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H_2O_2 Sensitivity of K_v Channels in Hypoxic Pulmonary Vasoconstriction: Experimental Conditions Matter

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Abstract

Hypoxic pulmonary vasoconstriction (HPV) optimizes gas exchange but, when impaired, can result in life-threatening hypoxemia. Moreover, under conditions of generalized alveolar hypoxia, HPV can result in pulmonary hypertension. Voltage-gated K^+ channels (K_v channels) are key to HPV: a change in the intracellular hydrogen peroxide (H_2O_2) levels during acute hypoxia is assumed to modulate these channels' activity to trigger HPV. However, there are longstanding conflicting findings on whether H_2O_2 inhibits or activates K_v channels. Therefore, we hypothesized that H_2O_2 affects K_v channels depending on the experimental conditions, i.e., the H_2O_2 concentration, the channel's subunit configuration or the experimental clamping potential in electrophysiological recordings. Therefore, cRNAs encoding the $K_v1.5$ channel and the auxiliary $K_v\beta$ subunits ($K_v\beta1.1$, $K_v\beta1.4$) were generated via in vitro transcription before being injected into *Xenopus laevis* oocytes for heterologous expression. The K^+ currents of homomeric (Kv1.5) or heteromeric ($K_v1.5/K_v\beta1.1$ or $K_v1.5/K_v\beta1.4$) channels were assessed by two-electrode voltage clamp. The response of the K_v channels to H_2O_2 was markedly dependent on (a) the clamping potential, (b) the H_2O_2 concentration, and (c) the K_v channel's subunit composition. In conclusion, our data highlight the importance of the choice of experimental conditions when assessing the H_2O_2 sensitivity of K_v channels in the context of HPV, thus providing an explanation for the long-lasting controversial findings reported in the literature.

Keywords: hypoxic pulmonary vasoconstriction (HPV); K_v channels; $K_v\beta$ subunits; hydrogen peroxide (H_2O_2); two-electrode voltage clamp (TEVC); *Xenopus laevis* oocytes

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1. Introduction

Hypoxic pulmonary vasoconstriction (HPV) is a physiological mechanism of the mammalian lung that optimizes gas exchange. By rapidly matching perfusion to ventilation, HPV ensures optimal oxygenation of the blood during local alveolar O_2 shortage, i.e., hypoxia [1–3]. Impairment of this vital mechanism can result in insufficient blood oxygenation and thus O_2 undersupply to the organs. Such fatal hypoxemia due to disturbed HPV can occur in various clinical scenarios. This includes patients afflicted with pneumonia, those with acute respiratory distress syndrome (ARDS), or those requiring mechanical ventilation, e.g., COVID-19 patients during the recent pandemic [4]. Additionally, systemic administration of pulmonary vasodilatory agents, such as prostacyclin, has been associated

with similar adverse outcomes due to impaired HPV [2,5]. Moreover, imbalanced HPV is considered to provoke high-altitude pulmonary edema (HAPE), a life-threatening condition [5–7], and also acquires pathophysiological significance in situations with prolonged hypoxia, e.g., at high altitudes or in lung diseases such as sleep apnea or chronic obstructive pulmonary disease (COPD) [8–10]. In these conditions, generalized and consistent alveolar hypoxia can occur, leading to the constriction of the blood vessels in the entire lung. If hypoxia persists, the subsequent onset of vascular remodeling processes leads—in addition to vasoconstriction—to the manifestation of pulmonary hypertension, which can ultimately result in right heart failure.

Although HPV was already described in 1946 by von Euler and Liljestrand, the underlying molecular mechanisms are not yet fully known [3,11]. A mandatory step in HPV is the hypoxia-induced inhibition of O_2-sensitive voltage-gated potassium (K_v) channels in pulmonary artery smooth muscle cells (PASMCs) [12–16]. K_v channels are composed of four α subunits forming a central pore in the membrane and four auxiliary β subunits to fine-tune the channel's activity [13,17]. By mediating constant K^+ leakage, K_v channels significantly contribute to maintaining the cellular membrane potential [16,18,19]. It is undisputed that hypoxia induces the closure of K_v channels, which in turn depolarizes the membrane potential of PASMCs, thereby increasing the Ca^{2+} influx via the activation of voltage-gated Ca^{2+} channels [2,20,21]. The resulting increase in intracellular Ca^{2+} induces the constriction of the individual cells and thus the pulmonary arterial vessels. Although much progress has been made in understanding the signaling pathways underlying O_2 sensing in HPV, the molecular mechanisms by which hypoxia affects the K_v channel activity are still not fully resolved [13].

A proposed signaling mechanism linking hypoxia to the K_v channel function in PASMCs involves reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) in particular [3,13,22–24]. Although there is a consensus on the involvement of ROS in hypoxia-induced K_v channel inhibition, there is a long-lasting disagreement as to whether hypoxia induces an increase or decrease in ROS [2,13]. The original redox hypothesis of Archer, Weir, and colleagues proposes that under normoxic conditions, a certain level of intracellular ROS, i.e., H_2O_2, maintains the K_v channels in an oxidized open state. Conversely, acute hypoxia causes an increase in the $NADH/NAD^+$ ratio and a concomitant decrease in the H_2O_2 level that shifts the cellular redox state to a more reduced state, resulting in K_v channel inhibition and subsequent vasoconstriction [12,17,25–27]. In contrast, Paul Schumacker's lab presented an opposing concept, often referred to as the mitochondrial ROS theory, which describes a hypoxia-induced increase in the intracellular ROS levels being causative for the inhibition of K_v channels [13,28]. This theory was not only confirmed by previous studies by our research group, but we could also identify the signaling molecule responsible as H_2O_2 [3] and further decipher the role of mitochondria in this regard [3,22]. Although there is a large body of evidence for increased ROS levels during acute hypoxia underlying HPV, both theories emphasize that the K_v channels are inhibited by the indisputable hypoxia-induced change in the intracellular H_2O_2 levels [3,13].

Consequently, several studies investigated the influence of H_2O_2 on K_v channel activity. In line with the discrepancies between the two distinct theories, contrary results were also reported by distinct research groups in this context. While some studies showed an activation of the K_v channels in response to H_2O_2 [29–31], other groups observed an H_2O_2-induced inhibition of these channels [3,32–34]. Upon thorough examination of these reports, discrepancies in the experimental conditions became apparent, specifically the widely varying and occasionally nonphysiologically high concentrations of H_2O_2 utilized. Furthermore, the studies were conducted at distinct experimental clamping potentials and on very different cell types, including various primary cells as well as

overexpression systems that may be featured by different subunit compositions of K_v channels. In particular, the auxiliary $K_v\beta$ subunits are differentially expressed in the pulmonary circulation [35]. The reported correlation between their expression level and the strength of HPV suggests a role for these subunits in O_2 sensitivity [35]. Thus, the presence or absence of $K_v\beta$ subunits could be responsible for the different responses of K_v channels to H_2O_2, thereby delivering a potential explanation for the contrary results described in the literature.

Considering the controversy as to whether H_2O_2 inhibits or activates K_v channels, we hypothesized that H_2O_2 regulates K_v channel activity (a) in a concentration-dependent manner and/or (b) in a manner dependent on the molecular composition of the channel, i.e., the presence or absence of auxiliary $K_v\beta$ subunits.

2. Results

2.1. Homomeric Kvβ Subunits Do Not Form Functional Kv Channels

To address these questions, we selected the $K_v1.5$ channel as a suitable candidate. $K_v1.5$ has long been recognized as a channel involved in HPV [36,37]. In line with this, mice lacking $K_v1.5$ exhibited impaired HPV [38], which could be fully restored by its subsequent introduction via gene transfer in vivo [39]. To address the H_2O_2 sensitivity of $K_v1.5$, this subunit was heterologously expressed either as a homomeric or heteromeric channel, i.e., in combination with either $K_v\beta1.1$ or $K_v\beta1.4$ subunits in *Xenopus laevis* oocytes, which are supposed to not express endogenous K_v channels [40,41]. The potassium (K^+) currents were assessed via the two-electrode voltage clamp technique (TEVC) by applying depolarizing 200 ms voltage pulses from a holding potential of −60 mV to +50 mV in 10 mV steps. Neither water-injected control oocytes (Figure 1a) nor oocytes injected with the auxiliary subunits $K_v\beta1.1$ (Figure 1b) or $K_v\beta1.4$ (Figure 1c) alone exhibited voltage-dependent currents when depolarized. In contrast, oocytes injected with cRNA-encoding $K_v1.5$ subunits showed typical rapid outward K^+ currents upon depolarization to potentials more positive than −20 mV (Figure 1d). In agreement with previous studies, the homomeric $K_v1.5$ currents exhibited no detectable inactivation in response to such short pressure pulses [42]. Co-expression with $K_v\beta1.1$ altered the $K_v1.5$ activity by inducing a rapid voltage-dependent inactivation (Figure 1e), while co-expression with $K_v\beta1.4$ lowered the currents' amplitude (Figure 1f). The final application of 4-aminopyridine (4-AP) largely inhibited the K^+ currents (Figure 1d–f), which confirmed the successful expression of the three K_v channel combinations.

2.2. Evaluation of H_2O_2 Decomposition in the Experimental Setup

Beside the temperature and the presence of decomposing impurities, the stability of H_2O_2 in aqueous solution is affected by the pH value [43]. For optimum stability, the pH of pure H_2O_2 is below pH 4.5, while increasing it to above pH 5 sharply increases the decomposition of H_2O_2 into water and oxygen. Since the pH value of the oocyte Ringer's solution (ORi) used in our experiments was at a physiological value of pH 7.4, we first tested the decomposition of H_2O_2 in our experimental setup via an Amplex Red Hydrogen Peroxide/Peroxidase Assay. As schematically shown in Figure 2a, the ORi in the reservoir was analyzed directly after adding H_2O_2 to the perfusate (input). Afterwards, the perfusion was started to deliver the H_2O_2-containing ORi to the recording chamber. The second sample was then taken from the recording chamber 90 s post starting the perfusion—which corresponds to the time point at which the K^+ currents were recorded in the electrophysiological experiments. We observed that at the time point of current recording, approximately 50% of the H_2O_2 of the distinct input concentrations was decomposed (Figure 2b).

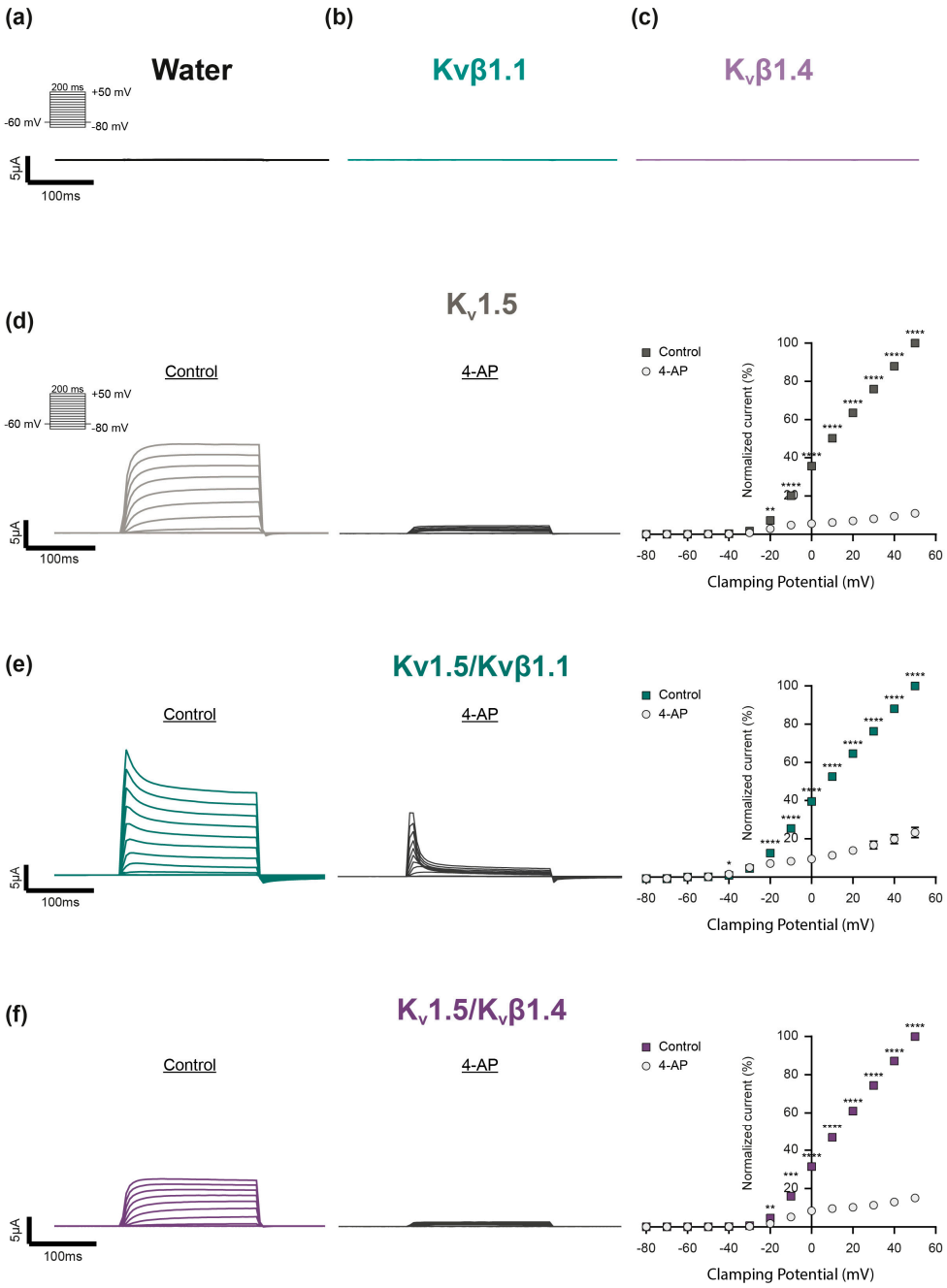

Figure 1. Expression and electrophysiological characterization of distinct K_v channel combinations in *Xenopus laevis* oocytes. (**a**–**c**) Representative current traces of control oocytes exposed to depolarizing voltage steps. Neither (**a**) water (black) nor (**b**) homomeric K_vβ1.1 (light turquois) or (**c**) K_vβ1.4 subunits (light purple) built functional channels. (**d**–**f**) Electrophysiological characterization of (**d**) homomeric K_v1.5 channels (gray), (**e**) K_v1.5 co-expressed with the auxiliary subunits K_vβ1.1 (K_v1.5/K_vβ1.1, dark turquoise) or (**f**) together with K_vβ1.4 (K_v1.5/K_vβ1.4, dark purple). Current traces were elicited by depolarizing voltage pulses (200 ms) between −80 mV and +50 mV in 10 mV steps from a holding potential of −60 mV before (Control) and after application of the K_v channel inhibitor 4-aminopyridine (4-AP) to validate the successful K_v channel expression. The current–voltage relationship (I–V curve) was determined by plotting the averaged current amplitude (plateau) against the corresponding voltage step (K_v1.5: n = 13; K_v1.5/K_vβ1.1: n = 12; K_v1.5/K_vβ1.4: n = 13). The mean K_v current amplitudes were each normalized relatively to the current value at + 50 mV under control conditions (no 4-AP). Data were statistically analyzed by 2-way ANOVA and the uncorrected Fisher's least significant difference test for multiple comparisons (*: $p \leq 0.05$; **: $p \leq 0.01$, ***: $p \leq 0.001$; ****: $p \leq 0.0001$).

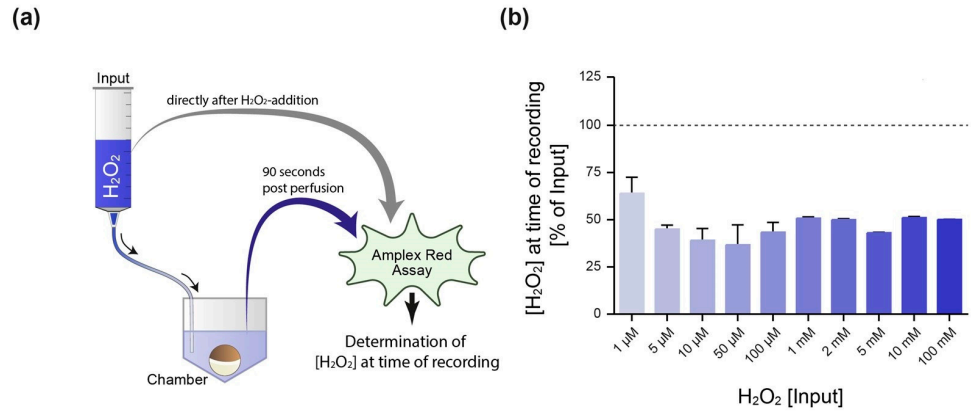

Figure 2. Determination of the H_2O_2 concentration present at the time point of electrophysiological recording. (**a**) Schematical illustration of the experimental setup. Oocyte Ringer's solution (ORi) was supplemented with H_2O_2 within the perfusion reservoir (input). One sample was taken directly after H_2O_2 addition (input), while the second sample was extracted from the recording chamber 90 s post starting the perfusion—which represents the time point at which the electrophysiological recording was performed. Both samples were subjected to an Amplex Red Hydrogen Peroxide/Peroxidase Assay to determine the H_2O_2 decomposition for distinct concentrations within the experimental setup, i.e., the H_2O_2 concentration at the time of recording (**b**). The dotted line represents the input concentration (100%).

2.3. Experimental Approach to Evaluate the Effect of H_2O_2 on K_v Channel Activity

For evaluation of the effect of H_2O_2 on K_v channel activity, K_v currents were elicited by depolarizing voltage pulses between −80 mV and +50 mV in 10 mV steps from a holding potential of −60 mV in the absence (Control, Figure 3a) and after the addition of H_2O_2 (Figure 3b). As shown in Figure 3c, H_2O_2 (10 mM) significantly activated K^+ currents at all the clamping potentials of −30 mV and more positive—with a prominent effect at −20 mV, a value within the physiological range of the membrane potential of PASMCs under hypoxia [3,23]. Therefore, two distinct voltage steps were chosen for statistical analysis in all the following experiments: the one that lies within the physiological range of the membrane potential of PASMCs (−20 mV) and one at a positive membrane potential, which was characterized by the strongest H_2O_2-induced activation (+50 mV).

2.4. The Effect of H_2O_2 on Homomeric K_v1.5 Channels Is Voltage- and Concentration-Dependent

To assess the effect of distinct H_2O_2 concentrations on the activity of homomeric K_v1.5 channels, H_2O_2 was applied in concentrations between 0.01 µM and 100 mM (Figure 4a,b), using a new oocyte and freshly prepared H_2O_2 for each individual experiment. As previously described, the K^+ current amplitudes were analyzed at step potentials of −20 mV (Figure 4a) and +50 mV (Figure 4b) to determine the potential voltage-dependency of the H_2O_2 effect. In our investigation, we observed dependencies on both voltage and concentration: while low H_2O_2 concentrations (ranging from 0.1 µM to 1 mM) did not affect the activity of K_v1.5 channels at a physiological step potential of −20 mV (Figure 4a), these same concentrations were found to inhibit the channels at a positive step potential (+50 mV, Figure 4b). Interestingly, higher H_2O_2 concentrations (10 mM and above) were observed to enhance the K^+ currents at −20 mV (Figure 4a), yet no such effect was noted for the same concentrations at +50 mV (Figure 4b). These findings underscore a dual relationship involving both the voltage- and concentration-dependency of H_2O_2 modulation on K_v1.5 channel activity.

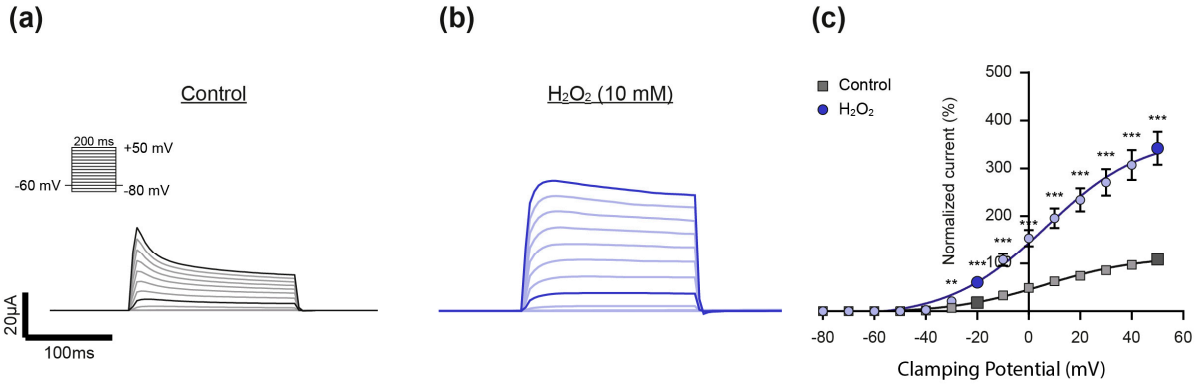

Figure 3. Experimental approach to evaluate the effect of H_2O_2 on K_v channel activity. (**a**) Representative current trace of a K_v-channel-expressing oocyte that was exposed to depolarizing voltage steps in the (**a**) absence (Control, gray) and (**b**) presence of H_2O_2 (10 mM, blue), as well as (**c**) the corresponding statistical analysis. The current–voltage relationship (I–V curve) was determined by plotting the averaged current amplitude (plateau) against the corresponding voltage step (control: n = 12; H_2O_2: n = 11). The I–V curve was fitted using the Boltzmann equation. No differences in the V_{50} (Control: 7.1 ± 1.6 mV, H_2O_2; 6.1 ± 1.4 mV; $p = 0.6523$) and gating charge (q) (control: 1.4 ± 0.038 e_0, H_2O_2: 1.4 ± 0.028 e_0; $p = 0.4252$) were observed in response to H_2O_2. The mean K_v current amplitudes were each normalized relatively to the current amplitude in the control condition at +50 mV. H_2O_2 increased the channel's activity at clamping potentials more positive than −30 mV, with a prominent effect at −20 mV, a value within the physiological range of the membrane potential of PASMCs. Therefore, clamping potentials of −20 mV and +50 mV (characterized by the strongest H_2O_2-induced activation) were chosen as the clamping potentials for analysis in all the following experiments (respective current traces and data points are highlighted in darker colors). Data were statistically analyzed by 2-way ANOVA and uncorrected Fisher's least significant difference test for multiple comparisons (**: $p \leq 0.01$, ***: $p \leq 0.001$).

2.5. Co-Expression of K_v1.5 with $K_v\beta$ Subunits Modulates the Channel's Response to H_2O_2

To determine whether co-expression with accessory β subunits alters the channel's response to H_2O_2, K_v1.5 was co-expressed with the β-isoform $K_v\beta$1.1 and the channels' response to H_2O_2 was assessed by TEVC (Figure 4c,d). While low H_2O_2 concentrations (0.01 µM–10 µM) did not affect the activity of the K_v1.5/$K_v\beta$1.1 channels at a step potential of −20 mV (Figure 4c), the same concentrations significantly inhibited the K_v1.5/$K_v\beta$1.1 currents at +50 mV (see enlargement in Figure 4d). Importantly, this inhibition was induced even with the lowest H_2O_2 concentration, i.e., 0.01 µM. In contrast, application of higher H_2O_2 concentrations (1 mM to 100 mM) significantly and concentration-dependently increased the K^+ currents at both step potentials, i.e., −20 mV and +50 mV (Figure 4c,d).

To assess whether distinct isoforms differently modulate the response of K_v1.5 to H_2O_2, an additional $K_v\beta$ isoform ($K_v\beta$1.4) was co-expressed with K_v1.5 (Figure 4e,f). In contrast to the homomeric K_v1.5 and heteromeric K_v1.5/$K_v\beta$1.1 channels, this channel combination was significantly inhibited by low H_2O_2 concentrations at −20 mV (Figure 4e). Importantly, this inhibition occurred under physiologically relevant conditions in terms of both the clamping potential and the H_2O_2 concentration. Notably, K_v1.5/$K_v\beta$1.4 was the only channel combination that remained unaffected by low H_2O_2 concentrations at a depolarized clamping potential (+50 mV) while being inhibited when exposed to high concentrations of 500 µM and above (Figure 4f).

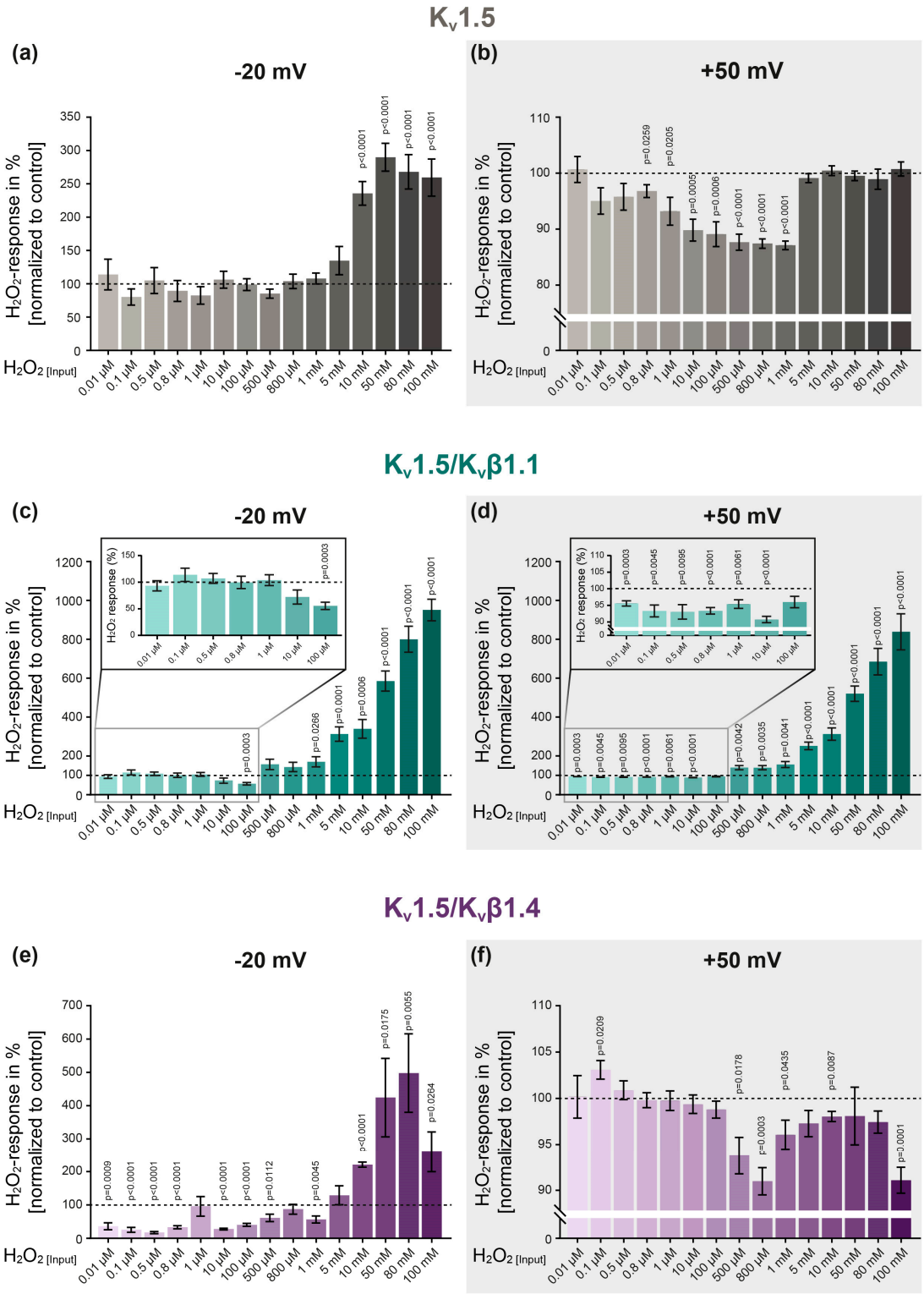

Figure 4. The effect of H_2O_2 on K_v channels is dependent on the concentration, step potential and ion channel subunit composition. (**a**) The effect of increasing H_2O_2 concentrations on homomeric $K_v1.5$ channels at step potentials of −20 mV and (**b**) +50mV (light gray background). (**c**,**d**) Assessment of the H_2O_2 response of heteromeric $K_v1.5/K_v\beta1.1$ as well as (**e**,**f**) $K_v1.5/K_v\beta1.4$ channels. The H_2O_2 response was assessed by normalizing the current amplitude upon H_2O_2 application to the control conditions (represented by the dotted line). A new oocyte and freshly prepared H_2O_2 were used for each experiment. Number of experiments per H_2O_2 concentration: n = 8–13 ($K_v1.5$); n = 8–13 ($K_v1.5/K_v\beta1.1$); n = 6–13 ($K_v1.5/K_v\beta1.4$). For statistical analysis, one-sample *t*-tests were performed, with $p \leq 0.05$ being considered indicative of significance. Group data are expressed as the mean ± SEM.

3. Discussion

It is well accepted that a shift in the intracellular ROS levels in response to acute hypoxia inhibits K_v channels in PASMCs, thereby triggering HPV. However, although the signaling molecule responsible could be identified as H_2O_2 [3], long-lasting divergences and contradictory reports exist in the literature on whether H_2O_2 inhibits or activates K_v channels. We hypothesized that these contrary observations depend on (a) the experimental clamping potential, (b) the H_2O_2 concentration utilized and/or (c) the K_v channel subunit composition, i.e., the presence of auxiliary $K_v\beta$ subunits. Therefore, the aim of the present study was to determine whether H_2O_2 modulates K_v channel activity in a concentration-dependent manner, and at the same time, to address the potential role of the accessory β subunits $K_v\beta1.1$ and $K_v\beta1.4$ as well as the experimental clamping potential in H_2O_2 sensitivity. For our studies, we heterologously expressed $K_v1.5$ as a homomeric channel and in combination with either $K_v\beta1.1$ or $K_v\beta1.4$, respectively, before exposing them to H_2O_2 at various concentrations between 0.01 µM and 100 mM. We then analyzed the K_v currents at two distinct clamping potentials, i.e., (a) at −20 mV, which falls within the physiological resting membrane potential of PASMCs and (b) at +50 mV, which was characterized by the highest voltage-induced current.

As an expression system, we used *Xenopus laevis* oocytes that are supposed to not express endogenous 4-AP-sensitive K_v channels [40,41]. This assumption was confirmed in our study as neither the water-injected control oocytes, nor the oocytes injected with $K_v\beta$ subunits alone exhibited voltage-induced currents upon depolarization, thereby confirming the choice of the expression system as ideally suited for investigating the effect of H_2O_2 on defined K_v channel combinations.

3.1. The Reaction of K_v Channels to H_2O_2 Depends on the Experimental Clamping Potential

K_v currents are typically measured at positive clamping potentials of up to +70 mV [29,34], which not only ensures channel activation but also maximizes the driving force for K^+ efflux, thereby facilitating current detection and analysis. However, such positive clamping potentials do not reflect physiological conditions, as the membrane potentials in PASMCs typically range from approximately −50 mV to −10 mV [3,44]. As a result, the K_v channel behavior observed at such strongly positive (depolarized) voltages may not fully reflect their natural activation, inactivation, or response to pharmacological modulation under physiological conditions. In line with this, we observed a clear voltage-dependency in the response to H_2O_2 for all three channel combinations, i.e., $K_v1.5$, $K_v1.5/K_v\beta1.1$ and $K_v1.5/K_v\beta1.4$ (see Figure 4). For example, at a depolarizing voltage step of +50 mV, the homomeric $K_v1.5$ channels were inhibited by low concentrations of H_2O_2 (0.8 µM–1 mM), while higher H_2O_2 concentrations (5 mM–100 mM) had no effect on the channels' activity. In contrast, at a physiological clamping potential of −20 mV, the channels exhibited a completely different response pattern, being unaffected by low H_2O_2 concentrations but strongly activated by H_2O_2 concentrations of 10 mM and above. Similar observations of voltage-dependent effects were also made for the heteromeric channels, i.e., $K_v1.5/K_v\beta1.1$ and $K_v1.5/K_v\beta1.4$. These data clearly indicate that the response of the K_v channels to H_2O_2 is markedly dependent on the experimental clamping potential.

However, it is important to consider that—in contrast to *Xenopus laevis* oocytes—in both primary cells and overexpression systems (particularly mammalian cell lines), endogenous ion channels might be active at physiological clamping potentials and contribute to the recorded currents, thus potentially confounding the detection and analysis of K_v-channel-specific responses. Furthermore, at physiological clamping potentials, the electrochemical driving force for K^+ is diminished, resulting in reduced current amplitudes and less pronounced differences, thereby potentially masking subtle differences that are more apparent

under depolarized conditions. Thus, the choice of clamping protocol constitutes a critical experimental parameter that can profoundly affect the biophysical properties and pharmacological response of K_v channels to H_2O_2.

3.2. The Effect of H_2O_2 on K_v Channel Activity Is Concentration-Dependent

The estimation of the intracellular concentration of H_2O_2 is based on the kinetics of production and elimination, while its determination is still technically difficult [45,46]. The physiological estimate of the cytosolic H_2O_2 concentration has been reported to be in the pico- to nanomolar range, e.g., 2.2 nM [47], 1–700 nM [48], 1–10 nM [46] or even in the picomolar range [47,49]. Consequently, H_2O_2 concentrations above the nanomolar range are regarded as pathological levels that are related to oxidative stress [46].

In previous studies investigating the effect of H_2O_2 on K_v channel activity, various concentrations of H_2O_2 were applied, resulting in contradictory outcomes. Higher concentrations, ranging from 0.1 to 10 mM, were reported to activate K_v channels [29–31]. In contrast, other studies employing lower concentrations, i.e., between 5 µM and 400 µM, observed an inhibitory effect on K_v channel activity [32–34]. These divergent observations prompted us to hypothesize that the concentration of H_2O_2 used under experimental conditions underlies the reported inconsistencies in H_2O_2-mediated K_v channel modulation, i.e., whether H_2O_2 inhibits or activates K_v channels. Based on this rationale, we applied a broad range of H_2O_2 concentrations, spanning from physiological to pathophysiological levels (10 nM to 100 mM).

At a physiological clamping potential of −20 mV, we observed the clear concentration-dependency of H_2O_2 on the homomeric $K_v1.5$ channel activity: whereas H_2O_2 did not affect the $K_v1.5$ activity at physiological concentrations, an activation of up to 200% was observed in response to pathophysiological concentrations of 10 mM and above. As discussed in the previous paragraph, a different response pattern was observed at a depolarized clamping potential (+50 mV), although there was still a clear difference between the physiological and pathophysiological H_2O_2 concentrations. Interestingly, the channel's response to high doses of H_2O_2 appeared to be an "all or nothing" effect as no difference was observed between the concentrations applied. This indicates that oxidation by H_2O_2 above a certain level might induce a conformational change within the channel structure that increases the channel's activity. However, although $K_v1.5$ has been implicated in HPV and is therefore presumed to be inhibited by hypoxia-induced H_2O_2 release, we did not observe an inhibition of homomeric $K_v1.5$ in response to H_2O_2 at physiological clamping potentials—regardless of the H_2O_2 concentration applied. Given that the accessory β subunits are reported to exert regulatory functions on K_v channels, including modulation of their redox sensitivity [50], we next investigated their influence on the H_2O_2 sensitivity of $K_v1.5$ channels.

3.3. $K_v\beta$ Subunits Modulate the Response of $K_v1.5$ to H_2O_2

Interestingly, the H_2O_2-induced activation in response to pathophysiological concentrations above 10 mM could be further potentiated by the co-expression of $K_v1.5$ with $K_v\beta1.1$. In these heteromeric channels ($K_v1.5/K_v\beta1.1$), H_2O_2 of 1 mM and above induced a channel activation of up to 900%, which in contrast to the homomeric $K_v1.5$ channels, was clearly concentration-dependent. This observation suggests that the accessory β subunits not only alter the channel's activity per se, as described in several studies [51–53], but also equip them with the ability to fine-tune the kinetics of $K_v1.5$ channels in response to H_2O_2, most likely as a result of their oxidoreductase properties [50]. However, an H_2O_2-induced inhibition of the channel, as would be necessary for HPV initiation, was only observed in response to an H_2O_2 concentration of 100 µM—which, however, is not in the assumed physiological range of intracellular H_2O_2 concentrations [46].

The co-expression of $K_v1.5$ with the auxiliary isoform $K_v\beta1.4$ rendered the channel sensitive to inhibition by physiological H_2O_2 concentrations—even as low as 10 nM. Most interestingly, this inhibition was observed at a physiological clamping potential—thereby closely reflecting the physiological conditions present in PASMCs under hypoxia. Given that H_2O_2 was decomposed by approximately 50% under our experimental conditions, this suggests that $K_v1.5/K_v\beta1.4$ channels can be inhibited by H_2O_2 concentrations of less than 10 nM. Sommer et al. reported in a study from our laboratory that 124 nM H_2O_2 inhibits the K_v currents in primary mouse PASMCs [3], thereby contributing to the initiation of HPV. This finding is consistent with the present data, which demonstrate that even lower concentrations of H_2O_2 can significantly inhibit K_v currents, an effect that is—at least for Kv1.5—critically dependent on the clamping potential and the ion channel composition, particularly the presence of the auxiliary subunit $K_v\beta1.4$.

It should be noted, however, that in addition to $K_v1.5$, a variety of other K_v channel subunits contribute to HPV initiation and further studies would be required to investigate the effect of H_2O_2 on these channels as well. However, our study clearly highlights the importance of carefully selecting experimental parameters—particularly the necessity of utilizing physiological conditions—when attempting to elucidate a physiological process.

3.4. Conclusions

In conclusion, our findings indicate that the response of K_v channels to H_2O_2 is markedly dependent on (a) the experimental clamping potential, (b) the H_2O_2 concentration and (c) the K_v channel subunit composition. Thus, our data clearly highlight the importance of the choice of experimental conditions when assessing the H_2O_2 sensitivity of K_v channels in the context of HPV, thus providing an explanation for the long-lasting controversial findings reported in the literature.

4. Materials and Methods

4.1. Generation of cRNA for Heterologous Expression of K_v Channels

Plasmids encoding murine $K_v1.5$ (NM_145983.2), $K_v\beta1.1$ (NM_010597.4) or $K_v\beta1.4$ (NM_001403872.1) in a pTNT expression vector (Promega, Madison, WI, USA) were purchased from the Eurofins gene synthesis service (Eurofins, Ebersberg, Germany) and transformed into competent JM109 *E. coli* cells (Promega, Madison, WI, USA) for amplification. Upon subsequent plasmid purification using the GenElute Plasmid Miniprep Kit (Sigma Aldrich, St Louis, MO, USA) according to the manufacturer's instructions, the purified plasmids were used for cRNA synthesis via in vitro transcription using the mMESSAGE mMACHINE T7 kit (Invitrogen, Waltham, MA, USA). The resulting full-length capped cRNAs were then purified using the MEGAclear transcription cleaning kit (Invitrogen, Waltham, MA, USA). The cRNAs were stored at –80 °C and diluted in nuclease-free water immediately prior to injection.

4.2. Preparation, Storage and Injection of Xenopus Laevis Oocytes

Xenopus laevis oocytes at stages V–VI were purchased from Ecocyte Bioscience (Dr. Lohmann Diaclean GmbH, Dortmund, Germany) and plated individually in a 96 Conical Bottom MicroWell Plate (Fischer Scientific, Schwerte, Germany) filled with modified Barth's solution (MBS, in mM: NaCl 88, KCl 1, $CaCl_2$ 0.4, $Ca(NO_3)_2$ 0.33, $MgSO_4$ 0.8, TRIS-HCl 5, $NaHCO_3$ 2.4, pH 7.4, Dr. Lohmann Diaclean GmbH, Dortmund, Germany) that was supplemented with sodium pyruvate (2.5 mM, Sigma Aldrich, St Louis, MO, USA), penicillin (20 μg/mL, Sigma Aldrich, St Louis, MO, USA) and streptomycin (25 μg/mL, Sigma Aldrich, St Louis, MO, USA). The oocytes were stored in a digital mini-incubator (Gilson, Middleton, WI, USA) at 5–8 °C until use.

At 24–48 h prior to the electrophysiological recordings, the oocytes were injected with K_v1.5: 0.1 ng/oocyte; K_vβ1.1: 1.75 ng/oocyte; K_vβ1.4: 0.1 ng/oocyte in a total injection volume of 10 nL/oocyte (for K_v1.5 and K_v1.5/K_vβ1.4, resp.) and 17.5 nL/oocyte (for K_v1.5/K_vβ1.1) using the Roboinject fully automated injection system (Multichannel Systems, Reutlingen, Germany). Therefore, the injection needle was filled with mineral oil (Sigma Aldrich, St Louis, MO, USA) before being mounted onto the device. Afterwards, the cRNAs that were diluted in nuclease-free water were drawn into the injection needle and injected into the oocytes. The impalement and injection depth for the cRNAs were defined for all the samples as 550 µm and 450 µm, respectively. The injected oocytes were incubated for 24 h at 18 °C for heterologous expression.

4.3. Electrophysiological Recordings via Two-Electrode Voltage Clamp (TEVC)

The K_v currents of the cRNA injected oocytes were recorded via the two-electrode voltage clamp (TEVC) technique using an automated TEVC system (Roboocyte2, Multichannel Systems, Reutlingen, Germany). Therefore, the glass capillaries of the measure head were filled with 1 M KCl. Only electrodes with tip resistances between 300 and 800 kΩ were used for the experiments. All the electrophysiological recordings were carried out at room temperature (20–22 °C) under continuous perfusion with oocyte Ringer's solution (ORi; Normal Frog Ringer, Ecocyte Biosciences, composition in mM: NaCl 64, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 26). The ORi inside the perfusion reservoir was continuously gassed with 21% O_2, 5.3% CO_2, 73.7 N_2 for pH stabilization at pH 7.4. Due to the rapid decomposition of H_2O_2 at physiological pH values, the H_2O_2 (Sigma Aldrich, St Louis, MO, USA) was freshly prepared directly before the start of each individual experiment. Being a strong oxidizing agent, H_2O_2 has the potential to corrode Ag/AgCl electrodes upon direct contact, which would manifest in a shift of the baseline current. Anticipating this potential issue, a rigorous maintenance protocol involving regular and frequent re-chlorination and replacement of the electrodes was implemented. Additionally, the baseline current was continuously monitored to ensure stability throughout the experiments.

For the electrophysiological recordings, the oocytes were voltage-clamped at a holding potential of −60 mV. The current amplitudes were elicited by depolarizing voltage pulses in 10 mV increment steps starting from −80 mV to +50 mV and 200 ms duration.

4.4. Experimental Design and Analysis

As a current rundown was already observed in the control recordings (two recordings in succession with an interval of 90 s between the two recordings), a different procedure was used. Two sets of experiments were performed for each experiment. As a control, two recordings were performed in the absence of H_2O_2 (control), separated by 90 s. In the second set of experiments, only one control recording was performed, before H_2O_2 was delivered to the recording chamber for 90 s. The recording taken after the 90 s (+H_2O_2) was then compared with the second current trace from the control measurements (−H_2O_2). The recordings +/−H_2O_2 were always performed on oocytes obtained from the same individual. The mean K_v current amplitudes were normalized relatively to the current at +50 mV in the control experiment. All the current traces were leak-subtracted prior to statistical evaluation. The current–voltage relationship (I–V curve) was obtained by plotting the averaged plateau current against the respective clamping potential. The specific blocker 4-aminopyrimidine (4-AP, 10 mM) was added at the end of experiment to validate the K_v channel expression.

4.5. Amplex Red Hydrogen Peroxide/Peroxidase Assay

To determine the decomposition of H_2O_2 inside the experimental system, H_2O_2 was added to the perfusate and delivered to the recording chamber via the perfusion system.

At 90 s post starting the perfusion, a sample was taken from the recording chamber, representing the time point where the electrophysiological recording (+H_2O_2) was performed. The H_2O_2 concentration (input, i.e., [H_2O_2] present at time of recording) was determined by Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The assay was performed according to the supplier's instructions. The absorbance was measured at 560/590 nm using a microplate reader TECAN Spark 10M (Tecan Trading AG, Männedorf, Switzerland). The manuscript consistently reports the input H_2O_2 concentration. However, it should be considered that—due to the decomposition of H_2O_2—the effective concentration at the oocyte during the electrophysiological recordings is lower (see Figure 2).

4.6. Statistical Analysis

The statistical differences were assessed via two-way ANOVA and the uncorrected Fisher's least significant difference test for multiple comparisons or one-sample *t*-tests. All the analyses were considered statistically significant at $p \leq 0.05$. Data are expressed as the mean ± SEM. The statistical analysis was performed using Prism 10 (GraphPad Software Inc., San Diego, CA, USA).

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Abbreviations

The following abbreviations are used in this manuscript:

ARDS	acute respiratory distress syndrome
cRNA	complementary ribonucleic acid
HPV	hypoxic pulmonary vasoconstriction
I–V curve	current–voltage relationship
K_v channel	voltage-gated potassium channel
nM	nanomolar
ORi	oocyte Ringer's solution
PASMC	pulmonary arterial smooth muscle cell
ROS	reactive oxygen species
TEVC	two-electrode voltage clamp
4-AP	4-aminopyridine

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