maximal depletion of Ca^{2+} from the endoplasmic reticulum stores. The direct inhibition of SERCA activity by thapsigargin (Breittmayer et al. 1993) is known to result in the generation of activatory signals.

Isoform-specific anti-SERCA monoclonal antibodies can detect two distinct enzyme species that are co-expressed in the same cells(Papp et al. 1992).The IID8 antibody recognizes the SERCA 2b isoform at 100kDa, whereas the PLIM430 antibody, obtained by immunizing with purified platelet internal membrane preparations(Hack et al. 1988), reacts with a distinct, 97-kDa pump species, temporarily designed as $SERCA_{PLIM430}$(Papp et al. 1992). The biochemical characteristics and the intracellular localization of these two pump species are different(Papp et al. 1992), suggesting that they play functionally distinct roles within the same cell.$SERCA_{PLIM430}$ is believed to be a variant of SERCA 3, the expression of which is restricted to cells of hemopoietic origin(Papp et al. 1992). $SERCA_{PLIM430}$, is specifically associated with the InsP3 mobilizable calcium pool(Papp et al. 1993). The down-modulation of $SERCA_{PLIM430}$ during activation may lead to decreased filling of this pool. It may contribute to the maintenance of the activated state or may alter the apoptotic potential of the cell.

1.4.2 The mitochondria

The mitochondria can regulate Ca^{2+} homeostasis, acted as a Ca^{2+} sink and sequester large amounts of Ca^{2+} quickly and release it slowly after Ca^{2+} influx subsides. Expression of a uniporter in the mitochondrial inner membrane and a Na^{+}/Ca^{2+} exchanger allow for Ca^{2+} uptake and release, respectively. By importing Ca^{2+} in the immediate vicinity of CRAC entry sites, mitochondria are able to reduce Ca^{2+}-dependent inactivation of CRAC channels, thereby increasing CRAC activity and the amplitude of $[Ca^{2+}]i$ signals. In addition, Ca^{2+} export from mitochondria contributes substantially to $[Ca^{2+}]i$ during CRAC activity. The increased function of Ca^{2+} uptake by the mitochondria would result in an apparent decrease the initial InsP3-mediated release of Ca^{2+} from internal stores.Ca^{2+} released from the endoplasmic reticulum is rapidly taken up by mitochondria located in close proximity(Rizzuto et al. 1993).

1.4.3 The CRAC channels

1.4.3.1 The structure components

1.4.3.1.1 STIM

The endoplasmic reticulum as a capacitor, can lead to the term “store-operated Ca^{2+} (SOC) entry”(Parekh & Putney 2005). SOC entry carries a highly Ca^{2+}-selective, nonvoltage-gated, inwardly rectifying current termed the “CRAC current”(Parekh & Putney 2005) .Lymphocytes use SOC entry as the main mode of Ca^{2+} influx. The best

characterized SOC channels in lymphocytes include CRAC channels. Although the majority of studies are focused on the molecular identity of CRAC channels (Lewis 2007), the complexity of the calcium response in T cells suggests the expression of more than one type of plasma membrane calcium channel. The molecular identity of the CRAC channel is completely still unclear, potential gene candidates include the TRP gene superfamily of ion channels (Sano et al. 2001; Cui et al. 2002; Gamberucci et al. 2002). It has been identified STIM1 (stromal interaction molecule1), the resident of endoplasmic reticulum as Ca^{2+} sensor and Orai1as the pore-forming subunit of CRAC channels (Zhang et al. 2005). The combined overexpression of STIM1 and ORAI1 leads to massive increase in CRAC currents, whereas expression of the individual proteins has less effect or may even be inhibitory (Soboloff et al. 2006b). After store Ca^{2+} depletion, Ca^{2+} unbind from STIM and STIM proteins form oligomers in the endoplasmic reticulum membrane and then move to regions of endoplasmic reticulum–plasma membrane apposition and accumulate in puncta next to the plasma membrane that coincide with sites of Ca^{2+} entry and contain small clusters (puncta) of STIM1 and ORAI1 localized together (Wu et al. 2006; Liou et al. 2007). Once it is immediately adjacent to the plasma membrane, i.e. within 10~25 nm observed using electron microscopy (Wu et al. 2006), STIM triggers CRAC channels to open, allowing Ca^{2+} to enter the cell. Whether there is a direct, physiologically relevant physical interaction between STIM1and ORAI1 remains controversial. The mechanism by which STIM activate CRAC channels in the plasma membrane is not comepletely understood.

STIM1 has a closely related homolog, STIM2. A study using mice with a T cell specific deficiency in STIM proteins confirmed the role of STIM1 is essential for store-operated Ca^{2+} entry (SOCE) and CRAC function, and revealed that STIM2 contributes to sustained Ca^{2+} influx into cells(Oh-Hora et al. 2008). By conditional disruption of the STIM1 and STIM2 genes in mice, the research showed both STIM proteins contribute to the nuclear translocation of NFAT in T cells (Oh-Hora et al. 2008). STIM1-deficient T cells completely lack Ca^{2+}-dependent cytokine expression (Oh-Hora et al. 2008). However, STIM2-deficient naïve T cells showed normal SOCE and cytokine production. This reflects the fact that STIM2 constitutes only a very small proportion of total STIM protein in T cells(Oh-hora & Rao 2008). STIM1 is a single spanning transmembrane protein that resides mainly in the endoplasmic reticulum and the plasma membrane. STIM1 has been identified as the “missing link” that connects intracellular store depletion to the opening of CRAC channels in T cells. STIM1 is a

ubiquitously expressed membrane protein that has been found to regulate CRAC channel function in a positive fashion (Liou et al. 2005; Roos et al. 2005). The function of STIM2 has remained somewhat controversial, with some early reports assigning it a positive function in SOCE and others suggesting a negative regulatory function (Soboloff et al. 2006a). STIM2, but not STIM1, regulates mainly basal cytosolic and endoplasmic reticulum Ca^{2+} concentrations in some cells (Brandman et al. 2007), but there was a report not to support this opinion (Oh-Hora et al. 2008). STIM2 differs from STIM1 in that it is already partially active at basal endoplasmic reticulum Ca^{2+} concentrations and becomes activated earlier during endoplasmic reticulum store depletion, before substantial decreases in endoplasmic reticulum Ca^{2+} concentrations (Brandman et al. 2007).

1.4.3.1.2 ORAI

ORAI1 is a small protein with four transmembrane domains (Vig et al. 2006). The ORAI1 protein has been shown to function as a Ca^{2+} channel in lymphocytes and is very likely part of the endogenous CRAC channel complex (DeHaven et al. 2007; Lis et al. 2007).ORAI1 has two homologous proteins, ORAI2 and ORAI3, in mice. ORAI2 and ORAI3 are also capable of forming a CRAC-like current when expressed together with STIM1 in heterologous cells(DeHaven et al. 2007; Lis et al. 2007). Both ORAI2 and ORAI3 have been shown to be able to conduct CRAC currents in in vitro overexpression systems (DeHaven et al. 2007; Lis et al. 2007). Overexpression of ORAI2 or ORAI3 together with STIM1 also gives rise to large currents similar, but not identical, to I_{CRAC} (DeHaven et al. 2007; Lis et al. 2007), indicating that ORAI2 and ORAI3 may form Ca^{2+}-permeable ion channels. ORAI proteins can interact with one another (Gwack et al. 2007). ORAI1can also form heteropolymers with ORAI2 and ORAI3 and possibly some TRPC channel subunits (Ong et al. 2007).

1.4.3.2 The physiological properties

CRAC channels are highly selective for Ca^{2+} entry. CRAC channels show a preference for Ca^{2+} over Na^{+} in physiological solutions that is estimated at ~1000:1 (Prakriya & Lewis 2003).CRAC channels are the Ca^{2+} influx ways with a characteristic inwardly rectifying current-voltage relationship (Prakriya & Lewis 2003).Even fairly large monovalent cations can permeate through the CRAC channel, a property shared with voltage-gated Ca^{2+} channels when external divalent ions are removed (Lepple-Wienhues & Cahalan 1996; Kerschbaum & Cahalan 1998). In cells, Ca^{2+}

entry occurs mainly through CRAC channels. In resting T cells, the number of CRAC channels that regulate Ca^{2+} influx is surprisingly low (10~15 channels per cell) and is up-regulated (~150 per cell) in activated T cells (Fomina et al. 2000). It is remarkable that 75% of all activation- regulated genes show a dependence on Ca^{2+} influx through plasma membrane via CRAC channels (Feske et al. 2001).

1.4.3.3 The activation mechanism

CRAC channels are activated by Ca^{2+} store depletion(Zweifach & Lewis 1993), and inactivated by store refilling (Zweifach & Lewis 1995). CRAC channels are activated indirectly by stimuli that result in depletion of Ca^{2+} from intracellular stores, including SERCA pump inhibitors such as thapsigargin. The CRAC channels activation mechanism is controversial. The mechanism of store-operated Ca^{2+} entry through CRAC channels could be: (1) “Conformational coupling” of proteins which sense store depletion (such as, InsP3 receptors, STIM1) to components of the CRAC channel. After the depletion of Ca^{2+} from endoplasmic reticulum, STIM1oligomerizes and translocates to discrete puncta at endoplasmic reticulum- plasma membrane junctions, thus activating ORAI1, the pore-forming subunit of the CRAC channel(Luik et al. 2006; Stathopulos et al. 2006; Liou et al. 2007); (2) An insertional model in which CRAC channels located in intracellular vesicles traffic to the plasma membrane in response to store depletion; (3) A diffusible messenger model in which CRAC channels are gated by a small, diffusible Ca^{2+} influx factor that is released from depleted stores. The conformational coupling hypothesis (Irvine 1987; Berridge 1995), proposes a direct physical link between the InsP3-sensitive Ca^{2+} store and the surface membrane. The InsP3 receptor senses endoplasmic reticulum Ca^{2+} depletion and tells the surface membrane Ca^{2+} channel to open. Besides its indirect role in emptying Ca^{2+} stores, a direct role for the InsP3 receptor in the regulation of TRP channels was suggested (Kiselyov et al. 1998). CIF (Calcium influx factor) was isolated from store-depleted Jurkat T cells and shown to evoke Ca^{2+} signals when applied to cells (Randriamampita & Tsien 1993). However, this material was later shown to activate nonspecific currents rather than I_{CRAC} (Bird et al. 1995), and this hypothesis has fallen into some disfavor.

1.4.4 ARC channels

ARC channels represent a novel arachidonate-regulated pathway for the entry of Ca^{2+}. But they are not the only Ca^{2+} channels whose activity is influenced by arachidonic

acid.ARC channels were highly Ca^{2+}-selective conductances whose biophysical features are superficially similar to the CRAC channels, such as a small macroscopic conductance, and voltage-independent gating. Perhaps the most extensively characterized of the store-independent pathways is that mediated by ARC channel (Mignen & Shuttleworth 2000). Interestingly, it was found that the sustained elevated levels of $[Ca^{2+}]$I that result from this activation of the store-operated channels act to turn-off the ARC channels (Mignen et al. 2001). STIM1 could be a key regulator of the store-independent ARC channels (Mignen et al. 2007). Orai proteins also form the ARC channel pore, but do so as a heteromeric assembly of both Orai1 and Orai3 (but not Orai2) subunits (Mignen et al. 2008).

1.4.5 P_2X receptors

The binding of extracellular ATP to P_2X receptors, which are ATP-gated ion channels, induces the influx of extracellular Ca^{2+}(North 2002).Among the seven known P_2X receptor subtypes (P_2X_{1-7}), $P2X_7$ receptors are particularly highly expressed in immune tissues (Glass et al. 2000; North 2002).In T cells, P_2X_7 receptors initiate Ca^{2+}-dependent downstream signals that lead to T-cell activation and proliferation (Budagian et al. 2003).Activation of P_2X7 receptors has also been implicated in the production of proinflammatory cytokines and in the regulation of cell proliferation and cell death(Baricordi et al. 1999; Mehta et al. 2001). Prolonged stimulation of P_2X_7 receptors with high concentrations of ATP induces cellular apoptosis (Yoon et al. 2007).Osmotic stress induces the release of endogenous ATP, which achieves extracellular concentrations of ~10μM (Yip et al. 2007). In contrast, apoptosis induced by P_2X_7 receptor in $CD4^+$ and $CD8^+$ T cells occurs only when extracellular ATP concentrations exceed 100μM (Aswad & Dennert 2006).Jurkat T cells release ATP within seconds after the stimulation and that the extracellular ATP concentrations generated near the cell surface are sufficient to activate P_2X_7 receptors of the cells (Ralevic & Burnstock 1998).Released ATP can be converted to ADP, AMP, and adenosine by Jurkat cells (Yip et al. 2007).Aswad et al. (2006) showed that the abundance of functional P_2X_7 receptors expressed by T cells correlates with the degree of apoptosis that is induced in response to ATP stimulation.

1.4.6 Voltage-gated Ca^{2+} channels in plasma membrane

Voltage-gated Ca^{2+} channels are heteromultimeric proteins whose conformations are sensitive to changes in the electrical potential across the plasma membrane(Hofmann

et al. 1999). The potential role of voltage-gated Ca^{2+} channels in T lymphocyte is controversial. Initial support for the presence of voltage-dependent-like Ca^{2+} channels in T lymphocytes came when Densmore et al. (1996) identified an electrically responsive current in the plasma membrane of Jurkat T lymphocytes. Several studies have provided further evidence to support the expression of voltage-gated Ca^{2+} channels in T lymphocytes. RT-PCR analysis has shown that transcripts L-type voltage-dependent calcium channels (VDCCs) are expressed in Jurkat T cells. Savignac et al. (2001) demonstrated that murine T cell hybridomas express L-type Ca^{2+} channel mRNA and protein. L-type Ca^{2+} channel antagonists, dihydropyridine (DHP) derivatives, have been used to indicate the existence of channels with L-type Ca^{2+} channel properties in lymphocytes (Gomes et al. 2004). The synthetic 1,4-DHP, nifedipine, can be a potent suppressor of T lymphocyte proliferation.

1.4.7 Ins (1,4,5)P_3R in plasma membrane

Plasma membrane Ins(1,4,5)P3 receptor Ca^{2+} channels, similar to endoplasmic reticulum-associated Ins(1,4,5)P3 receptors, contribute to Ca^{2+} influx in T cells (Khan et al. 1992). Although the three chief Ins(1,4,5)P3 receptor isoforms(IP_3R1,IP_3R2,and IP_3R3) have been identified at the cell surface, Ins(1,4,5)P3-activated Ca^{2+} currents were undetectable in the plasma membrane of cultured T-cell lines (Zweifach & Lewis 1993; Patterson et al. 2004).The role of plasma membrane InsP3Rs is unknown.InsP3R3 can be expressed on the external surface of the plasma membrane of T and B cells(Khan et al. 1992), InsP3R3 may be expressed as an integral plasma membrane protein and function as all or part of a store-operated Ca^{2+} channel(Putney 1997).

1.4.8 PMCA enzyme

PMCA is a transmembrane enzyme that extrudes Ca^{2+} from the cytoplasm to the extracellular environment. In T cells, PMCA is considered the primary Ca^{2+} extrusion mechanism(Balasubramanyam et al. 1993),but the mechanism is unknown. PMCA serves two main important functions: it allows larger Ca^{2+} rises during brief CRAC channel activity, which might be very important during Ca^{2+} oscillations and, at the same time, limits the amplitude of $[Ca^{2+}]i$ during prolonged Ca^{2+} influx, results in long term stability of the Ca^{2+} signal (Feske et al. 2005),which might protect the cells from apoptosis.

1.4.9 K^+ channels

1.4.9.1 The categorization

1.4.9.1.1 The Ca^{2+}-activated K^+ channels

There is a subfamily of four K_{Ca} genes called SKCa1~3 and IKCa1. Two different Ca^{2+}-activated K^+ channels, a small-conductance channel found particularly in Jurkat T cells and an intermediate conductance channel found in murine T cells, as well as in B lymphocytes (Grissmer et al. 1993; Partiseti et al. 1993). Each IKCa1, SKCa2, and SKCa3 channels are opened in response to a rise in intracellular Ca^{2+}.The increased number of IKCa1 channels in the activated cells serves as an important positive feedback regulator of the membrane potential. At the resting $[Ca^{2+}]_i$ level, all of the K_{Ca} channels are closed, but a 10-fold rise, to 1μM, opens them all(Cahalan et al. 2001). A variety of non-peptidyl compounds and peptides inhibit KCa3.1 channels. Ca^{2+}-activated K^+ channels can be regulated by intracellular pH (Riquelme et al. 1997). KCa3.1 lacks a voltage-sensor.The opening of the channels is not affected significantly by the membrane potential(Grissmer et al. 1993).The opening of KCa3.1 channels is highly sensitive to the intracellular Ca^{2+} concentration. The activation threshold of the channels is between 200 and 300nM $[Ca^{2+}]$i, half-maximal activation occurs between 300 and 450nM $[Ca^{2+}]$i and maximal activation of the conductance requires 1μM $[Ca^{2+}]$i (Grissmer et al. 1993; Verheugen et al. 1995). Since the resting $[Ca^{2+}]$i is below the activation threshold of the IKCa1 channels these channels are silent in intact resting T lymphocytes. Ca^{2+} does not bind directly to the channel, instead, it is the Ca^{2+}-binding protein calmodulin(CaM) bound to the C-terminus of IKCa1 subunits that mediates the rise in $[Ca^{2+}]$i to the channel (Fanger et al. 1999). From the key features of IKCa1 activation, it is clear that the channels do not contribute significantly to the membrane potential in resting T lymphocytes but exert a powerful influence when $[Ca^{2+}]$i is elevated(Grissmer et al. 1993).

1.4.9.1.2 The voltage-gated K^+ channels

K_V channels, by the voltage dependence, serve to protect the cell against depolarization. K_V channels normally set the resting potential of T lymphocytes at 250 to 255 mV. By recording from a variety of murine thymocyte and mature T cell subsets, it was possible to distinguish three distinct K_V channels (Decoursey et al. 1987; Lewis & Cahalan 1988). The most important of these K_V channels is the N-type channel,

which is encoded by the Kv1.3 gene. Functional lymphocyte K_V channels are composed of four identical Kv1.3 subunits(MacKinnon 1991). The closely related Kv1.1 gene is present in mouse thymocytes, while the distantly related Kv3.1 gene is present in resting $CD8^+$ mouse T cells and is abnormally overexpressed in $CD4^-CD8^-$TCR1 T cells from autoimmune mice. Kv1.3 is the dominant conductance in resting T cells, and it is a potential target for preventing an immune response. Due to their greater abundance, Kv1.3 channels are more important than IKCa1 channels in regulating the membrane potential of resting T cells. Selective blockade of Kv1.3 channels (but not IKCa1) chronically depolarizes the membrane of resting cells, attenuates Ca^{2+} entry, and suppresses the signaling cascade leading to cytokine production and cell proliferation(Koo et al. 1997; Ghanshani et al. 2000).The activation threshold of Kv1.3 channels is about -60mV, the open probability increases with increasing voltage and reaches saturation at -10 to 0mV. During long depolarized periods Kv1.3 channels enter a non-conducting inactivated state (Marom & Levitan 1994), from which they recover only in tens of seconds even at membrane potentials as negative as -120mV.

1.4.9.2 The expression

T cells express K^+ channels in a pattern that depends on the activation/ differentiation status of the cells. In murine T cells, both K_V and K_{Ca} channels vary dramatically in various subsets of T cells. Immature CD4+CD8+ mouse thymocytes express ~200-300 Kv1.3 channels, but this number decreases dramatically during maturation (Lewis & Cahalan 1988). Mature CD4+ T cells downregulate Kv1.3 expression to ~20 Kv1.3 channels/cell, while CD8+ cells turn off Kv1.3 and turn on a different channel, called Kv3.1. Among mouse T helper cells, Th1 mouse T cells express higher levels of IKCa1 channels compared to Th2 cells and the increased number is partly responsible for the higher calcium rise in Th1 cells following thapsigargin stimulation(Fanger et al. 2000).

1.4.9.3 The functions

The electrochemical driving force for Ca^{2+} influx is provided by the cation efflux through K^+ channels: hyperpolarisation increases the driving force for Ca^{2+} influx while depolarization decreases it. The principal K^+ channels in T cells include voltage -dependent Kv1.3 and Ca^{2+}-activated IKCa1 channels.Kv1.3 channels maintain membrane potential and therefore the driving force on Ca^{2+}: in turn Ca^{2+} entry and the

resulting increased [Ca^{2+}]i levels are thought to activate the opening of Ca^{2+}-activated IKCa1 channels. The two T-cell K^+ channels are attractive targets for immunomodulation because of their functionally restricted tissue distribution and their critical role in T-cell activation. Moreover, K^+ channels play a role in lymphocyte motility and chemotaxis that is inhibited by blocking the Kv1.3 channel with various peptide toxins or by substance P(Levite et al. 2000).

1.4.10 The TRP channels

1.4.10.1 The categorization,expressions,and functional properties and mechnisms of TRP channels

TRP channel superfamily can be divided into seven families: TRPC, TRPM, TRPV, TRPA, TRPP, TRPML,and TRPN. The Ca^{2+} influx channels of the TRP family compromise all the TRPCs and TRPVs, TRPM1, 2, 3, 6, 7, 8, TRPA1, TRPP2, 3, 5, and TRPML1, 2, 3. Investigations of TRP protein expression are difficult because of its relatively low abundance and the limited availability of specific and high-affinity antibodies, lack of specific blockers, broad expression patterns, huge amounts of TRP subtypes, the overlapping electrophysiological characteristics, heteromultimerization, and poorly understood mechanisms of activation.TRPC1, TRPC3, TRPC5 and TRPC6 have all been reported to be expressed in Jurkat T lymphocytes(Garcia & Schilling 1997). TRPC3/6/7 is involved in receptor-operated Ca^{2+} entry, which can be activated directly by diacylglycerol (DAG) in protein kinase C(PKC)-independent manner (Dietrich et al. 2005).The primary B lymphocytes express mRNA for TRPV2, TPRV4(Liu et al. 2005).Most TRP family members are permeable to both Na^+ and Ca^{2+}. All of them are permeable to Ca^{2+} except TRPM4 and TRPM5, whereasTRPV5 and TRPV6 are highly Ca^{2+} permeable(Venkatachalam & Montell 2007). It is possible that TRP proteins are accessory subunits in a Ca^{2+} channel complex.TRP channels contribute to changes in [Ca^{2+}]i by providing Ca^{2+} entry pathways, by modulating the driving force for the Ca^{2+} entry, and very likely also by providing intracellular pathways for Ca^{2+} release from cellular organelles, in some cases (e.g. TRPV1 and TRPM8) (Turner et al. 2003; Zhang & Barritt 2004). TRPC3 and TRPC6 are likely to be subunits of store-operated non-selective cation channel(Su et al. 2001).TRP channels are multifunctional sensors of environmental cues in the form of physical and chemical stimuli, including intra- and extracellular messengers, intracellular ligand binding, chemical, mechanical force, temperature, and osmotic stress, and some probably by the filling state of intracellular Ca^{2+} stores. It is not clear whether TRP

channels respond to depletion of stores. So far, the functional role of TRP proteins in lymphocytes is still unclear. The hypotheses about the mechanism of TRP gating are included: (1) the receptor-operated hypothese. Phosphatidylinositol 4,5-bisphosphate (PIP2) is likely to modulate gating of some TRP channels, but it is not a unifying mechanism of TRP channel activation. (2) Store-operated hypothesis: depleted Ca^{2+} stores somehow gate the entry of external Ca^{2+} to replenish the deficit(Putney & McKay 1999).

1.4.10.2The Ca^{2+} gating of temperature-sensitive channels

The six heat-sensitive channels include TREK-1, TRPV1~4, and TRPM8. TRPM8 functions as a cold sensor, responding to decreases in temperature below 22°C.The heat-sensitive channels are show a much greater sensitivity for heat than standard biochemical reactions. TRPV1~4 are non-selective cation channels which are thermosensitive, although TRPV1 and TRPV4, can also be activated by numerous other stimuli (Benham et al. 2002). Four TRP channels belonging to the TRPV subfamily are activated by heating, with characteristic activation temperatures ranging from warm temperatures (>25°C for TRPV4;>31° C for TRPV3)(Guler et al. 2002; Smith et al. 2002) to heat (>43°C for TRPV1)(Caterina et al. 1997)and noxious heat (>52°C for TRPV2)(Caterina et al. 1999). Expression of TRPV1 was demonstrated in murine dendritic cells by Western blot analysis and RT-PCR (Basu & Srivastava 2005) and TRPV2 was found in murine mast cells (Stokes et al. 2004), TRPV3 and TRPV4, but expression in lymphocytes has not been investigated.

1.4.10.3 The members of TRP channels

1.4.10.3.1 TRPC1 channels

There are seven TRPC channels, TRPC1~7.TRPC1 is essentially ubiquitously expressed. TRPC1 has most often been reported to form diverse channels, ranging from relatively Ca^{2+} selective to nonselective, in a variety of cell types (Villereal 2006). TRPC1 may be the strongest candidate of all TRPs to form store operated nonselective cation channels (Liu et al. 2003; Parekh & Putney 2005).It could be an important component of SOC in B cells (Mori et al. 2002). In B-lymphocytes (Mori et al. 2002), TRPC1 provides an important route for agonist-, growth factor-, and PKC-induced Ca^{2+} entry. It is possible to link TRPC1 to the IP3 receptor (Yuan et al. 2003).However, others have found TRPC1 to be insensitive to thapsigargin- or

IP3-induced store depletion when expressed alone (Lintschinger et al. 2000) One result of the study by Mori et al. (2002) was unexpected: there was no SOC current in 80% of the TRPC1 deficient cells, but normal SOC currents in the other 20%. Another surprising defect of the TRPC1-deficient B cells was the reduced B cell Ca^{2+} mobilization.

1.4.10.3.2 TRPM2 channels

TRPM2 forms a nonselective cation channel permeable to mainly Na^+ and Ca^{2+}, as well as to K^+ and Cs^+. Adenosine 5'-diphosphoribose (ADPR) activates Ca^{2+}/cation influx through TRPM2 channels in Jurkat T cells (Gasser et al. 2006).TRPM2 is regulated by the intracellular Ca^{2+} concentration with low-level activation at around 100 nM and maximal activation around 600 nM (McHugh et al. 2003). TRPM2 has been proposed to function as a sensor of the cellular redox status, and implicated in oxidative stress/reactive oxygen species (ROS)- and TNF-α-mediated Ca^{2+} influx and cell death (Hara et al. 2002).TRPM2 mRNA expression was found in Jurkat T cells by RT-PCR (Sano et al. 2001).TRPM2 is highly expressed in the brain, and is also found in a variety of peripheral cell types(Kraft & Harteneck 2005). Gasser et al. (2006) demonstrated that high concentrations of concanavalin A can elevate endogenous ADPR levels in Jurkat T cells, which in turn activates TRPM2 and subsequent cell death.

1.4.10.3.3 TRPM4 channels

TRPM4 is a voltage-gated and Ca^{2+}- activated nonselective channel that is not permeable to Ca^{2+} and that has been shown to depolarize the membrane potential following its activation through a rise in $[Ca^{2+}]i$ (Launay et al. 2002). This depolarization, caused by influx of Na^+, reduces the electrochemical gradient driving Ca^{2+} entry in nonexcitable cells and thus modulates Ca^{2+} oscillations as the important negative feedback mechanisms (Vennekens & Nilius 2007). TRPM4 can depolarize Jurkat T cells at the peak of Ca^{2+} flux after activation (Vennekens & Nilius 2007). The depolarization would then recruit voltage dependent K^+ currents (Kv1.3), which would tend to repolarize the membrane potential and also aid in the closure of TRPM4 channels, because the open probability of TRPM4 channels is reduced at negative membrane voltages (Hofmann et al. 2003; Nilius et al. 2003). The repolarization would reestablish the driving force for Ca^{2+} influx through I_{CRAC} so that the next oscillation in $[Ca^{2+}]i$ can take place. The Ca^{2+} sensitivity of TRPM4 is regulated by

cytosolic ATP, PKC-dependent phosphorylation and calmodulin(Nilius et al. 2005). TRPM4 expression was shown by Western blot in Jurkat T cells as well as in Molt-4 T lymphoblasts (Vennekens & Nilius 2007). But,in primary human leukocytes, TRPM4 could not be detected by Northern blot technology (Nilius et al. 2003).Launay et al. (2007) detected TRPM4-mediated currents in Jurkat T cells by either perfusing free Ca^{2+} concentrations of up to 800 nM into the cells or by stimulation with phytohemagglutinin(PHA). Using a siRNA approach, they showed that down-regulation of TRPM4 increased PHA-activated [Ca^{2+}]i and interleukin (IL)-2 production in Jurkat T cells.

1.4.10.4 TRPM7 channels

TRPM7 consists of an ion channel domain and an α-kinase domain, whose role for channel modulation or signaling is controversial (Nadler et al. 2001).The function of the kinase domain is poorly understood and its substrates have not been identified. TRPM7 is almost certainly a housekeeping divalent-permeable cation channel likely to be responsible for Mg^{2+} homeostasis in lymphocytes.TRPM7 passes little inward current under physiological conditions, is permeant to both Ca^{2+} and Mg^{2+}, and is inhibited by 0.6mM intracellular free Mg^{2+}(Nadler et al. 2001; Runnels et al. 2002). The cellular knockout of TRPM7 in lymphocytes was found to be lethal, probably because the cellular Mg^{2+} homeostasis was grossly disturbed (Nadler et al. 2001).

1.4.10.5 TRPV3 channels

It is activated by innocuous (>30~33° C) warm temperatures, which does not activate any of the other TRPVs(Smith et al. 2002). Whatever the precise thermal sensitivity of the channel, from a parallel comparison of TRPV1 and TRPV3, it appears that TRPV3 has a lower temperature threshold than TRPV1(Smith et al. 2002).In TRPV3 knockout mice, responses to innocuous and noxious heat, but not to other sensory modalities, are dramatically diminished (Moqrich et al. 2005).Thermal sensitivity depended on the thermal history of the cell and this may be one reason why there is some variation in the reported threshold of activation of TRPV3, ranging from 23°C (Xu et al. 2002) through 35°C (Peier et al. 2002) to 39°C (Smith et al. 2002).

1.4.10.6 TRPV4 channels

TRPV4 is widely expressed in multiple excitable and non-excitable peripheral cell types(Nilius et al. 2004).TRPV4 can be activated by surprisingly diverse stimuli,

including moderate heat (TRPV4 is constitutively active at normal body temperature), shear stress(Watanabe et al. 2002b).Comparison of the heat-evoked current with that activated in the same cells by the TRPV4 agonist, 4α-phorbol 12,13-didecanoate (4-Apdd) (Watanabe et al. 2002a), supported the heat activated current was through TRPV4 channels (Watanabe et al. 2002b).It was reported that responses to osmotic stress increased significantly at body temperature compared to room temperature (Liedtke et al. 2000).The TRPV4 channel was originally described as an osmosensor, opening in response to hypotonic swelling of the cell (Liedtke et al. 2000; Strotmann et al. 2000).

1.4.10.7 TRPV5 and TRPV6 channels

The molecular identities of CRAC channels remain unclear. So far, the best candidates for CRAC channels are members of the TRP superfamily (Sano et al. 2001; Yue et al. 2001; Cui et al. 2002), such as, TRPV5 and TRPV6.All TRP channels are nonselective with $P_{Ca}/P_{Na} \leq 10$, with the exception of the monovalent-selective TRPM4 and TRPM5.The permeability ratios P_{Ca}/P_{Na} for TRP channels vary considerably, ranging from 0.3 for TRPM2 to >100 for TRPV5 and TRPV6.TRPV5 and TRPV6 are the only highly Ca^{2+} selective channels over other cations in the TRPV family (Owsianik et al. 2006), and these channels are tightly regulated by $[Ca^{2+}]i$ and essential for Ca^{2+} reabsorption. It is unclear if TRPV6 forms functional ion channels in T cells at all. TRPV6 has also been proposed to function as a SOC, although this has been disputed in later studies(Voets et al. 2001; Nilius 2003).Like CRAC channels, because TRPV6 shares some of the biophysical properties of CRAC channels and was found to be activated by depletion of intracellular Ca^{2+} stores,It was postulated TRPV6 constitute the pore forming unit if not the whole CRAC channel complex (Yue et al. 2001). Cui et al. (2002) showed with over expression and antisense strategies that TRPV6 can work as a CRAC-like channel in Jurkat T cells. But, that idea has been challenged by other studies. TRPV6 were found to be similar but not identical to CRAC channels .TRPV6 alone does not account for several properties of I_{CRAC} (Voets et al. 2001).Subsequent studies with overexpressed TRPV6 did not confirm that TRPV6 was related to endogenous CRAC channels. The properties of TRPV6 and CRAC channels were found to be different (Voets et al. 2001; Bodding et al. 2002).And the selective CRAC channel inhibitor BTP2 (Ishikawa et al. 2003) had no effect on TRPV6 activity (He et al. 2005; Schwarz et al. 2006). Both TRPV5 and TRPV6 are voltage-dependently blocked by extracellular Mg^{2+}. Intracellular Mg^{2+} also exerts a voltage-dependent block (Voets et al. 2001; Voets et al. 2003). TRPV5

expression was reported in Jurkat T cells using RT-PCR, and expression of TRPV6 was found in Jurkat T cells using Northern blot and RT-PCR technology (Cui et al. 2002) but not in human leukocytes using Northern blot technology(Wissenbach et al. 2001), which could be a problem of low expression in leukocytes.

1.5 The mechanism of store-operated Ca^{2+} entry (SOCE)

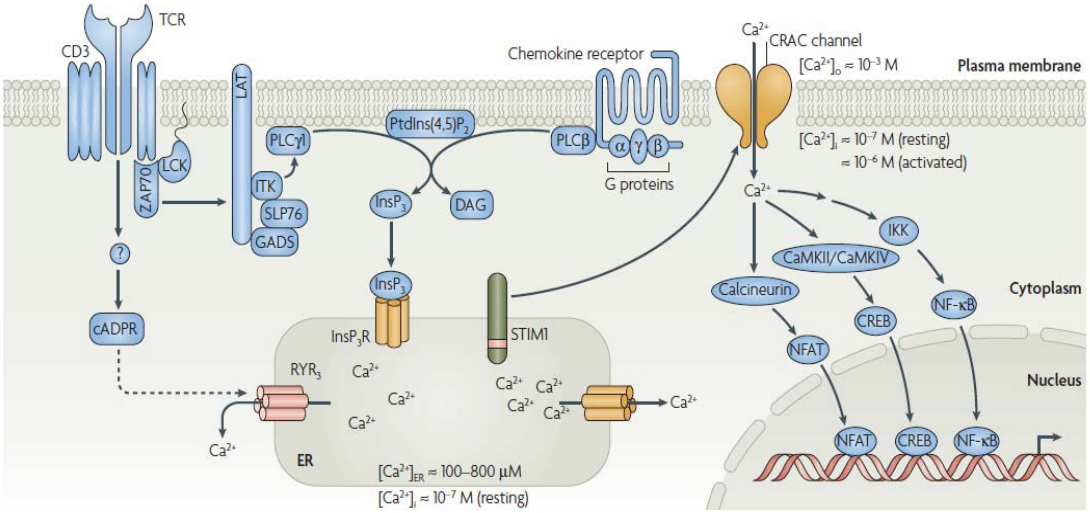

***Figure 2**-Store-operated Ca^{2+} entry (SOCE) through CRAC channels in T cells. Antigen recognition through the TCR results in the activation of protein tyrosine kinases, such as LCK and ZAP70, which initiate phosphorylation events of adaptor proteins, such as SLP76 and LAT. This leads to the recruitment and activation of the TEC kinase ITK and PLC γ 1. Similarly, binding of G-protein-coupled chemokine receptors results in the activation of PLC β. PLC β and PLC γ 1 catalyse the hydrolysis of the membrane PtdIns(4,5)P2 to inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG). InsP3 binds to and opens InsP3 receptors (InsP3Rs) in the membrane of the ER, resulting in the release of Ca^{2+} from intracellular Ca^{2+} stores. A decrease in the Ca^{2+} content of the ER is 'sensed' by STIM1, which in turn activates CRAC channels in the plasma membrane. Ca^{2+} influx though CRAC channels and elevated intracellular Ca^{2+} concentration activate Ca^{2+}-dependent enzymes, such as calcineurin, and thereby transcription factors, such as NFAT, NF-κB and CREB. cADPR, cyclic ADP ribose; CaMK, calmodulin-dependent kinase; GADS, growth-factor-receptor-bound- protein-2-related adaptor protein; IKK, inhibitor of NF-κB kinase; RYR3, ryanodine receptor 3.From: Feske S. Calcium signalling in lymphocyte activation and disease. Nat Rev Immunol. 2007;7(9):690-702.*

The model of strore-operated Ca^{2+} entry has been well described by Fiske (2007), as shown in Figure 2.Store depletion-induced CRAC channel activation has generally

been considered an 'all or none' event at a threshold concentration of IP3(Parekh et al. 1997).Once the CRAC channel is activated, the Ca^{2+} influx is driven by the cell's negative resting potential, and the positively charged Ca^{2+} ions flow into the cell. Although the mechanisms of Ca^{2+} release from the intracellular stores within T lymphocytes are well characterized, the Ca^{2+}entry pathway from extracellular sources into T lymphocytes still remains elusive (Haverstick et al. 1991). Multiple channels are probably involved in calcium entry to T cells. It is possible that these channels function sequentially or independently, and further studies are required to resolve the issue.

1.6 The encodement of Ca^{2+} signals and the expression of transcription factors

1.6.1 The encodement of Ca^{2+} signals

The Ca^{2+} influx through the plasma membrane has two components, one being the net amount of Ca^{2+} entering the cytosol and the other being the Ca^{2+} oscillation frequency. The two components can determine specificity of the pattern of Ca^{2+}-dependent gene expression (Dolmetsch et al. 1998).The activation of specific transcriptional pathways can be differentially "tuned" to particular frequencies or intensities of Ca^{2+} signaling within cells (Dolmetsch et al. 1998). The amplitude and frequency of oscillations dictate the signaling properties of Ca^{2+} and promotes different biological outcomes (Berridge et al. 2000). The intensity and shape of Ca^{2+} signals are essential in setting the threshold for different transcription programs (Crabtree 2001). The relationship between Ca^{2+} dynamics and different gene expression pathways was further examined (Dolmetsch et al. 1997) by using thapsigargin to clamp cytosolic Ca^{2+} to varying levels and oscillatory frequencies. By monitoring the history of Ca^{2+} oscillations followed by a snapshot of gene expression at varying times, it became clear that the frequency of Ca^{2+} oscillations positively correlated with the probability of gene expression, even when the averaged Ca^{2+} responses showed little difference(Cahalan & Chandy 2009). Because calcium release is critically dependent on SERCA enzymes, its activity is instrumental in the shaping of the amplitude, the intensity and the duration of cellular calcium signals (Higgins et al. 2006; Bertram & Arceo 2008) and therefore of cell activation (Clementi et al. 1994; Negulescu et al. 1994; Premack et al. 1994).

The cellular Ca^{2+}-signaling systems can unspecifically be activated by different physical and chemical factors. The calcium signal is transmitted from the cytosol into

the nucleus via multiple calcium signaling modules. The events need sustained Ca^{2+} influx to keep cytoplasmic Ca^{2+} concentrations at higher than basal levels for several hours. The duration and amplitude of cytosolic Ca^{2+} flux required for activation of each transcription factor varies(Dolmetsch et al. 1997). The activation of NFAT and NF-κB is sensitive to the frequency of $[Ca^{2+}]i$ oscillations(Hogan et al. 2003). NF-κB is preferentially activated by low-frequency Ca^{2+} oscillations, whereas both NFAT and NFκB are activated by high-frequency oscillations. Ca^{2+} oscillations may serve to drive gene expression efficiently but without the dangerous consequence for cells of prolonged elevation of cytosolic Ca^{2+}. NFAT on the other hand requires lower $[Ca^{2+}]i$ than NF-κB for its activation(Ledbetter et al. 1987)but this low $[Ca^{2+}]i$ rise needs to be present for an extended time. Ca^{2+}signals are not a binary switch but contain waves of information that need to be decoded (Dolmetsch et al. 1998). The different Ca^{2+} binding proteins and Ca^{2+}-activated transcription factors have different affinities for Ca^{2+}. An important question is whether or not the cell is able to decode the different types of $[Ca^{2+}]i$ signals. Ca^{2+} signals can be distinguished by whether short-term or chronic functions are affected. Short-term functions are generally influenced within minutes and are independent of new gene expression. The chronic functions include cell proliferation, gene expression (Hogan et al. 2003).

1.6.2 The expression of NFAT, NF-κB and AP-1 in Ca^{2+}-dependent manner

The transcription factors can being activated downstream in a Ca^{2+}-dependent manner. Activation of NF-κB, AP-1, and NFAT, that play crucial roles in orchestrating multiple responses in T cells including proliferation, differentiation, and apoptosis, is dependent on Ca^{2+} mobilization. For example, the induction of the transcription of the gene coding for IL-2(Rothenberg & Ward 1996), or the α-chain of the IL-2 receptor (Leonard et al. 1985) leads to a profound reorganization of the structure and function of the cell. Ca^{2+} signalling activates NFAT, a family of transcription factors consisting of five members: NFAT1, NFAT2, NFAT3, NFAT4 and NFAT5. Except for NFAT5, all NFAT proteins are regulated by calcium signals(Hogan et al. 2003). NFATs have been associated with the expression of many cytokine genes, as well as with the expression of immunoregulatory surface molecules, which control the productive T-cell-dependent immune response. The NFAT proteins are activated a rise in $[Ca^{2+}]i$. NFAT-dependent gene transcription is only poorly activated in response to a single pulse of high intracellular Ca^{2+} levels but requires prolonged elevation of Ca^{2+} levels for several hours (Dolmetsch et al. 1997).A sustained Ca^{2+} signal ranging from a

concentration of~200 nM to>1μM for up to 48 h is necessary to activate NFAT (Lewis 2001)Even after its translocation into the nucleus, NFAT requires a sustained increase in Ca^{2+} concentration(Crabtree & Olson 2002). Ca^{2+}, through Ca^{2+}-dependent kinases, also modulates AP-1 activity. In lymphocytes, AP-1 acts together with the transcription factors NFAT and NF-κB to modulate gene expression. The initial $[Ca^{2+}]i$ spike is sufficient to activate NF-κB(Dolmetsch et al. 1997). A transient increase in intracellular Ca^{2+} concentration is sufficient for activation and subsequent target gene expression of NF-κB (Dolmetsch et al. 1997).

1.7 The aims of this study

Dual-wavelength Ca^{2+} indicator, fura-2 can allow accurate measurement of intracellular Ca^{2+} concentrations in whole cell suspensions. The ratiometric readout is probably one of the simplest, yet most widely used methods in the attempt to measure intracellular Ca^{2+} concentration. According to Hirst et al. (1999),when the whole cell suspensions loaded with fura-2 are placed into a cuvette-based fluorescence spectrometer for the measurement (emission wavelength: 510nm, excitation wavelength: 340/340nm), the cells are stimulated with agonists to enable temporal fluorescence response and concentration-response curves are constructed. In the presence of saturating Ca^{2+}, the maximum fura-2 fluorescence can be observed at the wavelength of 340nm and during the Ca^{2+}-free conditions at the wavelength of 380nm.The concentration of free intracellular Ca^{2+} is proportional to the ratio of fluorescence at 340/380nm,and then $[Ca^{2+}]i$ is calculated following the formula of Grynkiewicz et al(1985).We have known, some polyclonal mitogens (Con A,PHA), monoclonal antibodies (OKT3), and inhibitors (thapsigargin), are known to elicit $[Ca^{2+}]i$ in lymphocytes, all these stimuli can be used as the convenient pharmaceutical tools to investigate cellular functions.

Cell proliferation also can serve as one important functional parameter to immune cell functions. There was some debate regarding the interpretation of traditional cell proliferation assays. Some researchers suggested when using traditional cell proliferation methods, the change in relative composition of lymphocytes is the most likely factor contributing to the reduction in mitogen response observed after exercise (Mosmann 1983; Gabriel et al. 1992). For exact quantitative evaluation of immune cell proliferation responses, CFSE can be used as an ideal tool to monitor cell proliferation (Quah et al. 2007) with a high fluorescent intensity of exceptionally, low variance, low cell toxicity(Quah & Parish 2010). The combination of CFSE-

labeling technique and the analysis method of flow cytometry make it possible not only to measure the cell division generation but also to calculate the number of division cells in each generation(Quah & Parish 2010).

The effects of exercise on the immune functions are very complicated. The increased knowledge on immune function modulation with exercise is relevant to health of public and athletes. The changes induced by exercise in the immune functions could be mediated via a number of cellular mechanisms, but intracellular molecular basis of exercise to improve the immune function still isn't so clear. It has been well known that signal transduction is fundamental for aspects of immune functions and for the development of immunity. Understanding how exercise stimuli and intracellular signals are transmitted in an efficient and reliable way inside a cell is important to our understanding of such a biological process, in which exercise influences the immune functions. The knowledge of signal transduction has grown tremendously in the past decade. It has been well known that Ca^{2+} controls a great range of functional processes within cells, and $[Ca^{2+}]i$ is regulated by the matching Ca^{2+}-regulating systems. Dysregulations of calcium homeostasis are critically involved in cellular dysfunction, apoptosis and diseases (Krebs 1998; Mooren & Kinne 1998). It is anticipated that research in Ca^{2+} signal transduction will have an important role in exercise immunity area. In previous publications, the most exciting research findings that have taken place are reviewed by many experts in the research field of Ca^{2+} signal transduction.

After acute exercise, the immune functions subject to transient changes. The period after exercise is of particular importance, because many immune parameters do not return to resting levels within several hours after exercise. The immune-depression after exercise is most pronounced when the exercise is continuous, prolonged, of moderate to high intensity(Gleeson 2006). However, there were few studies that assessed the mechanism by which exercise induced functional change in lymphocytes. This project focused on the effect of exercise on intracellular Ca^{2+} homeostasis which represents an important link between cell activation and functional response. We have known that acute bouts of high intensity exercise deleteriously alter $[Ca^{2+}]i$ of lymphocytes in human (Mooren et al. 2001), but the long term effects of large volumes of moderate intensity exercise upon intracellular Ca^{2+} signalling processes within lymphocytes is unknown. Thus, this research sought to investigate whether or not exercise (including chronic voluntary exercise and acute exercise)

could affect intracellular Ca^{2+} homeostasis under basal and stimulated conditions, expression of intracellular Ca^{2+} homeostasis-regulating factors and the abilities of mitogen-induced lymphocytes proliferation in order to uncover the potential mechanism by which exercise influences immune functions.

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2 Material and methods

2.1 Experimental animals

The research was accomplished with male CD1 Swiss mice, and the mice were used in the age of 10~14 weeks, and weighing 27.0 ± 2.8g, housed in the animal facilities of the University of Münster (Münster, Germany) and the Department of Sports Medicine of Justus-Liebig University (Giessen, Germany). The animal rooms were controlled for photoperiod 12h/12h light/dark cycles (Lights on at 07:00).Mice were bred in standard cages (4~6 per cage for the control mice and 1 per cage for the exercise mice) at the temperature of 21±1°C under ad libitum food conditions (the standard chow). All the experiments were approved by the local Animal Care and Use Committee.

In order to construct the chronic exercise animal models which were described by Avula et al.(2001), the mass-matched male mice were assigned to the control group and the chronic voluntary exercise group, randomly. For experiments assessing wheel running activity, mice were singly housed in cages with unlimited access to a running wheel (diameter: 11cm). The magnetic counters, Sigma BC 500, could measure the daily distance run and the total distance run. Each week, the total distance was recorded for each exercise animal, and then the magnetic counter would be reset. All the mice were kept for more than 84 days. At the end of the experimental period, the animals were killed by cervical dislocation under isoflurane anesthesia.

The animal model with a single bout of exhasutive exercise with high-intensity has been described by Krüger et al.(2009).Aerobic capacity was determined by using a treadmill spirometry (custom made). All animals were first acclimatized to the treadmill. Maximal oxygen consumption ($\dot{V}_{O2max}$) and maximal running speed (V_{max}) of mice were tested at least 4 days before starting the experiments. After 10 min of acclimatization in the treadmill chamber, mice performed a continuous, progressive exercise test until exhaustion. The test uptake started at 0.20 m/s; every 3 min, the speed was increased by 0.05m/s. Mice of the control group were exposed to treadmill noise without running, while mice of the intensive exercise test group performed a running test at 80% $\dot{V}_{O2max}$ corresponding to 0.33 m/s until exhaustion. Mice were killed by cervical dislocation under isoflurane anesthesia at various points

of time after the exercise tests (immediately, 3 h, and 24 h).

2.2 Laboratory instruments

Table 1.All laboratory instruments used for experiments in this thesis are listed with notice of corresponding suppliers:

Instruments	**Company name**
Biofuge Fresco	Heraeus, Hanau, Germany
Biofuge Pico	Heraeus, Hanau, Germany
Culture dish	BD Biosciences, Heidelberg, Germany
Cell Strainer	BD Falcon, Bedford, Massachusetts,USA
Fluorescence spectrometer	Deltascan PTI, Canada
EPICS XL flow cytometer	Beckman Coulter, Fullerton, CA,USA
Filter tips	Braun, Melsungen, Germany
Hera cell incubator	Heraeus, Hanau, Germany
Hematology analyzer	Sysmex Corporation,Norderstedt,Germany
Hera safe, clean bench	Heraeus, Hanau, Germany
iCycler	Bio-Rad, Munich, Germany
Ice machine, Scotsman AF-100	Scotsman Ice Systems, Vernon Hills, IL,USA
Magnetic stirrer model L-71T	Thomas Scientific, Swedesboro,USA
NanoDrop® ND-1000 UV-Vis spectrophotometer	NanoDrop Technologies,Rockland,USA
pH meter	Windaus Labortechnik, Clausthal-Zellerfeld, Germany
Pipettes 1µl,2.5 µl, 10 µl, 20 µl, 100 µl, 200 µl and1000 µl	Eppendorf, Hamburg, Germany
TRIO-thermoblock	Biometra, Göttingen, Germany
Sigma BC 500	Sigma Sport, Neustadt, Germany
Universal 320r centrifuge	Hettich ,Tuttlingen ,Germany

2.3 Chemicals

Table 2.General chemicals used in this thesis are listed with notice of corresponding suppliers：

Chemicals	Company name
Concanavalin A(Con A)	Sigma-Aldrich, USA
Carboxyfluorescein succinimidyl ester (CFSE)	Molecular Probes Inc., USA
Digitonin	Sigma-Aldrich, USA
Dimethyl sulfoxide(DMSO)	Carl Roth, Germany
Dulbecco's modified Eagle's medium (DMEM)	PAA, Austria
Ethanol	Riedel-de-Haën, Germany
Ethylene glycol bis (β-aminoethyl) -ether N, N, N', N' tetraacetic acid (EGTA)	Carl Roth,Germany
Fetal bovine serum (FBS)	PAA, Austria
Fura-2AM	Life Technologies GmbH,Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Roth, Germany
iTaq™ Universal SYBR® Green supermix	Bio-Rad, USA
Muromonab CD3 (OKT3)	Janssen-Cilag GmbH,Germany
NaCl,KCl,Na_2HPO_4,Glucose, $MgCl_2 \cdot 6H_2O$	
$CaCl_2$	Sigma-Aldrich, USA
Oligo(dT)12-18 primer	Invitrogen, Germany
Penicillin/Streptomycin	PAN Biotech, Germany
PBS Dulbecco	Biochrom AG, Germany
Phytohemagglutinin PHA-M	Sigma-Aldrich, USA
PE/Cy7 anti-mouse CD3	Biolegend, USA
qRT-PCR primers	Operon, Germany
RNeasy kit	Qiagen, Germany
RT-PCR kit	Invitrogen, Germany
RT-PCR primers(see table 3)	Operon, Germany
SuperScript II reverse transcriptase	Invitrogen, Germany
Taq DNA polymerase	Eppendorf, Germany
Trypan blue	Sigma, Germany
Thapsigargin(TG)	Sigma-Aldrich, USA

Table 3. List of primers for real time PCR

Gene	Symbol	Forward primer (5'-3')		Reverse primer (5'-3')	
Stromal interaction molecule 1	STIM1	TGAAGAGTCTACCGAAGCAGA	(Tm=57.9°C)	AGGTGCTATGTTTCACTGTTGG	(Tm=58.4°C)
Stromal interaction molecule 2	STIM2	ATGCTGCTCTTCGGGTTGTT	(Tm=57.3°C)	GCGACATACAGGGATCTGTCAG	(Tm=62.1°C)
ORAI calcium release-activated calcium modulator 1	ORAI1	AACGAGCACTCGATGCAGG	(Tm=58.8°C)	GGGTAGTCATGGTCTGTGTCC	(Tm=61.8°C)
ORAI calcium release-activated calcium modulator 2	ORAI2	GACCAAGTACCAGTACCCTCA	(Tm=59.8°C)	GCAAACAGATGCACGGCTAC	(Tm=59.4°C)
ORAI calcium release-activated calcium modulator 3	ORAI3	GGCTACCTGGACCTTATGGG	(Tm=61.4°C)	GCAGGCACTAAATGCCACC	(Tm=58.8°C)
Inositol 1,4,5-trisphosphate receptor 1	IP3R1	GTTGGGGACCGTCATCCAATA	(Tm=59.8°C)	AAAACCAGGACCCTTCATTTCC	(Tm=58.4°C)
Inositol 1,4,5-triphosphate receptor 2	IP3R2	CCTCGCCTACCACATCACC	(Tm=61.0°C)	TCACCACTCTCACTATGTCGT	(Tm=57.9°C)
Inositol 1,4,5-triphosphate receptor 3	IP3R3	AAGTACGGCAGCGTGATTCAG	(Tm=59.8°C)	CACGACCACATTATCCCCATTG	(Tm=60.3°C)
ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2	SERCA2	GAGAACGCTCACACAAAGACC	(Tm=59.8°C)	ACTGCTCAATCACAAGTTCCAG	(Tm=58.4°C)
ATPase, Ca^{2+} transporting, plasma membrane	PMCA1	TGAAGGAGCTGCGATCCTCTT	(Tm=59.8°C)	CTGTTCCTGCTCAATTCGACT	(Tm=57.9°C)
Ryanodine receptor 1, skeletal muscle	RYR1	GCCTTTGACGTGGGATTACAG	(Tm=59.8°C)	CCCCAACTCGAACCTTCTCTC	(Tm=61.8°C)
Ryanodine receptor 2, cardiac	RYR2	ATTATGAAGGTGGTGCCGTATCA	(Tm=58.9°C)	TTCCACTCCACGCGACTCTTA	(Tm=59.8°C)
Ryanodine receptor 3	RYR3	CCAACACCGTTGAAAATGGCG	(Tm=59.8°C)	GCACGTTAGATACATTCCACTGA	(Tm=58.9°C)
Purinergic receptor P2X, ligand-gated ion channel, 1	P2X1	GAAAGCCCAAGGTATTCGCAC	(Tm=59.8°C)	TCCACCTCTACAGGACACCAG	(Tm=61.8°C)
Purinergic receptor P2X, ligand-gated ion channel, 7	P2X7	GCACCGTCAAGTGGGTCTT	(Tm=58.8°C)	CAGGCTCTTTCCGCTGGTA	(Tm=58.8°C)
Purinergic receptor P2Y, G-protein coupled 1	P2Y1	AGGCAACAGCGTGGCTATC	(Tm=58.9°C)	TGGTAGGGTGAGCACGTATAAAA	(Tm=58.8°C)
Purinergic receptor P2Y, G-protein coupled 2	P2Y2	TGTTGCCCGTGTCCTATGG	(Tm=58.8°C)	GCGTAGAGAGAGTCCGAAACTG	(Tm=62.1°C)
Purinergic receptor P2Y, G-protein coupled 12	P2Y12	TTTCAGATCCGCAGTAAATCCAA	(Tm=57.1°C)	GGCTCCCAGTTTAGCATCACTA	(Tm=60.3°C)
Purinergic receptor P2Y, G-protein coupled 13	P2Y13	CTCTGGGTGTTCGTCCACATC	(Tm=61.8°C)	GTGTGAGTCGGAAAGGATTTTGA	(Tm=58.9°C)
Purinergic receptor P2Y, G-protein coupled, 14	P2Y14	AGCAGATCATTCCCGTGTTGT	(Tm=57.9°C)	AGCCACCACTATGTTCTTGAGA	(Tm=58.4°C)
Pyrimidinergic receptor P2Y, G-protein coupled, 6	P2Y6	GACCTGATGTATGCCTGTTCAC	(Tm=60.3°C)	CAGGATGCTGCCATGTAGATTG	(Tm=60.3°C)
Transient receptor potential cation channel, subfamily C, member 1	TRPC1	ATCATCGGCCAAAACGATCAT	(Tm=55.9°C)	GCAGCTAAAATAACAGGTGCGA	(Tm=58.4°C)
Transient receptor potential cation channel, subfamily C, member 2	TRPC2	GCCATGTGGTGTCATTTTCCT	(Tm=57.9°C)	GTTGTCCAGTCTTGTCCTGAG	(Tm=59.8°C)
Transient receptor potential cation channel, subfamily C, member 3	TRPC3	GCGAGCAAGAACTGCGAGAT	(Tm=59.4°C)	TGCACCACCTCGTACTTATGG	(Tm=59.8°C)
Transient receptor potential cation channel, subfamily C, member 6	TRPC6	GCTTCCGGGGTAATGAAAACA	(Tm=57.9°C)	GTATGCTGGTCCTCGATTAGC	(Tm=59.8°C)
Transient receptor potential cation channel, subfamily M, member 1	TRPM1	CTGGGGCATGGTGGAGAAC	(Tm=61.0°C)	TGAGTGTGGGAATTGTTGAGC	(Tm=57.9°C)
Transient receptor potential cation channel, subfamily M, member 2	TRPM1	CCAATCTCCGACGAAGCAATAG	(Tm=60.3°C)	CGGGAATCCATGAGCTAAGGTT	(Tm=60.3°C)
Transient receptor potential cation channel, subfamily M, member 4	TRPM4	AGTGGGAGGGACTGGAATTGA	(Tm=59.8°C)	GGCATCCTCTATCCGCTTTAAC	(Tm=60.3°C)

Transient receptor potential cation channel, subfamily M, member 5	TRPM5	CCTCCGTGCTTTTTGAACTCC	(Tm=59.8°C)	CATAGCCAAAGGTCGTTCCTC	(Tm=59.8°C)
Transient receptor potential cation channel, subfamily M, member 7	TRPM7	AGGATGTCAGATTTGTCAGCAAC	(Tm=58.9°C)	CCTGGTTAAAGTGTTCACCCAA	(Tm=58.4°C)
Transient receptor potential cation channel, subfamily V, member 3	TRPV3	ACAGGTTCATCAACGCTGAGT	(Tm=57.9°C)	CACCCGCTGCTATAAGCACT	(Tm=59.4°C)
Transient receptor potential cation channel, subfamily V, member 4	TRPV4	AAACCTGCGTATGAAGTTCCAG	(Tm=58.4°C)	CCGTAGTCGAACAAGGAATCCA	(Tm=60.3°C)
Transient receptor potential cation channel, subfamily V, member 5	TRPV5	TGCTGCTATAATGCTGATGGAG	(Tm=58.4°C)	GCACGGACTAGGTTCACATTCT	(Tm=60.3°C)
Transient receptor potential cation channel, subfamily V, member 6	TRPV6	GACCAGACACCTGTAAAGGAAC	(Tm=60.3°C)	AGACACAGCACATGGTAAAGC	(Tm=57.9°C)
Calcium channel, voltage-dependent, L type, alpha 1S subunit	Cav1.1	TCAGCATCGTGGAATGGAAAC	(Tm=57.9°C)	GTTCAGAGTGTTGTTGTCATCCT	(Tm=58.9°C)
Calcium channel, voltage-dependent, L type, alpha 1C subunit	Cav1.2	CTACAGAAACCCATGTGAGCAT	(Tm=58.4°C)	CAGCCACGTTGTCAGTGTTG	(Tm=59.4°C)
Calcium channel, voltage-dependent, L type, alpha 1D subunit	Cav1.3	CCCAAAGAAAACGTCAGCAATAC	(Tm=58.9°C)	GAGGGATAAACAGAAAAGGGCA	(Tm=58.4°C)
Calcium channel, voltage-dependent, alpha 1F subunit	Cav1.4	ATGTCGGAATCTGAAGTCGGG	(Tm=59.8°C)	ACCGCCACAGTCTTGTGTTT	(Tm=57.3°C)
Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	Cav2.1	TTGAGGCTGGAATTAAGATCGTG	(Tm=58.9°C)	CTCAGTGTCCGTAGGTCAAAC	(Tm=59.8°C)
Calcium channel, voltage-dependent, N type, alpha 1B subunit	Cav2.2	GAAAGTGGATCAAGGAGTCGC	(Tm=59.8°C)	CGCTAGGCGTAGCATAGAGG	(Tm=61.4°C)
Calcium channel, voltage-dependent, R type, alpha 1E subunit	Cav2.3	AAGACCCCAATGTCTCGAAGA	(Tm=57.9°C)	TGGAAGATGAACCCTAGAGCC	(Tm=59.8°C)
Calcium channel, voltage-dependent, T type, alpha 1G subunit	Cav3.1	AGATCCCTCTAGCTGAGATGGA	(Tm=60.3°C)	GGGCACTAAGTAAGAGTGTGTG	(Tm=60.3°C)
Calcium channel, voltage-dependent, T type, alpha 1H subunit	Cav3.2	GGCACGAGGGAAGGATACTCT	(Tm=61.8°C)	GTGACGAAGTAGACGGGGGA	(Tm=61.4°C)
Calcium channel, voltage-dependent, beta 4 subunit	Cavβ4	GATCCGGCAAGAGCGAGAAC	(Tm=61.4°C)	GAACGGGCACATCTTCGTC	(Tm=58.8°C)
Cholinergic receptor, nicotinic, alpha polypeptide 7	α7 nACh	AGTTTTAACCACCAACATTTGGC	(Tm=57.1°C)	TTTTCACTCCGGGGTACTCAG	(Tm=59.8°C)
Mitochondrial calcium uptake 1	MCU	CTTAACACCCTTTCTGCGTTGG	(Tm=60.3°C)	AGCATCAATCTTCGTTTGGTCT	(Tm=56.5°C)
ATPase, Ca^{2+} transporting, type 2C, member 1	ATP2C1	ATTGTGTGCGTGAAGGAAAACT	(Tm=56.5°C)	AAATAAGCGTAAGTCCGCAGG	(Tm=57.9°C)
Calmodulin 1	Calm1	CAGCGCACAACGCAGGT	(Tm=57.0°C)	TTCAGCAATCTGCTCTTCAGTCAG	(Tm=64.0°C)
Potassium channel, subfamily K, member 5	Kcnk5	TCTTCATCGTGTGGGGTGTCC	(Tm=63.0°C)	ATAGGGCGTGGTAGTTGGCAC	(Tm=63.0°C)
Potassium intermediate/small conductance calcium-activated subfamily N, member 4	Kcnn4	TGGTTCGTGGCCAAGCTGTA	(Tm=60.0°C)	AGTGTGTCTGTGAGGTGCCC	(Tm=63.0°C)
Heat shock protein 1A	HSPa1a	CATCCTGATGGGGGACAAGT	(Tm=60.0°C)	GTGGAGTTGCGCTTGATGAG	(Tm=60.0°C)
Calsequestrin 2	Casq2	GCGCCCAGAGGACATGTTTG	(Tm=63.0°C)	TTGTCCCGGGCAACCTGTTT	(Tm=60.0°C)

2.4 Buffers and solutions

Table 4.The buffers and solutions for intracellular calcium measurement

A: The measurement media: basispuffer (BP)

Composition	Molar concentration	Mass-volume concentration	
NaCl	140mM	8.1816g/1000ml	4.0908g/500ml
KCl	3mM	0.2234g/1000ml	0.1117g/500ml
Na_2HPO_4	0.4mM	0.0568g/1000ml	0.0284g/500ml
HEPES(H)	10mM	2.3830g/1000ml	1.1915g/500ml
Glucose	5mM	0.9010g/1000ml	0.4505g/500ml
$MgCl_2 \cdot 6H_2O$	1mM	0.2033g/1000ml	0.1017g/500ml
$CaCl_2$	0.8mM	0.1470g/1000ml	0.0735g/500ml
pH=7.4	NaOH(adjustment)		

B: The measurement media: Ca^{2+} free-solution

Ca^{2+} free-PBS solution with 0.1mM EGTA	
$CaCl_2$ solution	Application of 0.8mM

C: Fluorescent probes

	Molar concentration	Mass-volume concentration
Furo-2AM	1mM DMSO (Stock)	0.998ml DMSO/1mg

D: Stimulants

	Molar concentration	Application concentration
Thapsigargin	10mM DMSO (Stock)	Application of 1.7,3.3,10μM,
Concanavalin A	2.5mg/ml PBS (Stock)	Application of 10,20,or 40μg/ml
Phytohemagglutinin	4mg/ml PBS (Stock)	Application of 10,20,or 40μg/ml
Anti-CD3 antibody	1mg/ml (Stock)	Application of 5,10,or 20μg/ml

E: Calibrations

	Stock concentration	Application concentration
Digitonin	0.5mM,1.54mg/2.5ml BP (Stock)	Application of 10mM
EGTA	1M, 3.804g/10ml BP (Stock)	Application of 10mM

Table 5.The solution for cell proliferation assay

A: Washing solution of CFSE
PBS with 10%(v/v) FBS

B: Cell culture solution		
Composition	Stock concentration	Application concentration
DMEM		90% (v/v)
Fetal bovine serum (FBS)		10% (v/v)
Pen-strep		100μg/ml penicillin and 100μg/ml streptomycin
Concanavalin A	1mg/ml DMEM (Stock)	Application of 10μg/ml
Phytohemagglutinin	1mg/ml DMEM (Stock)	Application of 10μg/ml

2.5 Lymphocyte isolation

The concrete operations were as shown as follows: add 1ml PBS to a culture dish; kill mice and cut out spleen from mouse; place the spleen into a cell strainer, and mash the spleen through the cell strainer(Pore size: 100μm) into the culture dish by the plunger end of the syringe; rinse the cell strainer with 1ml PBS into the dish and discard the strainer; add 3ml Biocoll separation solution (Density:1.077g/ml) into a 15ml conical and transfer cells onto the solution by using pipette, very carefully; spin for 20 min at 2,000 rpm by using centrifuge; remove the lymphocyte layer from the solution; wash cells twice by adding 8ml PBS ;spin at 1500 rpm for 10 min; discard the supernatant and resuspend the cells in 1ml PBS. For the proliferation experiments, cells would be isolated under the sterile conditions. Cell viability was 98%, as assessed by Trypan blue, whereas purity was 95%, as determined by the forward/sideward-scatter mode. Lymphocytes were counted by the automatic hematology analyzer.

2.6 Measurement of $[Ca^{2+}]i$

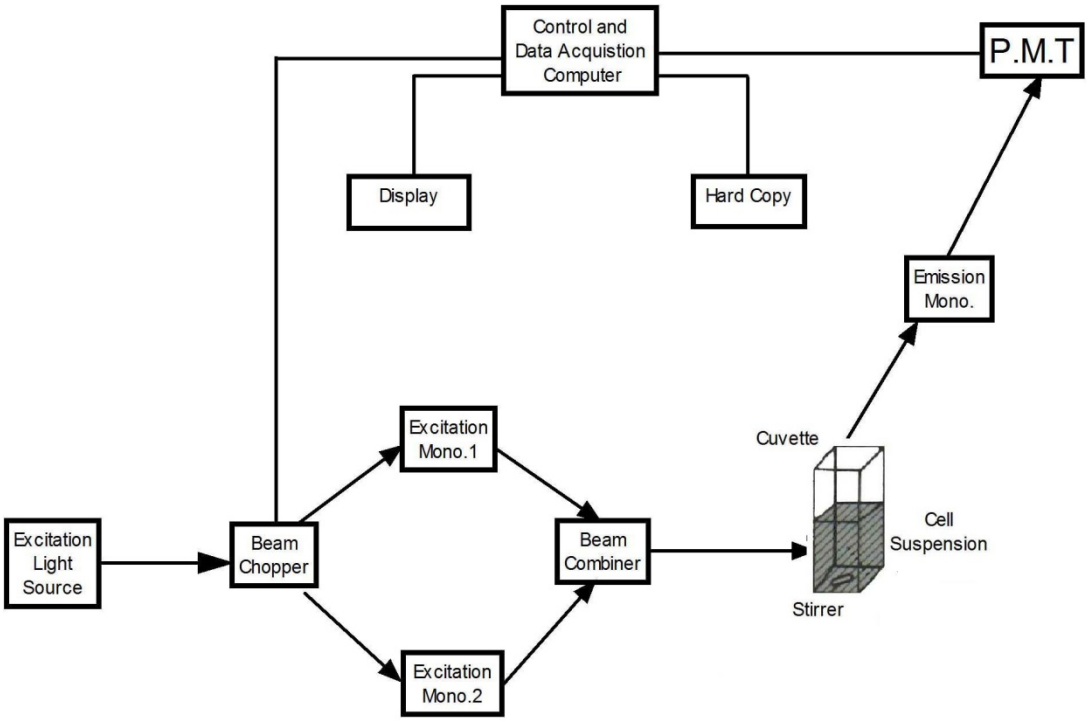


Figure 3-*Schematic diagram of a dual excitation wavelength fluorometer.Fura-2 shifts its emission wavelength on binding* Ca^{2+} *and requires a dual emission system. A single light source with either a rapid chopping device which alternates the excitation light through two separate static monochromators. P.M.T: photomultiplier tube. From: McCormack JG, Cobbold PH (1991) Cellular calcium: a practical approach. IRL Press (Oxford, UK).*

The measurement system is shown in Figure 3.The method was described by Mooren et al. (2001),1ml calcium medium with 9×10^6 cells was supplemented with 5μl Fura-2AM diluted by DMSO(1mM), and incubated for 30 min at room temperature. Excess dye was removed by three-time centrifugation (1,500 rpm for 10 min), and then resuspend cells in PBS, cells are stored on ice until use. Fluorescent signals from Fura-2AM loaded cell suspensions were monitored with a fluorescence spectrometer at 37°C by using temperature-controlled stirred cuvette at excitation wavelengths of 340 (absoption maximum of the Ca^{2+}-fura-2 complex) and 380 nm (the isobestic wavelength), whereas emitted light is monitored at 510 nm.

Autofluorescence of the cuvette, solution,etc. was determined before the experiment and subtracted automatically. Cells were routinely examined within 2h after dye loading. For each experiment, 2.7×10^6 cells were used for the measurement. In order

to measure $[Ca^{2+}]i$ in Ca^{2+} buffer, after 100 seconds for the measurement of resting $[Ca^{2+}]i$, the different concentration of stimulus was applied for 650 seconds. For determination of $[Ca^{2+}]i$ in free Ca^{2+} PBS with 0.1mM EGTA, after initiative 100 seconds for measurement of $[Ca^{2+}]i$, the stimulant was added into the measurement media with the activation for 250 seconds, and then $CaCl_2$ was supplemented into the cuvette with the final Ca^{2+} concentration for 0.8mM and recorded for 350 seconds.

According to the equation of Grynkiewicz et al(1985),$[Ca^{2+}]i$ was calciulated as follows: $[Ca^{2+}]i= (R-R_{min})/(R_{max}-R)\times K_d \times F$, with a K_d of Fura-2 for calcium of 220 nM, and where R is the ratio of fluorescence of the sample at 340 and 380 nm. R_{max} and R_{min} are the ratios for Fura-2 at these wavelengths in the presence of saturating Ca^{2+} (after application of 10 mM digitonin) and during Ca^{2+}-free conditions (after addition of EGTA, 10 mM final concentration), respectively; and F is the ratio of fluorescence intensity at 380 nm during Ca^{2+}-free conditions to the fluorescence intensity at 380 nm during Ca^{2+}-saturating conditions.

2.7 The proliferation measurement of CD3+ T cells

According to the protocol of Quah et al.,(2007) cells were resuspended in 1ml DMEM with 10% (v/v) heat-inactivated fetal bovine serum and a cell concentration of 6 x 10^6/ml; carefully placed cell solution in the bottom of a fresh 15 ml conical tube; Laid the tube horizontally; Carefully added 110µl of PBS to the top of the tube ensuring it didn't make contact with the cell solution; resuspended 1.1µl of the 5 mM stock of CFSE in the 110µl PBS; quickly capped the tube and inverted and vortexed well to get quick uniform mixing of the solutions; incubated cells for 5 min at room temperature and covering the tube with aluminum foil. Labeling was terminated by adding 8 ml 20°C PBS with 10%(v/v) FBS to quench the free CFSE for 5 min at room temperature; sedimenting by centrifugation at 2,500 rpm for 5 min at 20° C and discarding the supernatant; repeated wash twice; unstimulated, Con A,or PHA-stimulated (at concentrations of 5 µg/ml) cells were plated at 2×10^6 cells/per culture dish, and five replicate samples; 2ml DMEM supplemented with antibiotics (100µg/ml penicillin and 100µg/ml streptomycin), and 10% (v/v) FBS; the cells are incubated in a 5% CO_2 incubator at 37°C for 72 hours; cultured cells were then harvested, washed twice with PBS, then analyzed using a flow cytometer using anti-CD3-PE Abs to label $CD3^+$ T cells by acquiring a minimal of 10,000 events from each sample. The proliferation index is calculated as the sum of the cells in all generations divided

by the estimated number of original parent cells. Forward and side scatter profiles were monitored to evaluate the level of cell death.

2.8 RNA expression analysis by quantitative real-time RT-PCR

2.8.1 RNA isolation

All real-time RT-PCR experiments were performed in the laboratories of Prof. Dr. Eveline Baumgart-Vogt and received the technical support and help of consumptive material. In this study, total RNA was isolated from frozen lysed cells using the RNeasy Mini Kit from Qiagen, according to the manufacturer's protocol. In addition, possible DNA contaminations were removed by DNase digestion using RNase-Free DNase Set in the context of RNA isolation. The isolated RNA was redissolved in RNase free water and stored at -80°C untill use. The quantity and integrity of the isolated RNA was measured with a NanoDrop ND-1000 UV-Vis spectrophotometer.

2.8.2 Reverse transcription

First-strand cDNA was synthesized from 0.5~1 µg total RNA with 0.8 µl (25 ×,100mM each) dNTP mixtures,1 µl (50 U/µl) MultiScribe™ Reverse Transcriptase, 2 µl (10×) RT Random Primers, 2 µl (10×) RT Buffer, and sterile distilled water(added to 20 µl) by using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions.

2.8.3 Real time-PCR

For quantification of mRNA expressions, real-time PCR was performed with the iQ SYBR Green Supermix in an iCycler according to the manufacturer's suggestions. Following conditions were used, 1 cycle at 95°C for 3 min, followed by 42 cycles at 95°C for 15 s (denaturation), 61°C for 30 s (annealing) and 72°C for 30 s (elongation), followed by a dissociation curve. All primer pairs were intron-spanning and tested for optimal annealing temperatures. Respective primer sequences and melting temperatures are summarized in Table 3. The constitutively expressed gene β-actin was used as reference for normalization of cDNA levels in each experiment. Melting/dissociation curve analysis was used to control for amplification specificity. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (Ct). After calculation of ΔCt (difference between the Ct value of X_{target} and the Ct value of β-actin; $\Delta Ct=Ct_{target} - Ct_{\beta\text{-}aCtin}$), the fold change between exercise

and control was calculated using the formula $2^{-\Delta(\Delta Ct)}$, in which $\Delta(\Delta Ct)=(\Delta Ct_{\text{exercise,target}} - \Delta Ct_{\text{control,target}})$ or $(\Delta Ct_{\text{exercise II, target}} - \Delta Ct_{\text{exercise I,target}})$.

2.9 Statistical analysis

Data are analyzed using the SPSS statistical analysis program. For statistical evaluation, the means of the samples were compared using One-way ANOVA test and $P<0.05$ was considered as significant unless indicated otherwise. In all cases, data are presented as the mean± SEM.

3 Results

3.1 Part I: The effect chronic voluntary exercise on lymphocytes and functions

3.1.1 The effect of chronic voluntary exercise on the basal $[Ca^{2+}]_i$ of splenic lymphocytes

The basal $[Ca^{2+}]i$ levels of lymphocytes were determined to be 62.1±5.1nM in the control group and 98.9±7.2nM in the chronic exercise group(Fig. 4).There were a highly significant difference between the chronic exercise group and the control group ($P<0.001$, $n=62$).

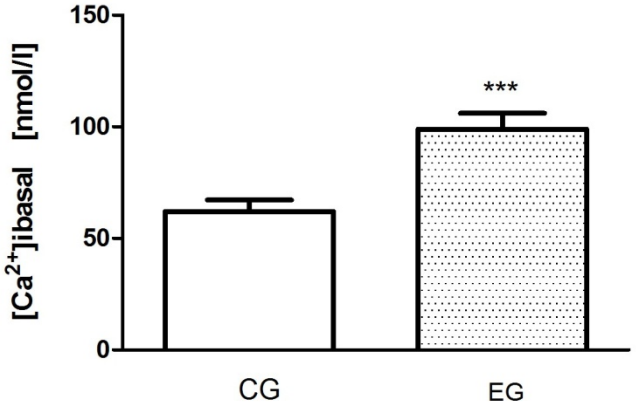


Figure 4-*The effect of chronic voluntary exercise on basal $[Ca^{2+}]i$ of murine splenic lymphocytes. The data was expressed as mean±SEM in the control group (CG) and the chronic exercise group (EG). ***P<0.001 vs CG.*

3.1.2 The effect of chronic voluntary exercise on the agonists-induced intracellular Ca^{2+} transients

3.1.2.1 PHA

3.1.2.1.1 The change of PHA-induced $[Ca^{2+}]$ipeak and $\Delta[Ca^{2+}]$ipeak in Ca^{2+} buffer

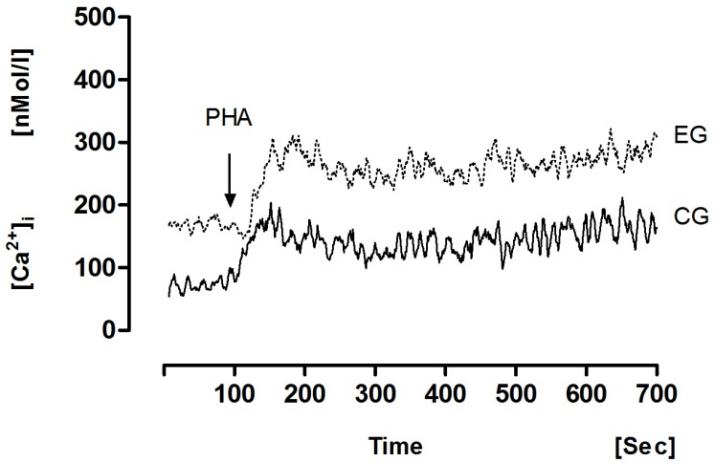
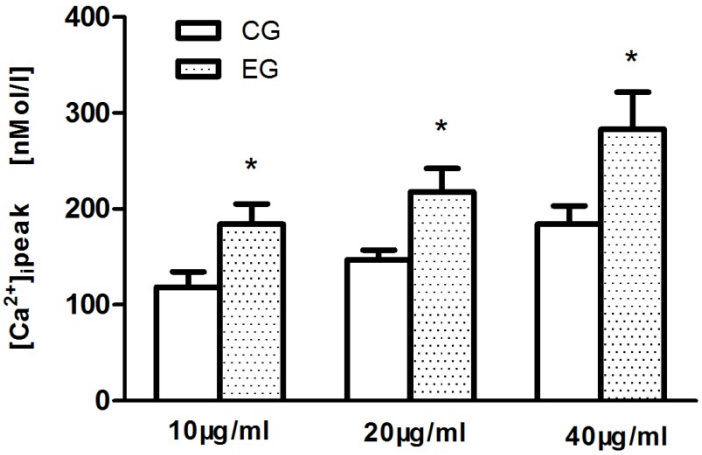


Figure 5-*The tracings of PHA-induced intracellular Ca^{2+} increase of splenic lymphocytes from the control group and the chronic voluntary exercise group in calcium buffer. Time is plotted on the x axis and $[Ca^{2+}]i$ is shown on the y axis. The representative tracings show PHA induced the change of intracellular Ca^{2+} concentration in the control group (CG, solid line) and the chronic exercise group (EG, dotted line).Arrows show when PHA were applied.*



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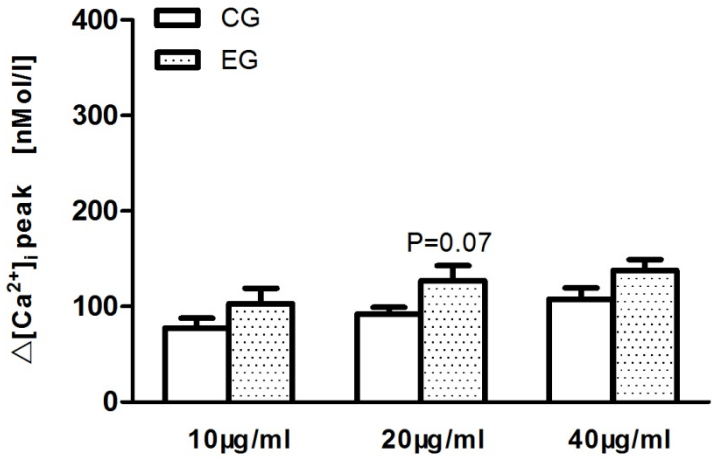


B

Figure 6-*The effect of chronic voluntary exercise on PHA-induced [Ca^{2+}]ipeak and Δ[Ca^{2+}]ipeak of lymphocytes in calcium buffer. A: The figures show that the maximal level of intracellular Ca^{2+} concentration of lymphocytes with the stimulation of PHA. B: The figures show that PHA-induced Δ[Ca^{2+}]ipeak of lymphocytes. Data are mean±SEM derived from 5 or 6 separate experiments. Note: [Ca^{2+}]ipeak is elevated immediately and reached a maximum after the addition of PHA; Δ[Ca^{2+}]ipeak is calculated according to the equation: Δ[Ca^{2+}]ipeak= [Ca^{2+}]i peak - [Ca^{2+}]i basal. The chronic exercise group (EG) is compared with the control group (CG) by using the same dose of PHA as the stimuli.*

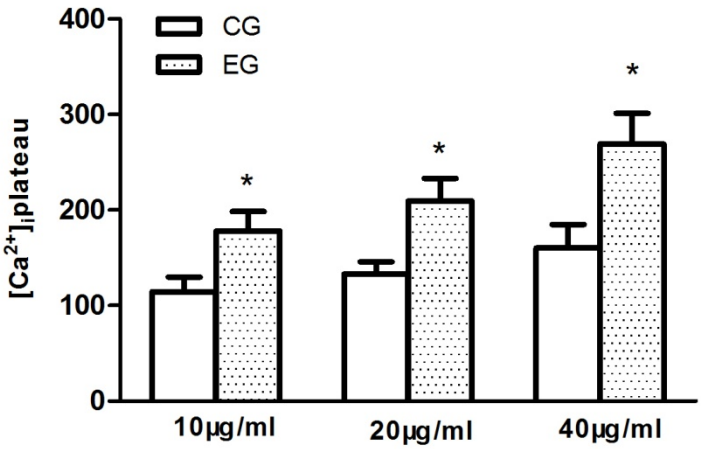
The unspecific mitogenic lectin, such as PHA and Con A, can stimulate lymphocytes, and activate phospholipase C-γ(PLC-γ) to produce inositol1,4,5-trisphosphate (InsP3) that binds to the specific receptors of endoplasmic reticulum and triggers the initial increase of intracellular Ca^{2+} concentration that contributes to an intracellular Ca^{2+} transient "upburst", followed by a sustained elevation in intracellular Ca^{2+} concentration involving the Ca^{2+} influx through the store-operated Ca^{2+} channels. The elevation of [Ca^{2+}]i in lymphocytes treated with mitogen shows two phases: [Ca^{2+}]i in cells is elevated immediately and reached a maximum level tens of seconds after the addition of stimulant, i.e. [Ca^{2+}]ipeak (the first phase); then decreased approximately ten of seconds after [Ca^{2+}]ipeak, but won't return to the basal level and has a steady state of sustained Ca^{2+} concentration, i.e.[Ca^{2+}]iplateau (the second phase).

The effect of various doses of PHA on intracellular Ca^{2+} levels was studied in this study. As shown in Fig.6A,following the addition of 10μg/ml PHA beginning at 100s, [Ca^{2+}]ipeak had a significant difference between the control group (118.6±16.0nM) and the chronic exercise group (184.0±21.2nM)($P<0.05$,n=5);and a significant

difference existed between the two groups (146.7±10.5nM, 217.9±24.3nM, respectively) by using 20μg/ml PHA for the activation ($P<0.05$,n=6); and there also was a significant difference between the two groups(184.2±19.0nM, 283.0±38.7nM, respectively, $P<0.05$,n=5),when cells exposed to 40μg/ml PHA.

To exclude the effect of different resting Ca^{2+} levels, the change in $[Ca^{2+}]i$ was calculated by subtracting the basal $[Ca^{2+}]i$ from the $[Ca^{2+}]ipeak$ after stimulation, i.e. $\Delta[Ca^{2+}]ipeak= [Ca^{2+}]ipeak-[Ca^{2+}]ibasal$. As shown in Fig.6B, cells exposed to PHA (10μg/ml, 20μg/ml, and 40μg/ml) exhibited a rapid and concentration-dependent rise in intracellular Ca^{2+} levels. The chronic exercise group was compared with the control group at the same dose, we found that $\Delta[Ca^{2+}]ipeak$ hadn't a significant difference between the control group (77.5±10.5nM, 92.1±7.2nM, and 107.8±11.6nM, respectively for the above doses) and the chronic exercise group (103.1±16.1nM, 127.2±15.7nM, and 138.0±11.2nM, respectively for the above doses) ($P>0.05$,n≥5). However, it need be reminded that there was almost a significant difference between the two groups at the PHA dose of 20μg/ml ($P=0.07$, n=6).

3.1.2.1.2 The change of PHA-induced $[Ca^{2+}]iplateau$ and $\Delta[Ca^{2+}]iplateau$ in Ca^{2+} buffer



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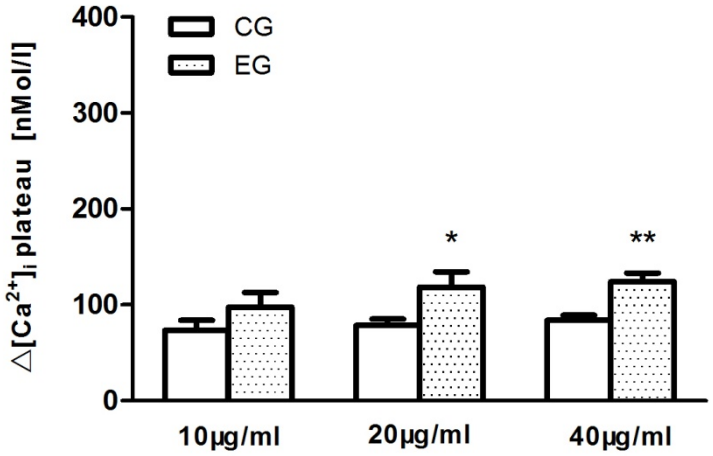


B

Figure 7- *The effect of chronic voluntary exercise on PHA-induced [Ca^{2+}]iplateau and Δ[Ca^{2+}]iplateau of lymphocytes in calcium buffer. A: The figure shows that the level of intracellular Ca^{2+} of lymphocytes with the stimulation of PHA. B: The figure shows that the change of intracellular Ca^{2+} level of lymphocytes with the stimulation of PHA.Data are mean±SEM derived from 5 or 6 separate experiments. Note that Δ[Ca^{2+}]iplateau is calculated according to the equation: Δ[Ca^{2+}]iplateau =[Ca^{2+}]iplateau-[Ca^{2+}]ibasal. The chronic exercise group (EG) is compared with the control group (CG) under the situation with the same dose.*P<0.05, **P<0.01 vs the control group by using the same dose of PHA as the stimuli.*

As shown in Fig.7A, when 10μg/ml, 20μg/ml and 40μg/ml PHA was used respectively for the activation, the increase of [Ca^{2+}]i reached a steady state of 114.5±15.5nM, 130.2±15.2nM, 160.2±24.5nM in the control group, and 178.2±20.3nM, 209.4±23.7nM, 269.3±31.9nM in the chronic exercise group, respectively for the above doses, the significant difference can be checked out between the two groups at the same dose($P<0.05$, $n\geq5$).

Δ[Ca^{2+}]iplateau was calculated according to the equation: Δ[Ca^{2+}]iplateau= [Ca^{2+}]iplateau-[Ca^{2+}]ibasal. As shown in Fig.7B, after using 10μg/ml PHA for the activation, the increase in [Ca^{2+}]i reached a plateau above resting of 73.4±10.5nM for the control group and 97.4±15.4nM for the chronic exercise group, Δ[Ca^{2+}]iplateau hadn't a significant difference between the two groups($P>0.05$,$n=5$). When 20μg/ml and 40μg/ml PHA was used for the activation respectively, the significant difference and the high significant difference, respectively for the above two doses, existed between the control group (78.6±6.5nM, 83.8±5.4nM) and the chronic exercise group (118.7±15.8nM, 124.2±8.8nM), respectively for the above doses ($P<0.05$ or $P<0.01$,

n≥5).

3.1.2.1.3 The change of PHA-induced $[Ca^{2+}]i$ and $\Delta[Ca^{2+}]i$ in Ca^{2+}-free PBS solution with 0.1mmol/L EGTA

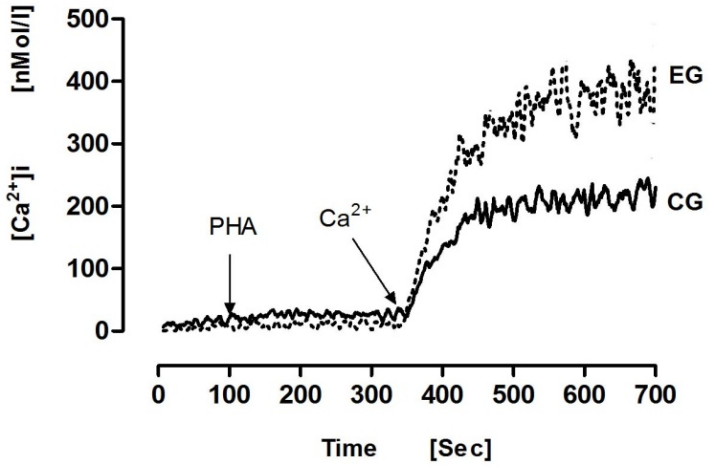
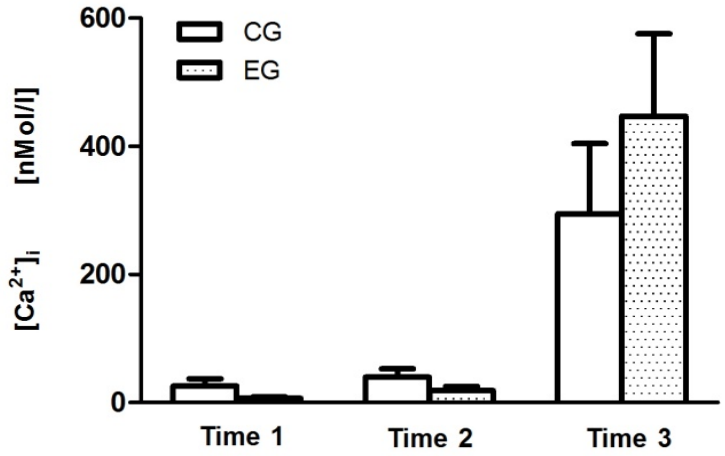


Figure 8- *The tracings of 40μg/ml PHA -induced calcium response of lymphocytes in Ca^{2+} free PBS solution with 0.1mM EGTA. Time is plotted on the x axis and $[Ca^{2+}]i$ is shown on the y axis. The representative tracings showing PHA- induced the change of $[Ca^{2+}]i$ in the control group (CG, solid line) and the chronic exercise group (EG, dotted line). Two arrows show when stimulants and Ca^{2+} were applied, respectively. A representative experiment out of five independently performed experiments is shown, respectively.*



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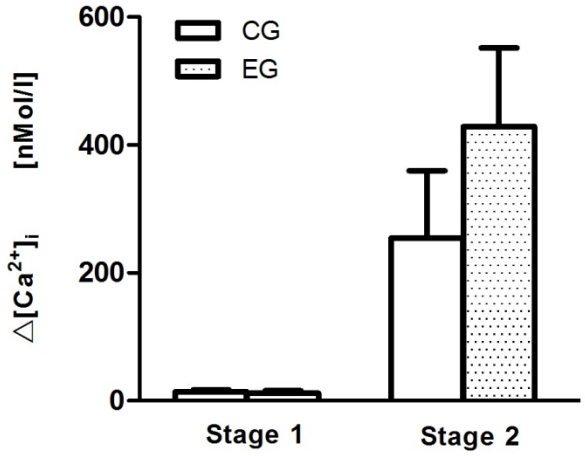


B

Figure 9-*The effect of chronic voluntary exercise on PHA-induced intracellular calcium response of lymphocytes in Ca^{2+} free PBS solution with 0.1mM EGTA. CG: The control group EG: the chronic exercise group. The figures show that the basal $[Ca^{2+}]i$ in PBS solution (Time1), the intracellular Ca^{2+} level for PHA-induced Ca^{2+} release of intracellular stores(Time 2) and it's variation amplitude (stage1) ,intracellular Ca^{2+} level after the addition of Ca^{2+} into the measurement solution(Time 3) and it's variation amplitude (stage2). Data are mean±SEM derived from 5 separate experiments. Note that $\Delta[Ca^{2+}]i$ was calculated according to the equation: $\Delta[Ca^{2+}]i = [Ca^{2+}]i$ agonist- $[Ca^{2+}]i$ basal. *P<0.05 vs the control group by using the same dose of PHA as the stimuli.*

To evaluate that the elevation in $[Ca^{2+}]i$ was induced by Ca^{2+} influx from the extracellular space, only a small increase by PHA could be observed when using Ca^{2+}-free PBS solution containing 0.1mM EGTA. The application of EGTA, which are applied before cells were stimulated with mitogen, could prevent extracellular Ca^{2+}influx, and deplete intracellular stores due to continued unidirectional efflux of Ca^{2+} from the cells. In this study, after the supplementation with 0.8mM of $CaCl_2$ into PBS solution, intracellular Ca^{2+} concentration was rapidly elevated to reach high levels as obtained with the calcium buffer. These results suggest that the further increase in agonist-induced $[Ca^{2+}]i$ depends on extracellular Ca^{2+} influx and not on the intracellular pools. As shown in Fig.9A-Time 3,when cells were exposed to 40μg/ml PHA and Ca^{2+} was added into the measurement solution, there wasn't a significant difference of PHA-induced $[Ca^{2+}]i$ existed between the control group (294.6±109.9nM) and the exercise group(447.4±128.6nM) ($P>0.05$,n=5). As shown in Fig.9B-Stage 2, $\Delta[Ca^{2+}]i$ also had not a significant difference between the control group and the exercise group (254.6±104.9nM, 428.8±122.8nM, respectively)($P>0.05$,n=5).The

study showed that PHA-induced activation of cells required the presence of extracellular Ca^{2+}, because only a transient change was observed nominally in Ca^{2+}-free PBS solution with 0.1mMEGTA(as shown in Fig.9B-stage 1,the change of PHA-induced release of intracellular Ca^{2+}stores were 14.18±2.85nM, 11.96±4.00nM in the control mice and the chronic exercise mice, respectively).

3.1.2.2 Con A

3.1.2.2.1 The change of Con A-induced [Ca^{2+}]ipeak and Δ[Ca^{2+}]ipeak in Ca^{2+} buffer

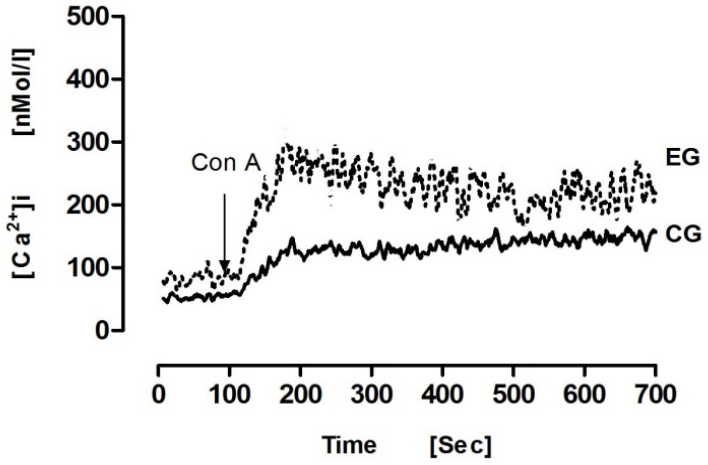
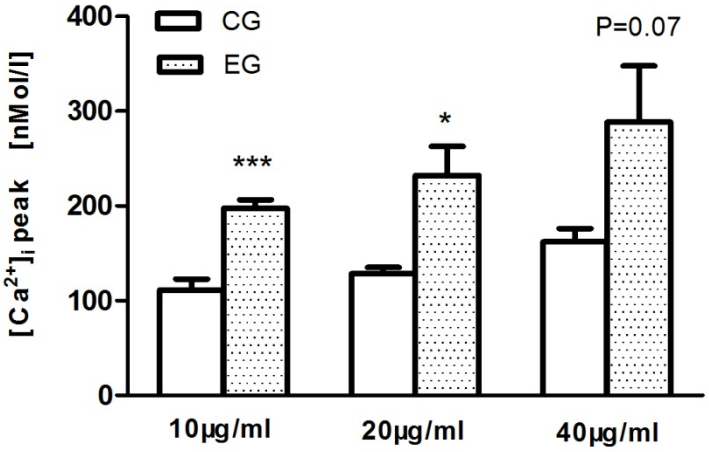


Figure 10- *The tracings of Con A-induced intracellular calcium response of splenic lymphocytes in calcium buffer. Time is plotted on the x axis and [Ca^{2+}]i is shown on the y axis. Representative tracings show Con A-induced the increase of intracellular Ca^{2+} concentration in the control group (CG, solid line) and the chronic voluntary exercise group (EG, dotted line). Arrows show when Con A were applied. A representative experiment out independently performed experiments is shown.*



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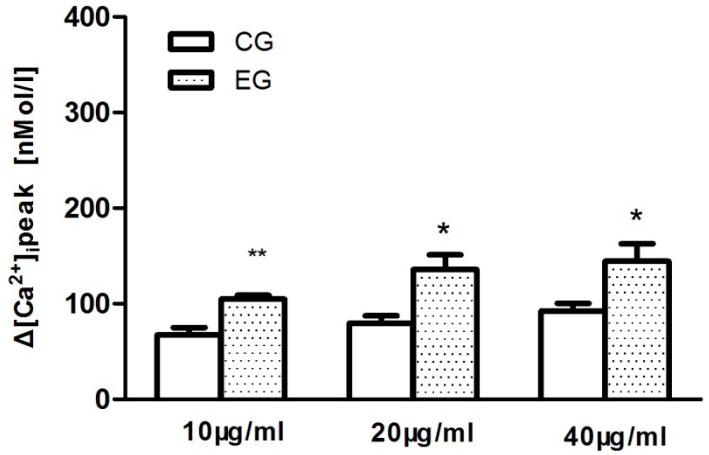


B

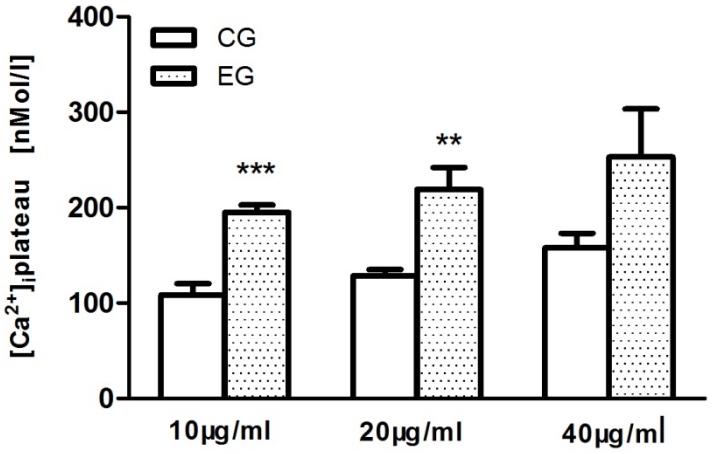
Figure 11*- The effect of chronic voluntary exercise on Con A-induced [Ca^{2+}]ipeak and Δ[Ca^{2+}]ipeak of splenic lymphocytes in calcium buffer. The figures show that the level of intracellular Ca^{2+} and the change of [Ca^{2+}]i of lymphocytes with the stimulation of Con A. Data are mean±SEM derived from 5 separate experiments. Note: [Ca^{2+}]i is elevated immediately and reached a maximum after the addition of Con A, i.e. [Ca^{2+}]ipeak; Δ[Ca^{2+}]ipeak is calculated according to the equation: Δ[Ca^{2+}]ipeak= [Ca^{2+}]i peak-[Ca^{2+}]i basal. The chronic exercise group (EG) is compared with the control group (CG) under the situation with the same dose. *P<0.05, ** P<0.01, ***P<0.001vs the control group by using the same dose of Con A as the stimulant.*

The dosage-dependent changes and time-dependent changes in Con A-induced calcium transients were similar to the results with PHA, we could observe the same pattern of [Ca^{2+}]i changes. More high concentration of Con A to administrate, and more high [Ca^{2+}]i spike could be observed. As shown in Fig.11A,when cells were

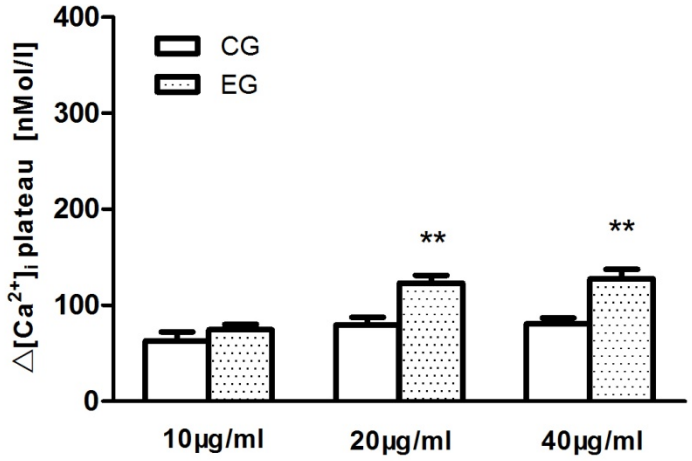
stimulated by 10μg/ml Con A, a high significant difference of Con A-induced $[Ca^{2+}]$ipeak existed between the control group (111.3±11.6nM) and the exercise group (197.3±9.2nM) (P<0.001,n=5). After used 20μg/ml Con A for the activation, $[Ca^{2+}]$ipeak of exercise groups(232.0±30.8nM) had a significant difference compared to the control group (128.5±6.6nM)(P<0.05, n=5). However, there was a close significant difference between the control group (162.2±13.8nM) and the exercise group (288.6± 59.4nM) (P=0.07,n=5),when used 40μg/ml Con A for the activation.

As shown in Fig.11B, 10μg/ml, 20μg/ml, and 40μg/ml Con A induced change of $[Ca^{2+}]$ipeak(△$[Ca^{2+}]$ipeak) in both of the control group(67.5±7.6nM, 79.6±7.8nM, and 92.6±7.9nM, respectively for the above doses) and the exercise group(104.7±4.0nM, 135.9±15.2nM, and 144.6±18.2nM,respectively for the above doses) ,and there were the significant difference between the control group and exercise group under the situation by using the same dose Con A as the stimulant (P<0.05,or 0.01, n=5).

3.1.2.2.2 The change of Con A-induced $[Ca^{2+}]$iplateau and Δ$[Ca^{2+}]$iplateau in Ca^{2+} buffer



A



B

Figure 12*- The effect of chronic voluntary exercise on Con A-induced [Ca^{2+}]iplateau and Δ[Ca^{2+}]iplateau of lymphocytes in calcium buffer. The figures show that the level of intracellular Ca^{2+} and the change of [Ca^{2+}]i of lymphocytes with the stimulation of Con A. Data are mean±SEM derived from 5 or 6 separate experiments. Note that Δ[Ca^{2+}]iplateau is calculated according to the equation:Δ[Ca^{2+}]iplateau=[Ca^{2+}]iplateau-[Ca^{2+}]ibasal. The chronic exercise group (EG) is compared with the control group (CG) under the situation with the same dose. **P<0.01, ***P<0.001 vs the control group by using the same dose of Con A as the stimulant.*

As shown in Fig.12A, when administrated Con A of 10μg/ml, 20μg/ml, and 40μg/ml, [Ca^{2+}]iplateau could be calculated in the control group (108.4±12.2nM, 128.5±6.6nM, and158.1±15.2nM, respectively for the above doses) and in the chronic exercise group(195.1±7.6nM, 219.1±23.2nM, and 253.1±50.5nM, respectively for the above doses), and the significant difference existed between the control group and the chronic exercise group under the same dose(10μg/ml, 20μg/ml, and 40μg/ml) (P<0.05 or 0.01, n=5).

As shown in Fig.12B, when cells were stimulated by Con A of 10μg/ml, 20μg/ml, and 40μg/ml, the increase in [Ca^{2+}]i reached a plateau above the resting 62.8±9.5nM, 79.6±7.8nM, and 80.9±6.0nM in the control group ,and 74.5±5.8nM, 123.0±8.2nM,and 127.4±10.2nM in the chronic exercise group, respectively for the above doses, there were the high significant difference between the two groups under the situation with the same doses(20μg/ml and 40μg/ml)(P<0.01, n=5).However, there was significant difference between the two groups at the dose of 10μg/ml.

3.1.2.2.3 The change of Con A-induced $[Ca^{2+}]i$ and $\Delta[Ca^{2+}]i$ in Ca^{2+}-free PBS solution with 0.1mmol/L EGTA

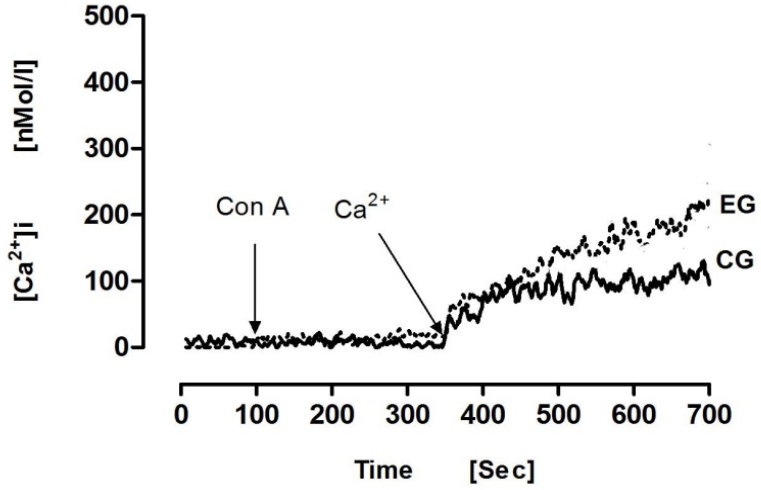
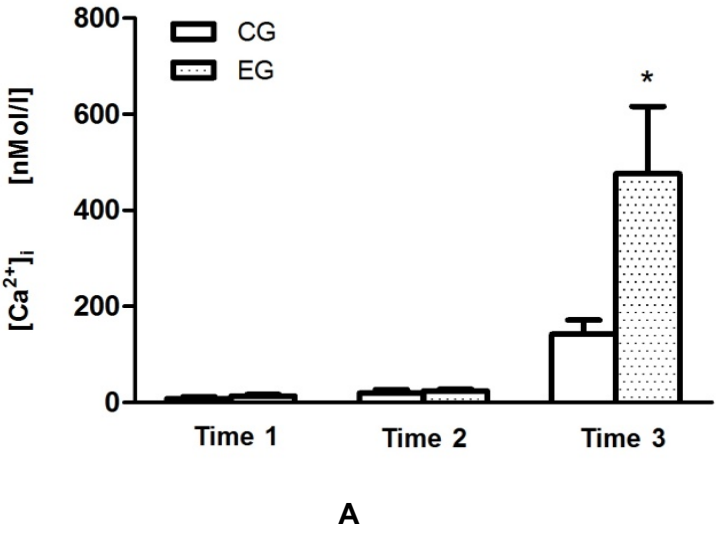


***Figure 13**- The tracings of 40μg/ml Con A -induced calcium response of lymphocytes in Ca^{2+} free PBS solution with 0.1mM EGTA. The representative tracings showing Con A -induced the change of $[Ca^{2+}]i$ in the control group (CG, solid line) and the chronic exercise group (EG, dotted line). Time is plotted on the x axis and $[Ca^{2+}]i$ is shown on the y axis. Two arrows show when stimulants and Ca^{2+} were applied, respectively. A representative experiment out of five independently performed experiments is shown, respectively.*



A

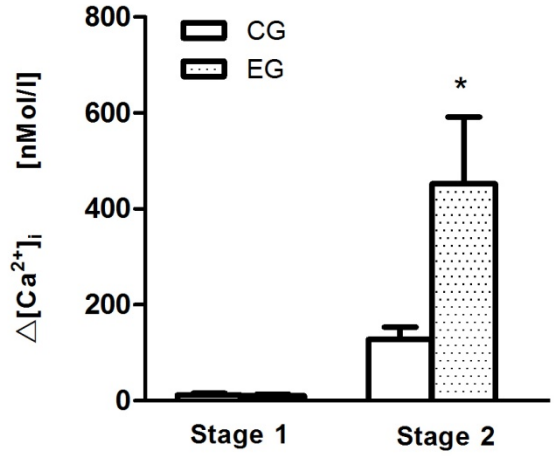


Figure 14*- The effect of chronic voluntary exercise on Con A-induced intracellular calcium response of lymphocytes in Ca^{2+} free PBS solution with 0.1mM EGTA. CG: the control group, EG: the chronic exercise group. The figures show that the basal $[Ca^{2+}]i$ (Time1), the intracellular Ca^{2+} level from Con A-induced Ca^{2+} release of intracellular stores(Time 2) and it's variation amplitude (stage1) ,intracellular Ca^{2+} level after the addition of Ca^{2+} into the measurement solution(Time 3) and it's variation amplitude (stage2). Data are mean±SEM derived from 5 separate experiments. Note that $\Delta[Ca^{2+}]i$ was calculated according to the equation: $\Delta[Ca^{2+}]i = [Ca^{2+}]i$ agonist- $[Ca^{2+}]i$ basal. *P<0.05 vs the control group.*

As shown in Fig.14A-Time 3, cells were exposed to 40μg/ml Con A in Ca^{2+}-free PBS solution with 0.1mmol/L EGTA, followed the addition of Ca^{2+}into the measurement solution. The significant difference of Con A-induced $[Ca^{2+}]i$ existed between the control group (142.0±67.0nM) and the exercise group (476.3±312.8nM) ($P<0.05$,n=5), and $\Delta[Ca^{2+}]i$ also had the significant difference between the two groups (127.8±57.0nM, 452.2±311.3nM, respectively) ($P<0.05$,n=5) as shown in Fig.14B-Stage 2. The findings confirm that release of Ca^{2+} from intracellular calcium pools is mainly responsible for the early Ca^{2+} rise, and that influx of extracellular Ca^{2+} is necessary for a sustained $[Ca^{2+}]i$ elevation. From the above results about Con A, we suggest that Con A is a stronger mitogenic lectin to induce the increase of $[Ca^{2+}]i$ in lymphocytes than PHA is.

3.1.2.3 OKT-3

3.1.2.3.1 The change of OKT3-induced $[Ca^{2+}]i$ and $\Delta[Ca^{2+}]i$ in Ca^{2+} buffer

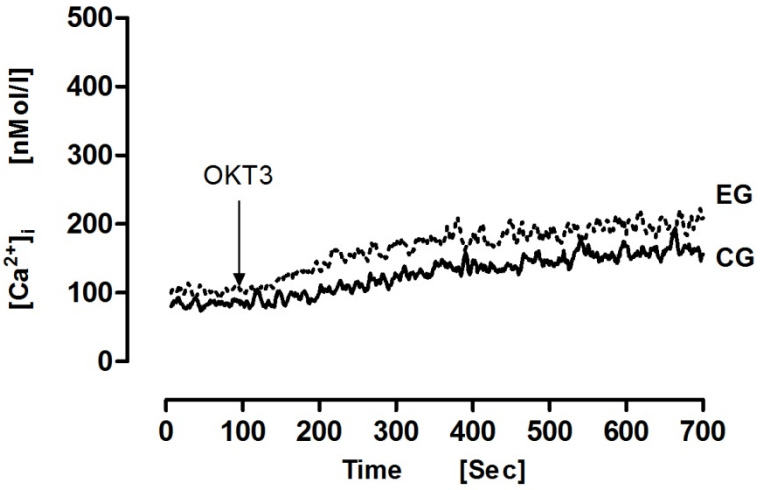
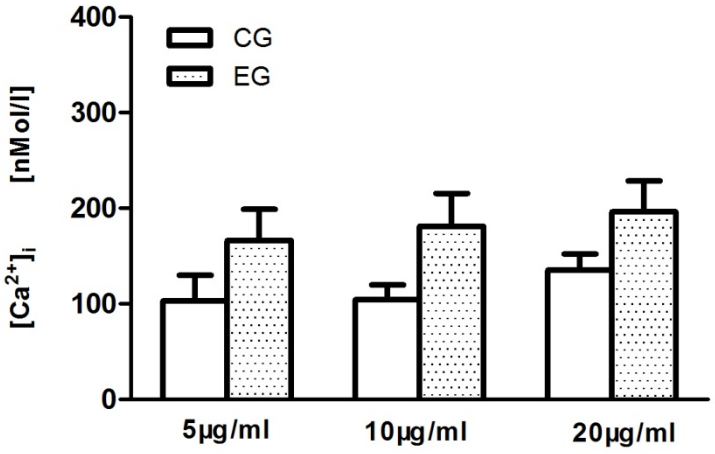
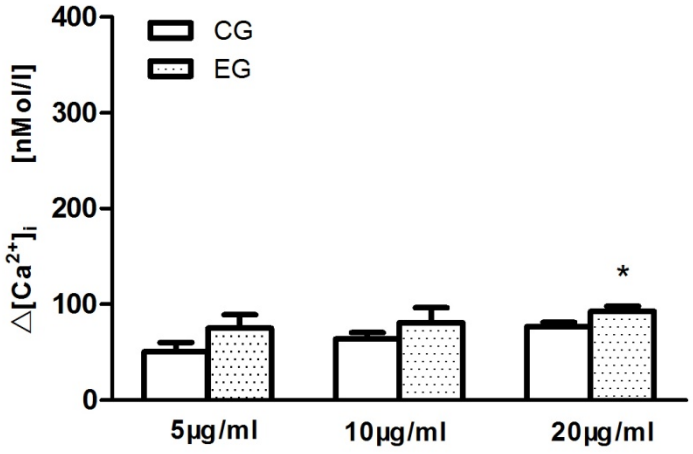


***Figure 15**- The tracings of OKT3-induced intracellular calcium response of splenic lymphocytes in calcium buffer. Time is plotted on the x axis and $[Ca^{2+}]i$ is shown on the y axis. The representative tracings showg OKT3 induced the increase of $[Ca^{2+}]i$ in the control group (CG, solid line) and the chronic voluntary exercise group (EG, dotted line). Arrows show when OKT3 were applied. The representative experiment out independently performed experiments is shown.*



A



B

Figure 16*- The effect of chronic voluntary exercise on OKT3-induced calcium response of lymphocytes in calcium buffer. Data are mean±SEM derived from 5 separate experiments. The figures show that the level of intracellular Ca^{2+} and the change of $[Ca^{2+}]i$ of lymphocytes with the stimulation of OKT3.Bar chart diagram summarizes the results of the entire group. The chronic exercise group (EG) is compared with the control group (CG) under the situation with the same dose. Note that $\Delta[Ca^{2+}]i$ is calculated according the equation: $\Delta[Ca^{2+}]i = [Ca^{2+}]i$agonist $-[Ca^{2+}]i$basal. *P<0.05 vs the control group.*

Stimulating the complex of T-cell receptor (TCR) and CD3 protein with an anti-CD3 antibody induced an increase of $[Ca^{2+}]i$ in T cells. The specific monoclonal antibodies (OKT-3) can activate PLC-γ via tyrosine phosphorylation producing in turn InsP3 from phosphatidylinositol (4,5) bisphosphate(PIP2) that liberates Ca^{2+} from the endoplasmic reticulum. In this study, as shown in Fig.16A, when lymphocytes were exposed to OKT3 (5μg/ml, 10μg/ml, and 20μg/ml) exhibited a rapid and dose-dependent rise in intracellular Ca^{2+} levels, but there weren't a obvious $[Ca^{2+}]i$ spike after activated by OKT3, and OKT3-induced $[Ca^{2+}]i$ by using the above dosed respectively, were 103.1±26.6nM, 104.4±15.3nM, and 135.1±17.2nM in the control group ,and 166.3±32.6nM, 181.1±34.5nM, and 196.1±32.4nM in the exercise group. There was no significant difference between the two groups at the same dose (P>0.05, n=5).

As shown in Fig.16B, after the addition of OKT-3 (5μg/ml,10μg/ml, and 20μg/ml, respectively), the increase in $[Ca^{2+}]i$ reached a value above the resting 50.5±9.8nM, 63.8±6.7nM, and 76.7±4.4nM in the control group ,and 75.3±13.8nM, 80.9±15.5nM, and 92.8±5.3nM in the exercise group, respectively for the above doses, there were

the significant difference between the control group and the chronic exercise group by using 20μg/ml OKT-3 as the stimuli($P<0.05$, n=5). But there was no significant difference between the two groups at the other same dose ($P>0.05$, n=5).

3.1.2.3.2 The change of OKT3-induced $[Ca^{2+}]i$ and $\Delta[Ca^{2+}]i$ in Ca^{2+}-free PBS solution with 0.1mmol/L EGTA

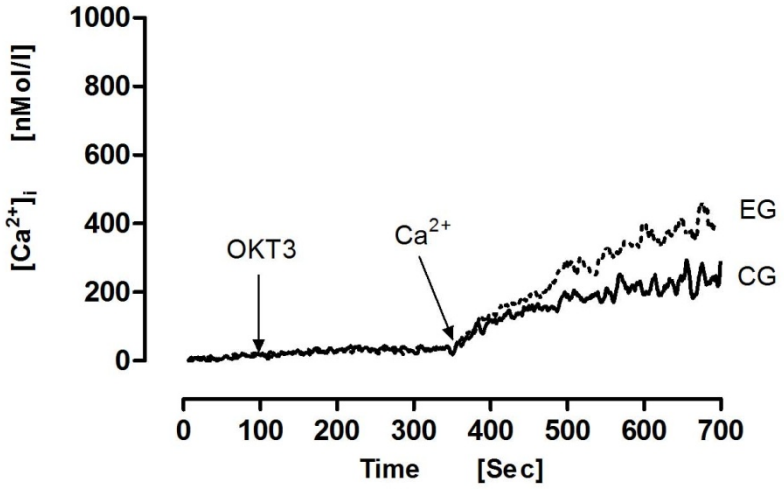
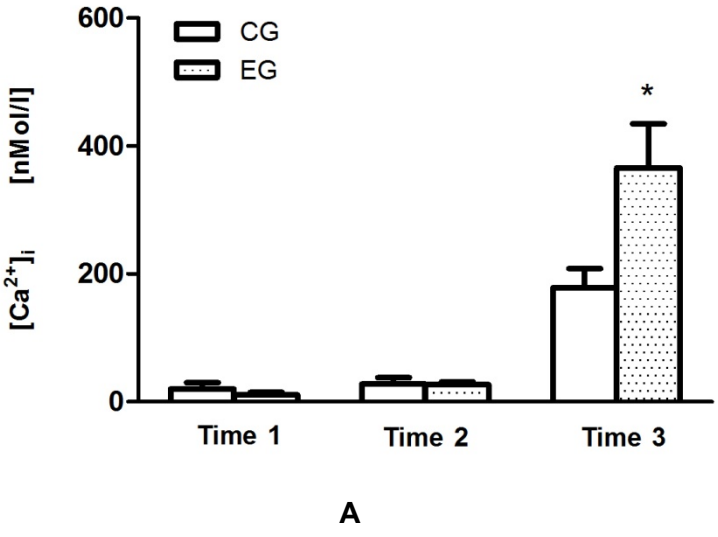


***Figure 17**- The tracings of 20μg/ml OKT-induced calcium response of lymphocytes in Ca^{2+} free PBS with 0.1mM EGTA. The representative tracings showing OKT3- induced the change of $[Ca^{2+}]i$ in the control group (CG, solid line) and the chronic exercise group (EG, dotted line). Time is plotted on the x axis and $[Ca^{2+}]i$ is shown on the y axis. Two arrows show when stimulants and Ca^{2+} were applied, respectively. A representative experiment out of five independently performed experiments is shown, respectively.*



A

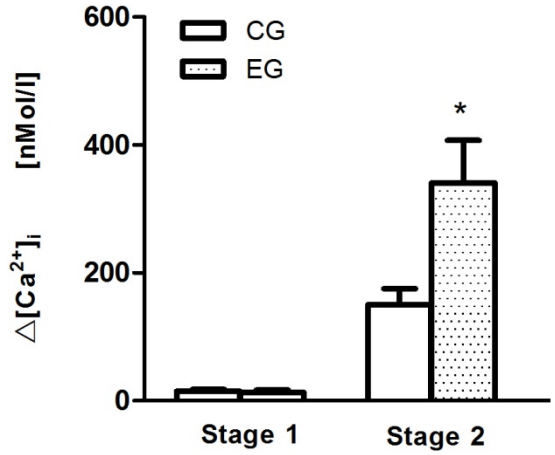


***Figure 18**-The effect of chronic voluntary exercise on 20μg/ml OKT3-induced intracellular calcium response of lymphocytes in Ca^{2+} free PBS solution with 0.1mM EGTA. CG: the control group, EG: the chronic exercise group. The figures show that the basal $[Ca^{2+}]i$ (Time1), the intracellular Ca^{2+} level from OKT3 -induced Ca^{2+} release of intracellular stores(Time 2) and it's variation amplitude (stage1) ,intracellular Ca^{2+} level after the addition of Ca^{2+} into the measurement solution(Time 3) and it's variation amplitude (stage2). Data are mean±SEM derived from 5 separate experiments. Note that $Δ[Ca^{2+}]i$ was calculated according to the equation: $Δ[Ca^{2+}]i= [Ca^{2+}]iagonist- [Ca^{2+}]ibasal$. *P<0.05 vs the control group.*

To evaluate external Ca^{2+} participation in the intracellular Ca^{2+} increase observed in lymphocytes, cells were exposed to OKT-3(10μg/ml) in the absence of external Ca^{2+} (Ca^{2+}-free PBS solution with 0.1 mmol/L EGTA). As shown in Fig.18, there wasn't a significant difference of OKT3-induced $[Ca^{2+}]i$ existed between the control group (178.0±30.4nM) and the exercise group(366.0±68.9nM) ($P>0.05$, n=5), however, $Δ[Ca^{2+}]i$ had the significant difference between the two groups (150.3±24.8nM, 340.7±66.5nM, respectively) ($P<0.05$,n=5). Compared to PHA and Con A, OKT3 had a slow effect on the elevation in intracellular Ca^{2+} concentration in lymphocytes. The results suggest that the Ca^{2+}response pattern of cells activated by OKT-3 wasn't the same than PHA and Con A.

3.1.2.4 Thapsigargin

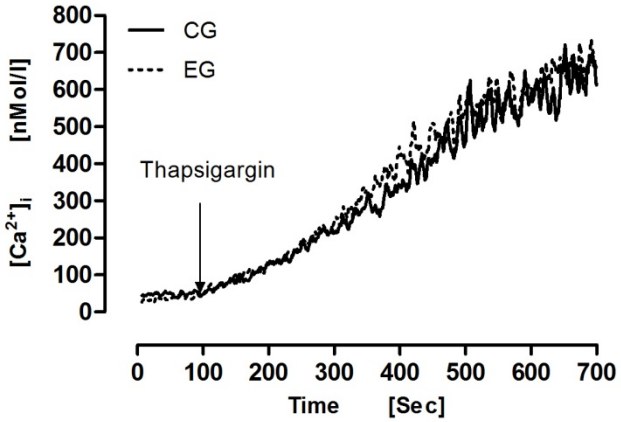
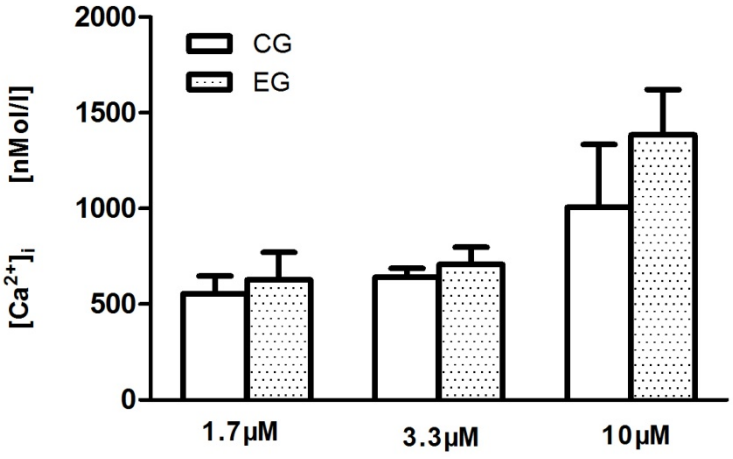
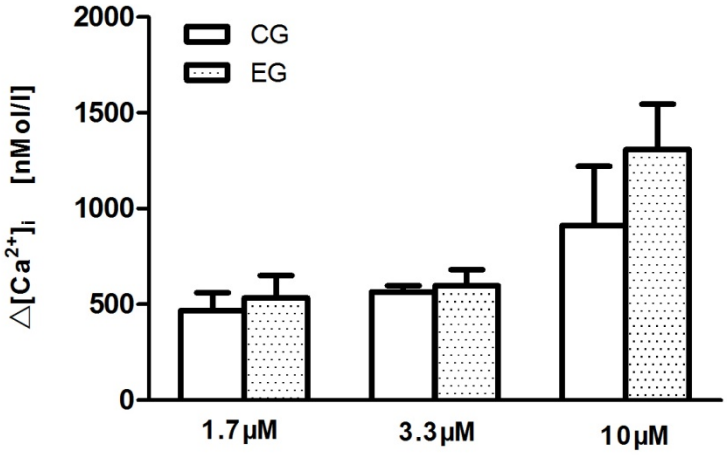


***Figure 19**-The tracings of thapsigargin-induced intracellular calcium response of splenic lymphocytes in calcium buffer. Time is plotted on the x axis and $[Ca^{2+}]i$ is shown on the y axis. The representative tracings show thapsigargin-induced the increase of $[Ca^{2+}]$ in the control group (CG, solid line) and the chronic exercise group (EG, dotted line). Arrows show when thapsigargin were applied. A representative experiment out five independently performed experiments is shown.*



A



B

Figure 20-*The effect of chronic voluntary exercise on thapsigargin-induced intracellular calcium response of splenic lymphocytes in calcium buffer. The figures show that the level of intracellular Ca^{2+} and the change of $[Ca^{2+}]i$ of lymphocytes with the stimulation of thapsigargin. Data are mean±SEM derived from 5 or 6 separate experiments. Bar chart diagram summarizes the results of the entire group. The chronic exercise group (EG) is compared with the control group (CG) under the situation with the same dose. Note that $Δ[Ca^{2+}]i$ is calculated according to the equation: $Δ[Ca^{2+}]i=[Ca^{2+}]iagonist- [Ca^{2+}]ibasal$. The chronic exercise group was compared with the control group.*

Thapsigargin, a specific inhibitor of the Ca^{2+}-ATPase of the endoplasmic reticulum is used to directly release the Ca^{2+} stores of endoplasmic reticulum by inhibiting the ATP-dependent Ca^{2+} pump responsible for Ca^{2+} release from the intracellular Ca^{2+} stores, and induce a further rise in $[Ca^{2+}]i$ due to Ca^{2+} influx by store-operated calcium entry, largely attributed to Ca^{2+}-release activated Ca^{2+} current into cells. The cells were stimulated with thapsigargin and the increase of $[Ca^{2+}]i$ could lead to Ca^{2+} depletion of these Ca^{2+} organelles. This Ca^{2+} influx is therefore not directly related to the initial ligand-receptor interaction. In this study, treatment of isolated murine splenic lymphocytes with 1.7μM, 3.3μM, and 10μM thapsigargin, respectively, caused a sustained increase in intracellular Ca^{2+} in the cytosol, and the Ca^{2+} mobilization induced by thapsigargin was concentration dependent.

As shown as Fig.20A, when cells were stimulated by thapsigargin (1.7μM, 3.3μM, and 10μM,respectively) for 650s,the maximal $[Ca^{2+}]i$ in the control group (554.5±92.8nM, 640.6±47.6nM, and 1006.2± 328.0nM, respectively for the above doses) and in the exercise group(628.3±143.0nM, 708.0±89.5nM, and 1384.6±237.1nM, respectively

for the above doses) hadn't a significant difference that existed between the control group and the chronic exercise group under the situation with the same dose($P>0.05$, $n=5$).

As shown in Fig.20B, the increase in $[Ca^{2+}]i$ above the resting $[Ca^{2+}]i$ were 467.2±93.1nM, 565.9±32.2nM, and 912.6±307.4nM in the control group, and 534.2±116.6nM, 597.2±83.0nM, and 1309.1±256.6nM in the chronic exercise group, respectively for the above doses, there wasn't the significant difference between the control group and the chronic exercise group under the situation with the same doses($P>0.05$, $n=5$).From the above results about thapsigargin, this study suggested that the administration of thapsigargin has the stronger function to increase $[Ca^{2+}]i$ of T cells, compared to the other agonists, i.e. PHA,Con A and OKT3. The mobilization of intracellular Ca^{2+} pools by thapsigargin is a sufficient signal to trigger the opening of cell surface Ca^{2+}-permeable structures (i.e.Ca^{2+} channels) since restoration of external Ca^{2+} instantaneously raised $[Ca^{2+}]i$ to high values.

3.1.3 The effect of chronic voluntary exercise on the proliferation of $CD3^+$ T cells

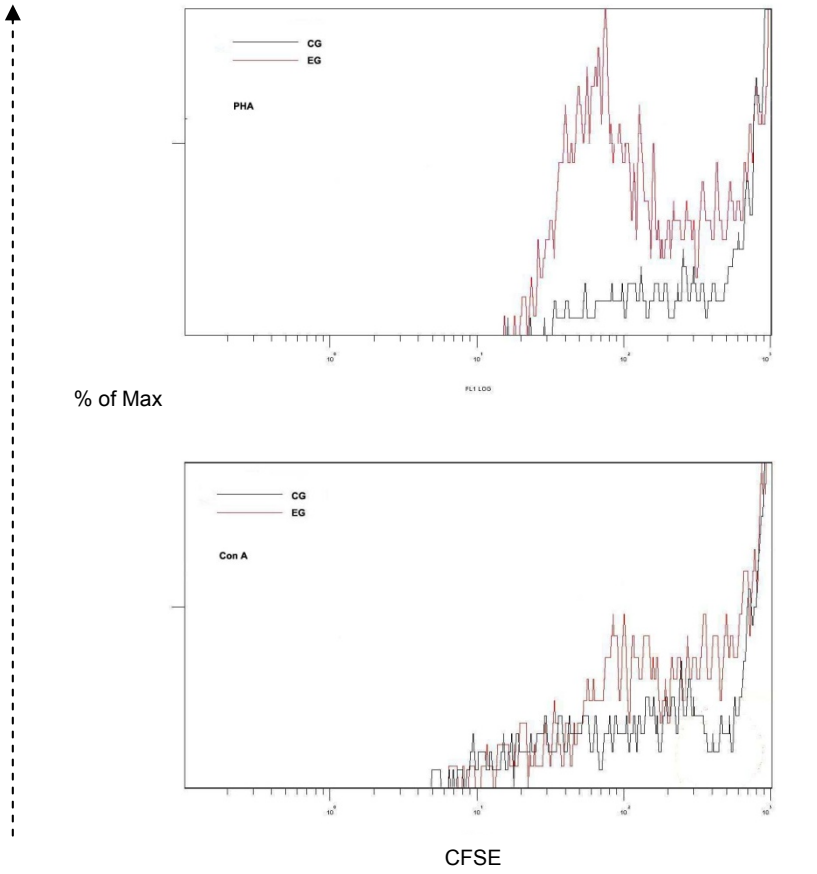


Figure 21*-Demonstration of the effect of CFSE labeling CD3+ T cells from the control group (CG, black line) and the chronic voluntary exercise group (EG, red line) to respond to PHA and Con A, respectively. CD3+ T cells were labeled with CFSE for 5 min at 20 °C in protein-free PBS and then PBS containing 5% (v/v) FCS was added to halt the reaction, and then assessed for their ability to proliferate by using flow cytometry after the CFSE labeling cells with stimulation of PHA or Con A were cultured for 72 hours. In the pictures, a representative experiment out of seven independently performed experiments is shown, respectively.*

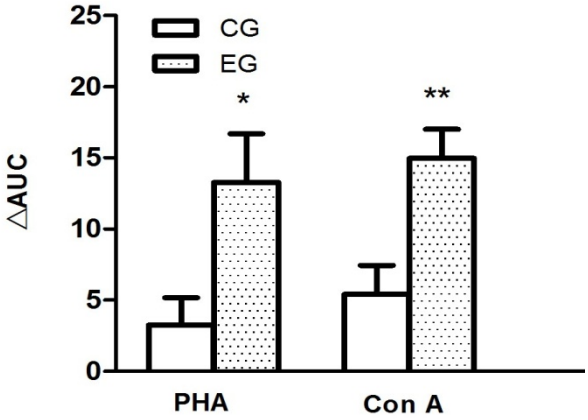


Figure 22- *The effect of chronic voluntary exercise on the ability of mitogens-induced splenic lymphocytes proliferation. CG: the control group, EG: the chronic exercise group. Cells (2×10^6) with CFSE labeling were incubated for 72 h in Dulbecco's Modified Eagle Medium (DMEM) under the same experimental conditions. Samples were treated with Con A or PHA (10μg/ml) throughout the experiment. Note that AUC is the area under curve that the x-axis denotes cellular generations, and the y-axis represents the percentage of cells in each generation. △AUC was calculated according to the equation: △AUC=AUC1 (that reflects the level of cell proliferation with the stimulation of mitogens)- AUC2(that reflects basal proliferation state of cell without stimulation of mitogens). *P<0.05, **P<0.01 vs the control group (n=7).*

The cells with the double labeling of CFSE and PE/Cy7 anti-mouse CD3 automatically were calculated the proportions of cells in each generation, up to the 6th generation by using flow cytometry. △AUC was calculated according to the equation: △AUC=AUC1(that reflects the level of mitogen-induced cell proliferation)- AUC2(that reflects basal state of sample before mitogen induced cell proliferation). Note that AUC is the area under curve that the x-axis denotes cellular generations, and the y-axis represents the percentage of cells in each generation.

As shown as Fig.22, when CD3+ T cells were stimulated by PHA and Con A (10μg/ml),

△AUC in the control group (3.25±1.93, 3.99±0.75,respectively for the above stimuli) and in the chronic exercise group(13.28±3.42,14.81±2.12, respectively for the above stimuli) had a significant difference or highly significant difference that existed between the control group and the chronic exercise group (P<0.05,or 0.01, n=7).

3.1.4 The effect of chronic voluntary exercise on the Ca^{2+}-regulating genes mRNA expression in splenic lymphocytes

3.1.4.1 The expression of CRAC channels

As shown in Fig.23A, compared with the non-exercise mice, STIM1, ORAI1, and ORAI2 mRNA expression in the chronic exercise group, was significantly downregulated 45.6±8.4%, 42.6±10.8%, 35.2±6.5% (P<0.01, P<0.05, and P=0.067, respectively,n=5), and the STIM2 and ORAI3 mRNA expression was downregulated 52.4±34.7%, 14.2±16.4%, respectively (P>0.05,n=5).

3.1.4.2 The expression of voltage-gated Ca^{2+} channels

In Fig.23B, compared with the control group, the expression of Cav1.2,Cav2.3 in the chronic exercise group was significantly downregulated 72.0±6.2%,82.7±4.4% (P=0.059,P<0.01,respectively, n=5),and the expression of Cav1.3, Cav2.1, Cav3.1, and Cav β 4 was downregulated 27.6±12.0%, 31.5±32.3%, 66.1±11.9%, and 61.6±11.7%, respectively (P>0.05, n=5).

3.1.4.3 The expression of purinergic receptors

In Fig.23C, the P2X7 and P2Y14 mRNA expression in the chronic exercise group was significantly downregulated 47.7±12.1%, 35.8±10.2% (P=0.056, P<0.05, n=5, respectively); the expression of P2X1, P2Y1, P2Y2, P2Y6, P2Y12, and P2Y13 was downregulated 35.2±12.2%,46.7±10.5%,35.0±14.7%,46.2±12.4%,32.4±18.2%,and 26.8±7.5%, respectively, in comparison with the non-exercise group (P>0.05, n=5).

3.1.4.4 The expression of K+ channels

As shown in Fig.23D, compared with the non-exercise group, the Kcnn4 mRNA expression in the chronic exercise mice was downregulated 17.7±15.8% (P>0.05, n=5), however the expression of Kcnk5 was upregulated 17.8±23.0% (P>0.05, n=5).

3.1.4.5 The expression of intracellular Ca^{2+} release channels

In Fig.23E, in comparison with the control group, the IP3R2 mRNA expression in chronic exercise mice was significantly downregulated 58.3±3.9% (P<0.01, n=5), and the expression of IP3R1, IP3R3 and RYR2 was downregulated 28.6±7.7%, 24.4±12.7% and 5.7±20.9%, respectively (P>0.05, n=5).

3.1.4.6 The expression of TRP channels

In Fig.23F, compared to the control group, TRPM1, TRPM5, TRPV4, TRPC1 mRNA expression in the chronic exercise group was significantly downregulated 56.8±7.9%, 57.5±8.6%, 57.1±10.0%, 83.1±7.1% (P=0.066, P<0.05, P<0.01, and P<0.05, n=5, respectively), and the expression of TRPM2, TRPM4, TRPM7,TRPC2,TRPC3, TRPC6 was downregulated 48.6±5.2%, 28.6±10.3%,17.3±5.5%,19.2±13.4%, 42.0±19.8%%, 36.9±13.1%, respectively (P>0.05, n=5). Even though the expression of TRPV6 was upregulated 33.8±18.3%, in the chronic exercise group, there was no significant difference between the two groups (P>0.05, n=5).

3.1.4.7 The expression of Ca^{2+}pumps

As shown in Fig.23G, compared to the control group, the PMCA mRNA expression in the chronic exercise group was downregulated 5.3±16.7% (P>0.05, n=5), and the SERCA mRNA expression was upregulated 27.1±12.2% (P>0.05, n=5).

3.1.4.8 The expression of Ca^{2+}-regulating genes in intracellular Ca^{2+}store

In Fig.23H, compared to the control group, the MCU mRNA expression in the chronic exercise group was significantly downregulated 50.4±13.2% (P<0.05, n=5), and the expression of ATP2C1 was upregulated 22.2±17.3% (P>0.05, n=5).

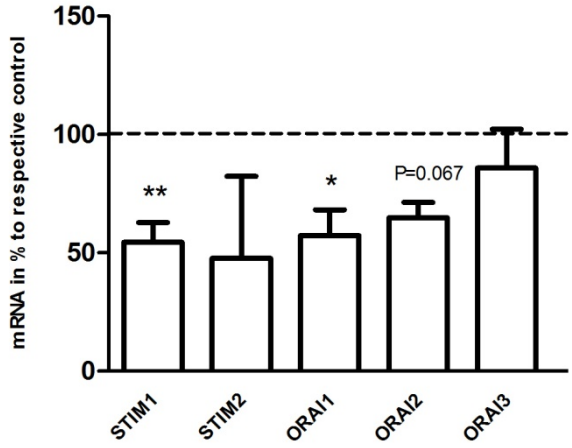
3.1.4.9 The expression of Calm1 and Hspa1a

As shown in Fig.23G, compared to the control group, the Calm1 and Hspa1a mRNA expression in the chronic exercise group was downregulated 24.5±11.5%, 47.8±19.9% respectively (P>0.05, n=5).

3.1.4.10 The expression of other Ca^{2+}regulating genes

The expression of RYR1, RYR3, TRPV3, TRPV5, Cav1.1, Cav1.4, Cav2.2, Cav3.2,

α7 nACh, and Casq2 mRNA wasn't be successfully detected in murine splenic lymphocytes.



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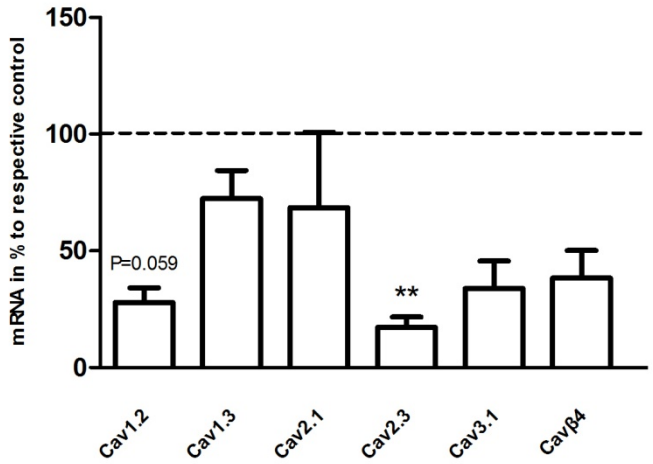

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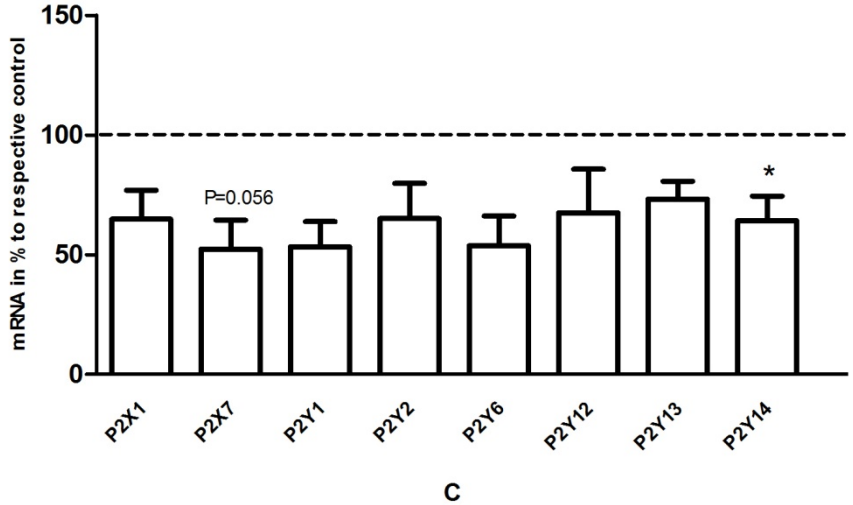
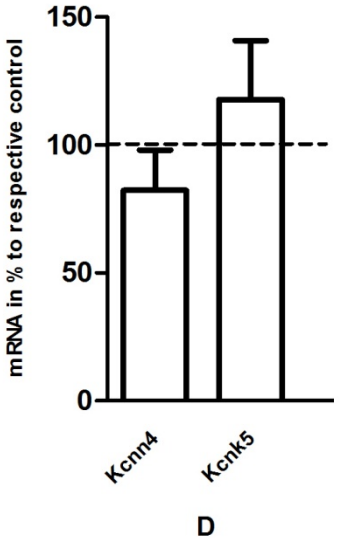
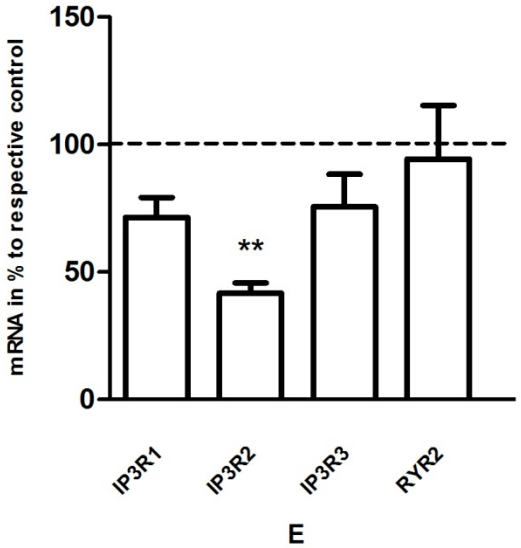


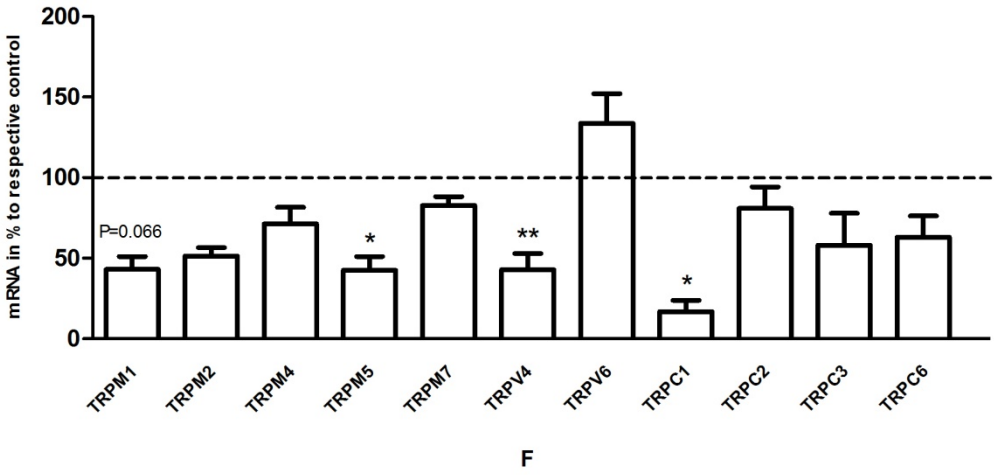
C


mRNA in % to respective control
150
100
50
0
Kcnn4
Kcnk5

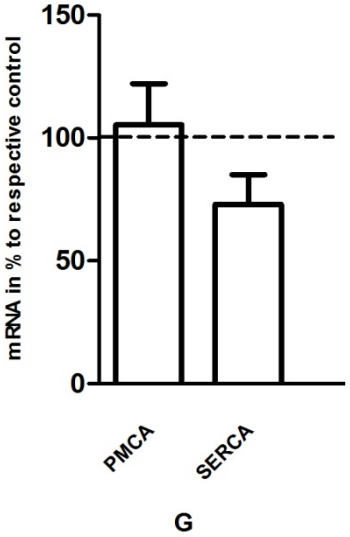
D


mRNA in % to respective control
150
100
50
0
**
IP3R1
IP3R2
IP3R3
RYR2

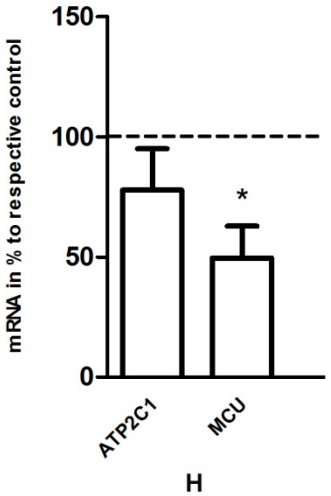
E


mRNA in % to respective control
200
150
100
50
0
P=0.066
*
**
*
TRPM1
TRPM2
TRPM4
TRPM5
TRPM7
TRPV4
TRPV6
TRPC1
TRPC2
TRPC3
TRPC6

F


mRNA in % to respective control
150
100
50
0
PMCA
SERCA

G


mRNA in % to respective control
150
100
50
0
*
ATP2C1
MCU

H

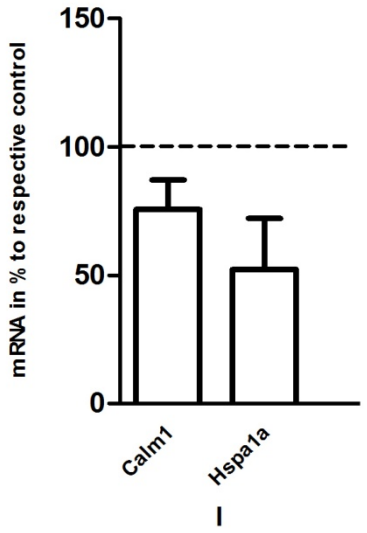


Figure 23*- The effects of chronic voluntary exercise on Ca^{2+}- regulating gene expression of murine splenic lymphocytes. RNA samples were isolated from cells from 5 mice for each group. Relative quantification of target genes mRNA expression was evaluated by quantitative real-time PCR and the experiments were carried out according to materials and methods. The relative levels of target gene mRNA expression were normalized against the mRNA expression of internal housekeeping gene (β-actin). The expression of β-actin mRNA was not significantly different between the tested cells allowing a direct comparison (data not shown).In these figures, data shown are representative of 5 independent experiments. Note that columns and error bars represent (mRNA in % to respective control ±SEM), and levels of significance (*P<0.05, ** P<0.01 compared with the control group) are indicated.*

3.2 Part II: The effect of acute exercise on splenic lymphocytes and functions

3.2.1The effect of acute exercise on the basal $[Ca^{2+}]i$ of splenic lymphocytes

As shown as in Fig.24, before any stimulus, basal $[Ca^{2+}]i$ levels of lymphocytes were determined to be 45.45±5.14nM in the non-exercise group. Immediately after exercise, the basal $[Ca^{2+}]i$ were elevated to 49.65±6.23 nM (P>0.05,n=14). In the-3 hours after exercise-group, the basal $[Ca^{2+}]i$ were highly significantly increased to 101.00±30.92nM compared with the non-exercise group (P<0.01,n=11).And also a close significant difference existed between the-3 hours after exercise-group and the-immediately after exercise-group (P=0.08,n=11).After 24 hours, the basal $[Ca^{2+}]i$

levels were decreased to 46.00±15.17nM, but there were no significant difference, when compared to other groups (P>0.05,n=8).

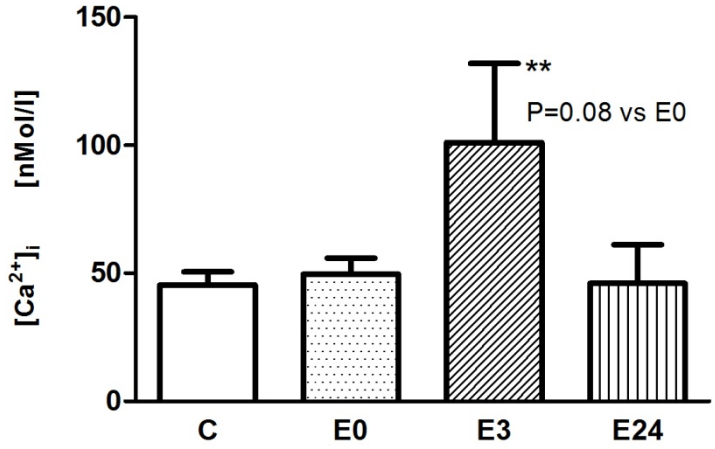


***Figure 24**-Basal [Ca^{2+}]i of splenic lymphocytes at the control group(C), immediately after (E0), 3 h after (E3), and 24 h (E24) after a single bout of exhaustive exercise with high intensity.**P<0.01 vs C, P=0.08 vs E0 (n=28).*

3.2.2 The effect of acute exercise on the agonist-induced intracellular Ca^{2+} transients

3.2.2.1 Con A

3.2.2.1.1 The change of Con A-induced [Ca^{2+}]ipeak and Δ[Ca^{2+}]ipeak in Ca^{2+} buffer

As shown as in Fig.26A, when cells were stimulated by 40μg/ml Con A, the significant difference of Con A-induced [Ca^{2+}]ipeak existed between the control group (156.5±15.7nM) and the-3 hours after exercise-group (390.7±201.8nM) (P<0.05,n=5). The Con A-induced [Ca^{2+}]ipeak could be decided in the-immediately after exercise-group (232.0±30.8nM) and the-24 hours after exercise-group (163.3±47.0 nM), but there was no significant difference between any other two groups except for the comparison that was mentioned before.

As shown as in Fig.26B,Con A-induced Δ[Ca^{2+}]ipeak in the control group, the-immediately after exercise-group,the-3 hours after exercise-group, and the-24 hours after exercise-group, were 104.9±13.6nM, 80.7±14.3nM, 246.5±137.7nM, and 117.3±37.6nM, respectively. There was a close significant difference between the-3 hours after exercise-group and the control group (P=0.08, n=5), and there was no

significant difference between any other two groups (P>0.05, n=7).

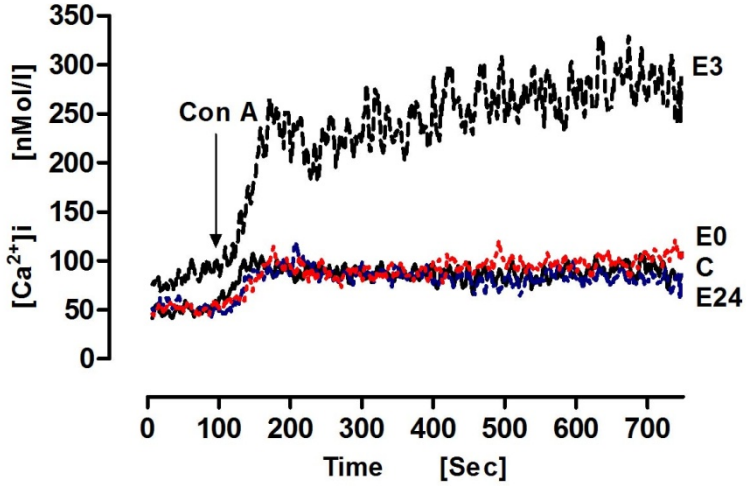
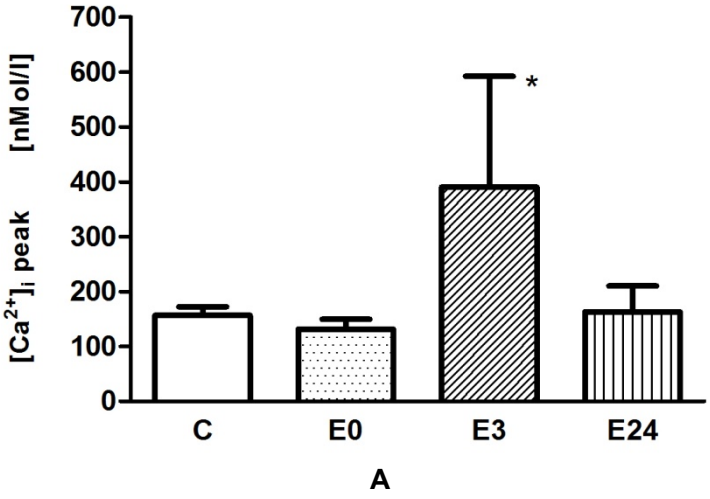


Figure 25-*The tracings of Con A-induced intracellular calcium response change of lymphocytes at different time point after acute exercise in calcium buffer. Time is plotted on the x axis and [Ca^{2+}]i is shown on the y axis. Representative tracings show Con A-induced the increase of intracellular Ca^{2+} concentration in different time points. C: the control group (black solid line), E0: immediately after exercise (red dotted line), E3:3 h after exercise (black dashed line), and E24:24 h after exercise (blue dashed dotted line). Arrows show when Con A were applied. A representative experiment out independently performed experiments is shown.*



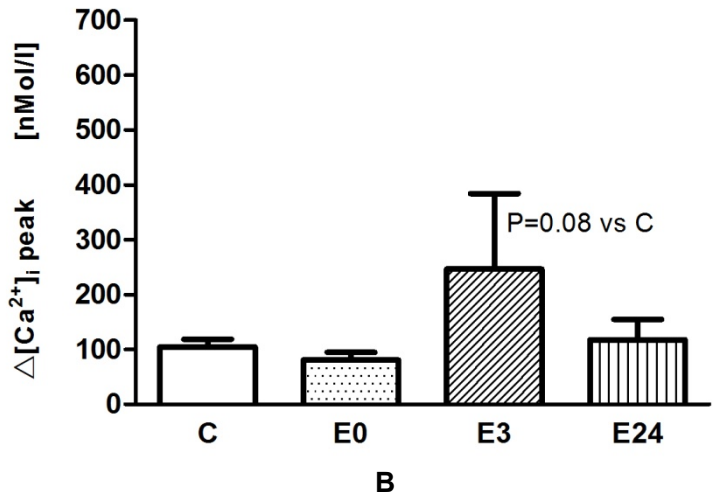


Figure 26- *The effect of acute exercise on 40μg/ml Con A-induced [Ca^{2+}]ipeak and △[Ca^{2+}]i of splenic lymphocytes in calcium buffer. C: the control group, E0: immediately after exercise, E3:3 h after exercise, and E24:24 h after exercise. Bar chart diagram summarizes the results of the entire group, and shows that the level of intracellular Ca^{2+} and the change of [Ca^{2+}]i of lymphocytes with the stimulation of Con A. Data are mean±SEM derived from 5 separate experiments. Note: [Ca^{2+}]i is elevated immediately and reached a maximum after the addition of Con A, i.e. [Ca^{2+}]ipeak; Δ[Ca^{2+}]ipeak is calculated according to the equation: Δ[Ca^{2+}]ipeak= [Ca^{2+}]i peak - [Ca^{2+}]i basal. *P<0.05 or P=0.08 vs C(n=5).*

3.2.2.1.2 The change of Con A-induced [Ca^{2+}]iplateau and Δ[Ca^{2+}]iplateau in Ca^{2+} buffer

As shown as in Fig.27A, there was a significant difference of Con A-induced [Ca^{2+}]iplateau existed between the control group (146.9±15.3nM) and the-3 hours after exercise-group (408.7±197.8nM) (P<0.05,n=5). The Con A-induced [Ca^{2+}]iplateau was decided in the-immediately after exercise-group (121.9±19.4nM) and the-24 hours after exercise-group (157.0±48.4nM), but there was no significant difference between any other two groups except for the comparison that was mentioned before.

As shown as in Fig.27B, Con A-induced Δ[Ca^{2+}]iplateau in the control group, the-immediately after exercise-group,the-3 hours after exercise-group, and the-24 hours after exercise-group, were 95.32±14.34nM, 70.91±15.27nM, 264.4±133.3nM, and 110.0±39.23nM, respectively. There were the significant difference between the-3 hours after exercise-group and the control group (P<0.05, n=5), but there was no significant difference between any other two groups (P>0.05, n=7).

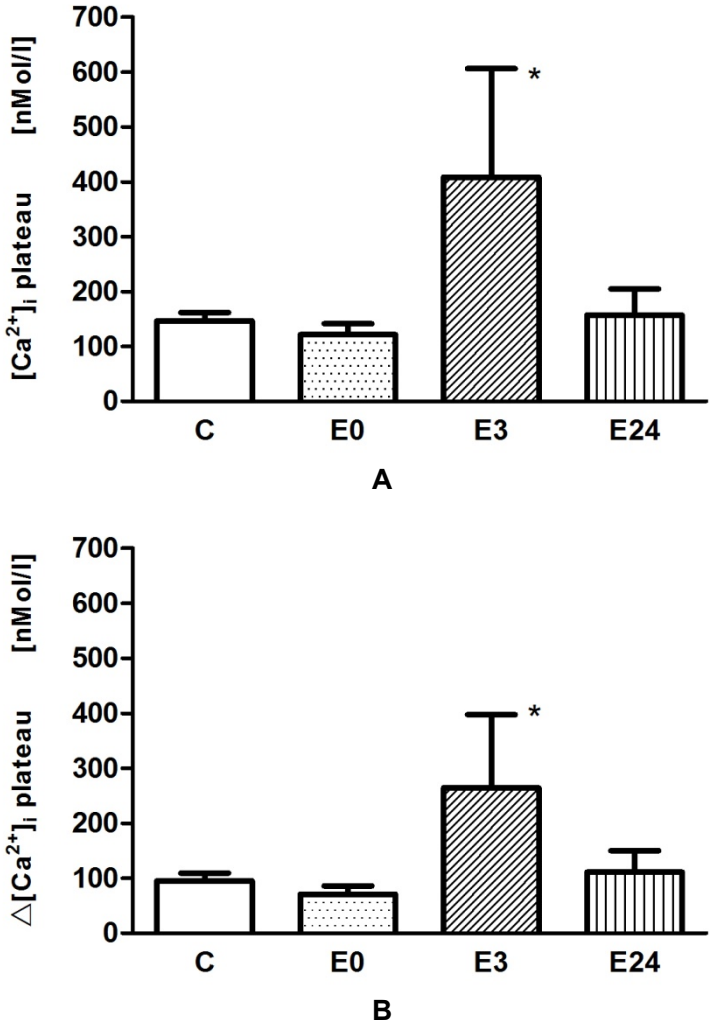


***Figure 27**-The effect of acute exercise on 40μg/ml Con A-induced [Ca^{2+}]iplateau and Δ[Ca^{2+}]iplateau of splenic lymphocytes in calcium buffer. C: the control group, E0: immediately after exercise, E3:3 h after exercise, and E24:24 h after exercise. Bar chart diagram summarizes the results of the entire group, and shows that the level of intracellular Ca^{2+} and the change of [Ca^{2+}]i of lymphocytes with the stimulation of Con A. Data are mean±SEM derived from 5 separate experiments.Note that [Ca^{2+}]iplateau refers to a minimum level, the steady state of [Ca^{2+}]i after the emergence of [Ca^{2+}]ipeak; Δ[Ca^{2+}]iplateau is calculated according to the equation: Δ[Ca^{2+}]iplateau=[Ca^{2+}]iplateau-[Ca^{2+}]ibasal. *P<0.05 vs the control group (n=5).*

3.2.2.1.3 The change of Con A-induced [Ca^{2+}]i and Δ[Ca^{2+}]i in Ca^{2+}-free PBS solution with 0.1mmol/L EGTA

As shown in Fig. 29A, cells were exposed to 40μg/ml Con A in Ca^{2+}-free PBS with 0.1mM EGTA for 250s, then Ca^{2+} was added into the reaction solution. A significant difference of Con A-induced [Ca^{2+}]i existed between the control group (193.3±46.6nM)

and the-3 hours after exercise-group (568.3±201.6nM) (P<0.05, n=8).There also was a significant difference between the-immediately after exercise-group(155.4±28.0nM) and the-3 hours after exercise-group(P<0.05, n=8). Compared with the-3 hours after exercise-group, Con A-induced $[Ca^{2+}]i$ was significantly decreased in the-24 hours after exercise-group(152.3±42.4nM) (P=0.08,n=8). There was no significant difference between any other two groups except for the comparison that was mentioned before.

As shown in Fig.29B, Con A-induced $\Delta[Ca^{2+}]i$ existed a significant difference between the control group and the-3 hours after exercise-group (175.0±46.2nM, 548.9± 200.2nM, respectively) (P<0.05,n=8).Compared with the-immediately after exercise-group (136.0±28.0nM), the value of $\Delta[Ca^{2+}]i$ was significantly enhanced in the-3 hours after exercise-group(P<0.05,n=8).At 24 hours after acute exercise, it was significantly decreased to 134.1±42.0nM (P=0.08,n=7). But there was no significant difference between any other two groups except for the comparison that was mentioned before.

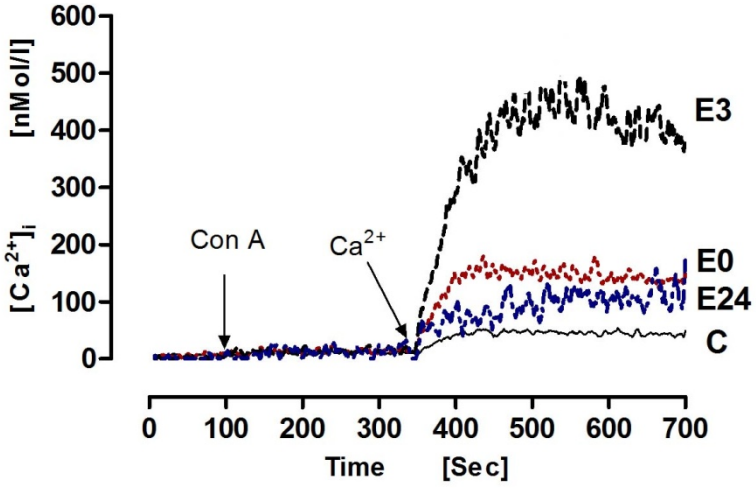


Figure 28-*The tracings of Con A -induced intracellular calcium response of lymphocytes at different time point after acute exercise in Ca^{2+} free PBS solution with 0.1mM EGTA. Time is plotted on the x axis and $[Ca^{2+}]i$ is shown on the y axis. Representative tracings showing Con A induced the increase of intracellular Ca^{2+} concentration in different time points. C: the control group (black solid line), E0: immediately after exercise (red dotted line), E3:3 h after exercise (black dashed line), and E24:24 h after exercise (blue dashed dotted line). Arrows show when Con A were applied. A representative experiment out independently performed experiments is shown.*

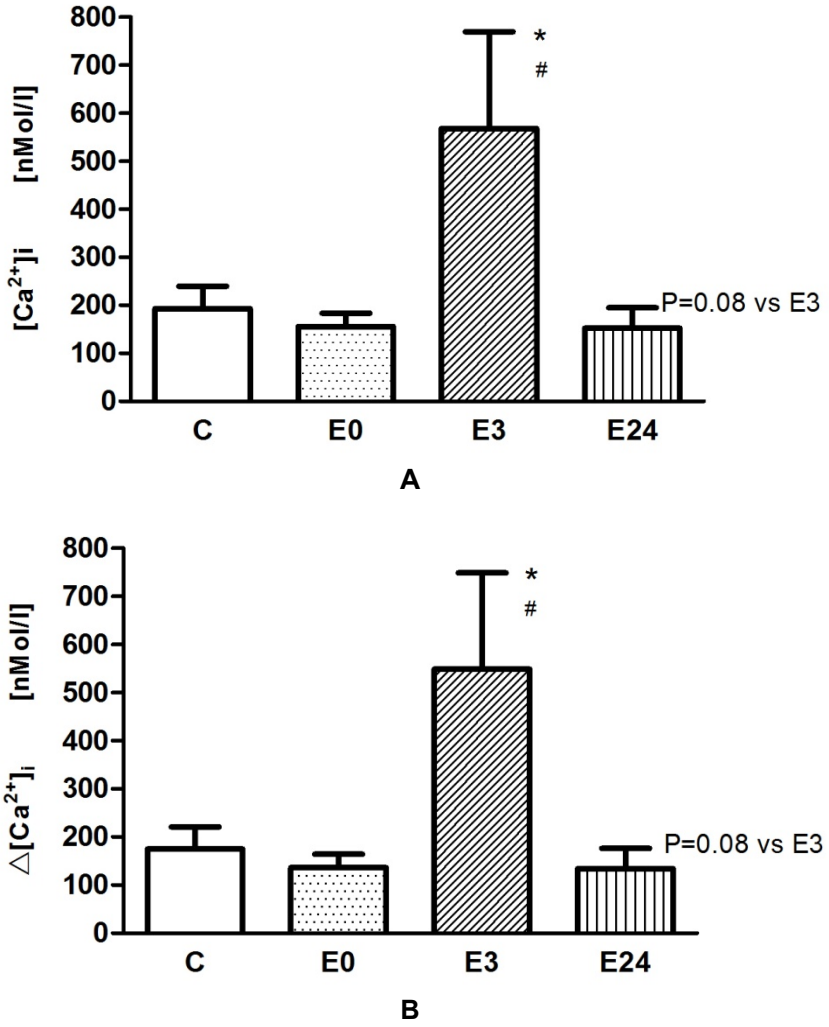


***Figure 29**- The effect of acute exercise on Con A-induced calcium response of murine splenic lymphocytes in Ca^{2+} free PBS solution with 0.1mM EGTA. Bar chart diagram summarizes the results of the entire group. Time points are C: the control group, E0: immediately after exercise, E3:3 h after exercise, and E24:24 h after exercise. Data are mean±SEM derived from 7 separate experiments. Note that on the x-axis, the change in $[Ca^{2+}]i$ is plotted, which is calculated according the following equation:$\triangle[Ca^{2+}]i= [Ca^{2+}]iagonist- [Ca^{2+}]ibasal$. *P<0.05 vs C, $^{\#}$P<0.05 vs E0, P = 0.08 vs E3 (n=7).*

3.2.2.2 OKT-3

3.2.2.2.1 The change of OKT3-induced $[Ca^{2+}]i$ and $\Delta[Ca^{2+}]i$ in Ca^{2+} buffer

As shown as in Fig.31A, when cells were stimulated by 20μg/ml OKT-3, a significant difference of OKT3-induced $[Ca^{2+}]i$ existed between the-3 hours after exercise-group (307.1±94.9nM) and the control group (103.6±26.9nM)(P<0.05,n=6). Compared with the-immediately after exercise-group (98.8±19.0nM),OKT3-induced $[Ca^{2+}]i$ also was

significantly enhanced in the-3 hours after exercise-group (P<0.05,n=6).But there was no significantly difference between the-immediately after exercise-group and the control group.

As shown as in Fig.31B, the values of OKT3-induced Δ[Ca^{2+}]i in the control group, the-immediately after exercise-group,the-3 hours after exercise-group, were 144.5±18.5nM, 304.4±64.1nM,304.4±64.1nM, respectively. There were a highly significant difference between the-3 hours after exercise-group and the control group, and the-3 hours after exercise-group and the-immediately after exercise-group (P<0.05, n=6), but there was no significant difference between the-immediately after exercise-group and the control group.

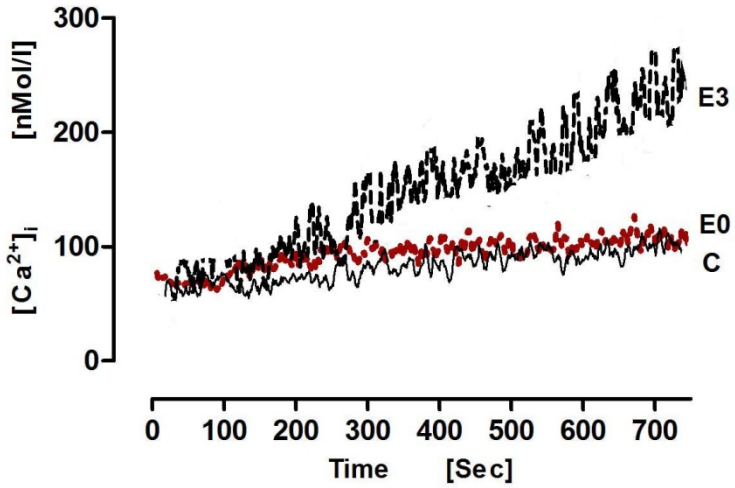


***Figure 30**-The tracings of OKT3-induced intracellular calcium response of splenic lymphocytes at different time point after acute exercise in calcium buffer. Time is plotted on the x axis and [Ca^{2+}]i is shown on the y axis. Representative tracings show OKT3-induced the increase of intracellular Ca^{2+} concentration in different time points. C: the control group (black solid line), E0: immediately after exercise (red dotted line), E3:3 h after exercise (black dashed line). Arrows show when OKT3 were applied. A representative experiment out independently performed experiments is shown.*

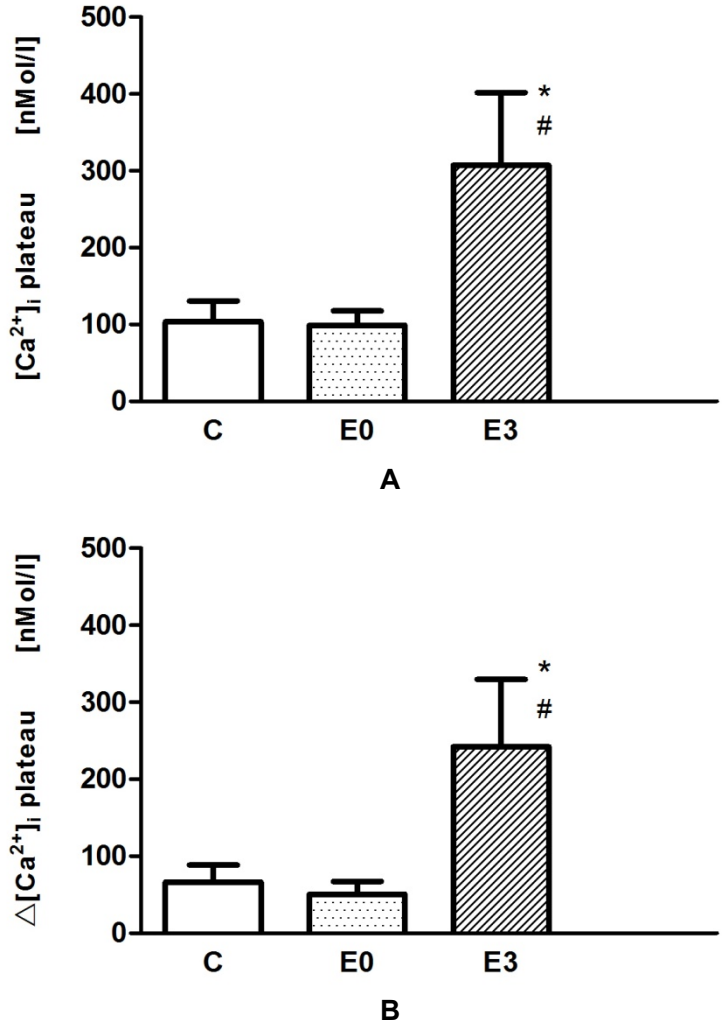


***Figure 31**- The effect of acute exercise on OKT3-induced [Ca^{2+}]i and Δ[Ca^{2+}]i of lymphocytes in calcium buffer. C: the control group, E0: immediately after exercise, E3:3 h after exercise. Bar chart diagram summarizes the results of the entire group, and show that the level of intracellular Ca^{2+} and the change of [Ca^{2+}]i of lymphocytes with the stimulation of OKT3. Data are mean±SEM derived from 7 separate experiments. Note that Note that Δ[Ca^{2+}]iplateau is calculated according the equation: Δ[Ca^{2+}]i=[Ca^{2+}]iagonist-[Ca^{2+}]ibasal. *P<0.05 vs the control group (n=7).*

3.2.2.2.2 The change of OKT3-induced [Ca^{2+}]i and Δ[Ca^{2+}]i in Ca^{2+}-free PBS solution with 0.1mmol/L EGTA

As shown in Fig. 33A, cells were exposed to 20μg/ml OKT3 in Ca^{2+}-free PBS with 0.1mmol/L EGTA for 250s, then Ca^{2+} was added into the reaction solution. The highly significant difference of OKT3-induced [Ca^{2+}]i existed between the-3 hours after exercise-group (320.8±66.6nM) and the control group (130.9± 14.8nM)(P<0.01,n=6). There also was a significant difference between the-3 hours after exercise-group and

the-immediately after exercise-group (155.4±28.0nM) (P<0.05, n=6).There was no significant difference between the-immediately after exercise-group and the control group.

As shown in Fig.33B, the value of $\Delta[Ca^{2+}]i$ existed a significant difference between the control group and the-3 hours after exercise-group (175.0±46.2nM, 548.9±200.2nM, respectively)(P<0.01,n=8).Compared with the-immediately after exercise-group (136.0±28.0nM), the value of $\Delta[Ca^{2+}]i$ was significantly enhanced in the-3 hours after exercise-group(P<0.01,n=8). There was no significant difference between any other two groups except for the comparison that was mentioned before.

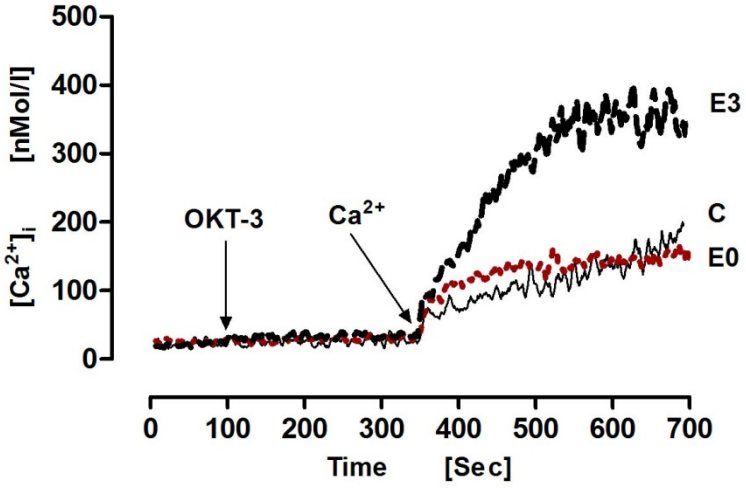


***Figure 32**- The tracings of OKT3-induced intracellular calcium response of murine splenic lymphocytes in Ca^{2+} free PBS solution with 0.1mM EGTA. Time is plotted on the x axis and $[Ca^{2+}]i$ is shown on the y axis. Representative tracings showing OKT3 induced the increase of intracellular Ca^{2+} concentration in different time points. C: the control group (black solid line), E0: immediately after exercise (red dotted line), E3:3 h after exercise (black dashed line). Arrows show when OKT3 were applied. A representative experiment out independently performed experiments is shown.*

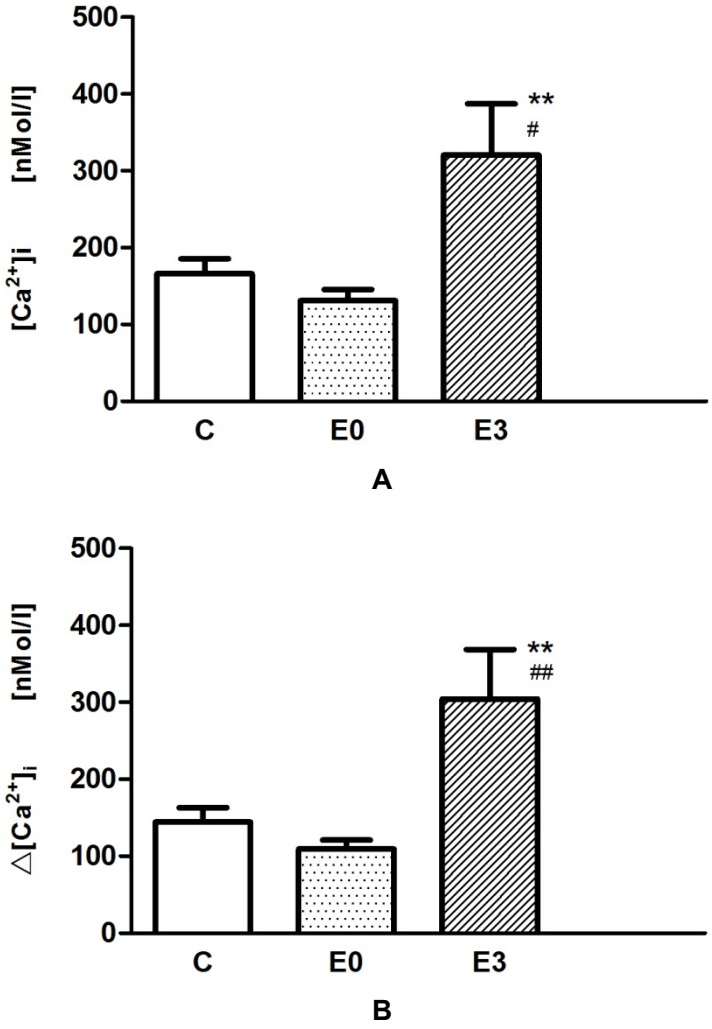
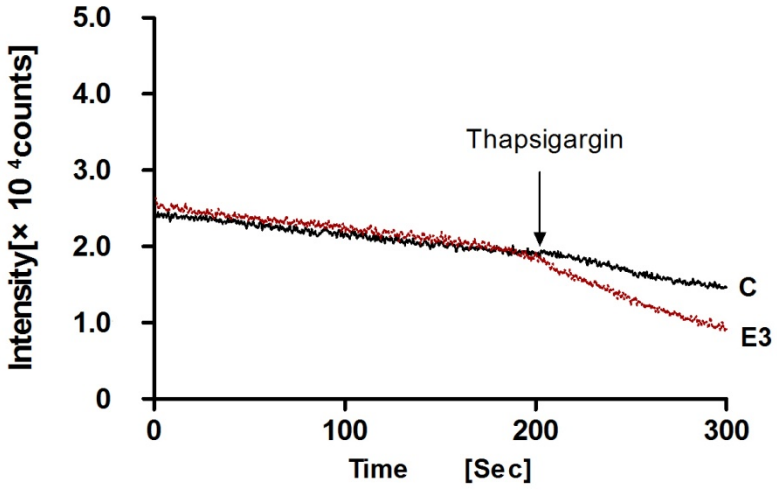


Figure 33*-The effect of acute exercise on OKT3-induced [Ca^{2+}]i and △[Ca^{2+}]i of lymphocytes in Ca^{2+} free PBS solution with 0.1mM EGTA. Bar chart diagram summarizes the results of the entire group. Time points are C: the control group, E0: immediately after exercise, E3:3 h after exercise. Data are mean±SEM derived from 6 separate experiments. Note that on the x-axis, the change in [Ca^{2+}]i is plotted, which is calculated according the following equation:△[Ca^{2+}]i= [Ca^{2+}]iplateau-[Ca^{2+}]ibasal. **P < 0.01 vs C,$^{\#}$P < 0.05 or $^{\#\#}$P < 0.01 vs E0(n=6).*

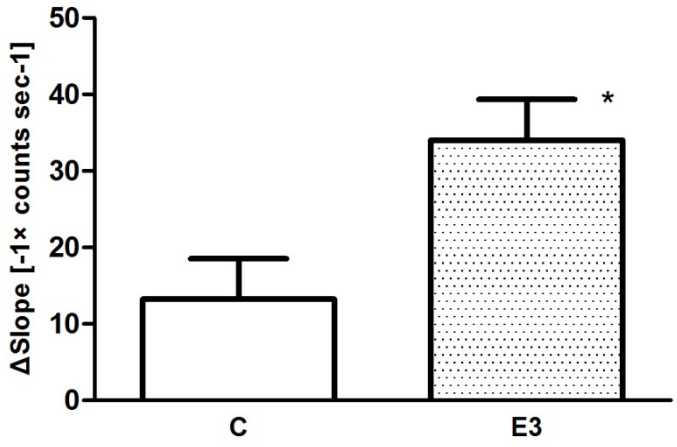
3.2.3 The effect of acute exercise on the Ca^{2+} influxes cross the plasma membrane of splenic lymphocytes

Mn^{2+} has been widely used as a tracer to study divalent cation, e. g. Ca^{2+} influx. The opening of store-operated calcium entry channels in lymphocytes by acute exercise was supported by this study carried out with Mn^{2+} as a surrogate permeable ion for Ca^{2+}, and measuring the rate of the Mn^{2+}-induced a progressive quench of cytosolic Fura-2 fluorescence. As can be seen in Fig. 34A, when the excitation wavelength is

set at the isosbestic point for Fura-2 (360nm) and the emission is recorded at 510 nm, it can be followed the quenching of the fluorescent probe in the presence of Mn^{2+} solution by addition of thapsigargin. Under this condition, there is a more large decay of the fluorescence in the-3 hours after exercise-group after the addition of thapsigargin (red dotted line), when compared to the control group (black line) in Fig. 34A,which can be readily explained that there was more entrance of Mn^{2+} through the store-operated calcium entry channels with the subsequent fluorescence quenching of the intracellular calcium indicator in the-3 hours after exercise-group compared with the control group. The initial rate of fluorescence quenching was assessed by measuring the “slopes 1” of Fura-2 fluorescence deline (A basal Mn^{2+} quenching, corrected for the “slope 2” of the Fura-2 signal decrease after stimulant was applied). △slope is an ideal index used to evaluate the rate of the quenching Fura-2 fluorescence by transmembranous Mn^{2+} influx. As shown as in Fig.34B, acute exercise can significantly cause △slope from the control group(13.23±5.30) to the-3 hours after exercise-group(34.02±5.33)($P<0.05$,n=7).



A



B

***Figure 34**-Effect of a single bout of exhaustive exercise with high intensity on the Mn^{2+} influx in lymphocytes. Entry of extracellular $MnCl_2$ was detected by its quenching effect on the Ca^{2+}-insensitive Fura-2 fluorescence signal, excited at 360 nm (isosbestic point), and the emission (F360) recorded at 510 nm in panel A gives an example of the manganese influx measurements. Firstly, the fluorescence of cells in Ca^{2+} buffer with 10mM $MnCl_2$ was evaluated for 200 seconds, then thapsigargin (10 μM) is added into the solution. A precipitous decline curve could be watched, which indicates quenching of the dye by Mn^{2+} influx into the cell via the store-operated calcium channel. This Mn^{2+} influx is an indicator of Ca^{2+} influx and is increased in the-3 hours after exercise-animals (red dotted line) in comparison to those from the control group (black continuous line). The histograms in panel B summarize the results from 7 experiments and indicate that Mn^{2+} influx after thapsigargin stimulation be significantly increased in the-3 hours after exercise-group. Note:△Slope= Slope2-Slope1. Slope1 indicated that Mn^{2+} quenching of fura-2 under resting conditions because of an Ca^{2+} leak influx in unstimulated cells. Slope 2 indicated that Mn^{2+} quenching of fura-2 when thapsigargin stimulated the cells.*

3.2.4 The effect of a single bout of exhaustive exercise with high intensity on the proliferation of $CD3^+$ T cells

As shown as Fig.35, 36, when CD3+ T cells were stimulated by PHA and Con A (10μg/ml), △AUC in the control group (3.054±0.452, 5.893±0.590, respectively for the above stimuli) and in the-3 hours after exercise-group (1.918±0.281, 2.641±0.402, respectively for the above stimuli) had a significant difference or highly significant difference that existed between the control group and the-3 hours after exercise-group ($P<0.05$,or 0.001, n=10).The results suggested that a single bout of exhaustive exercise with high intensity could downregulate the ability of mitogen-induced cell proliferation of splenic lymphocytes at the 3rd hour after acute exercise.

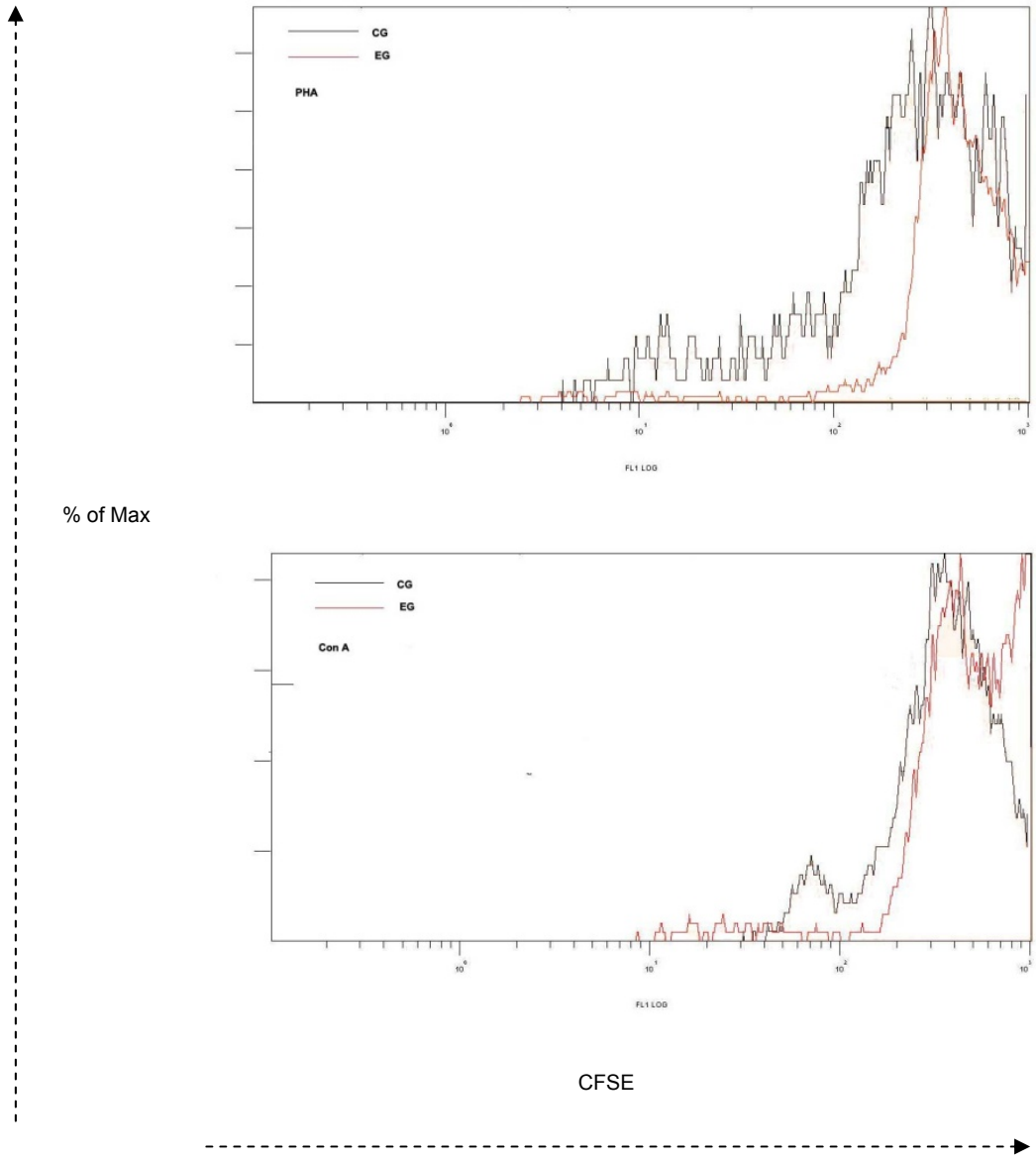


***Figure 35**-Demonstration of the effect of CFSE labeling CD3+ T cells from the control group (CG, black line) and the-3 hours after exercise-group (EG, red line) to respond to the polyclonal mitogens, PHA and Con A, respectively. CD3+ T cells were labeled with CFSE for 5 min at 20 °C in protein-free PBS and then PBS containing 5% (v/v) FCS was added to halt the reaction, and then assessed for their ability to proliferate by using flow cytometry after the CFSE labeling cells with stimulation of PHA or Con A were cultured for 3d.In the pictures, a representative experiment out of seven independently performed experiments is shown, respetively.*

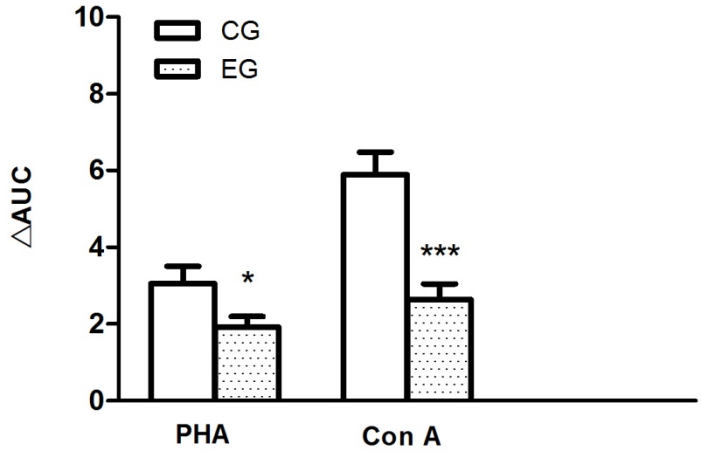


Figure 36-*The change of mitogens-induced lymphocytes proliferation in the-3 hours after exercise-group (EG) compared with the control group (CG). Cells (2×10^6) with CFSE labeling were incubated for 72 h in Dulbecco's Modified Eagle Medium (DMEM) under the same experimental conditions. Samples were treated with Con A or PHA (10μg/ml) throughout the experiment. Note that AUC is the area under curve that the x-axis denotes cellular generations, and the y-axis represents the percentage of cells in each generation. △AUC was calculated according to the equation:△AUC=AUC1(that reflects the level of cell proliferation with the stimulation of mitogens)-AUC2(that reflects basal proliferation state of cell without the stimulation of mitogens). *P<0.05, **P<0.01 vs the control group (n=16).*

3.2.5 The effect of a single bout of exhaustive exercise with high intensity on intracellular Ca^{2+}homeostasis-regulating gene mRNA expression in splenic lymphocytes

3.2.5.1The change of Ca^{2+}-regulating gene mRNA expression of splenic lymphocytes in the-3 hours after exercise-group

3.2.5.1.1 The expression of CRAC channels

As shown in Figure 37A, compared with the control group, STIM1and ORAI1 expression in the-3 hours after exercise-group was downregulated 14.0±11.9%, 12.9±14.4% (P>0.05, n=5),and the ORAI2 expression was upregulated 2.9±14.1% (P>0.05,n=5).

3.2.5.1.2 The expression of Ca^{2+}pumps

As shown in Figure37B, compared with the non-exercise group, the PMCA and SERCA expression in the-3 hours after exercise-group, was significantly

downregulated 35.5±6.1%, 30.0±7.2%,respectively (P<0.01,or 0.05,n=5).

3.2.5.1.3 The expression of TRP channels

In Figure37C, in the-3 hours after exercise-group, the TRPC1 expression was significantly downregulated 62.1±4.8% (P<0.01, n=5), and the expression of TRPM5 and TRPV4 was downregulated 49.9±7.6% and 3.6±18.7%, respectively, in comparison with the non-exercise group (P>0.05, n=5). Although TRPV6 expression was upregulated 19.5±12.7%, there is no significant difference compared with the control mice (P>0.05, n=5).

3.2.5.1.4 The expression of intracellular Ca^{2+} release channels

In Figure37D, in comparison with the control mice, the IP3R2 expression in the-3 hours after exercise-group was significantly upregulated 128.5±31.6% (P<0.01, n=5), and the expression of RYR2 was upregulated 124.6±55.2% (P>0.05, n=5).

3.2.5.1.5 The expression of Ca^{2+}-regulating genes in intracellular Ca^{2+}store

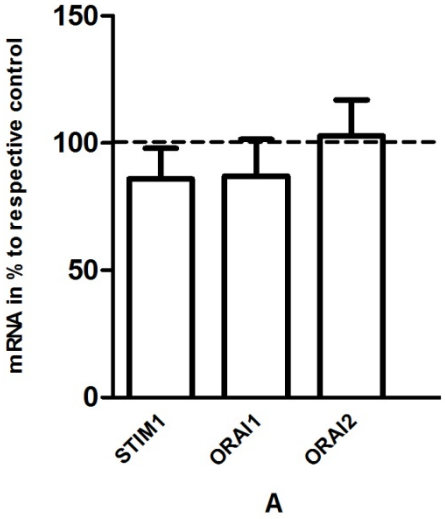
In Figure37E, the ATP2C1 and MCU expression in the-3 hours after exercise-group compared with the control group was upregulated 21.5±17.4%, 47.6±21.9%, respectively (P>0.05, n=5).

3.2.5.1.6 The expression of Cav2.3 and P2X7

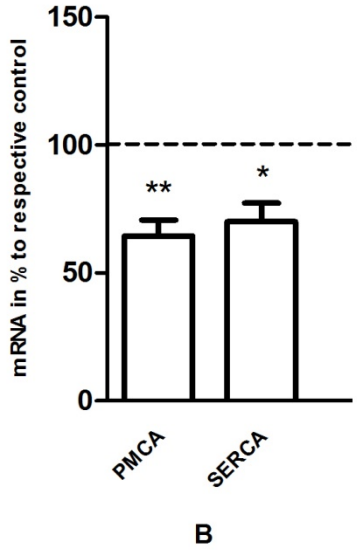
As shown in Figure37F, compared with the non-exercise mice, the P2X7 expression in the-3 hours after exercise-group, was significantly downregulated 35.1±2.8%(P<0.05,n=5). Cav2.3 expression in the-3 hours after exercise-group, was downregulated 37.9±11.5%.

3.2.5.1.7 The expression of Calm1, Hspa1a and K+ channels

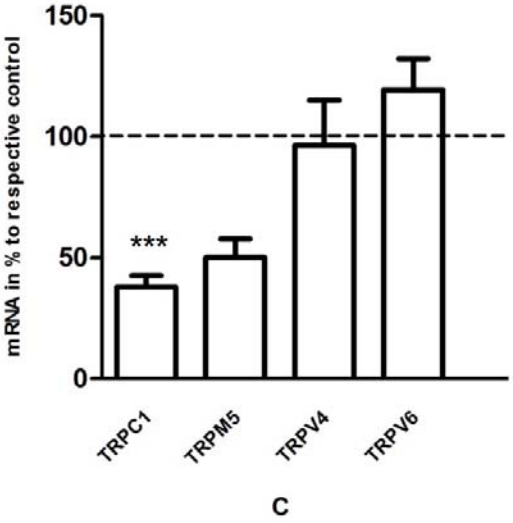
In Figure37G, compared with the non-exercise mice, the Calm1,Hspa1a and Kcnk5 expression in the-3 hours after exercise-group was upregulated 3.8±6.3%, 6.5±16.5%,and 58.5±33.7% respectively (P>0.05,n=5).


mRNA in % to respective control
150
100
50
0
STIM1
ORAI1
ORAI2

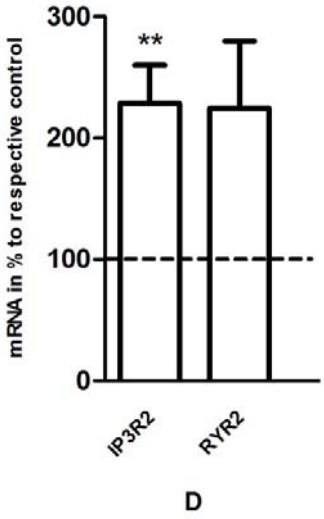
A


mRNA in % to respective control
150
100
50
0
**
*
PMCA
SERCA

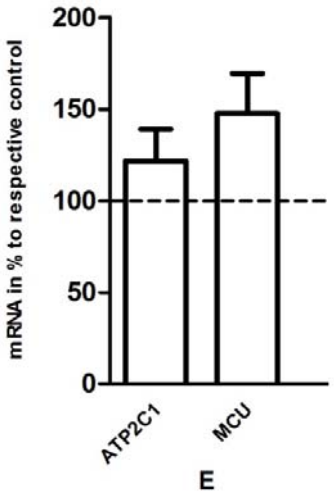
B


mRNA in % to respective control
150
100
50
0
***
TRPC1
TRPM5
TRPV4
TRPV6

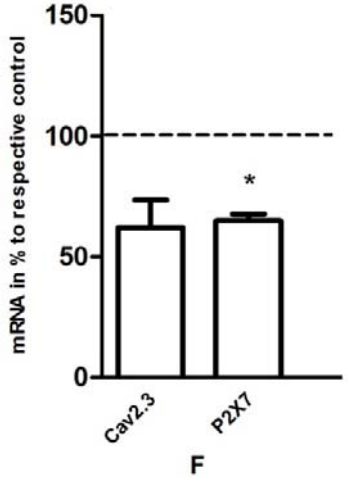
C


mRNA in % to respective control
300
200
100
0
**
IP3R2
RYR2

D


mRNA in % to respective control
200
150
100
50
0
ATP2C1
MCU

E


mRNA in % to respective control
150
100
50
0
*
Cav2.3
P2X7

F

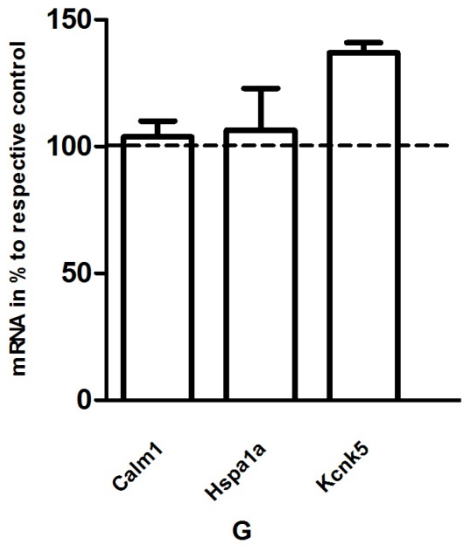


Figure 37*-The effects of acute exercise on intracellular Ca^{2+} homeostasis-regulating gene expression of murine splenic lymphocytes at the 3rd hour after exercise. RNA samples were isolated from cells from 5 mice for each group. Relative quantification of target genes mRNA expression was evaluated by quantitative real-time PCR and the experiments were carried out according to materials and methods. The relative levels of target gene mRNA expression were normalized against the mRNA expression of internal housekeeping gene (β-actin). The expression of β-actin mRNA was not significantly different between the tested cells allowing a direct comparison (data not shown).In these figures, data shown are representative of 5 independent experiments. Note that columns and error bars represent (mRNA in % to respective control ±SEM), and levels of significance (*P<0.05, ** P<0.01 compared with the control group) are indicated.*

3.2.5.2 The change of Ca^{2+}-regulating gene mRNA expression of splenic lymphocytes in the-24 hours after exercise-group

3.2.5.2.1 The expression of CRAC channels

As shown in Figure38A, compared with the non-exercise mice, STIM1and ORAI1 mRNA expression of lymphocytes in the-24 hours after exercise-group, was downregulated 12.3±4.0%, 4.4±6.3% (P>0.05, n=5),and the ORAI2 mRNA expression in the exercise group was upregulated 2.2±15.3% (P>0.05,n=5).

3.2.5.2.2 The expression of Ca^{2+}pumps

As shown in Figure38B, compared with the non-exercise mice, the SERCA mRNA expression of lymphocytes in the-24 hours after exercise-group, was significantly

downregulated 18.9±6.9% (P=0.074,n=5). PMCA mRNA expression in the-24 hours after exercise-group was downregulated 11.1±16.0% (P>0.05,n=5).

3.2.5.2.3 The expression of TRP channels

In Figure38C, the TRPM5 mRNA expression of lymphocytes in the-24 hours after exercise-group was significantly downregulated 66.1±12.0% (P<0.05, n=5), and the expression of TRPC1 and TRPV6 was downregulated 10.4±13.4%, 28.2±16.8%, respectively, in comparison with the non-exercise group (P>0.05, n=5). Although TRPV4 mRNA expression was upregulated 41.5±24.3% in the exercise mice, there is no significant difference compared with the control mice (P>0.05, n=5).

3.2.5.2.4 The expression of intracellular Ca^{2+} release channels

In Figure38D, in comparison with the control mice, the IP3R2 mRNA expression of lymphocytes in the-24 hours after exercise-group was significantly upregulated 134.8±35.1% (P<0.01, n=5), and the expression of RYR2 was upregulated 12.6±30.7% (P>0.05, n=5).

3.2.5.2.5 The expression of Ca^{2+}-regulating genes in intracellular Ca^{2+}store

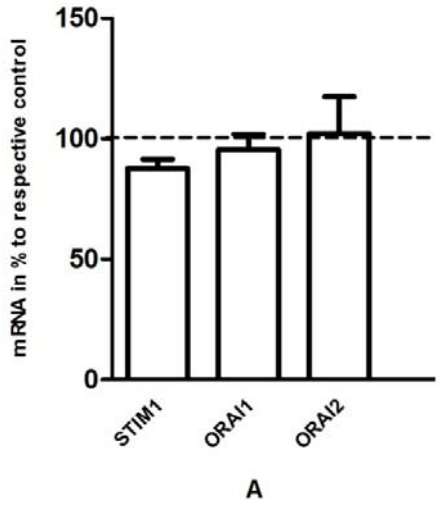
In Figure38E, the ATP2C1 and MCU mRNA expression of lymphocytes in the exercise group compared to the control group was significantly upregulated 62.6±9.9%, 46.5±5.9%, respectively (P<0.001, or 0.01, n=5).

3.2.5.2.6 The expression of Cav2.3 and P2X7

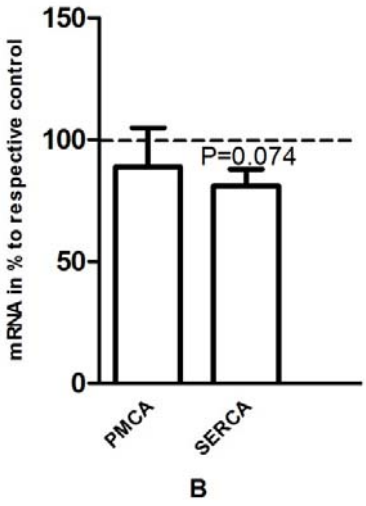
As shown in Figure38F, the Cav2.3 mRNA expression of lymphocytes in the-24 hours after exercise-group compared with the non-exercise mice, was significantly upregulated 116.1±42.7%(P<0.05,n=5). P2X7 mRNA expression in the-24 hours after exercise-group was downregulated 13.8±11.2%.

3.2.5.2.7 The expression of Calm1, Hspa1a and Kcnk5

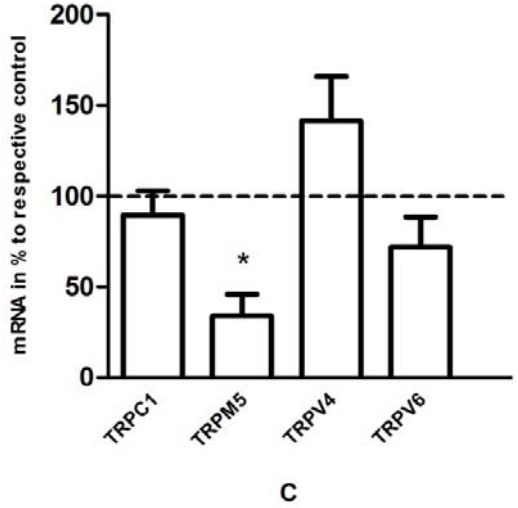
As shown in Figure38H, the Calm1 and Hspa1a mRNA expression of lymphocytes in the-24 hours after exercise-group relative compared with the non-exercise mice, was downregulated 13.3±4.6%, 47.2±31.4% (P>0.05,n=5). The Kcnk5 mRNA expression of lymphocytes in the-24 hours after exercise-group compared with the control group was significantly upregulated 59.6±4.7 % (P<0.05, n=5).


mRNA in % to respective control
150
100
50
0
STIM1
ORAI1
ORAI2

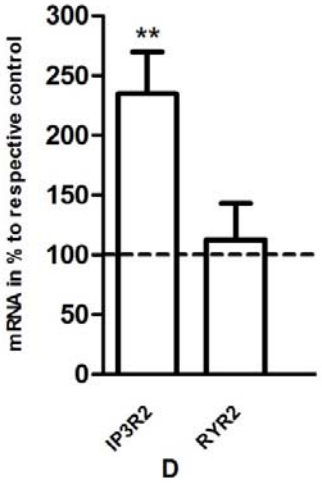
A


mRNA in % to respective control
150
100
50
0
P=0.074
PMCA
SERCA

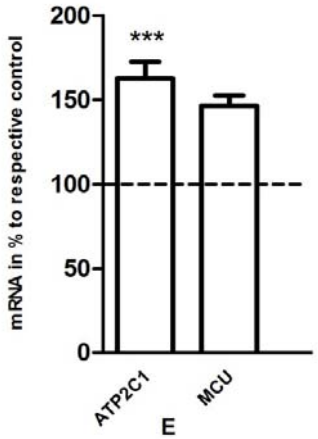
B


mRNA in % to respective control
200
150
100
50
0
*
TRPC1
TRPM5
TRPV4
TRPV6

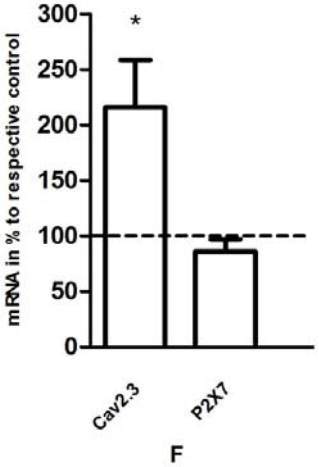
C


mRNA in % to respective control
300
250
200
150
100
50
0
**
IP3R2
RYR2

D


mRNA in % to respective control
200
150
100
50
0
***
ATP2C1
MCU

E


mRNA in % to respective control
300
250
200
150
100
50
0
*
Cav2.3
P2X7

F

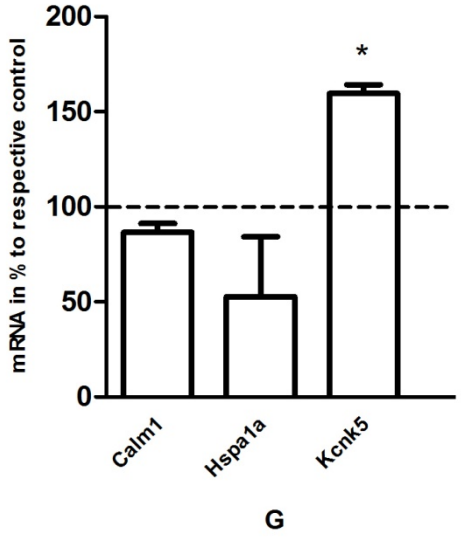


Figure 38*-The effects of acute exercise on intracellular Ca^{2+} homeostasis- regulating gene expression of murine splenic lymphocytes at the 24th hour after exercise. RNA samples were isolated from cells from 5 mice for each group. Relative quantification of target genes mRNA expression was evaluated by quantitative real-time PCR and the experiments were carried out according to materials and methods. The relative levels of target gene mRNA expression were normalized against the mRNA expression of internal housekeeping gene (β-actin). The expression of β-actin mRNA was not significantly different between the tested cells allowing a direct comparison (data not shown).In these figures, data shown are representative of 5 independent experiments. Note that columns and error bars represent (mRNA in % to respective control ±SEM), and levels of significance (*P<0.05, ** P<0.01 compared with the control group) are indicated.*

3.2.5.3 The change of intracellular Ca^{2+}homeostasis-regulating gene mRNA expression of splenic lymphocytes in the-24 hours after exercise-group compared with in the-3 hours after exercise-group

3.2.5.3.1 The expression of CRAC channels

As shown in Figure 39A, compared with in the-3 hours after exercise-group, STIM1and ORAI1 mRNA expression of splenic lymphocytes in the-24 hours after exercise-group, was upregulated 2.0±4.6%, 9.8±7.2% (P>0.05, n=5),and the ORAI2 mRNA expression in the-24 hours after exercise-group was downregulated 0.6±14.9% (P>0.05,n=5).

3.2.5.3.2 The expression of Ca^{2+}pumps

As shown in Figure 39B, compared with the the-3 hours after exercise-group, the PMCA and SERCA mRNA expression of lymphocytes in the-24 hours after exercise-group, was upregulated 37.9±24.9%,15.8±9.8%(P>0.05,n=5).

3.2.5.3.3 The expression of TRP channels

In Figure 39C, compared with the-3 hours after exercise-group, the TRPC1 mRNA expression of lymphocytes in the-24 hours after exercise-group, was significantly upregulated 136.0±35.2% (P<0.01, n=5), and the expression of TRPV6 was downregulated 39.9±14.0% (P<0.05, n=5). TRPV4 mRNA expression was upregulated 46.7±25.2%, and TRPM5 mRNA expression was downregulated 32.5±23.9% in the-24 hours after exercise-group(P>0.05, n=5).

3.2.5.3.4 The expression of intracellular Ca^{2+} release channels

In Figure 39D, in comparison with in the-3 hours after exercise-group, the IP3R2 mRNA expression of lymphocytes in the-24 hours after exercise-group was upregulated 2.7±15.4%(P>0.05,n=5), and the expression of RYR2 was downregulated 49.9±13.7%(P>0.05, n=5).

3.2.5.3.5 The expression of Ca^{2+}-regulating genes in intracellular Ca^{2+}store

In Figure 39E, the ATP2C1 mRNA expression of lymphocytes in the-24 hours after exercise-group compared with the-3 hours after exercise-group was significantly upregulated 33.8±8.1% (P=0.075, n=5),however, the MCU mRNA expression was downregulated 0.7±4.0%(P>0.05, n=5).

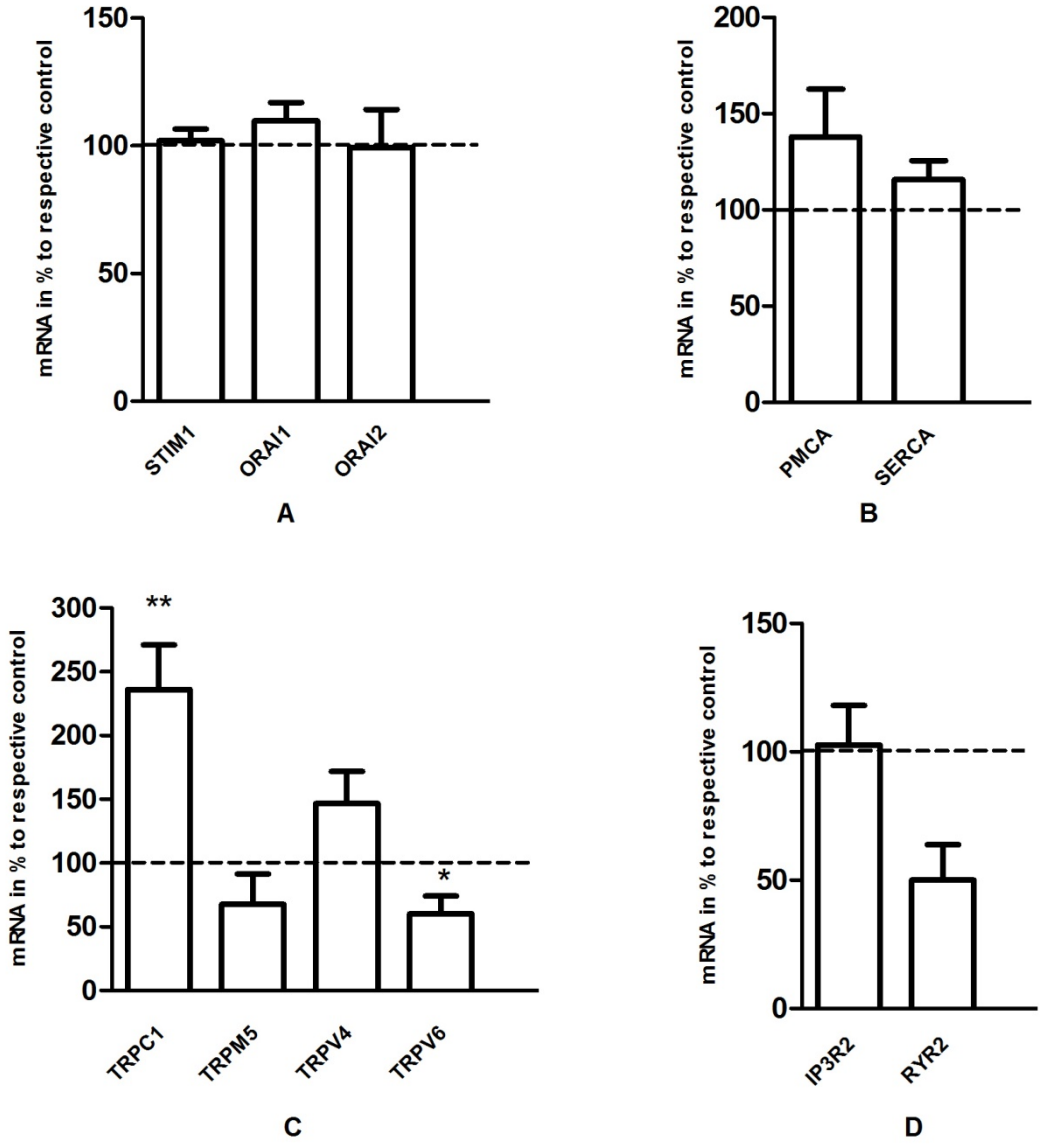
3.2.5.3.6 The expression of Cav2.3 and P2X7

As shown in Figure 39F, the Cav2.3 mRNA expression of lymphocytes in the-24 hours after exercise-group compared with the-3 hours after exercise-group, was significantly upregulated 248.1±68.7%(P<0.01,n=5).P2X7 mRNA expression in the-24 hours after exercise-group was upregulated 37.9±24.9% (P>0.05,n=5).

3.2.5.3.7 The expression of Calm1, Hspa1a and Kcnk5

As shown in Figure 39G, the Calm1 and Hspa1a mRNA expression of lymphocytes in

the-24 hours after exercise-group, was significantly upregulated 16.5±4.4%, and 75.6±14.5% (P=0.06, and P<0.01, respectively, n=5), Kcnk5 mRNA expression in the-24 hours after exercise-group was upregulated 0.7±2.9 % (P>0.05, n=5).



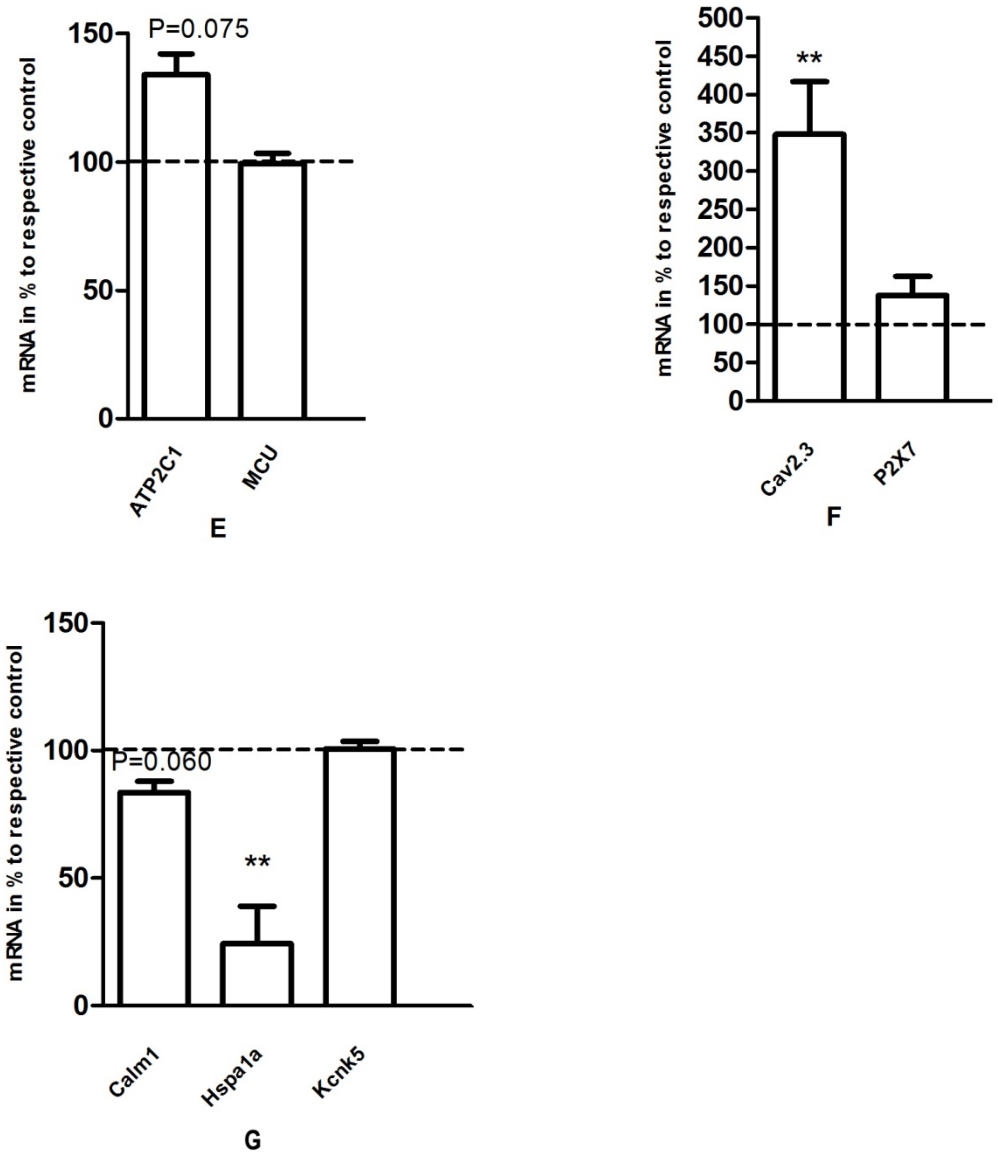


Figure 39- *The change of intracellular Ca^{2+} homeostasis-regulating gene expression of murine splenic lymphocytes from the 3rd hour to the 24th hour after acute exercise. RNA samples were isolated from cells from 5 mice for each group. Relative quantification of target gene mRNA expression was evaluated by quantitative real-time PCR and the experiments were carried out according to materials and methods. The relative levels of target gene mRNA expression were normalized against the mRNA expression of internal housekeeping gene (β-actin). The expression of β-actin mRNA was not significantly different between the tested cells allowing a direct comparison (data not shown).In these figures, data shown are representative of 5 independent experiments. Note that columns and error bars represent (mRNA in % to respective control ±SEM), and levels of significance (*P<0.05, ** P<0.01 compared with the control group) are indicated.*

4 Discussion

4.1 Part I: chronic voluntary exercise enhanced the sensitivity of mitogens or anti-CD3 antibodies-evoked transmembrane Ca^{2+} influx and immune function in murine splenic lymphocytes

4.1.1 Chronic voluntary exercise elevated the basal $[Ca^{2+}]i$ of splenic lymphocytes

The development of fluorescent indicators of intracellular free Ca^{2+} ion concentration and the dual-wavelength measurement mode initiated a revolution in studies of intracellular Ca^{2+} homeostasis and signal transduction. The specificity of indicator for Ca^{2+} and lack of sensitivity to the most physiological ions offer the most straight forward and accessible of the available techniques of measuring $[Ca^{2+}]i$ in living cells which could be loaded into cells in a non-disruptive manner(McCormack & Cobbold 1991). The dual-wavelength measurement of intracellular Ca^{2+} concentration can considerably reduce the effects of dye concentration, uneven dye loading, leakage of dye, illumination intensity, photobleaching, and cell thicknesses in mixed populations by providing varying more stable and reproducible results with the improved signal-to noise and the better temporal resolution. When cells are loaded with fura-2, the significant Ca^{2+} buffering or damping of Ca^{2+} transients at intracellular Ca^{2+} concentrations won't be caused by fura-2, because of high efficiency of fluorescence from this indicator. And fura-2 has a high constant, K_d for Ca^{2+} and less Mg^{2+} sensitivity, and it is used to detect intracellular Ca^{2+} concentrations in the tens of micromolar and measure intracellular Ca^{2+} concentration accurately (McCormack & Cobbold 1991).

However, when the dye binds Ca^{2+}, the true level of $[Ca^{2+}]i$ could be perturbed. This problem is directly related to the level of dye loading. But fura-2 offer much higher efficiency of fluorescence, reducing the required loading level.Ca^{2+} buffering by fura-2 tends not to have much effect on basal or steady state elevated level of $[Ca^{2+}]i$. Ca^{2+} buffering by fura-2 alter the kinetics of Ca^{2+} changes and buffer transient $[Ca^{2+}]i$ response. But this problem can be solved by using suitable controls and calibrations(McCormack & Cobbold 1991). In this study, the operating essential for intracellular Ca^{2+} measurement are followed: the conditions of cell isolation,Fura-2 loading and measurement of fluorescence were controlled the same states no matter

the control group or the exercise group. So the disadvantage from fura-2 as the indicator could be minimized in this study.

According to Mooren et al.(1998),[Ca^{2+}]i is a result of balance between intracellular Ca^{2+} up-regulated, Ca^{2+} down-regulated, and Ca^{2+}- buffered mechanisms. [Ca^{2+}]i can be increased by release from intracellular Ca^{2+} stores and Ca^{2+} influx through Ca^{2+} channels in plasma membrane, passively along its electrochemical gradient, which consists of the electrical potential and a difference in the transmembrane chemical concentration. Voltage-gated and Ca^{2+}- activated K^+ channels contribute to the hyperpolarization of the membrane potential that maintains the electrochemical gradient and the driving force of Ca^{2+} influx(Inada et al. 2006). The extracellular Ca^{2+} concentration at rest is usually much higher than the intracellular level, and there is almost 10^4 fold Ca^{2+} concentration difference(Clapham 1995),which is found on plasma membrane impermeable to Ca^{2+}, Ca^{2+} sequestration by endoplasmic reticulum (ER) and extrusion by plasma membrane (PM),and buffering by intracellular Ca^{2+} binding proteins (Carafoli 1987). This study suggests that chronic voluntary exercise can induce the increase of basal [Ca^{2+}]i from 62.1±5.1nM to 98.9±7.2nM ($P<0.001$, $n=62$). As we known, exercise is a special stressor.There were some previous studies that addressed the effect of stress on the basal [Ca^{2+}]i in lymphocytes. Csermely et al. (1995) reported chronic overcrowding reduced the basal [Ca^{2+}]i of splenic T lymphocytes from young and aged CBA/CA mice. Eckert et al. (1998) found the basal [Ca^{2+}]i didn't differ among lymphocytes from aged controls and Alzheimer's patients. Broadbent and Gass (2006) suggested an increase in intracellular Ca^{2+} signalling during hot weather and endurance training may decrease [Ca^{2+}]i through altered intracellular signalling, possibly to maintain lymphocyte function during heat stress. So these studies implied the effects of chronic voluntary exercise and forced-stress or exercise on basal [Ca^{2+}]i of lymphocytes could be different, they have different functional mechanisms.

The results from this study didn't support that chronic voluntary exercise raised the basal [Ca^{2+}]i of lymphocytes by influencing the K^+ channel genes, Kcnn4 and Kcnk5 expression. However, this study suggested that chronic voluntary exercise downregulated TRPM5 expression, which can produce a depolarising generator potential (Chaudhari & Roper 2010).It was possible to increase the membrane potential hyperpolarization by weakening the function of depolarization in plasma membrane.Intracellular Ca^{2+} stores play the important role in regulating intracellular

Ca^{2+} homeostasis, which include endoplasmic reticulum, mitochondria, Golgi apparatus and so on(Chanat & Huttner 1991; Krause 1991). The intracellular Ca^{2+} stores can control the amplitude, length, localization and propagation of cytosolic Ca^{2+} elevations (Krause 1991). Two different channels, Inositol 1,4,5-trisphosphate(InsP3) receptor- linked and ryanodine receptor (RyRs)-linked Ca^{2+} channels can regulate the release of Ca^{2+} from endoplasmic reticulum(Mikoshiba 1993; Ehrlich et al. 1994; Ashley 1995). MCU (mitochondria Ca^{2+} uniporter) is highly selective ion channel (Kirichok et al. 2004), which play a role in Ca^{2+} uptake into mitochonchia and involve in regulating intracellular Ca^{2+} transients. In this study, chronic voluntary exercise significantly downregulated the expression of IP3R2. It seem that this study didn't support that chronic voluntary exercise influenced the basal $[Ca^{2+}]i$ by changing the expression of Ca^{2+} release channels. However MCU expression was significantly downregulated. It could mean that Ca^{2+} uptake into mitochondria was decreased, so it is possible that the downregulation of MCU expression reduced the function of Ca^{2+} uptake and then served as an intracellular Ca^{2+} concentration of up-regulated mechanism in lymphocytes.

For the transmembrane Ca^{2+} influx, several Ca^{2+} entry pathways have been suggested including voltage-gated Ca^{2+} channels, receptor-operated Ca^{2+} channels, store-operated Ca^{2+} channels, and stretch-activated Ca^{2+} channels(Meldolesi & Pozzan 1987; Penner et al. 1993). In this study, the expression of CRAC channel (STIM1, ORAI1, and ORAI2), voltage-gated Ca^{2+} channels (Cav1.2 and Cav2.3), purinergic receptors (P_2X7, P2Y14), TRP channel (TRPM1, TRPV4, and TRPC1) was significantly downregulated. These results didn't support the exercise-induced the increase of basal $[Ca^{2+}]i$ in lymphocytes was because of upregultion of Ca^{2+} channel expression on plasma membrane. On the opposite of Ca^{2+} up-regulated mechanism, $[Ca^{2+}]i$ can be decreased by two different Ca^{2+} pump (ER and PM Ca^{2+}-ATPase superfamilies). Calcium pumps use the energy of ATP to transport Ca^{2+} against its electrochemical gradient. However in this study, chronic voluntary exercise didn't change the Ca^{2+}-ATPase expression. Therefore the increase of basal $[Ca^{2+}]i$ couldn't reflect that chronic voluntary exercise-induced change of Ca^{2+} pumps expression.

4.1.2 Chronic voluntary exercise boosted mitogens or OKT3-induced intracellular Ca^{2+} response in splenic lymphocytes

In resting lymphocytes, Ca^{2+} channels seem to be functionally closed, but rapidly

opened by treatment with agonists, such as mitogens, anti-CD3 antibodies, and thapsigargin. The Ca^{2+} release from the intracellular is the primary response to these agonists stimuli, and a sustained or oscillatory rise in $[Ca^{2+}]i$ is required to drive subsequent transmembrane Ca^{2+} influx. OKT-3, or mitogens (including PHA and Con A) can bind to the T cell receptor/CD3 complex or a great deal of membrane glycoproteins on the surface of cells, and induces the change of permeability on the plasma membrane, then activates the relative signaling molecules, and generates diacylglycerol(DAG) and InsP3. Both of the two products can serve as the second messengers. InsP3 can cause the release of Ca^{2+} from intracellular stores and a sustained Ca^{2+} influx from the extracellular space, while DAG can activate protein kinase C, a key enzyme involved in mRNA production for proteins. The Ca^{2+} influx through plasma membrane Ca^{2+} channels into cells is the key mechanism to change intracellular Ca^{2+} transients. In this study, when the measurement of fluorescence were performed in the medium, free Ca^{2+} PBS solution with 0.1mM EGTA, and pretreated with OKT3,PHA,and Con A, respectively, only negligible change of agonists-induced $[Ca^{2+}]i$ could be detected. It could mean that intracellular Ca^{2+} stores didn't dedicate a great proportion to the increase value of agonists-induced $[Ca^{2+}]i$ even they are very important to cause the sustained transmembrane Ca^{2+} influx and the main intracellular Ca^{2+} source was the extracellular space. Furthermore, after the addition of Ca^{2+} into the measurement medium, a stronger Ca^{2+} influx from the external space into cells could be detected.

This study suggests that chronic voluntary exercise had a remarkable effect to raise PHA, Con A, or OKT3-induced Ca^{2+} transients in lymphocytes. There were other studies to address the effect of chronic stress or diseases on agonists-induced Ca^{2+} transients in lymphocytes before. Csermely et al (1995) has investigated the effect of chronic overcrowding on lectin-stimulated intracellular Ca^{2+} transients of splenic T lymphocytes from young and aged CBA/CA mice and this inadequate adaptation in the calcium metabolism of T lymphocytes may significantly contribute to the diminished immune response of the aged in stress. Silberman et al(2005) studied the effect of chronic mild stress (CMS) exposure on the early intracellular pathways involved in T cells after stimulation with mitogen and found that mitogen stimulation of T lymphocytes from CMS-exposed mice resulted in a reduction of the intracellular Ca^{2+} concentration rise. Sei et al.(1991)reported that chronic restraint stress (2 hours daily for up to 21 days) resulted in a significant suppression of mitogen-stimulated increases in $[Ca^{2+}]i$ in CD4+ T cells at 3 and 7 days, but not at 21 days;CD8+ T cells

were unaffected by chronic stress; Chronic stress (for 7 days) had a modest suppressive effect on mitogen-induced Ca^{2+} responses in B cells, and the findings indicated that the inhibitory effects of stress on calcium mobilization in lymphocytes may be an early event mediating stress-induced immunosuppression. Sayeed (1996) suggested the generation of the sepsis-related T lymphocyte responses emanate from alterations in intracellular Ca^{2+} homeostasis and this decrease in TCR-related Ca^{2+} mobilization evidently contributes to the suppression of T lymphocyte proliferation during sepsis. Ibarreta et al. (1997) reported Alzheimer lymphoblasts show higher antibodies-induced elevation of $[Ca^{2+}]i$ than controls in Alzheimer disease lymphoblasts. The kinetics of Ca^{2+} replenishment of Ca^{2+}- depleted cells showed a higher accumulation of cytosolic Ca^{2+} in Alzheimer disease than in control lymphoblasts. And the authors concluded that Alzheimer disease lymphoblasts had a lower Ca^{2+} buffering capacity than normal cells, probably because of changes in availability or intrinsic functional properties of the intracellular Ca^{2+}-binding structures. Eckert et al. (1998) found the activation-induced Ca^{2+} responses differed among lymphocytes from aged controls and Alzheimer's patients, and Ca^{2+} mobilization in lymphocytes is specifically impaired in lymphocytes from patients with vascular dementia. However, they observed a delayed Ca^{2+} response of Alzheimer's disease lymphocytes after PHA stimulation indicating an impaired function of Ca^{2+} influx-controlling mechanisms. Eckert et al. (1996) identified no relevant change of the PHA-induced Ca^{2+} elevations in lymphocytes, which is not supporting the assumption of general alterations of cellular Ca^{2+} regulation in Alzheimer's patients. So basically, the reduced agonists-induced intracellular Ca^{2+}transients in lymphocytes could be found during chronic stress and some diseases. The improved effects of chronic exercise on agonists-induced intracellular Ca^{2+} response could imply the different functional mechanisms that distinguished between chronic voluntary exercise and the other chronic stress.

However, in this study, chronic voluntary exercise didn't influence thapsigargin-induced intracellular Ca^{2+} response. As we know, store-operated Ca^{2+} channels are the major source of intracellular Ca^{2+} and store-operated Ca^{2+} entry is initiated by the depletion of intracellular Ca^{2+} stores, and its activation can be independent of InsP3 production. During the whole process from the beginning time when PHA, Con A, and OKT3 acted on the cellular surface to the ending time when intracellular stores Ca^{2+} release and transmembrane Ca^{2+} influx was upburst, the enhancement of key cascade molecules of Ca^{2+} signaling transduction, InsP3 could become a focus how

chronic voluntary exercise induced the increase of intracellular Ca^{2+} response. The increased number and sensitivity of Ca^{2+} signaling molecules might become the cause of the increase of intracellular stores Ca^{2+} release and transmembrane Ca^{2+} influx into cells, because that InsP3-independent of intracellular Ca^{2+} response induced by thapsigargin hadn't the significant difference between the exercise group and control group, and this could mean the key point that induced the enhanced intracellular Ca^{2+} response in the chronic exercise group and made a difference from the non-exercise group was InsP3 signaling cascade. The enhanced InsP3 signaling cascade with lower Ca^{2+} channel expression could mean that the chronic voluntary exercise enhanced the sensibility of Ca^{2+} signaling transduction. The enhanced sensitivity is the result of chronic voluntary exercise- induced immune adaptation.

4.1.3 Chronic voluntary exercise improved mitogen-induced cell proliferation in splenic lymphocytes

Cell proliferation is the increase of cell number as a result of cell growth and division, and is a very sensitive indicator of stress. The proliferation assay determines the cell number that is growing in the absence or presence of certain affecting agents. The functional capability of lymphocytes can be evaluated through mitogen-induced cell proliferation experiments in vitro. When immune cells are stimulated by a foreign antigen, the capability for cell proliferation is a vital element of immunological adaptation. The polyclonal mitogen, such as PHA and Con A, these plant lectins bind to cell surface receptors and activate lymphocytes, and can be are usually used to measure the ability of cell proliferation. Lymphocytes can be activated by mitogen, then proliferate, and increase the number of cells capable of mounting a response to stimulation. Determination of the proliferative response of lymphocytes on stimulation with various mitogens in vitro is a well-established assay to examine the functional capacity of T cells and B cells (Nieman 1997). Early studies examined the influence of exercise training on splenocyte proliferation in response to polyclonal mitogens (Nasrullah & Mazzeo 1992). Kwak (2006) showed the splenic lymphocytes of the trained mice had much greater proliferative activity than those of the untrained mice. MacNeil et al.(1991) studied the effects of intensity and duration of exercise on immune cell proliferation as a measure of immunologic function in men of defined fitness.

In sports medicine area, the focus of cell proliferation experiments is to investigate the response exercise induced the change of immune cell proliferation capability. The use

of CFSE as a cell proliferation measuring dye has had a great impact on immunological studies. About chronic exercise, there was a research that addressed effects of moderate endurance exercise and training on in vitro lymphocyte proliferation (Rhind et al. 1996).The ability of lymphocyte proliferation may be enhanced by non-stressful chronic exercise and/or moderate exercise training, whereas intense or prolonged exercise may suppress proliferative responses. Tharp and Preuss (1991) showed that the proliferative response of splenic lymphocyte to Con A, was significantly higher in moderate exercise-trained rats (45 min of treadmill running at a speed of 22m/min in 5 days/week during 8 weeks). The regular exercise with moderate intensity may increase CD4+ proliferation, thus enhancing the CD4+ lymphocyte antigenic response and decreasing the risk of infection(Bruunsgaard & Pedersen 2000). Coleman and Rager(1993)demonstrated that the voluntary wheel running for 8 weeks in rats significantly enhanced lymphocyte proliferative responses to Con A. However, forced running exercise training (treadmill running at 75% maximal running capacity for 1 h day^{-1}, 5 days $week^{-1}$, for 15 weeks) was reported to suppress the proliferative response in 8-month-old rats(Nasrullah & Mazzeo 1992). Cell proliferation is a highly regulated and coordinated process in which Ca^{2+} signaling could be a key event that may involve a wide variety of intracellular signal transduction pathways. In this study, by combing CFSE loading and flow cytometry technology, the enhanced mitogen-induced cell proliferation capability after chronic voluntary exercise could be decided. The mechanism could be relative to enhanced Ca^{2+} signaling transduction.

4.1.4 The downregulation of Ca^{2+}-regulating gene expression protected lymphocytes from intracellular Ca^{2+} overload

As it was shown in this study, chronic voluntary wheel running training can cause the elevation of basal[Ca^{2+}]i in lymphocytes. However, too much transmembrane Ca^{2+} inflow may induce intracellular Ca^{2+} overload and cause the impairment and apoptosis in cells(Roy & Hajnoczky 2008). Previous reports have indicated that Ca^{2+} overload is a final common pathway of cell death(Dong et al. 2006). In order to maintain intracellular Ca^{2+} homeostasis and control the appropriate intracellular Ca^{2+} level, it is necessary to employ a negative feedback mechanism to balance the tendency of gradually increased intracellular Ca^{2+} concentration during chronic exercise. Negative feedback is homeostatic feedback mechanism, a reaction that causes a decrease in function after some kind of stimuli. The negative feedback mechanism make a self-regulating process, and can produce stability and reduce the Ca^{2+} signal output

back to its normal range of fluctuation. Ca^{2+} channels play an important role to regulate the entry of Ca^{2+} influx from the extracellular space and the Ca^{2+} release from the intracellular stores. In this study, chronic voluntary exercise reduced the expression of intracellular calcium upregulating genes, such as STIM1, Cav2.3, TRPV4, and IP3R2, ORAI1, and TRPC1. The Ca^{2+} channel gene expression downregulation could have a physiological significance to control the intracellular Ca^{2+} homeostasis.

4.1.5 The mechanism by which chronic voluntary exercise induced the changes of intracellular Ca^{2+}-related physiological parameters

4.1.5.1 The neuroendocrine modulation

The immune system is closely linked to the neuroendocrine system. The neuroendocrine system has been thought to participate in the maintenance of homeostasis of immune cells, because the immune cells have numerous receptors for neuroendocrine factors, and immune cell function can be modulated by the neuroendocrine factors. The most primary (bonemarrow, thymus) and secondary lymphoid tissues (including spleen, gut-associated lymphoid tissue) receive dense sympathetic nervous system(SNS) innervations(Fleshner 2000).Since both SNS and the hypothalamic-pituitary-adrenal(HPA) axis are influenced by exercise, it is likely that the exercise-induced immune changes are driven, at least in part, by hormonal responses. In exercise, the organism needs to re-establish homeostatic equilibrium by the hormonal system. There is a bidirectional network of polyfactorial interactions, which exists between the neuroendocrine and the immune system and employs a mutual biochemical language. Except hormones, various messengers, including neurotransmitters and cytokines, regulate cellular and humoral immunity. For example, glucocorticoids, catecholamine, growth hormone, ACTH and prolactin, are increased in response to exercise in a sport-type, dose- and intensity-dependent manner, and lymphoid cells contain receptors for these hormones. By blocking the interaction of stress hormones, the detrimental effects of stress can be minimized (Fleshner et al. 1996).During exercise, Ca^{2+} transduction passway should be a target that neuroendocrine signals modulate immunity. The increase of chronic exercise-induced basal $[Ca^{2+}]i$ might be a result of modulation of neuroendocrine and adaptation of cellular functions.

4.1.5.2 Oxidative stress

Physical exercise is associated with oxidative stress, an imbalance between oxidant and antioxidant systems that involves the production and release of reactive oxygen species (ROS) that can induce damage in the cellular composition. Even moderate exercise also can increase ROS production (Ji et al. 1993) by enhancing mitochondrial oxygen consumption and exceeding the capacity of antioxidant defenses. But chronic exercise can increase the resistance against the toxic effects of ROS by adaptable increasing the activity of antioxidant enzymes (Leeuwenburgh & Ji 1998). It is possible that chronic exercise inevitably induced the production of ROS, and then attacks the plasma membrane, then causes the increasing of $[Ca^{2+}]i$. The increased levels of ROS could be necessary for the adaptable enhancement of chronic exercise-induced cellular functions of lymphocytes.

4.1.5.3 Cross-talk between skeletal muscle and immune tissues

Skeletal muscle is also an endocrine organ, and it has the capacity to express some cytokines(Pedersen & Febbraio 2008). Skeletal muscle may communicate with immune cells in relation to mediation of chemical messengers. Muscle can produce ATP, which is released during exercise. Extracellular ATP activates purinergic receptors that could elevate Ca^{2+}influx in lymphocytes. Physical exercise can cause the other cellular stress reaction, such as hyperosmosis, hyperthermia, pH alterations, ischemia, osmotic change, mechanical stimulation, energy depletion, and hypoxia which might cause release of Ca^{2+} from endoplasmic reticulum, leading to activation of plasma membrane Ca^{2+}-permeable store-operated channels. Osmotic stress and mechanical stimulation can induce the release of cellular ATP (Loomis et al. 2003; Yip et al. 2007). The binding of extracellular ATP to P_2X or P_2Y receptors, which are ATP-gated ion channels, induces the influx of extracellular Ca^{2+} (North 2002).Heat can activate TRPV channels with characteristic warm temperature. Heat shock protein, HSP72, levels increase in response to heat stress and induce increases in intracellular calcium concentration(Fehrenbach & Northoff 2001).

4.2 Part II: A single bout of endurance exercise with high intensity might cause "delayed" intracellular Ca^{2+} upburst and impairment of cellular function in murine splenic lymphocytes

4.2.1 The time-dependent intracellular Ca^{2+} transients change and Ca^{2+}-regulating gene expression in lymphocytes after intensive and exhaustive exercise

This study suggested that the basal [Ca^{2+}]i, and Con A or OKT3-induced intracellular Ca^{2+} transients in lymphocytes was increased at the 3rd hour after intensive and exhaustive exercise. Interestingly, IP3R2 gene mRNA expression was elevated 128.5%, PMCA and SERCA pump expression was downregulated. It was possible to increase the basal [Ca^{2+}]i by reducing Ca^{2+}pump expression, which play the key role to maintain low intracellular Ca^{2+} concentration and homeostasis. The downregualtion of Ca^{2+} pumps function might mean that the self-protection mechanism that prevented lymphocytes from intracellular Ca^{2+} load was weaken. This study suggested that an impairment of the Ca^{2+} pumps could account for the increased basal [Ca^{2+}]i level. Moreover the expression of IP3R2 was upregulated at the 3rd after exercise, this could mean there was more transmembrane Ca^{2+} influx into cytoplasm and Ca^{2+} release from intracellular store through IP3R2 channels at rest and then elevated the basal[Ca^{2+}]i in lymphocytes. The downregulation of important ion channel, TRPC1 expression might serve as the opposite mechanism to balance the tendency to increase intracellular Ca^{2+} concentration, and therefore a new intracellular Ca^{2+} homeostasis was constructed. This study has characterized tranmembrane Ca^{2+} influx indirectly by using Fura-2 quenching by Mn^{2+} as a surrogate permeable ion for calcium, which enter cells by the same influx passway (Sage et al. 1989; Jacob 1990), and measuring the rate of the Mn^{2+}-induced quench of intracellular Fura-2 fluorescence. Mn^{2+} quench studying is a powerful tool in investigating Ca^{2+}entry in cells.The ability of agonist to stimulate Mn^{2+} entry is shown in Fig. 33.At the start of the experiment there was a basal rate of fluorescence decay, due to photobleaching and Fura-2 leakage from the cell. The experiments done by measuring the Fura-2 quench by Mn^{2+} also support that intensive exercise could improve the effect of intensive exercise on the capacitative calcium entry at the 3rd hour after intensive and exhaustive exercise through Ca^{2+} channels in plasma membrane, such as IP3R-linked Ca^{2+} channels.

Sei et al. (1991) reported that acute restraint stress modulated mitogen-induced

increases in $[Ca^{2+}]i$ in mouse spleen cells. Dual-color analysis of lymphocyte subpopulations demonstrated that acute (2 hour) restraint stress suppressed mitogen-stimulated increases in $[Ca^{2+}]i$ in CD4+ T cells, but enhanced $[Ca^{2+}]i$ in CD8+ T cells. Within T lymphocyte subpopulations, acute stress predominantly affected CD4+ T cells, which may induce a functional reversal of the CD4/CD8 ratios in vivo. Such a reversal could result in suppression of a variety of immune responses such as lymphocyte proliferation and antigen-specific antibody production. These findings indicate that the inhibitory effects of stress on calcium mobilization in lymphocytes may be an early event mediating stress-induced immunosuppression. Han et al. (2010) investigated the effects of acute heat stress on the concentration of $[Ca^{2+}]i$ in splenic lymphocytes from broiler chickens and showed that acute heat stress caused a significant increase in $[Ca^{2+}]i$ and enhanced Con A-stimulated lymphocyte proliferation significantly. The results suggested that the effect of acute heat stress to increase the $[Ca^{2+}]i$ in lymphocytes might be an early event that enhances Con A-stimulated T-cell proliferation. Velbinger et al. (2000) tested for the effects of acute stress on the Ca^{2+} signaling in helpless rats, as compared to non-stressed rats and found that mitogen-induced Ca^{2+} signaling only tended to be reduced in helpless rats. However, when helpless rats were submitted to acute immobilization stress, Ca^{2+} signaling appeared to be significantly blunted, whereas the same stressor did not affect Ca^{2+} signaling in the non-helpless control rats. It is hypothesized that blunted Ca^{2+} signaling, as assessed in spleen T-lymphocytes of helpless rats, may be a correlate of the increased vulnerability of helpless rats to acute stressors. Others have found that an acute bout of exhaustive exercise decreases $[Ca^{2+}]i$ in lymphocyte simmediately post-exercise, which may avert lymphocyte responses and allow a “window of opportunity”for infection immediately post-exercise (Mooren et al. 2001). Mooren et al. (2001) found that the proliferative response in lymphocytes returned to, and remained at, pre-exercise concentration 1 and 24 h after acute, exhaustive exercise, suggesting that normal immune function was restored after 1 h. It is possible that the normalization of proliferative responses within 24 h of exercise maintains a normal lymphocyte response and reduces the risk of infection.

From the beginning of exercise to its ending, the basal $[Ca^{2+}]i$, and Con A and OKT3-induced intracellular Ca^{2+} transients in lymphocytes didn’t change much finally. However, compared with the-immediately after exercise-group, the basal $[Ca^{2+}]i$, OKT3-induced intracellular Ca^{2+} transients in lymphocytes were elevated in the-3 hours after exercise group. So it could be defined that the 3 hours after a single bout

of endurance exercise with high intensity were the critical time for the change of intracellular Ca^{2+} transients and its triggering change of Ca^{2+}-regulating gene expression. There was a study to report that the high increases of malondialdehyde (MDA) levels were found in spleen at the 3rd hour after acute exercise (Kruger et al. 2009), which is one of the most frequently used indicators of lipid peroxidation. At the 24th hour after exercise, the basal $[Ca^{2+}]i$, Con A-induced intracellular Ca^{2+} transients in lymphocytes has been returned to the original non-exercise state. However, Ca^{2+}pump expression still was downregulated; IP3R2 gene expression was upregulated; interestingly, as the important Ca^{2+} uptake factors of intracellular stores and the homeostasis-regulated mechanism, ATP2C1 and MCU expression was increased. It could mean the function of Ca^{2+} uptake in intracellular stores was enhanced.As the regulation factors of plasma membrane potential difference, TRPM5 and Kcnk5 gene expression was downregulated. During the recovery from exercise, from the 3rd to 24th hour after exercise, Con A-induced intracellular Ca^{2+} transients was declined;TRPV6,Calm1and Hapa1a expression was downregulated, whereas TRPC1, ATP2C1 and Cav2.3 expression was increased. So far, the best candidates for CRAC channels are members of the TRP superfamily, such as highly Ca^{2+} selective channels, TRPV6.Calmodulin is intracellular Ca^{2+} binding protein, whose role in determining intracellular Ca^{2+} concentration can't be neglected. Hapa1a could be related with intracellular Ca^{2+}signaling transduction. So, even exercise has been terminated for 24 hours, its effect on intracellular Ca^{2+}response and Ca^{2+}-regulating gene expression was continued.

4.2.2 The acute exercise reduced the ability of mitogen-induced cell proliferation in splenic lymphocytes

Many studies have suggested that strenuous exercise downregulate the ability of cell proliferation in lymphocytes. The reduced murine splenic lymphocyte proliferation in response to Con A was associated with acute exercise stress(Randall Simpson et al. 1989). Shinkai et al.(1992) reported an even greater decrease after endurance race events. With regard to mitogenic activities of splenocytes in response to exhaustive exercise stress, all groups showed much lower lymphocyte proliferative activity when stimulated with media, Con A and LPS after exhaustive exercise stress(Hwang et al. 2007). Potteiger et al. (2001) showed a decrease in T cell proliferative ability after acute resistance training. Gleeson and Bishop (2005) reported a decrease in mitogen-stimulated T cell proliferation immediately after acute, intensive exercise. It was noted that the post-exercise values of the suppression of lymphocyte proliferation,

in comparison to corresponding pre-exercise values(Tian et al. 2012). Studies in humans indicated that the lymphocyte responses to PHA and Con A declined during 2h of moderate exercise and up to several hours after exercise (Nielsen & Pedersen 1997).High-intensity exercise was associated with reduced immune cell proliferative responses to mitogens (Shephard & Shek 1994; Nieman et al. 1995c). Mazzeo et al. (1998) suggested that, while having lower initial T cell numbers and PHA–induced immunoresponsiveness during a single bout of exercise is, in general, maintained in old when compared to young individuals. Dohi et al. (2001) suggested that the heavy resistance exercise protocol reduced the lymphocyte proliferative responses only in the stronger group of subjects, and this effect might be due to the high absolute total work and the greater exercise stress created by the resistance exercise protocol in the high strength group. Longitudinal training studies in previously sedentary people failed to show marked changes in T and B cell functions provided that blood samples were taken at least 24 h after the last exercise bout. In contrast, T and B cell functions appear to be sensitive to increases in training load in well-trained athletes, with reduced T cell proliferative responses (Walsh et al. 2011). The downregulation of cell proliferation ability in lymphocytes has been speculated that this may account for the apparent increased susceptibility of athletes to viral infections.

Although numerous studies report decreased mitogen- and antigen-stimulated T cell proliferation following acute exercise, the interpretation of these findings may be confounded by alterations in the relative proportion of cells in the circulation that can respond to stimulation(Walsh et al. 2011). Green et al. (2002) tested twelve well-trained male runners completed a 60-min exercise trial at 95% ventilatory threshold and a no-exercise control trial. Six blood samples were taken at each session: before exercise, midexercise, immediately after exercise, and 30, 60, and 90 min after exercise. Isolated PBMC and NK cell-depleted PBMC were stimulated with the mitogen phytohemagglutinin. In the PBMC cultures, there was a significantly lower mitogen response to phytohemagglutinin in exercise compared with the control condition immediately postexercise. There were no significant differences between the control and exercise conditions in NK cell-depleted PBMC cultures or in the responses adjusted for the percentage of CD3 cells. The findings do not support the view that T-lymphocyte function is reduced after exercise.

The alteration of cell proliferation ability in lymphocytes is relative to the exercise workload.Nehlsen-Cannarella et al. (1991) showed that the 45-min walk, in

comparison to rest in a seated position, was not associated with significant changes in spontaneous or Con A-stimulated lymphocyte proliferation.A trend for decreased PHA-stimulated lymphocyte proliferation in comparison to the rest condition, however, was seen 1.5 h following the exercise bout. Verde et al. reported (1992) PHA-stimulated lymphocyte proliferation was unchanged by 30 min of acute submaximal exercise, after 3 weeks of heavy training the same bout of exercise caused an 18% suppression of proliferation.However, Green et al. (2003) suggested that on an individual cell basis 1 h of exercise at 95% of ventilatory threshold did not alter the ability of T-lymphocytes (CD3+) or T-lymphocyte subsets (CD3+CD4+ and CD3+CD8+) to become activated and did not alter the ability of T-lymphocytes to proliferate.Lin et al. (1993) investigated the effects of exercise training and acute exercise on the immune system in rats. The mitogenic activity of spleen lymphocytes to Con A decreased as compared to the sedentary control. The immunomodulatory effect after acute exercise has been investigated and it showed profound enhancement of cell proliferation to Con A in mild and moderate exercise groups. The enhancing activity was less prominent after severe exercise. This indicates that chronic exercise training may cause the reduction of T cell activity while acute exercise manifests an enhancing effect. However, B cell proliferation was elevated in both chronic and acute exercise groups. Nieman et al. (1995b) reported Con A-stimulated lymphocyte proliferation (unadjusted) rose 50% above preexercise levels, but when these data were adjusted on a per T cell (CD3+) basis, no change from rest was observed.

In this study it has been very clear that the ability of mitogen-induced cell proliferation in lymphocyte was significantly downregulated at the 3rd hour after acute exercise. It means that acute exercise could cause the negative change of immunological adaptation. The PHA and Con A induced proliferative responses declinee after acute exercise might be related with “immunosuppression”. Therefore the high intracellular Ca^{2+} concentration with the low ability of cell proliferation in lymphocytes implied the toxic effect of the high frequency Ca^{2+} oscillatory. An increase in $[Ca^{2+}]i$ is among the earliest changes seen in mitogen-stimulated lymphocytes and is a consequence of signal transduction which usually results in the initiation of cell cycle progression. However, increased $[Ca^{2+}]i$ has also been correlated with the cytotoxicity that was caused by high frequency intracellular Ca^{2+}.

4.2.3 The mechanism of which acute exercise modulates intracellular Ca^{2+}homeostasis and immune functions

In this study, the mechanism by which intensive exercise regulated intracellular Ca^{2+} transients and cellular function in lymphocytes should involve in the free radical generation and oxidative stress response. As we know, free radicals are produced during almost all forms of exercise (Vollaard et al. 2005; Finaud et al. 2006; Fisher-Wellman & Bloomer 2009) by elevating the oxygen uptake and respiration, which in turn increases the generation of reactive oxygen species (ROS).And the exhaustive and/or strenuous exercise can cause oxidative stress in both animals and humans (Ji 1995; Vina et al. 2000).So both of free radical production and oxidative stress are greatly increased during high-intensity exercise(Aguilo et al. 2003; Goto et al. 2003; Finaud et al. 2006). The sources of ROS during exercise are multiple including the xanthine oxidase reaction, electron leakage from the mitochondrial electron transport chain, hemoglobin oxidation (Banerjee et al. 2003). The production of free radicals is related to type, intensity and duration of exercise. Intensive exercise was a strong inducible factor to cause tremendous free radical to produce.

Both of high- and moderate-intensity exercise may increase not only free radical production but also antioxidant capability. Increased levels of ROS induce oxidative damage and also the expression of antioxidant enzymes. Antioxidants are capable of accepting electrons from free radical. Antioxidants can protect immune cells against potentially damaging effects of oxidative stress. The antioxidant system plays a pivotal role in reducing the presence of free radicals and reactive oxygen species to ensure that a suitable environment is present. Antioxidants can be classified as either endogenous, those produced by the body, or exogenous, which come from outside the body. There is conflicting evidence (Lawler et al. 1993) whether acute exercise increases antioxidant levels(Alessio & Goldfarb 1988; Ji 1993). Whilst long-term high intensity exercise shows an up-regulation of antioxidant systems (Gomez-Cabrera et al. 2008; Sachdev & Davies 2008).During severe unaccustomed exercise such as ultra-endurance running the increase in ROS production may overwhelm cellular ability to increase antioxidant capacity. During moderate intensity exercise there is a concomitant increase in cellular antioxidant capacity enabling ROS to be reduced without increased oxidative stress (Mastaloudis et al. 2001; Knez et al. 2006). As it is well documented that concentrations of antioxidant enzymes are increased in endurance-trained athletes (Sen 1995; Wang & Huang 2005).

During exercise, the increased production of free radicals could exceed the defence capacity of antioxidant. Although the body is equipped with antioxidant defense, the problem arises when the level of free radicals increases so much that the antioxidant system of the body is not able to counteract them. The imbalance between the oxidative system and the antioxidant system causes oxidative damage to cells and tissue, which is referred as oxidative stress. The aerobic exercise could elicit oxidative stress, because there is an increase in oxygen flux and subsequent possible electron leakage from the mitochondria and electron leakage can in turn induce an increase in production of free radical production. In addition, there are a number of pathways through which exercise can increase oxidative stress, such as ischaemia/reperfusion injury, auto-oxidation of catecholamines, enhanced purine oxidation in muscle, increased haemoglobin turnover, high intracellular Ca^{2+} and extracellular glucocorticoid concentrations, xanthine oxidase-catalysed reaction hyperthermia and activation of inflammatory cells due to tissue damage and hypoxia.

Oxidative stress can lead to undesirable outcomes, and it can trigger many problems, such as chronic inflammation, apoptosis and necrosis of cells and tissues, tissue damage and enhanced susceptibility to opportunistic pathogens, may impair the immune response. Oxidative stress induced by exhaustive exercise could initiate apoptotic processes in lymphocytes and the loss of cellularity of lymphoid tissues, resulting in the lymphopenia observed after exhaustive exercise. Quadrilatero and Hoffman-Goetz (2005) reported that the oxidative stress associated with a single prolonged bout of exercise in mice induced thymocyte apoptosis. However, oxidative stress also can be associated with positive outcomes, and it is an important modulator in a number of adaptive responses to exercise. Oxidative stress has a vital role to play in a number of natural physiological processes necessary for some immune reactions response. The repeated transient increases in oxidative stress such as that observed in regular moderate exercise, has been shown to up-regulate antioxidant enzyme systems (Gomez-Cabrera et al. 2008). Oxidative stress is a common tool employed to explore and demonstrate exercise-induced changes in immune function. Adaptation to exercise training might decrease oxidative stress-associated DNA damage with exhaustive exercise(Niess et al. 1996). However, how moderate exercise affects the relationship of oxidative stress status with apoptosis in lymphocyte remains unclear.

In this study, the decrease of Ca^{2+} pumps expression implied weaken regulation

mechanism of intracellular Ca^{2+} homeostasis, and this could be the mechanism of exercise-induced increased Ca^{2+} signals. Even high intracellular Ca^{2+} concentration could be advantage to signaling transduction, too high intracellular Ca^{2+} can increase oxidative stress and oxidative stress can impair cellular functions. So the enhanced calcium aren't turned into an enhanced proliferation could mean exercise-induced the production of free radical serve as a blockade mechanism of intracellular signal transduction.

5 References

Aguilo A, Tauler P, Pilar Guix M et al. (2003). Effect of exercise intensity and training on antioxidants and cholesterol profile in cyclists. *J Nutr Biochem* **14**, 319-25.

Alessio HM, Goldfarb AH (1988). Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. *J Appl Physiol* **64**, 1333-6.

Ashley RH (1995). Intracellular calcium channels. *Essays Biochem* **30**, 97-117.

Aswad F, Dennert G (2006). $P2X_7$ receptor expression levels determine lethal effects of a purine based danger signal in T lymphocytes. *Cell Immunol* **243**, 58-65.

Avula CP, Muthukumar AR, Zaman K et al. (2001). Inhibitory effects of voluntary wheel exercise on apoptosis in splenic lymphocyte subsets of C57BL/6 mice. *J Appl Physiol* **91**, 2546-52.

Balasubramanyam M, Kimura M, Aviv A et al. (1993). Kinetics of calcium transport across the lymphocyte plasma membrane. *Am J Physiol* **265**, C321-7.

Banerjee AK, Mandal A, Chanda D et al. (2003). Oxidant, antioxidant and physical exercise. *Mol Cell Biochem* **253**, 307-12.

Baricordi OR, Melchiorri L, Adinolfi E et al. (1999). Increased proliferation rate of lymphoid cells transfected with the $P2X_7$ ATP receptor. *J Biol Chem* **274**, 33206-8.

Basu S, Srivastava P (2005). Immunological role of neuronal receptor vanilloid receptor 1 expressed on dendritic cells. *Proc Natl Acad Sci USA* **102**, 5120-5.

Benham CD, Davis JB, Randall AD (2002). Vanilloid and TRP channels: a family of lipid-gated cation channels. *Neuropharmacology* **42**, 873-88.

Berridge MJ (1995). Capacitative calcium entry. *Biochem J* **312 (Pt 1)**, 1-11.

Berridge MJ, Lipp P, Bootman MD (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* **1**, 11-21.

Bertram R, Arceo RC 2nd (2008). A mathematical study of the differential effects of two SERCA isoforms on Ca^{2+} oscillations in pancreatic islets. *Bull Math Biol* **70**, 1251-71.

Bird GS, Bian X, Putney JW Jr. (1995). Calcium entry signal? *Nature* **373**, 481-2.

Bodding M, Wissenbach U, Flockerzi V (2002). The recombinant human TRPV6 channel functions as Ca^{2+} sensor in human embryonic kidney and rat basophilic leukemia cells. *J Biol Chem* **277**, 36656-64.

Brandman O, Liou J, Park WS et al. (2007). STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca^{2+} levels. *Cell* **131**, 1327-39.

Breittmayer JP, Ticchioni M, Ferrua B et al. (1993). Ca^{2+}-ATPase inhibitors induce interleukin-2 synthesis and T cell proliferation. *Cell Immunol* **149**, 248-57.

Brenner IK, Severs YD, Shek PN et al. (1996). Impact of heat exposure and moderate, intermittent exercise on cytolytic cells. *Eur J Appl Physiol Occup Physiol* **74**, 162-71.

Broadbent S, Gass G (2006). Effects of endurance training on intracellular calcium concentration in T lymphocytes. *Eur J Appl Physiol* **98**, 242-9.

Bruunsgaard H, Pedersen BK (2000). Special feature for the Olympics: effects of exercise on

the immune system: effects of exercise on the immune system in the elderly population. *Immunol Cell Biol* **78**, 523-31.

Budagian V, Bulanova E, Brovko L et al. (2003). Signaling through $P2X_7$ receptor in human T cells involves p56lck, MAP kinases, and transcription factors AP-1 and NF-kappa B. *J Biol Chem* **278**, 1549-60.

Cahalan MD, Chandy KG (2009). The functional network of ion channels in T lymphocytes. *Immunol Rev* **231**, 59-87.

Cahalan MD, Wulff H, Chandy KG (2001). Molecular properties and physiological roles of ion channels in the immune system. *J Clin Immunol* **21**, 235-52.

Carafoli E (1987). Intracellular calcium homeostasis. *Annu Rev Biochem* **56**, 395-433.

Castilho RF, Kowaltowski AJ, Meinicke AR et al. (1995). Permeabilization of the inner mitochondrial membrane by Ca^{2+} ions is stimulated by t-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria. *Free Radic Biol Med* **18**, 479-86.

Caterina MJ, Rosen TA, Tominaga M et al. (1999). A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* **398**, 436-41.

Caterina MJ, Schumacher MA, Tominaga M et al. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816-24.

Chanat E, Huttner WB (1991). Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network. *J Cell Biol* **115**, 1505-19.

Chandrasekera PC, Kargacin ME, Deans JP et al. (2009). Determination of apparent calcium affinity for endogenously expressed human sarco(endo)plasmic reticulum calcium- ATPase isoform SERCA3. *Am J Physiol Cell Physiol* **296**, C1105-14.

Chaudhari N, Roper SD (2010). The cell biology of taste. *J Cell Biol* **190**, 285-96.

Cheng G, Liu BF, Yu Y et al. (1996). The exit from G(0) into the cell cycle requires and is controlled by sarco(endo)plasmic reticulum Ca^{2+} pump. *Arch Biochem Biophys* **329**, 65-72.

Choudhry MA, Ahmad S, Thompson KD et al. (1994). T-lymphocyte Ca^{2+} signalling and proliferative responses during sepsis. *Shock* **1**, 466-71.

Clapham DE (1995). Calcium signaling. *Cell* **80**, 259-68.

Clementi E, Martino G, Grimaldi LM et al. (1994). Intracellular Ca^{2+} stores of T lymphocytes: changes induced by in vitro and in vivo activation. *Eur J Immunol* **24**, 1365-71.

Coleman KJ, Rager DR (1993). Effects of voluntary exercise on immune function in rats. *Physiol Behav* **54**, 771-4.

Crabtree GR (2001). Calcium, calcineurin, and the control of transcription. *J Biol Chem* **276**, 2313-6.

Crabtree GR, Olson EN (2002). NFAT signaling: choreographing the social lives of cells. *Cell* **109 Suppl**, S67-79.

Csermely P, Penzes I, Toth S (1995). Chronic overcrowding decreases cytoplasmic free calcium levels in T lymphocytes of aged CBA/CA mice. *Experientia* **51**, 976-9.

Cui J, Bian JS, Kagan A et al. (2002). CaT1 contributes to the stores-operated calcium

current in Jurkat T-lymphocytes. *J Biol Chem* **277**, 47175-83.

Cyster JG, Goodnow CC (1995). Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. *Immunity* **2**, 13-24.

Dawson AP (1997). Calcium signalling: how do IP3 receptors work? *Curr Biol* **7**, R544-7.

Decoursey TE, Chandy KG, Gupta S et al. (1987). Two types of potassium channels in murine T lymphocytes. *J Gen Physiol* **89**, 379-404.

DeHaven WI, Smyth JT, Boyles RR et al. (2007). Calcium inhibition and calcium potentiation of Orai1, Orai2, and Orai3 calcium release-activated calcium channels. *J Biol Chem* **282**, 17548-56.

Delon J, Bercovici N, Liblau R et al. (1998). Imaging antigen recognition by naive CD4+ T cells: compulsory cytoskeletal alterations for the triggering of an intracellular calcium response. *Eur J Immunol* **28**, 716-29.

Densmore JJ, Haverstick DM, Szabo G et al. (1996). A voltage-operable current is involved in Ca^{2+} entry in human lymphocytes whereas I_{CRAC} has no apparent role. *Am J Physiol* **271**, C1494-503.

Di Pietro R, Alba Rana R, Sciscio A et al. (1996). Age- and training-related events in active T subpopulation. Changes in polyphosphoinositide metabolism during mitogenic stimulation. *Mech Ageing Dev* **90**, 103-9.

Dietrich A, Mederos y Schnitzler M, Kalwa H et al. (2005). Functional characterization and physiological relevance of the TRPC3/6/7 subfamily of cation channels. *Naunyn Schmiedebergs Arch Pharmacol* **371**, 257-65.

Dinarello CA (1997). Role of pro- and anti-inflammatory cytokines during inflammation: experimental and clinical findings. *J Biol Regul Homeost Agents* **11**, 91-103.

Dohi K, Mastro AM, Miles MP et al. (2001). Lymphocyte proliferation in response to acute heavy resistance exercise in women: influence of muscle strength and total work. *Eur J Appl Physiol* **85**, 367-73.

Dolmetsch RE, Lewis RS, Goodnow CC et al. (1997). Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* **386**, 855-8.

Dolmetsch RE, Xu K, Lewis RS (1998). Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392**, 933-6.

Dong Z, Saikumar P, Weinberg JM et al. (2006). Calcium in cell injury and death. *Annu Rev Pathol* **1**, 405-34.

Donnadieu E, Bismuth G, Trautmann A (1995). The intracellular Ca^{2+} concentration optimal for T cell activation is quite different after ionomycin or CD3 stimulation. *Pflugers Arch* **429**, 546-54.

Eckert A, Forstl H, Zerfass R et al. (1996). Lymphocytes and neutrophils as peripheral models to study the effect of beta-amyloid on cellular calcium signalling in Alzheimer's disease. *Life Sci* **59**, 499-510.

Eckert A, Forstl H, Zerfass R et al. (1998) Changes of intracellular calcium regulation in Alzheimer's disease and vascular dementia. *J Neural Transm Suppl* **54**, 201-10.

Ehrlich BE, Kaftan E, Bezprozvannaya S et al. (1994). The pharmacology of intracellular Ca^{2+}-release channels. *Trends Pharmacol Sci* **15**, 145-9.

Elphick GF, Greenwood BN, Campisi J et al. (2003a). Increased serum nIgM in voluntarily physically active rats: a potential role for B-1 cells. *J Appl Physiol* **94**, 660-7.

Elphick GF, Wieseler-Frank J, Greenwood BN et al. (2003b). B-1 cell (CD5+/CD11b+) numbers and nIgM levels are elevated in physically active vs. sedentary rats. *J Appl Physiol* **95**, 199-206.

Fanger CM, Ghanshani S, Logsdon NJ et al. (1999). Calmodulin mediates calcium-dependent activation of the intermediate conductance KCa channel, IKCa1. *J Biol Chem* **274**, 5746-54.

Fanger CM, Neben AL, Cahalan MD (2000). Differential Ca^{2+} influx, KCa channel activity, and Ca^{2+} clearance distinguish Th1 and Th2 lymphocytes. *J Immunol* **164**, 1153-60.

Fehrenbach E, Northoff H (2001). Free radicals, exercise, apoptosis, and heat shock proteins. *Exerc Immunol Rev* **7**, 66-89.

Ferry A, Rieu P, Laziri F et al. (1992). Effect of moderate exercise on rat T-cells. *Eur J Appl Physiol Occup Physiol* **65**, 464-8.

Ferry A, Rieu P, Laziri F et al. (1991). Immunomodulations of thymocytes and splenocytes in trained rats. *J Appl Physiol* **71**, 815-20.

Feske S (2007). Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol* **7**, 690-702.

Feske S, Giltnane J, Dolmetsch R et al. (2001). Gene regulation mediated by calcium signals in T lymphocytes. *Nat Immunol* **2**, 316-24.

Feske S, Prakriya M, Rao A et al. (2005). A severe defect in CRAC Ca^{2+} channel activation and altered K^+ channel gating in T cells from immunodeficient patients. *J Exp Med* **202**, 651-62.

Fill M, Copello JA (2002). Ryanodine receptor calcium release channels. *Physiol Rev* **82**, 893-922.

Finaud J, Lac G, Filaire E (2006). Oxidative stress : relationship with exercise and training. *Sports Med* **36**, 327-58.

Fisher-Wellman K, Bloomer RJ (2009). Acute exercise and oxidative stress: a 30 year history. *Dyn Med* **8**, 1.

Fleshner M (2000). Exercise and neuroendocrine regulation of antibody production: protective effect of physical activity on stress-induced suppression of the specific antibody response. *Int J Sports Med* **21 Suppl 1**, S14-9.

Fleshner M, Brennan FX, Nguyen K et al. (1996). RU-486 blocks differentially suppressive effect of stress on in vivo anti-KLH immunoglobulin response. *Am J Physiol* **271**, R1344-52.

Fomina AF, Fanger CM, Kozak JA et al. (2000). Single channel properties and regulated expression of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in human T cells. *J Cell Biol* **150**, 1435-44.

Gabriel H, Schwarz L, Born P et al. (1992). Differential mobilization of leucocyte and

lymphocyte subpopulations into the circulation during endurance exercise. *Eur J Appl Physiol Occup Physiol* **65**, 529-34.

Gamberucci A, Giurisato E, Pizzo P et al. (2002). Diacylglycerol activates the influx of extracellular cations in T-lymphocytes independently of intracellular calcium-store depletion and possibly involving endogenous TRP6 gene products. *Biochem J* **364**, 245-54.

Garcia RL, Schilling WP (1997). Differential expression of mammalian TRP homologues across tissues and cell lines. *Biochem Biophys Res Commun* **239**, 279-83.

Gasser A, Glassmeier G, Fliegert R et al. (2006). Activation of T cell calcium influx by the second messenger ADP-ribose. *J Biol Chem* **281**, 2489-96.

Ghanshani S, Wulff H, Miller MJ et al. (2000). Up-regulation of the IKCa1 potassium channel during T-cell activation. Molecular mechanism and functional consequences. *J Biol Chem* **275**, 37137-49.

Glass R, Townsend-Nicholson A, Burnstock G (2000). P2 receptors in the thymus: expression of P2X and P2Y receptors in adult rats, an immunohistochemical and in situ hybridisation study. *Cell Tissue Res* **300**, 295-306.

Gleeson M (2006). Immune system adaptation in elite athletes. *Curr Opin Clin Nutr Metab Care* **9**, 659-65.

Gleeson M (2007). Immune function in sport and exercise. *J Appl Physiol* **103**, 693-9.

Gleeson M, Bishop NC (2005). The T cell and NK cell immune response to exercise. *Ann Transplant* **10**, 43-8.

Gleeson M, Walsh NP, British Association of Sport and exercise science et al. (2012). The BASES expert statement on exercise, immunity, and infection. *J Sports Sci* **30**, 321-4.

Gomes B, Savignac M, Moreau M et al. (2004). Lymphocyte calcium signaling involves dihydropyridine-sensitive L-type calcium channels: facts and controversies. *Crit Rev Immunol* **24**, 425-47.

Gomez-Cabrera MC, Domenech E, Vina J (2008). Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. *Free Radic Biol Med* **44**, 126-31.

Goto C, Higashi Y, Kimura M et al. (2003). Effect of different intensities of exercise on endothelium-dependent vasodilation in humans: role of endothelium-dependent nitric oxide and oxidative stress. *Circulation* **108**, 530-5.

Gravina L, Ruiz F, Diaz E et al. (2012). Influence of nutrient intake on antioxidant capacity, muscle damage and white blood cell count in female soccer players. *J Int Soc Sports Nutr* **9**, 32.

Green KJ, Rowbottom DG, Mackinnon LT (2002). Exercise and T-lymphocyte function: a comparison of proliferation in PBMC and NK cell-depleted PBMC culture. *J Appl Physiol* **92**, 2390-5.

Green KJ, Rowbottom DG, Mackinnon LT (2003). Acute exercise and T-lymphocyte expression of the early activation marker CD69. *Med Sci Sports Exerc* **35**, 582-8.

Grissmer S, Nguyen AN, Cahalan MD (1993). Calcium-activated potassium channels in

resting and activated human T lymphocytes. Expression levels, calcium dependence, ion selectivity, and pharmacology. *J Gen Physiol* **102**, 601-30.

Grynkiewicz G, Poenie M, Tsien RY (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**, 3440-50.

Gueldner SH, Poon LW, La Via M et al. (1997). Long-term exercise patterns and immune function in healthy older women. A report of preliminary findings. *Mech Ageing Dev* **93**, 215-22.

Guler AD, Lee H, Iida T et al. (2002). Heat-evoked activation of the ion channel, TRPV4. *J Neurosci* **22**, 6408-14.

Gwack Y, Srikanth S, Feske S et al. (2007). Biochemical and functional characterization of Orai proteins. *J Biol Chem* **282**, 16232-43.

Hack N, Wilkinson JM, Crawford N (1988). A monoclonal antibody (PL/IM 430) to human platelet intracellular membranes which inhibits the uptake of Ca^{2+} without affecting the Ca^{2+} +Mg^{2+}-ATPase. *Biochem J* **250**, 355-61.

Han AY, Zhang MH, Zuo XL et al. (2010). Effect of acute heat stress on calcium concentration, proliferation, cell cycle, and interleukin-2 production in splenic lymphocytes from broiler chickens. *Poult Sci* **89**, 2063-70.

Hara Y, Wakamori M, Ishii M et al. (2002). LTRPC2 Ca^{2+}-permeable channel activated by changes in redox status confers susceptibility to cell death. *Mol Cell* **9**, 163-73.

Haverstick DM, Engelhard VH, Gray LS (1991). Three intracellular signals for cytotoxic T lymphocyte-mediated killing. Independent roles for protein kinase C, Ca^{2+} influx, and Ca^{2+} release from internal stores. *J Immunol* **146**, 3306-13.

He LP, Hewavitharana T, Soboloff J et al. (2005). A functional link between store-operated and TRPC channels revealed by the 3,5-bis(trifluoromethyl)pyrazole derivative, BTP2. *J Biol Chem* **280**, 10997-1006.

Higgins ER, Cannell MB, Sneyd J (2006). A buffering SERCA pump in models of calcium dynamics. *Biophys J* **91**, 151-63.

Hirst RA, Harrison C, Hirota K et al. (1999). Measurement of [Ca^{2+}]i in whole cell suspensions using fura-2. *Methods in molecular biology (Clifton, N J)* **114**, 31-9.

Hoffman-Goetz L (1998). Influence of physical activity and exercise on innate immunity. *Nutr Rev* **56**, S126-30.

Hoffman-Goetz L, Pedersen BK (1994). Exercise and the immune system: a model of the stress response? *Immunol Today* **15**, 382-7.

Hofmann F, Lacinova L, Klugbauer N (1999). Voltage-dependent calcium channels: from structure to function. *Rev Physiol Biochem Pharmacol* **139**, 33-87.

Hofmann T, Chubanov V, Gudermann T et al. (2003). TRPM5 is a voltage-modulated and Ca^{2+}-activated monovalent selective cation channel. *Curr Biol* **13**, 1153-8.

Hogan PG, Chen L, Nardone J et al. (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* **17**, 2205-32.

Hwang HJ, Kwak YS, Yoon GA et al. (2007). Combined effects of swim training and ginseng supplementation on exercise performance time, ROS, lymphocyte proliferation, and

DNA damage following exhaustive exercise stress. *Int J Vitam Nutr Res* **77**, 289-96.

Ibarreta D, Parrilla R, Ayuso MS (1997). Altered Ca^{2+} homeostasis in lymphoblasts from patients with late-onset Alzheimer disease. *Alzheimer Dis Assoc Disord* **11**, 220-7.

Inada H, Iida T, Tominaga M (2006). Different expression patterns of TRP genes in murine B and T lymphocytes. *Biochem Biophys Res Commun* **350**, 762-7.

Irvine RF (1987). Inositol phosphates and calcium entry. *Nature* **328**, 386.

Ishikawa J, Ohga K, Yoshino T et al. (2003). A pyrazole derivative, YM-58483, potently inhibits store-operated sustained Ca^{2+} influx and IL-2 production in T lymphocytes. *J Immunol* **170**, 4441-9.

Jacob R (1990). Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. *J Physiol* **421**, 55-77.

Jayaraman T, Marks AR (1997). T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. *Mol Cell Biol* **17**, 3005-12.

Jayaraman T, Ondriasova E, Ondrias K et al. (1995). The inositol 1,4,5-trisphosphate receptor is essential for T-cell receptor signaling. *Proc Natl Acad Sci USA* **92**, 6007-11.

Ji LL (1993). Antioxidant enzyme response to exercise and aging. *Med Sci Sports Exerc* **25**, 225-31.

Ji LL (1995). Oxidative stress during exercise: implication of antioxidant nutrients. *Free Radic Biol Med* **18**, 1079-86.

Ji LL, Katz A, Fu R et al. (1993). Blood glutathione status during exercise: effect of carbohydrate supplementation. *J Appl Physiol* **74**, 788-92.

Jonsdottir IH, Johansson C, Asea A et al. (1997). Duration and mechanisms of the increased natural cytotoxicity seen after chronic voluntary exercise in rats. *Acta Physiol Scand* **160**, 333-9.

Kerschbaum HH, Cahalan MD (1998). Monovalent permeability, rectification, and ionic block of store-operated calcium channels in Jurkat T lymphocytes. *J Gen Physiol* **111**, 521-37.

Khaidukov SV, Litvinov IS (2005). Calcium homeostasis change in CD4+ T lymphocytes from human peripheral blood during differentiation in vivo. *Biochemistry (Mosc)* **70**, 692-702.

Khan AA, Soloski MJ, Sharp AH et al. (1996). Lymphocyte apoptosis: mediation by increased type 3 inositol 1,4,5-trisphosphate receptor. *Science* **273**, 503-7.

Khan AA, Steiner JP, Klein MG et al. (1992). IP3 receptor: localization to plasma membrane of T cells and cocapping with the T cell receptor. *Science* **257**, 815-8.

Kirichok Y, Krapivinsky G, Clapham DE (2004). The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427**, 360-4.

Kiselyov K, Xu X, Mozhayeva G et al. (1998). Functional interaction between InsP3 receptors and store-operated Htrp3 channels. *Nature* **396**, 478-82.

Knez WL, Coombes JS, Jenkins DG (2006). Ultra-endurance exercise and oxidative damage : implications for cardiovascular health. *Sports Med* **36**, 429-41.

Kohut ML, Arntson BA, Lee W et al. (2004). Moderate exercise improves antibody response

to influenza immunization in older adults. *Vaccine* **22**, 2298-306.

Kohut ML, Boehm GW, Moynihan JA (2001). Prolonged exercise suppresses antigen-specific cytokine response to upper respiratory infection. *J Appl Physiol* **90**, 678-84.

Koo GC, Blake JT, Talento A et al. (1997). Blockade of the voltage-gated potassium channel Kv1.3 inhibits immune responses in vivo. *J Immunol* **158**, 5120-8.

Kraft R, Harteneck C (2005). The mammalian melastatin-related transient receptor potential cation channels: an overview. *Pflugers Arch* **451**, 204-11.

Krause KH (1991). Ca^{2+}-storage organelles. *FEBS Lett* **285**, 225-9.

Krebs J (1998). The role of calcium in apoptosis. *Biometals* **11**, 375-82.

Kruger K, Frost S, Most E et al. (2009). Exercise affects tissue lymphocyte apoptosis via redox-sensitive and Fas-dependent signaling pathways. *Am J Physiol Regul Integr Comp Physiol* **296**, R1518-27.

Kwak YS (2006). Effects of training on spleen and peritoneal exudate reactive oxygen species and lymphocyte proliferation by splenocytes at rest and after an acute bout of exercise. *J Sports Sci* **24**, 973-8.

Lacabaratz C, Corvazier E, Kovacs T et al. (1996). The PL/IM 430 and the N 89 antibodies recognize two distinct 97 kDa sarco/endoplasmic-reticulum Ca^{2+}-ATPase proteins. *Biochem J* **318 (Pt 1)**, 359-60.

Launay P, Fleig A, Perraud AL et al. (2002). TRPM4 is a Ca^{2+}-activated nonselective cation channel mediating cell membrane depolarization. *Cell* **109**, 397-407.

Launay S, Bobe R, Lacabaratz-Porret C et al. (1997). Modulation of endoplasmic reticulum calcium pump expression during T lymphocyte activation. *J Biol Chem* **272**, 10746-50.

Lawler JM, Powers SK, Visser T et al. (1993). Acute exercise and skeletal muscle antioxidant and metabolic enzymes: effects of fiber type and age. *Am J Physiol* **265**, R1344-50.

Ledbetter JA, June CH, Grosmaire LS et al. (1987). Crosslinking of surface antigens causes mobilization of intracellular ionized calcium in T lymphocytes. *Proc Natl Acad Sci USA* **84**, 1384-8.

Leeuwenburgh C, Ji LL (1998). Glutathone and glutathione ethyl ester supplementation of mice alter glutathione homeostasis during exercise. *J Nutr* **128**, 2420-6.

Leonard WJ, Kronke M, Peffer NJ et al. (1985). Interleukin 2 receptor gene expression in normal human T lymphocytes. *Proc Natl Acad Sci USA* **82**, 6281-5.

Lepple-Wienhues A, Cahalan MD (1996). Conductance and permeation of monovalent cations through depletion-activated Ca^{2+} channels (I_{CRAC}) in Jurkat T cells. *Biophys J* **71**, 787-94.

Levite M, Cahalon L, Peretz A et al. (2000). Extracellular K^+ and opening of voltage-gated potassium channels activate T cell integrin function: physical and functional association between Kv1.3 channels and beta1 integrins. *J Exp Med* **191**, 1167-76.

Lewis RS (2001). Calcium signaling mechanisms in T lymphocytes. *Annu Rev Immunol* **19**, 497-521.

Lewis RS (2007). The molecular choreography of a store-operated calcium channel. *Nature*

446, 284-7.

Lewis RS, Cahalan MD (1988). Subset-specific expression of potassium channels in developing murine T lymphocytes. *Science* **239**, 771-5.

Lewis RS, Cahalan MD (1995). Potassium and calcium channels in lymphocytes. *Annu Rev Immunol* **13**, 623-53.

Liedtke W, Choe Y, Marti-Renom MA et al. (2000). Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* **103**, 525-35.

Lin YS, Jan MS, Chen HI (1993). The effect of chronic and acute exercise on immunity in rats. *Int J Sports Med* **14**, 86-92.

Lintschinger B, Balzer-Geldsetzer M, Baskaran T et al. (2000). Coassembly of Trp1 and Trp3 proteins generates diacylglycerol- and Ca^{2+}-sensitive cation channels. *J Biol Chem* **275**, 27799-805.

Liou J, Fivaz M, Inoue T et al. (2007). Live-cell imaging reveals sequential oligomerization and local plasma membrane targeting of stromal interaction molecule 1 after Ca^{2+} store depletion. *Proc Natl Acad Sci USA* **104**, 9301-6.

Liou J, Kim ML, Heo WD et al. (2005). STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} influx. *Curr Biol* **15**, 1235-41.

Lis A, Peinelt C, Beck A et al. (2007). CRACM1, CRACM2, and CRACM3 are store-operated Ca^{2+} channels with distinct functional properties. *Curr Biol* **17**, 794-800.

Liu QH, Liu X, Wen Z et al. (2005). Distinct calcium channels regulate responses of primary B lymphocytes to B cell receptor engagement and mechanical stimuli. *J Immunol* **174**, 68-79.

Liu X, Singh BB, Ambudkar IS (2003). TRPC1 is required for functional store-operated Ca^{2+} channels. Role of acidic amino acid residues in the S5-S6 region. *J Biol Chem* **278**, 11337-43.

Loomis WH, Namiki S, Ostrom RS et al. (2003). Hypertonic stress increases T cell interleukin-2 expression through a mechanism that involves ATP release, P2 receptor, and p38 MAPK activation. *J Biol Chem* **278**, 4590-6.

Luik RM, Wu MM, Buchanan J et al. (2006). The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. *J Cell Biol* **174**, 815-25.

Mackinnon LT (1989). Exercise and natural killer cells. What is the relationship? *Sports Med* **7**, 141-9.

MacKinnon R (1991). Determination of the subunit stoichiometry of a voltage-activated potassium channel. *Nature* **350**, 232-5.

MacNeil B, Hoffman-Goetz L, Kendall A et al. (1991). Lymphocyte proliferation responses after exercise in men: fitness, intensity, and duration effects. *J Appl Physiol* **70**, 179-85.

Marks AR, Marx SO, Reiken S (2002). Regulation of ryanodine receptors via macromolecular complexes: a novel role for leucine/isoleucine zippers. *Trends Cardiovasc Med* **12**, 166-70.

Marom S, Levitan IB (1994). State-dependent inactivation of the Kv3 potassium channel. *Biophys J* **67**, 579-89.

Mastaloudis A, Leonard SW, Traber MG (2001). Oxidative stress in athletes during extreme endurance exercise. *Free Radic Biol Med* **31**, 911-22.

Mazzeo RS, Rajkumar C, Rolland J et al. (1998). Immune response to a single bout of exercise in young and elderly subjects. *Mech Ageing Dev* **100**, 121-32.

McCormack JG, Cobbold PH (1991). Cellular calcium: a practical approach. *IRL Press (Oxford, UK)*.

McHugh D, Flemming R, Xu SZ et al. (2003). Critical intracellular Ca^{2+} dependence of transient receptor potential melastatin 2 (TRPM2) cation channel activation. *J Biol Chem* **278**, 11002-6.

Mehta VB, Hart J, Wewers MD (2001). ATP-stimulated release of interleukin (IL)-1beta and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. *J Biol Chem* **276**, 3820-6.

Meldolesi J, Pozzan T (1987). Pathways of Ca^{2+} influx at the plasma membrane: voltage-, receptor-, and second messenger-operated channels. *Exp Cell Res* **171**, 271-83.

Mignen O, Shuttleworth TJ (2000). I(ARC), a novel arachidonate-regulated, noncapacitative Ca^{2+} entry channel. *J Biol Chem* **275**, 9114-9.

Mignen O, Thompson JL, Shuttleworth TJ (2001). Reciprocal regulation of capacitative and arachidonate-regulated noncapacitative Ca^{2+} entry pathways. *J Biol Chem* **276**, 35676-83.

Mignen O, Thompson JL, Shuttleworth TJ (2007). STIM1 regulates Ca^{2+} entry via arachidonate-regulated Ca^{2+}-selective (ARC) channels without store depletion or translocation to the plasma membrane. *J Physiol* **579**, 703-15.

Mignen O, Thompson JL, Shuttleworth TJ (2008). Both Orai1 and Orai3 are essential components of the arachidonate-regulated Ca^{2+}-selective (ARC) channels. *J Physiol* **586**, 185-95.

Mikoshiba K (1993). Inositol 1,4,5-trisphosphate receptor. *Trends Pharmacol Sci* **14**, 86-9.

Mikoshiba K (1997) The InsP3 receptor and intracellular Ca^{2+} signaling. *Curr Opin Neurobiol* **7**, 339-45.

Miyakawa T, Maeda A, Yamazawa T et al. (1999). Encoding of Ca^{2+} signals by differential expression of IP3 receptor subtypes. *EMBO J* **18**, 1303-8.

Mooren FC, Kinne RK (1998). Cellular calcium in health and disease. *Biochim Biophys Acta* **1406**, 127-51.

Mooren FC, Lechtermann A, Fromme A et al. (2001). Alterations in intracellular calcium signaling of lymphocytes after exhaustive exercise. *Med Sci Sports Exerc* **33**, 242-8.

Moqrich A, Hwang SW, Earley TJ et al. (2005). Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. *Science* **307**, 1468-72.

Moraska A, Fleshner M (2001). Voluntary physical activity prevents stress-induced behavioral depression and anti-KLH antibody suppression. *Am J Physiol Regul Integr Comp Physiol* **281**, R484-9.

Mori Y, Wakamori M, Miyakawa T et al. (2002). Transient receptor potential 1 regulates capacitative Ca^{2+} entry and Ca^{2+} release from endoplasmic reticulum in B lymphocytes. *J Exp Med* **195**, 673-81.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55-63.

Nadler MJ, Hermosura MC, Inabe K et al. (2001). LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. *Nature* **411**, 590-5.

Nasrullah I, Mazzeo RS (1992). Age-related immunosenescence in Fischer 344 rats: influence of exercise training. *J Appl Physiol* **73**, 1932-8.

Negulescu PA, Krasieva TB, Khan A et al. (1996). Polarity of T cell shape, motility, and sensitivity to antigen. *Immunity* **4**, 421-30.

Negulescu PA, Shastri N, Cahalan MD (1994). Intracellular calcium dependence of gene expression in single T lymphocytes. *Proc Natl Acad Sci USA* **91**, 2873-7.

Nehlsen-Cannarella SL, Nieman DC, Jessen J et al. (1991). The effects of acute moderate exercise on lymphocyte function and serum immunoglobulin levels. *Int J Sports Med* **12**, 391-8.

Nielsen HB, Pedersen BK (1997). Lymphocyte proliferation in response to exercise. *Eur J Appl Physiol Occup Physiol* **75**, 375-9.

Nieman DC (1997). Immune response to heavy exertion. *J Appl Physiol* **82**, 1385-94.

Nieman DC (2000). Special feature for the Olympics: effects of exercise on the immune system: exercise effects on systemic immunity. *Immunol Cell Biol* **78**, 496-501.

Nieman DC, Buckley KS, Henson DA et al. (1995a). Immune function in marathon runners versus sedentary controls. *Med Sci Sports Exerc* **27**, 986-92.

Nieman DC, Henson DA, Gusewitch G et al. (1993a). Physical activity and immune function in elderly women. *Med Sci Sports Exerc* **25**, 823-31.

Nieman DC, Henson DA, Sampson CS et al. (1995b). The acute immune response to exhaustive resistance exercise. *Int J Sports Med* **16**, 322-8.

Nieman DC, Miller AR, Henson DA et al. (1993b). Effects of high- vs moderate-intensity exercise on natural killer cell activity. *Med Sci Sports Exerc* **25**, 1126-34.

Nieman DC, Nehlsen-Cannarella SL, Fagoaga OR et al. (2000). Immune function in female elite rowers and non-athletes. *Br J Sports Med* **34**, 181-7.

Nieman DC, Nehlsen-Cannarella SL, Henson DA et al. (1998). Immune response to exercise training and/or energy restriction in obese women. *Med Sci Sports Exerc* **30**, 679-86.

Nieman DC, Pedersen BK (1999). Exercise and immune function. Recent developments. *Sports Med* **27**, 73-80.

Nieman DC, Simandle S, Henson DA et al. (1995c). Lymphocyte proliferative response to 2.5 hours of running. *Int J Sports Med* **16**, 404-9.

Niess AM, Hartmann A, Grunert-Fuchs M et al. (1996). DNA damage after exhaustive treadmill running in trained and untrained men. *Int J Sports Med* **17**, 397-403.

Nilius B (2003). From TRPs to SOCs, CCEs, and CRACs: consensus and controversies. *Cell*

Calcium **33**, 293-8.

Nilius B, Prenen J, Droogmans G et al. (2003). Voltage dependence of the Ca^{2+}-activated cation channel TRPM4. *J Biol Chem* **278**, 30813-20.

Nilius B, Prenen J, Tang J et al. (2005). Regulation of the Ca^{2+} sensitivity of the nonselective cation channel TRPM4. *J Biol Chem* **280**, 6423-33.

Nilius B, Vriens J, Prenen J et al. (2004). TRPV4 calcium entry channel: a paradigm for gating diversity. *Am J Physiol Cell Physiol* **286**, C195-205.

North RA (2002). Molecular physiology of P2X receptors. *Physiol Rev* **82**, 1013-67.

Oh-hora M, Rao A (2008). Calcium signaling in lymphocytes. *Curr Opin Immunol* **20**, 250-8.

Oh-Hora M, Yamashita M, Hogan PG et al. (2008). Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. *Nat Immunol* **9**, 432-43.

Ong HL, Cheng KT, Liu X et al. (2007). Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components. *J Biol Chem* **282**, 9105-16.

Oshimi Y, Oshimi K, Miyazaki S (1996). Necrosis and apoptosis associated with distinct Ca^{2+} response patterns in target cells attacked by human natural killer cells. *J Physiol* **495 (Pt2)**, 319-29.

Owsianik G, Talavera K, Voets T et al. (2006). Permeation and selectivity of TRP channels. *Annu Rev Physiol* **68**, 685-717.

Pahlavani MA, Cheung TH, Chesky JA et al. (1988). Influence of exercise on the immune function of rats of various ages. *J Appl Physiol* **64**, 1997-2001.

Papp B, Enyedi A, Paszty K et al. (1992). Simultaneous presence of two distinct endoplasmic-reticulum-type calcium-pump isoforms in human cells. Characterization by radio-immunoblotting and inhibition by 2,5-di-(t-butyl)-1,4-benzohydroquinone. *Biochem J* **288 (Pt 1)**, 297-302.

Papp B, Paszty K, Kovacs T et al. (1993). Characterization of the inositol trisphosphate-sensitive and insensitive calcium stores by selective inhibition of the endoplasmic reticulum-type calcium pump isoforms in isolated platelet membrane vesicles. *Cell Calcium* **14**, 531-8.

Parekh AB, Fleig A, Penner R (1997). The store-operated calcium current I(CRAC): nonlinear activation by InsP3 and dissociation from calcium release. *Cell* **89**, 973-80.

Parekh AB, Putney JW Jr. (2005). Store-operated calcium channels. *Physiol Rev* **85**, 757-810.

Partiseti M, Choquet D, Diu A et al. (1992). Differential regulation of voltage- and calcium-activated potassium channels in human B lymphocytes. *J Immunol* **148**, 3361-8.

Partiseti M, Korn H, Choquet D (1993). Pattern of potassium channel expression in proliferating B lymphocytes depends upon the mode of activation. *J Immunol* **151**, 2462-70.

Pate RR, Pratt M, Blair SN et al. (1995). Physical activity and public health. A recommendation from the Centers for Disease Control and Prevention and the American College of Sports Medicine. *JAMA* **273**, 402-7.

Patterson RL, Boehning D, Snyder SH (2004). Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annu Rev Biochem* **73**, 437-65.

Pedersen BK, Febbraio MA (2008). Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev* **88**, 1379-406.

Pedersen BK, Hoffman-Goetz L (2000). Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev* **80**, 1055-81.

Pedersen BK, Kappel M, Klokker M et al. (1994). The immune system during exposure to extreme physiologic conditions. *Int J Sports Med* **15 Suppl 3**, S116-21.

Pedersen BK, Saltin B (2006). Evidence for prescribing exercise as therapy in chronic disease. *Scand J Med Sci Sports* **16 Suppl 1**, 3-63.

Pedersen BK, Tvede N, Klarlund K et al. (1990). Indomethacin in vitro and in vivo abolishes post-exercise suppression of natural killer cell activity in peripheral blood. *Int J Sports Med* **11**, 127-31.

Peier AM, Reeve AJ, Andersson DA et al. (2002). A heat-sensitive TRP channel expressed in keratinocytes. *Science* **296**, 2046-9.

Penner R, Fasolato C, Hoth M (1993). Calcium influx and its control by calcium release. *Curr Opin Neurobiol* **3**, 368-74.

Phaneuf S, Leeuwenburgh C (2001). Apoptosis and exercise. *Med Sci Sports Exerc* **33**, 393-6.

Potteiger JA, Chan MA, Haff GG et al. (2001). Training status influences T-cell responses in women following acute resistance exercise. *J Strength Cond Res* **15**, 185-91.

Prakriya M, Lewis RS (2003). CRAC channels: activation, permeation, and the search for a molecular identity. *Cell Calcium* **33**, 311-21.

Premack BA, McDonald TV, Gardner P (1994). Activation of Ca^{2+} current in Jurkat T cells following the depletion of Ca^{2+} stores by microsomal Ca^{2+}-ATPase inhibitors. *J Immunol* **152**, 5226-40.

Putney JW Jr. (1997). Type 3 inositol 1,4,5-trisphosphate receptor and capacitative calcium entry. *Cell Calcium* **21**, 257-61.

Putney JW Jr., McKay RR (1999). Capacitative calcium entry channels. *Bioessays* **21**, 38-46.

Quadrilatero J, Hoffman-Goetz L (2005). N-acetyl-l-cysteine protects intestinal lymphocytes from apoptotic death after acute exercise in adrenalectomized mice. *Am J Physiol Regul Integr Comp Physiol* **288**, R1664-72.

Quah BJ, Parish CR (2010). The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor lymphocyte proliferation. *J Vis Exp*.

Quah BJ, Warren HS, Parish CR (2007). Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc* **2**, 2049-56.

Radak Z, Sasvari M, Nyakas C et al. (2001). Single bout of exercise eliminates the

immobilization-induced oxidative stress in rat brain. *Neurochem Int* **39**, 33-8.

Ralevic V, Burnstock G (1998). Receptors for purines and pyrimidines. *Pharmacol Rev* **50**, 413-92.

Randall Simpson JA, Hoffman-Goetz L, Thorne R et al. (1989). Exercise stress alters the percentage of splenic lymphocyte subsets in response to mitogen but not in response to interleukin-1. *Brain Behav Immun* **3**, 119-28.

Randriamampita C, Trautmann A (2004). Ca^{2+} signals and T lymphocytes;"New mechanisms and functions in Ca^{2+} signalling". *Biol Cell* **96**, 69-78.

Randriamampita C, Tsien RY (1993). Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature* **364**, 809-14.

Rhind SG, Shek PN, Shinkai S et al. (1996). Effects of moderate endurance exercise and training on in vitro lymphocyte proliferation, interleukin-2 (IL-2) production, and IL-2 receptor expression. *Eur J Appl Physiol Occup Physiol* **74**, 348-60.

Rink TJ, Deutsch C (1983). Calcium-activated potassium channels in lymphocytes. *Cell Calcium* **4**, 463-73.

Riquelme G, Diaz M, Sepulveda FV (1997). Possible thiol group involvement in intracellular pH effect on low-conductance Ca^{2+}-dependent K^+ channels. *Am J Physiol* **273**, C230-8.

Rizzuto R, Brini M, Murgia M et al. (1993). Microdomains with high Ca^{2+} close to IP3-sensitive channels that are sensed by neighboring mitochondria. *Science* **262**, 744-7.

Roos J, DiGregorio PJ, Yeromin AV et al. (2005). STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. *J Cell Biol* **169**, 435-45.

Rothenberg EV, Ward SB (1996). A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin 2 gene regulation. *Proc Natl Acad Sci USA* **93**, 9358-65.

Roy SS, Hajnoczky G (2008). Calcium, mitochondria and apoptosis studied by fluorescence measurements. *Methods* **46**, 213-23.

Runnels LW, Yue L, Clapham DE (2002). The TRPM7 channel is inactivated by PIP2 hydrolysis. *Nat Cell Biol* **4**, 329-36.

Sachdev S, Davies KJ (2008). Production, detection, and adaptive responses to free radicals in exercise. *Free Radic Biol Med* **44**, 215-23.

Sage SO, Merritt JE, Hallam TJ et al. (1989). Receptor-mediated calcium entry in fura-2-loaded human platelets stimulated with ADP and thrombin. Dual-wavelengths studies with Mn^{2+}. *Biochem J* **258**, 923-6.

Sano Y, Inamura K, Miyake A et al. (2001). Immunocyte Ca^{2+} influx system mediated by LTRPC2. *Science* **293**, 1327-30.

Savignac M, Badou A, Moreau M et al. (2001). Protein kinase C-mediated calcium entry dependent upon dihydropyridine sensitive channels: a T cell receptor-coupled signaling pathway involved in IL-4 synthesis. *FASEB J* **15**, 1577-9.

Sayeed MM (1996). Alterations in calcium signaling and cellular responses in septic injury.

New Horiz **4**, 72-86.

Scharenberg AM, Kinet JP (1998). PtdIns-3,4,5-P3: a regulatory nexus between tyrosine kinases and sustained calcium signals. *Cell* **94**, 5-8.

Schwarz EC, Wissenbach U, Niemeyer BA et al. (2006). TRPV6 potentiates calcium-dependent cell proliferation. *Cell Calcium* **39**, 163-73.

Sei Y, McIntyre T, Skolnick P et al. (1991). Stress modulates calcium mobilization in immune cells. *Life Sci* **49**, 671-6.

Sen CK (1995). Oxidants and antioxidants in exercise. *J Appl Physiol* **79**, 675-86.

Shephard RJ (1999). Biology and medicine of soccer: an update. *J Sports Sci* **17**, 757-86.

Shephard RJ, Shek PN (1994). Potential impact of physical activity and sport on the immune system--a brief review. *Br J Sports Med* **28**, 247-55.

Shephard RJ, Shek PN (1999). Effects of exercise and training on natural killer cell counts and cytolytic activity: a meta-analysis. *Sports Med* **28**, 177-95.

Shinkai S, Kohno H, Kimura K et al. (1995). Physical activity and immune senescence in men. *Med Sci Sports Exerc* **27**, 1516-26.

Shinkai S, Shore S, Shek PN et al. (1992). Acute exercise and immune function. Relationship between lymphocyte activity and changes in subset counts. *Int J Sports Med* **13**, 452-61.

Silberman DM, Zorrilla-Zubilete M, Cremaschi GA et al. (2005). Protein kinase C-dependent NF-kappaB activation is altered in T cells by chronic stress. *Cell Mol Life Sci* **62**, 1744-54.

Smith GD, Gunthorpe MJ, Kelsell RE et al. (2002). TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature* **418**, 186-90.

Soboloff J, Spassova MA, Hewavitharana T et al. (2006a). STIM2 is an inhibitor of STIM1-mediated store-operated Ca^{2+} Entry. *Curr Biol* **16**, 1465-70.

Soboloff J, Spassova MA, Tang XD et al. (2006b). Orai1 and STIM reconstitute store-operated calcium channel function. *J Biol Chem* **281**, 20661-5.

Spielmann G, McFarlin BK, O'Connor DP et al. (2011). Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. *Brain Behav Immun* **25**, 1521-9.

Stathopulos PB, Li GY, Plevin MJ et al. (2006). Stored Ca^{2+} depletion-induced oligomerization of stromal interaction molecule 1 (STIM1) via the EF-SAM region: An initiation mechanism for capacitive Ca^{2+} entry. *J Biol Chem* **281**, 35855-62.

Steensberg A, Toft AD, Bruunsgaard H et al. (2001). Strenuous exercise decreases the percentage of type 1 T cells in the circulation. *J Appl Physiol* **91**, 1708-12.

Stokes AJ, Shimoda LM, Koblan-Huberson M et al. (2004). A TRPV2-PKA signaling module for transduction of physical stimuli in mast cells. *J Exp Med* **200**, 137-47.

Strotmann R, Harteneck C, Nunnenmacher K et al. (2000). OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nat Cell Biol* **2**, 695-702.

Su Z, Csutora P, Hunton D et al. (2001). A store-operated nonselective cation channel in lymphocytes is activated directly by Ca^{2+} influx factor and diacylglycerol. *Am J Physiol Cell Physiol* **280**, C1284-92.

Sugawara H, Kurosaki M, Takata M et al. (1997). Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. *EMBO J* **16**, 3078-88.

Suzui M, Kawai T, Kimura H et al. (2004). Natural killer cell lytic activity and CD56(dim) and CD56(bright) cell distributions during and after intensive training. *J Appl Physiol* **96**, 2167-73.

Suzuki K, Tagami K (2005). Voluntary wheel-running exercise enhances antigen-specific antibody-producing splenic B cell response and prolongs IgG half-life in the blood. *Eur J Appl Physiol* **94**, 514-9.

Swierenga SH, Whitfield JF, Karasaki S (1978). Loss of proliferative calcium dependence: simple in vitro indicator of tumorigenicity. *Proc Natl Acad Sci USA* **75**, 6069-72.

Taylor CW, Genazzani AA, Morris SA (1999). Expression of inositol trisphosphate receptors. *Cell Calcium* **26**, 237-51.

Tharp GD, Preuss TL (1991). Mitogenic response of T-lymphocytes to exercise training and stress. *J Appl Physiol* **70**, 2535-8.

Tian Y, Nie J, Tong TK et al. (2012). The release of immunosuppressive factor(s) in young males following exercise. *Sensors (Basel)* **12**, 5586-95.

Timmerman LA, Clipstone NA, Ho SN et al. (1996). Rapid shuttling of NF-AT in discrimination of Ca^{2+} signals and immunosuppression. *Nature* **383**, 837-40.

Turner H, Fleig A, Stokes A et al. (2003). Discrimination of intracellular calcium store subcompartments using TRPV1 (transient receptor potential channel, vanilloid subfamily member 1) release channel activity. *Biochem J* **371**, 341-50.

Velbinger K, De Vry J, Jentzsch K et al. (2000). Acute stress induced modifications of calcium signaling in learned helpless rats. *Pharmacopsychiatry* **33**, 132-7.

Venkatachalam K, Montell C (2007). TRP channels. *Annu Rev Biochem* **76**, 387-417.

Vennekens R, Nilius B (2007) Insights into TRPM4 function, regulation and physiological role. *Handb Exp Pharmacol*, 269-85.

Verde T, Thomas S, Shephard RJ (1992). Potential markers of heavy training in highly trained distance runners. *Br J Sports Med* **26**, 167-75.

Verheugen JA, Vijverberg HP, Oortgiesen M et al. (1995). Voltage-gated and Ca^{2+}-activated K^+ channels in intact human T lymphocytes. Noninvasive measurements of membrane currents, membrane potential, and intracellular calcium. *J Gen Physiol* **105**, 765-94.

Vig M, Peinelt C, Beck A et al. (2006). CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. *Science* **312**, 1220-3.

Villereal ML (2006). Mechanism and functional significance of TRPC channel multimerization. *Semin Cell Dev Biol* **17**, 618-29.

Vina J, Gomez-Cabrera MC, Lloret A et al. (2000). Free radicals in exhaustive physical exercise: mechanism of production, and protection by antioxidants. *IUBMB Life* **50**, 271-7.

Voets T, Janssens A, Prenen J et al. (2003). Mg^{2+}-dependent gating and strong inward rectification of the cation channel TRPV6. *J Gen Physiol* **121**, 245-60.

Voets T, Prenen J, Fleig A et al. (2001). CaT1 and the calcium release-activated calcium channel manifest distinct pore properties. *J Biol Chem* **276**, 47767-70.

Vollaard NB, Shearman JP, Cooper CE (2005). Exercise-induced oxidative stress:myths, realities and physiological relevance. *Sports Med* **35**, 1045-62.

Walsh NP, Gleeson M, Shephard RJ et al. (2011). Position statement. Part one: Immune function and exercise. *Exerc Immunol Rev* **17**, 6-63.

Wang JS, Huang YH (2005). Effects of exercise intensity on lymphocyte apoptosis induced by oxidative stress in men. *Eur J Appl Physiol* **95**, 290-7.

Watanabe H, Davis JB, Smart D et al. (2002a). Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. *J Biol Chem* **277**, 13569-77.

Watanabe H, Vriens J, Suh SH et al. (2002b). Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. *J Biol Chem* **277**, 47044-51.

Watson RR, Moriguchi S, Jackson JC et al. (1986). Modification of cellular immune functions in humans by endurance exercise training during beta-adrenergic blockade with atenolol or propranolol. *Med Sci Sports Exerc* **18**, 95-100.

Wissenbach U, Niemeyer BA, Fixemer T et al. (2001). Expression of CaT-like, a novel calcium-selective channel, correlates with the malignancy of prostate cancer. *J Biol Chem* **276**, 19461-8.

Woods JA, Ceddia MA, Wolters BW et al. (1999). Effects of 6 months of moderate aerobic exercise training on immune function in the elderly. *Mech Ageing Dev* **109**, 1-19.

Woods JA, Keylock KT, Lowder T et al. (2009). Cardiovascular exercise training extends influenza vaccine seroprotection in sedentary older adults: the immune function intervention trial. *J Am Geriatr Soc* **57**, 2183-91.

Wu MM, Buchanan J, Luik RM et al. (2006). Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. *J Cell Biol* **174**, 803-13.

Wuytack F, Raeymaekers L, Missiaen L (2002). Molecular physiology of the SERCA and SPCA pumps. *Cell Calcium* **32**, 279-305.

Xu H, Ramsey IS, Kotecha SA et al. (2002). TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature* **418**, 181-6.

Yang H, Wang T, Li J et al. (2006). Decreasing expression of alpha1C calcium L-type channel subunit mRNA in rat ventricular myocytes upon manganese exposure. *J Biochem Mol Toxicol* **20**, 159-66.

Yip L, Cheung CW, Corriden R et al. (2007). Hypertonic stress regulates T-cell function by the opposing actions of extracellular adenosine triphosphate and adenosine. *Shock* **27**, 242-50.

Yoon MJ, Lee HJ, Lee YS et al. (2007). Extracellular ATP is involved in the induction of apoptosis in murine hematopoietic cells. *Biol Pharm Bull* **30**, 671-6.

Yuan JP, Kiselyov K, Shin DM et al. (2003). Homer binds TRPC family channels and is required for gating of TRPC1 by IP3 receptors. *Cell* **114**, 777-89.

Yue L, Peng JB, Hediger MA et al. (2001). CaT1 manifests the pore properties of the calcium-release-activated calcium channel. *Nature* **410**, 705-9.

Zhang L, Barritt GJ (2004). Evidence that TRPM8 is an androgen-dependent Ca^{2+} channel required for the survival of prostate cancer cells. *Cancer Res* **64**, 8365-73.

Zhang SL, Yu Y, Roos J et al. (2005). STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. *Nature* **437**, 902-5.

Zweifach A, Lewis RS (1993). Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proc Natl Acad Sci USA* **90**, 6295-9.

Zweifach A, Lewis RS (1995). Slow calcium-dependent inactivation of depletion-activated calcium current. Store-dependent and -independent mechanisms. *J Biol Chem* **270**, 14445-51.