Kv12.1 channels are not sensitive to GqPCR-triggered activation of phospholipase Cβ

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ABSTRACT

Kv12.1 K+ channels are expressed in several brain areas, but no physiological function could be attributed to these subunits so far. As genetically-modified animal models are not available, identification of native Kv12.1 currents must rely on characterization of distinct channel properties. Recently, it was shown in Xenopus laevis oocytes that Kv12.1 channels were modulated by membrane PI(4,5)P₂. However, it is not known whether these channels are also sensitive to physiologically-relevant PI(4,5)P₂ dynamics. We thus studied whether Kv12.1 channels were modulated by activation of phospholipase Cβ (PLCβ) and found that they were insensitive to receptor-triggered depletion of PI(4,5)P₂. Thus, Kv12.1 channels add to the growing list of K+ channels that are insensitive to PLCβ signaling, although modulated by PI(4,5)P₂ in Xenopus laevis oocytes.

INTRODUCTION

The ether-à-go-go-gene-like (Elk; Kv12) family of voltage-gated potassium (Kv) channels comprises three members (Kv12.1-Kv12.3) that are predominantly expressed in neurons of various brain regions [1-6]. Despite recent significant progress in understanding biophysical properties and characteristics of these channels [7-10], at present only little information is available on physiological functions of the family members. Genetic deletion and pharmacological inhibition revealed that Kv12.2 channels constitute subthreshold K+ conductance regulating excitability of pyramidal neurons in hippocampus [11], but no native physiological relevance could be attributed to Kv12.1 and Kv12.3 subunits so far. As Kv12.1 channels activate at hyperpolarized membrane potentials [7,8,10], it is reasonable to assume that these channels also modulate neuronal excitability.

A prominent and characteristic feature of Kv12.1 channels is a mode shift of activation (also referred to as pre-pulse facilitation or voltage-dependent potentiation) [7,8,12]. This biophysical phenomenon describes hysteresis of voltage-dependent channel activation that most probably is caused by time-dependent stabilization of the channel’s voltage sensor in a “relaxed” open state in response to depolarized (conditioning) holding potentials [13,14]. It was shown recently that prolonged depolarization of the membrane potential induces slow rearrangement of a structural interaction between domains in the C- and N-terminus of Kv12.1 channels [8]. This rearrangement apparently is coupled to channel gating and necessary for transition of Kv12.1 channels into the more stable gating mode that favors channel opening [8]. Accordingly, conditioning depolarisation of the membrane potential causes a large shift in voltage dependence of activation to hyperpolarized potentials and a slowing of deactivation of Kv12.1 channels [7,8,10]. This mode shift may constitute a biophysical adaptation to dampen excitability of neurons upon ongoing stimulation possibly also to prevent hyperexcitability in nervous tissue. Analogous mode shift of related Kv11.1 channels might contribute to repolarization of cardiac action potentials through slowing of channel deactivation [15,16], but to our knowledge physiological relevance of this mode shift in neurons has not been demonstrated.

Recently, it was shown that Kv12.1 channel activity was regulated through membrane-associated phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂)
that is a minor component of the inner leaflet of the plasma membrane and a well-known co-factor of many ion channels [17-19]. Li and colleagues showed that loss of phosphoinositides (PI) induced by excision of membrane patches from *Xenopus laevis* oocytes caused acceleration of Kᵥ12.1 channel activation and deactivation and a potentiation of steady-state K⁺ currents. Furthermore, excision of membrane patches expressing Kᵥ12.1 channels caused a shift of voltage dependence of channel activation to hyperpolarized potentials and almost completely eliminated voltage-dependent mode shift [7]. As application of a water soluble PI(4,5)P₂ analogue restored Kᵥ12.1 channel kinetics as well as voltage dependence, and partially brought-back mode shift, the authors consistently concluded that PI(4,5)P₂ in a bimodal way of action stabilized the open state, but at the same time inhibited voltage dependent activation of Kᵥ12.1 channels [7]. Accordingly, variations of membrane PI(4,5)P₂ levels might control excitability of neurons through modulating Kᵥ12.1 channel activity. Most interestingly, impact of PI(4,5)P₂ level changes on neuronal excitability might vary considerably with the general excitation status of the neuron, as the extent of mode shift apparently also was PI-dependent [7]. Unfortunately, it has not been shown yet whether Kᵥ12.1 channels were sensitive to physiologically-relevant PI(4,5)P₂ depletion at all. As activation of phospholipase C β (PLCβ) through Gq protein-coupled receptors (GqPCR) constitutes an important signaling pathway to deplete PI(4,5)P₂ in neurons [20,21], we studied whether Kᵥ12.1 channels were modulated by activation of Gq protein-coupled muscarinic receptors.

**Results**

**Analyzing voltage-dependent mode shift of Kᵥ12.1 channels**

We analyzed sensitivity to GqPCR signaling of human Kᵥ12.1 channels heterologously expressed in CHO cells. To be able to analyze whether voltage-dependent activation of Kᵥ12.1 channels was sensitive to activation of the GqPCR pathway, we first established appropriate voltage protocols to study voltage dependence of these channels. As recently reported [10], we applied depolarizing holding potentials (conditioning voltage; 200 ms) before a series of activating voltage steps (pulse potentials from −140 mV to +10 mV; 600 ms) (Figure 1(A,B)). We activated Kᵥ12.1 channels for as short as 600 ms, as we previously found that 600 ms allowed for steady-state activation of Kᵥ12.1 channels, whereas longer activating pulses attenuated mode shift due to longer time intervals at hyperpolarized potentials [10]. Accordingly, we also recorded tail currents at correspondingly depolarized potentials to minimize time at hyperpolarized voltages (Figure 1(B)). These voltage protocols elicited robust, outwardly-rectifying and voltage-dependent Kᵥ12.1 currents in CHO cells (Figure 1(A)). For conditioning voltages of −60 mV, half-maximal voltage (V₅₀) and slope factor of Kᵥ12.1 channel activation were −21.3 ± 2.1 mV and −14.6 ± 1.3 mV, respectively (n = 6; Figure 1(C-E)).

Accordingly, as reported by us and others [7,8,10] depolarizing conditioning potentials induced a large shift of voltage dependence to hyperpolarized potentials by about −50 mV. This shift of voltage dependence in response to depolarized holding potentials is generally referred to as mode shift of activation [12].

**Kᵥ12.1 channels are insensitive to physiological PI(4,5)P₂ depletion through PLCβ**

Recently, it was shown that activity of Kᵥ12.1 channels was modulated by PI in membrane patches excised from *Xenopus laevis* oocytes [7,8]. We thus set out to analyze in CHO cells whether these channels were also sensitive to stimulation of GqPCR signaling that physiologically leads to depletion of membrane PI(4,5)P₂ through activation of PLCβ. To this end, we overexpressed Gq-coupled muscarinic receptor type 1 (m1R) in CHO cells and activated the receptor by extracellular application of oxotremorine-M (Oxo-M; 10 µM). In this expression system, activation of recombinant m1R reconstitutes GqPCR signaling and induces depletion of PI(4,5)P₂ through endogenous PLCβ thereby producing second messengers diacylglycerol (DAG) and inositol-1,4,5-
trisphosphate (I(1,4,5)P$_3$) [e.g. 20,22–25]. At start, we co-expressed bona fide PI(4,5)P$_2$-sensitive homomeric K$_v$7.4 or K$_v$7.3 channels together with m1R. We utilized K$_v$7.3 channels carrying a A315T mutation in the pore region to increase whole cell currents without however affecting the channel’s sensitivity to PI(4,5)P$_2$ depletion (K$_v$7.3T [26,27]). We found that stimulation of m1R strongly reduced K$_v$7.4- and K$_v$7.3T-mediated currents (Figure 2(A,B)). It is well established that m1R-dependent inhibition of K$_v$7 channels mainly depends on PLCβ-dependent depletion of PI(4,5)P$_2$ [22,23,28,29] and that degree of inhibition corresponds to PI(4,5)P$_2$ affinity of the respective K$_v$7 isoform [30]. In line, activation of m1R caused stronger inhibition of K$_v$7.4 that exhibit lower PI (4,5)P$_2$ affinity than K$_v$7.3T channels [29–31]. These recordings demonstrated robust activation of G$_q$PCR signaling and substantial PLCβ-dependent PI(4,5)P$_2$ depletion in CHO cells upon stimulation of m1R. In contrast, K$_v$12.1-mediated steady-state currents elicited by voltages between -140 mV and +10 mV were not affected by stimulation of m1R through Oxo-M (Figure 2(A–E)). G$_q$PCR-dependent activation of PLCβ also did not change kinetics of activation (Figure 2(F)) and deactivation (Figure 2(G)) of human K$_v$12.1 channels in CHO cells. After 60 s of application of Oxo-M (10 µM), $V_h$ of activation was $-23.7 \pm 2.1$ mV and $-67.3 \pm 5.6$ mV for conditioning voltages of $-60$ mV and 0 mV, respectively (Figure 2(H,I)). Thus, also voltage dependence of human K$_v$12.1 channels was not affected by stimulation of PLCβ demonstrating that mode shift of K$_v$12.1 channels was not sensitive to activation of the pathway in CHO cells. To rule out that for
Figure 2. Human K\textsubscript{12.1} channels are insensitive to G\textsubscript{q}PCR/PLC\textsubscript{β} signaling.

(A + B) Activation of muscarinic G\textsubscript{q} protein-coupled receptor type 1 (m1R) by Oxo-M (10 µM) strongly inhibited bona fide PI(4,5)P\textsubscript{2}-sensitive K\textsubscript{7.4} and K\textsubscript{7.3} channels. In contrast, K\textsubscript{12.1} channels were resistant to stimulation of m1R. (A) shows representative K\textsubscript{7.4}- and K\textsubscript{12.1}-mediated whole cell currents before (black) and at the end of a 60 s application of 10 µM Oxo-M (red) (voltage protocol and scale bars as indicated). (B) shows a summary of recordings as presented in (A). (C-E) Steady-state K\textsubscript{12.1} currents elicited by voltage steps between −140 mV and +10 mV were not sensitive to activation of m1R through Oxo-M (10 µM). (F) Neither activating kinetics nor (G) deactivating kinetics of K\textsubscript{12.1} channels were altered upon activation of m1R (time constants were calculated from double-exponential fits to the activating current component and to deactivating tail currents). (C + D) show representative whole cell K\textsubscript{12.1} currents elicited by the voltage protocols indicated. To induce voltage-dependent mode shift of K\textsubscript{12.1} channels, voltage protocols consisted of a 200 ms conditioning potential step to (C) −60 mV or (D) 0 mV followed by 600 ms activating pulse potentials from −140 mV to +10 mV (10 mV increments). (H + I) Oxo-M-dependent activation of m1R did not change voltage-dependence or mode-shift of K\textsubscript{12.1} channels expressed in CHO cells. (H) shows a summary of the voltage dependence of human K\textsubscript{12.1} channels and (I) displays mean V\textsubscript{h} derived from Boltzmann fits to individual recordings as shown in (C) and (D) (solid lines in (H) represent Boltzmann fits to averaged data; Data with Oxo-M were analyzed at the end of 60 s Oxo-M application; control recordings are also shown in Figure 1). (J + K) In CHO cells coexpressing m1R together with K\textsubscript{7.4}, application of Oxo-M (10 µM) induced robust and reversible translocation of the optical PI(4,5)P\textsubscript{2}/I(1,4,5)P\textsubscript{3} biosensor PLC\textsubscript{δ1}-PH-mRFP from the membrane to the cytoplasm. PLC\textsubscript{δ1}-PH-RFP translocation was indistinguishable between cells coexpressing K\textsubscript{12.1} or K\textsubscript{7.4} channels together with m1R. Thus, PLC\textsubscript{β} activation was comparable in cells expressing K\textsubscript{7.4} or K\textsubscript{12.1}. (J) shows mean RFP fluorescence intensities measured in confocal sections averaged over a region of interest in the cytoplasm of CHO cells coexpressing PLC\textsubscript{δ1}-PH-RFP together with either K\textsubscript{12.1} or K\textsubscript{7.4}. (K) shows representative images of a CHO cell coexpressing K\textsubscript{12.1} together with m1R before (top), after 60 s application of Oxo-M (middle) and after wash out of the agonist (bottom). The scale bar represents 10 µm.
some reason depletion of PI(4,5)P$_2$ was reduced in CHO cells expressing K$_v$12.1 channels, we then directly assessed activity of PLCβ under our experimental conditions. We quantified putative PLCβ activity using pleckstrin homology (PH) domain of PLCδ$_1$ fused to RFP (PLCδ$_1$-PH-RFP) as genetically-encoded, optical biosensor that binds to membrane PI(4,5)P$_2$, but also to cytoplasmic I(1,4,5)P$_3$ [32]. PLCδ$_1$-PH-RFP associates to the plasma membrane, when resting PI(4,5)P$_2$ levels are high, and translocates into the cytoplasm in response to PLCβ-dependent decrease of membrane PI(4,5)P$_2$ and a corresponding increase of cytoplasmic I(1,4,5)P$_3$ [32,33]. Accordingly, changes of the fluorescence intensity at the membrane or in the cytoplasm are a measure for the activity of PLCβ during stimulation of G$_q$PCR [17,34]. Here, we used confocal imaging to monitor translocation of PLCδ$_1$-PH-RFP from membrane to the cytoplasm of transiently transfected CHO cells during application of Oxo-M (10 µM). In cells, coexpressing m1R together with PLCδ$_1$-PH-RFP and K$_v$7.4 channels, RFP-associated fluorescence in the cytoplasm strongly increased during activation of the G$_q$PCR. This increase of fluorescence indicated translocation of the sensor from membrane into cytoplasm and thus PLCβ-mediated PI(4,5)P$_2$ depletion (Figure 2(J)). Upon wash-out of Oxo-M, the signal recovered within a couple of minutes demonstrating reversibility of PI(4,5)P$_2$ depletion. When we activated m1R in cells coexpressing K$_v$12.1 channels, sensor translocation into the cytoplasm and its return to the plasma membrane were indistinguishable from cells expressing K$_v$7.4 (Figure 2(J,K)). These experiments demonstrated that PLCβ was strongly activated under our experimental conditions and that PLCβ activity and thus PI(4,5)P$_2$ depletion were the same in cells expressing K$_v$7.4 and K$_v$12.1 channels. Taking together these data showed that human K$_v$12.1 channels were not sensitive to activation of G$_q$PCR signaling and more importantly PLCβ-mediated PI(4,5)P$_2$ depletion in CHO cells.

**Recombinant K$_v$12.1 channels are insensitive to Ci-VSP-dependent PI(4,5)P$_2$ depletion**

To assess in more detail whether K$_v$12.1 channels were sensitive to PI(4,5)P$_2$ depletion in CHO cells, we utilized voltage-sensitive PI $5'$-phosphatase from *ciona intestinalis* (Ci-VSP). Upon depolarization of the membrane potential, Ci-VSP rapidly removes PI(4,5)P$_2$ and PI(3,4,5)P$_3$ from the membrane by dephosphorylation to PI(4)P and PI(3,4)P$_2$, respectively [35–39]. In these experiments, we selected cells for clear membrane localization of Ci-VSP-RFP-associated fluorescence and recorded K$_v$7 and K$_v$12.1 currents activated by depolarizing voltage steps from the holding potential of −80 mV to 0 mV or −20 mV, respectively (Figure 3(A)). After recording stable control current amplitudes for at least 30 s, the holding potential was depolarized for another 30 s to +80 mV to activate Ci-VSP. As shown in Figure 3(A,B), currents through homomeric K$_v$7.2 and K$_v$7.3$^T$ channels were strongly inhibited by activation of Ci-VSP. After 30 s of Ci-VSP activation at +80 mV, K$_v$7.2 and K$_v$7.3$^T$ currents were reduced to 27.8 ± 4.6% and 40.6 ± 1.9% of baseline current amplitudes, respectively (Figure 3(A,B)). This inhibition of K$_v$7-mediated currents, especially inhibition of K$_v$7.3$^T$ channels that exhibit higher PI(4,5)P$_2$ affinity than K$_v$7.2, demonstrated substantial depletion of membrane PI(4,5)P$_2$ upon activation of Ci-VSP, in line with many previous reports [e.g. 29, 38]. In these experiments, K$_v$7 currents returned to baseline within a minute after deactivation of Ci-VSP at hyperpolarized potentials demonstrating reversible PI(4,5)P$_2$ depletion and its resynthesis through endogenous PI kinases (Figure 3(B)) [38]. Interestingly after deactivation of Ci-VSP, K$_v$7.2-mediated currents transiently over-recovered before returning to baseline amplitudes indicating some kind of over-recovery of PI(4,5)P$_2$ levels possibly stimulated by Ci-VSP-induced PI(4,5)P$_2$ depletion [c.f. 38].

In contrast to K$_v$7 currents, K$_v$12.1 channels were almost completely resistant to activation of co-expressed Ci-VSP utilizing the same voltage protocol to activate the phosphatase. After 30 s at +80 mV, K$_v$12.1 current amplitudes were 92.7 ± 1.3% of control currents before voltage-dependent activation of Ci-VSP (n = 5; Figure 3(A,B)). In three out of five cells tested, we detected slight acceleration of the activating kinetics of K$_v$12.1 channels in response to stimulation of Ci-VSP (Figure 3(C)). As these changes of kinetics were small and not detectable in all cells, we did not examine the phenomenon any further.
In summary, \( \text{K}_\text{v}12.1 \) channels were not affected by Ci-VSP-dependent depletion of \( \text{PI}(4,5)\text{P}_2 \) in CHO cells. Thus, we conclude that \( \text{K}_\text{v}12.1 \) channels are not relevantly modulated by membrane \( \text{PI}(4,5)\text{P}_2 \) in this cell line. As Ci-VSP stoichiometrically converts \( \text{PI}(4,5)\text{P}_2 \) into \( \text{PI}(4)\text{P} \) thereby substantially increasing \( \text{PI}(4)\text{P} \) levels in the membrane [39], these data also demonstrated that \( \text{K}_\text{v}12.1 \) channels are not modulated by membrane-associated \( \text{PI}(4)\text{P} \).

**Discussion**

Activation of PLC\( \beta \) through \( \text{G}_q/11 \)-coupled receptors is an important intercellular signaling pathway that induces PI dynamics in neurons [17,18,21]. Although PLC\( \beta \) possibly also hydrolyses \( \text{PI}(4)\text{P} \) [20,40,41], it is well known that \( \text{PI}(4,5)\text{P}_2 \) is the prime substrate of its enzymatic activity in living cells [21]. Whereas actual changes in membrane-associated \( \text{PI}(4,5)\text{P}_2 \) might vary significantly depending on the receptor type, as well as on the activity of PLC\( \beta \) and \( \text{PI}(4,5)\text{P}_2 \) resynthesis pathways, it is generally believed that the signaling cascade affects cellular physiology also through depletion of \( \text{PI}(4,5)\text{P}_2 \) [20,21,32]. Thus, as \( \text{PI}(4,5)\text{P}_2 \) is an important cofactor for many ion channels, activation of PLC\( \beta \) is directly linked to neuronal excitability through \( \text{PI}(4,5)\text{P}_2 \) depletion and \( \text{PI}(4,5)\text{P}_2 \)-dependent modulation of ion channel activity. As one prominent example, stimulation of endogenous \( \text{G}_q \)-coupled muscarinic receptors induces significant depolarization of neuronal membrane potentials through \( \text{PI}(4,5)\text{P}_2 \)-dependent inhibition of \( \text{K}_v7 \) potassium channels [22,23,42].
Here, we evaluated whether human \( K_{\alpha} \), 12.1 channels were sensitive to physiological changes of \( \text{PI}(4,5)P_2 \) for two reasons: (i) As no physiological function could be assigned to \( K_{\alpha} \), 12.1 channels yet, novel properties of these channels might be useful to identify native \( K_{\alpha} \), 12.1-mediated currents in neurons [c.f. 10]. We thus considered PLC\( \beta \) sensitivity of \( K_{\alpha} \), 12.1 channels a potentially interesting feature fostering future attribution of neuronal \( K^+ \) currents to \( K_{\alpha} \), 12.1 channels. And (ii), recently it was shown for \( K_{\alpha} \), 12.1 channels that excision of membrane patches from \textit{Xenopus laevis} oocytes speeded activation and deactivation, shifted voltage dependence to hyperpolarized potentials and significantly attenuated mode shift [7]. Importantly, such excision of membrane patches into solution without ATP causes depletion of \( \text{PI}(4,5)P_2 \) (and possibly other PI species) through irreversible activation of phosphatases [19,24,28,31,43,44]. As application of a water-soluble \( \text{PI}(4,5)P_2 \) analogue restored \( K_{\alpha} \), 12.1 channel properties in the excised inside-out patches, these findings demonstrated \( \text{PI}(4,5)P_2 \) sensitivity of \( K_{\alpha} \), 12.1 channels [7]. We considered this \( \text{PI}(4,5)P_2 \) sensitivity especially interesting, as by attenuating mode shift and by affecting voltage dependence of these channels, impact of PLC\( \beta \) activation on the excitability of \( K_{\alpha} \), 12.1 expressing neurons might significantly vary depending on synaptic input and thus excitation status of the respective neuron. Utilizing m1R as G protein-coupled receptor to activate PLC\( \beta \) in an expression system, we studied sensitivity of human \( K_{\alpha} \), 12.1 channels to \( \text{G}_q \), PCR signaling. It has been shown in many studies that overexpression of a \( \text{G}_q \), PCR (such as m1R) adequately reconstitutes PLC\( \beta \) signaling in expression systems (including CHO cells used in our report). Through activation of endogenous PLC\( \beta \), stimulation of recombinant m1R substantially depletes \( \text{PI}(4,5)P_2 \) [20,22,23,24,25,30,34], produces reasonable amounts of DAG and \( I(1,4,5)P_3 \), induces \( I(1,4,5)P_3 \)-dependent \( Ca^{2+} \) release and activates downstream effectors of the cascade (e.g. protein kinase C, PKC) [20,22,30,34,45,46]. This has been used successfully to explore sensitivity of ion channels to physiological \( \text{PI}(4,5)P_2 \) depletion [20,22,29,30,47], but also the modulation of ion channels through second messengers downstream of PLC\( \beta \) activation [48–51]. Importantly, heterologous systems can even be utilized to dissect \( \text{PI}(4,5)P_2 \) dependence of ion channels from their sensitivity to second messengers produced during activation of PLC\( \beta \) [47,49,52]. Heterologous expression systems accordingly constitute a well-accepted model to study the \( \text{G}_q \), PCR pathway and \( \text{PI}(4,5)P_2 \) dependence of ion channels [17,18]. The stimulation of PLC\( \beta \) as well as voltage-dependent activation of Ci-VSP strongly inhibited \textit{bonafide} \( \text{PI}(4,5)P_2 \)-dependent \( K_{\beta} \) channels (c.f. Figures 2 and 3). Especially, the significant inhibition of \( K_{\alpha} \), 7.3\( ^T \) channels that exhibit considerably higher \( \text{PI}(4,5)P_2 \) affinity than \( K_{\alpha} \), 7.2 or \( K_{\alpha} \), 7.4 channels [29,30,38] demonstrated substantial \( \text{PI}(4,5)P_2 \) depletion under our experimental conditions. Translocation of the optical \( \text{PI}(4,5)P_2 \) biosensor PLC\( \delta_1 \)-PH-RFP from the membrane during stimulation of m1R additionally showed strong reduction of \( \text{PI}(4,5)P_2 \) levels in these experiments. However, \( K_{\alpha} \), 12.1 channels were not sensitive to this PLC\( \beta \)-mediated \( \text{PI}(4,5)P_2 \) depletion at all. Theoretically, a rise in DAG, \( I(1,4,5)P_3 \) and intracellular \( Ca^{2+} \) or activation of PKC could stimulate \( K_{\alpha} \), 12.1, which might counterbalance PI depletion thereby masking \( \text{PI}(4,5)P_2 \) sensitivity of these channels. However, although we consider such a mechanism rather unlikely, we want to point out that we cannot exclude sensitivity of \( K_{\alpha} \), 12.1 to these second messengers completely at present. \( K_{\beta} \), mediated currents are indeed reduced by PKC-dependent phosphorylation [48] and \( Ca^{2+} \)/calmodulin signaling [53], but importantly \( \text{G}_q \), PCR-dependent inhibition of these channels almost exclusively depends on \( \text{PI}(4,5)P_2 \) depletion and not on messengers produced by PLC\( \beta \) [23,29]. Based on these considerations, we conclude that \( K_{\alpha} \), 12.1 channels are resistant to stimulation of \( \text{G}_q \), PCR and importantly insensitive to physiologically-relevant \( \text{PI}(4,5)P_2 \) dynamics. This insensitivity of \( K_{\alpha} \), 12.1 channels to stimulation of PLC\( \beta \) is not surprising, as several other \( K^+ \) channels, including members of the \( K_{\alpha,1} \), \( K_{\alpha,2} \), \( K_{\alpha,3} \), and \( K_{\alpha,4} \) families, have been described to be not affected by stimulation of
the PLCβ pathway and of Ci-VSP [24,25]. However, it is surprising that for some of these Kv channels (Kv1.1, Kv1.4, Kv1.5, Kv3.4), just as for Kv12.1 channels, PI(4,5)P2 sensitivity was reported using excised patches from Xenopus laevis oocytes as experimental model [54,55]. Importantly however, like Kv12.1 these channels were resistant to activation of m1R and Ci-VSP in mammalian cell lines [24,25]. As comprehensively discussed by the Hille group [24,25], we do not think that our findings represent a discrepancy to results obtained in the frog oocytes (presented by Li and colleagues [7]). First, upon excision of inside-out patches levels of membrane PI(4,5)P2 might fall well below levels reached by physiological stimulation of PLCβ (and possibly even by activation of Ci-VSP) and other PI species may also be depleted during the patch excision. Conversely, perfusion of membrane patches with water soluble PI analogues might introduce super-physiological PI(4,5)P2 levels within or close to the membrane [25]. Therefore, on the one hand PI sensitivity of ion channels might be overestimated upon PI(4,5)P2 depletion through patch excision and subsequent application of exogenous PI(4,5)P2. On the other hand, the degree of PLCβ-dependent PI (4,5)P2 depletion might be just too low to induce relevant modulation of Kv12.1 channel activity. And second, as comprehensively discussed and pointed-out by Li and colleagues [7], Kv12.1 channels might exhibit low PI affinity and low selectivity between different PI species. This conclusion is supported by the fact that not only PI(4,5)P2 but probably also other PI species might be depleted after excision of the membrane patches from oocytes. This depletion of several PI species upon patch excision might affect ion channels with unselective PI specificity even stronger than a highly selective channel that recognizes only a certain PI species. Supporting this notion, in membrane patches excised from Xenopus oocytes mode shift of Kv12.1 channels was sensitive to application of PI(4)P, PI(4,5)P2 and PI (3,4,5)P3 [7]. This strongly indicated that Kv12.1 channels (in contrast to e.g. Kv7 channels) do not exhibit highly selective binding to a certain PI species, but rather general electrostatic interactions with several PI [7]. Completely in line, Kv12.1 channels were resistant to activation of Ci-VSP in CHO cells, which stoichiometrically converts PI(4,5)P2 and PI(3,4,5)P3 into PI(4)P and PI(3,4)P2, respectively [35–37,39]. Whilst depleting PI (4,5)P2 from the membrane, activation of Ci-VSP thus leaves constant the total PI concentration in the plasma membrane. At the same time however, Ci-VSP substantially increases PI (4)P levels [38,39], which revealed that activity of Kv12.1 channels is not relevantly modulated by PI(4)P. This low sensitivity to Ci-VSP activation (and PLCβ signaling) may as well point to lack of selectivity towards PI(4,5)P2 thereby indicating a rather general PI sensitivity of Kv12.1 channels. In line, EC50 values of Kv12.1 channels for application of exogenous PI(4,5)P2 expressed in Xenopus oocytes (approx. 10 μM [7]) were well in the range previously demonstrated for Kv7.3 channels [28]. However, most probably due to high PI(4,5)P2 specificity and selectivity, Kv7.3 channels are readily inhibited by activation of m1R and Ci-VSP, whereas Kv12.1 channels are not. In line, sensitivity of K+ channels to activation of Ci-VSP generally correlates well with their sensitivity towards receptor-triggered activation of PLC [29], which apparently is also true for Kv12.1 channels.

Thus, in summary, human Kv12.1 channels most probably exhibit unselective PI sensitivity thereby adding to the growing list of Kv channels resistant to stimulation of Gq/PLCβ-signaling and Ci-VSP-dependent PI(4,5)P2 depletion [c.f. 24, 25].

**Methods**

**Cell culture and transfection**

Chinese hamster ovary (CHO) dhFR− cells were maintained as previously reported [56]. In brief, cells were kept in MEM Alpha Medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (pen/strep) (all Invitrogen GmbH, Darmstadt, Germany) in a humidified atmosphere at 5% CO2 and 37°C. Transient transfection of CHO cells in culture was done with jetPEI
transfection reagent (Polyplus Transfection, Illkirch, France) and all experiments were performed at room temperature (22°C-25°C) approx. 24–48 h after transfection. The following vectors for transient expression in CHO cells were used: Kv7.2-pBK-CMV (gene: human KCNQ2; UniProt accession number: O43526), Kv7.3(A315T)-pBK-CMV (human KCNQ3(A315T); O43525), Kv7.4-pBK-CMV (human KCNQ4; P56696), Kv12.1(Elk1)-pcDNA3.1-IRES-eGFP (human KCNH8; Q96L42), human muscarinic receptor 1 (human M1R)-pSGHV0 (Q96RH1), Ci-VSP-mRFP-C1 (Q4W8A1), PLCδ1-PH-mRFP-C1 (amino acids 1–70; P51178) and pEGFP-C1 (transfection control; Addgene, Teddington, UK).

Electrophysiological recordings

Electrophysiological recordings (in the whole cell configuration) were performed with an Axopatch 200B amplifier (Molecular Devices, Union City, CA) in voltage-clamp mode [57]. Recordings were low-pass filtered at 2 kHz and sampled at 5 kHz. In the figures, voltage protocols are indicated and the dashed lines highlight zero current. Borosilicate glass patch pipettes (Sutter Instrument Company, Novato, CA, USA) used had an open pipette resistance of 2–3 MΩ after back-filling with intracellular solution containing (in mM) 135 KCl, 2.41 CaCl₂ (100 nM free Ca²⁺), 3.5 MgCl₂, 5 HEPES, 5 EGTA, 2.5 Na₂ATP, 0.1 Na₃GTP, pH 7.3 (with KOH), 290–295 mOsm/kg. Series resistance (Rs) typically was below 6 MΩ and compensated throughout the recordings (80–90%), and liquid junction potentials were not compensated (approx. −4 mV). For presentation whole cell currents were normalized to the cell capacitance (current density; pA/pF) or to baseline current amplitude (I/Iₖ). The extracellular solution contained (in mM) 144 NaCl, 5.8 KCl, 1.3 CaCl₂, 0.9 MgCl₂, 0.7 NaH₂PO₄, 10 HEPES and 5.6 D-glucose, pH 7.4 (with NaOH), 305–310 mOsm/kg.

Voltage-dependent activation of Ci-VSP

For coexpression of ion channels with Ci-VSP-RFP, cells were selected for clear membrane localization of RFP (attached to Ci-VSP). Kv channel-mediated currents were elicited every 5s with the voltage protocol indicated in the figure and Ci-VSP was activated in between those voltage steps by depolarizing the holding potential to +80 mV for a total of 30 s, as previously reported [47].

Confocal microscopy

Confocal imaging was performed with an upright LSM 710 – Axio Examiner.Z1 microscope equipped with a W Plan/Apochromat 20x/1.0 DIC M27 75 mm water immersion objective (Zeiss, Jena, Germany) [58]. Red fluorescent protein (RFP) was excited at 561 nm with a DPSS 561–10 laser (Zeiss) and fluorescence emission was sampled at 582–754 nm. Green fluorescent protein (GFP) was excited at 488 nm with an argon laser and fluorescence emission was recorded at 493–597 nm. The sample interval for time series was 5 s. In these experiments, Kv7.4 or Kv12.1 expressing cells were identified through coexpression of GFP or the GFP expression associated with the pcDNA3.1-IRES-eGFP plasmid, respectively.

Solutions and substances

Oxotremorine-M (Oxo-M) was purchased from Biotrend Chemikalien GmbH (Cologne, Germany) and was diluted to a concentration of 10 µM in extracellular solution. Oxo-M was applied locally through a custom-made application system via a glass capillary brought into close proximity to cells under investigation.

Data analysis

Patch clamp recordings were analyzed with IgorPro (Wavemetrics, Lake Oswego, OR) and the PatchMaster (HEKA) software. Voltage dependence of activation was derived from tail current amplitudes using voltage protocols indicated: Tail currents were fitted with a two-state Boltzmann function with \( I = I_{\text{min}} + (I_{\text{max}} - I_{\text{min}})/(1 + \exp ((V - V_{h})/s)) \), where \( I \) is current, \( V \) is the membrane voltage, \( V_{h} \) is the voltage at half maximal activation, and \( s \) describes the steepness of the curve [59]. Results are shown as conductance-voltage curves, obtained by normalizing to \( (I_{\text{max}} - I_{\text{min}}) \), obtained from fits to data of individual experiments. Time constants of activation and deactivation were
derived from double-exponential fits to deactivating current components at indicated potentials. Imaging time series were analyzed with confocal microscopy with Zen2009 (Zeiss) and IgorPro (Wavemetrics). PLC$_{δ1}$-PH-mRFP fluorescent intensities were derived after background subtraction from averages over a region of interest (ROI) in the cytoplasm of transfected cells and are presented as cytoplasmic F/F$_0$.

**Data presentation**

In electrophysiological experiments, $n$ represents the number of individual cells and accordingly the number of independent experiments (no pseudo-replication). In imaging experiments (c.f. Figure 2(f)), $n$ represents the number of individual cells and $ε$ denotes the number of independent experiments.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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