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BRIEF REPORT

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$K_v12.1$ channels are not sensitive to G_qPCR-triggered activation of phospholipase Cβ

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ABSTRACT

$K_v12.1$ 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 $K_v12.1$ currents must rely on characterization of distinct channel properties. Recently, it was shown in *Xenopus laevis* oocytes that $K_v12.1$ channels were modulated by membrane $PI(4,5)P_2$. However, it is not known whether these channels are also sensitive to physiologically-relevant $PI(4,5)P_2$ dynamics. We thus studied whether $K_v12.1$ channels were modulated by activation of phospholipase C β (PLCβ) and found that they were insensitive to receptor-triggered depletion of $PI(4,5)P_2$. Thus, $K_v12.1$ channels add to the growing list of K^+ channels that are insensitive to PLCβ signaling, although modulated by $PI(4,5)P_2$ in *Xenopus laevis* oocytes.

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Introduction

The ether-à-go-go-gene-like (Elk; K_v12) family of voltage-gated potassium (K_v) channels comprises three members ($K_v12.1$-$K_v12.3$) 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 $K_v12.2$ channels constitute subthreshold K^+ conductance regulating excitability of pyramidal neurons in hippocampus [11], but no native physiological relevance could be attributed to $K_v12.1$ and $K_v12.3$ subunits so far. As $K_v12.1$ 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 $K_v12.1$ 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 $K_v12.1$ channels [8]. This rearrangement apparently is coupled to channel gating and necessary for transition of $K_v12.1$ 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 $K_v12.1$ channels [7,8,10]. This mode shift may constitute a biophysical adaption to dampen excitability of neurons upon ongoing stimulation possibly also to prevent hyperexcitability in nervous tissue. Analogous mode shift of related $K_v11.1$ 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 $K_v12.1$ channel activity was regulated through membrane-associated phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$)

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[7] 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 $K_v12.1$ channel activation and deactivation and a potentiation of steady-state K^+ currents. Furthermore, excision of membrane patches expressing $K_v12.1$ 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 $PI(4,5)P_2$ analogue restored $K_v12.1$ channel kinetics as well as voltage dependence, and partially brought-back mode shift, the authors consistently concluded that $PI(4,5)P_2$ in a bimodal way of action stabilized the open state, but at the same time inhibited voltage dependent activation of $K_v12.1$ channels [7]. Accordingly, variations of membrane $PI(4,5)P_2$ levels might control excitability of neurons through modulating $K_v12.1$ channel activity. Most interestingly, impact of $PI(4,5)P_2$ 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 $K_v12.1$ channels were sensitive to physiologically-relevant $PI(4,5)P_2$ depletion at all. As activation of phospholipase C β (PLCβ) through G_q protein-coupled receptors (G_qPCR) constitutes an important signaling pathway to deplete $PI(4,5)P_2$ in neurons [20,21], we studied whether $K_v12.1$ channels were modulated by activation of G_q protein-coupled muscarinic receptors.

Results

Analyzing voltage-dependent mode shift of $K_v12.1$ channels

We analyzed sensitivity to G_qPCR signaling of human $K_v12.1$ channels heterologously expressed in CHO cells. To be able to analyze whether voltage-dependent activation of $K_v12.1$ channels was sensitive to activation of the G_qPCR 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 $K_v12.1$ channels for as short as 600 ms, as we previously found that 600 ms allowed for steady-state activation of $K_v12.1$ 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 $K_v12.1$ currents in CHO cells (Figure 1(A)). For conditioning voltages of −60 mV, half-maximal voltage (V_h) and slope factor of $K_v12.1$ channel activation were −21.3 ± 2.1 mV and −14.6 ± 1.3 mV, respectively (n = 6; Figure 1(C–E)). When cells were held at a depolarized conditioning potential of 0 mV, V_h of channel activation was -68.2 ± 5.2 mV and the slope factor was −8.1 ± 0.8 mV (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].

$K_v12.1$ channels are insensitive to physiological $PI(4,5)P_2$ depletion through PLCβ

Recently, it was shown that activity of $K_v12.1$ 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 G_qPCR signaling that physiologically leads to depletion of membrane $PI(4,5)P_2$ through activation of PLCβ. To this end, we overexpressed G_q-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 G_qPCR signaling and induces depletion of $PI(4,5)P_2$ through endogenous PLCβ thereby producing second messengers diacylglycerol (DAG) and inositol-1,4,5-

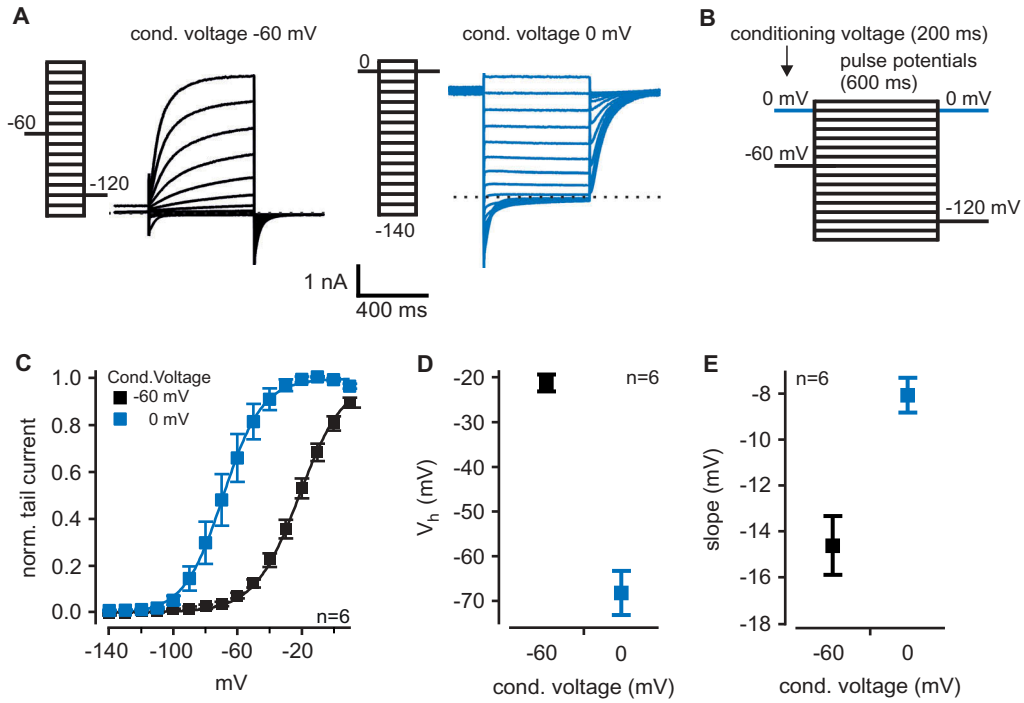

Figure 1. Mode shift of recombinant human $K_v12.1$ channels.

(A) Representative patch clamp recording of a CHO cell transiently transfected with human $K_v12.1$ channels activated by the voltage steps as indicated. **(B)** Voltage protocols consisted of a 200 ms conditioning potential step to −60 mV (*black*) or 0 mV (*blue*) followed by 600 ms activating pulse potentials from −140 mV to +10 mV (10 mV increments). Tail currents were elicited either at −120 mV or at 0 mV to minimize time at hyperpolarized membrane potentials (voltage protocols were established in a recent publication [10]). **(C-E)** Summary of voltage dependence of human $K_v12.1$ channels. **(C)** Voltage dependence was analyzed with Boltzmann fits to individual recordings as shown in (A). Solid lines in (C) represent Boltzmann fits to averaged data. Depolarized conditioning potential of 0 mV induced a large shift of voltage dependence to hyperpolarized potentials, as recently reported [10]. **(D)** shows mean V_h and **(E)** displays mean slope factor of channel activation in dependence of conditioning potentials of −60 mV and 0 mV (values were derived from Boltzmann fits shown in (C).

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 $K_v7.4$ or $K_v7.3$ channels together with m1R. We utilized $K_v7.3$ 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 ($K_v7.3^T$ [26,27]). We found that stimulation of m1R strongly reduced $K_v7.4$- and $K_v7.3^T$-mediated currents (Figure 2(A,B)). It is well established that m1R-dependent inhibition of K_v7 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_v7 isoform [30]. In line, activation of m1R caused stronger inhibition of $K_v7.4$ that exhibit lower $PI(4,5)P_2$ affinity than $K_v7.3^T$ channels [29–31]. These recordings demonstrated robust activation of G_qPCR signaling and substantial PLCβ-dependent $PI(4,5)P_2$ depletion in CHO cells upon stimulation of m1R. In contrast, $K_v12.1$-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_qPCR-dependent activation of PLCβ also did not change kinetics of activation (Figure 2(F)) and deactivation (Figure 2(G)) of human $K_v12.1$ channels in CHO cells. After 60 s of application of Oxo-M (10 μM), V_h of activation was −23.7 ± 2.1 mV and −67.3 ± 5.6 mV for conditioning voltages of −60 mV and 0 mV, respectively (Figure 2(H,I)). Thus, also voltage dependence of human $K_v12.1$ channels was not affected by stimulation of PLCβ demonstrating that mode shift of $K_v12.1$ channels was not sensitive to activation of the pathway in CHO cells. To rule out that for

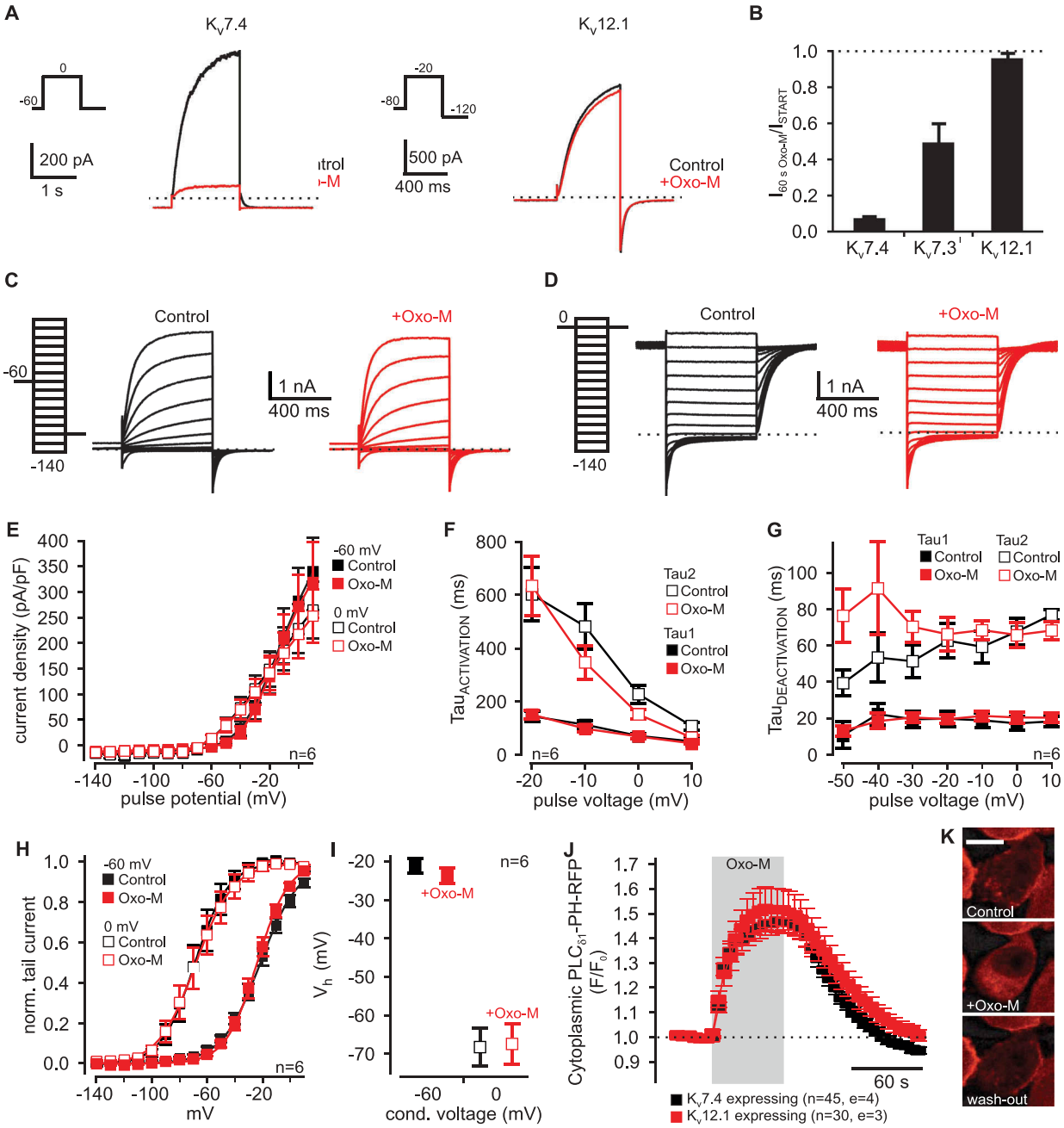

Figure 2. Human K_v12.1 channels are insensitive to G_qPCR/PLCβ signaling.

(A + B) Activation of muscarinic G_q protein-coupled receptor type 1 (m1R) by Oxo-M (10 µM) strongly inhibited *bona fide* PI(4,5)P_2-sensitive K_v7.4 and K_v7.3^T channels. In contrast, K_v12.1-mediated currents were resistant to stimulation of m1R. **(A)** shows representative K_v7.4- and K_v12.1-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_v12.1 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_v12.1 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_v12.1 currents elicited by the voltage protocols indicated. To induce voltage-dependent mode shift of K_v12.1 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_v12.1 channels expressed in CHO cells. **(H)** shows a summary of the voltage dependence of human K_v12.1 channels and **(I)** displays mean V_h derived from Boltzmann fits to individual recordings as shown in (C) an (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_v7.4, application of Oxo-M (10 µM) induced robust and reversible translocation of the optical PI(4,5)P_2/I(1,4,5)P_3 biosensor $PLC_{\delta1}$-PH-mRFP from the membrane to the cytoplasm. $PLC_{\delta1}$-PH-RFP translocation was indistinguishable between cells coexpressing K_v12.1 or K_v7.4 channels together with m1R. Thus, PLCβ activation was comparable in cells expressing K_v7.4 or K_v12.1. (J) shows mean RFP fluorescence intensities measured in confocal sections averaged over a region of interest in the cytoplasm of CHO cells coexpressing $PLC_{\delta1}$-PH-RFP together with either K_v12.1 or K_v7.4. (K) shows representative images of a CHO cell expressing K_v12.1 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 $K_v12.1$ channels, we then directly assessed activity of PLCβ under our experimental conditions. We quantified putative PLCβ activity using pleckstrin homology (PH) domain of $PLC_{\delta1}$ fused to RFP ($PLC_{\delta1}$-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]. $PLC_{\delta1}$-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_qPCR [17,34]. Here, we used confocal imaging to monitor translocation of $PLC_{\delta1}$-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 $PLC_{\delta1}$-PH-RFP and $K_v7.4$ channels, RFP-associated fluorescence in the cytoplasm strongly increased during activation of the G_qPCR. 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 $K_v12.1$ channels, sensor translocation into the cytoplasm and its return to the plasma membrane were indistinguishable from cells expressing $K_v7.4$ (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 $K_v7.4$ and $K_v12.1$ channels. Taking together these data showed that human $K_v12.1$ channels were not sensitive to activation of G_qPCR signaling and more importantly PLCβ-mediated $PI(4,5)P_2$ depletion in CHO cells.

Recombinant $K_v12.1$ channels are insensitive to Ci-VSP-dependent $PI(4,5)P_2$ depletion

To assess in more detail whether $K_v12.1$ 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_v7 and $K_v12.1$ 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 $K_v7.2$ and $K_v7.3^T$ channels were strongly inhibited by activation of Ci-VSP. After 30 s of Ci-VSP activation at +80 mV, $K_v7.2$ and $K_v7.3^T$ 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_v7-mediated currents, especially inhibition of $K_v7.3^T$ channels that exhibit higher $PI(4,5)P_2$ affinity than $K_v7.2$, 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_v7 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, $K_v7.2$-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_v7 currents, $K_v12.1$ 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, $K_v12.1$ 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 $K_v12.1$ 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.

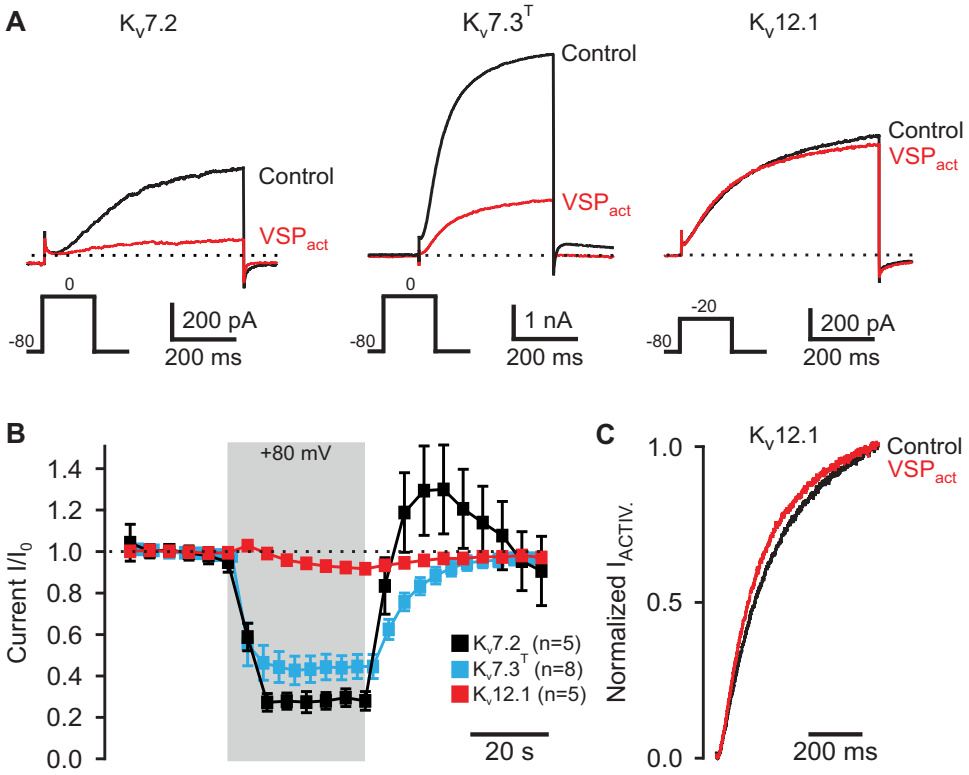

Figure 3. In CHO cells, human $K_v12.1$ channels are insensitive to activation of a voltage-sensitive $PI(4,5)P_2/PI(3,4,5)P_3$ 5-phosphatase from *ciona intestinalis*.

(A + B) Activation of Ci-VSP through 30 s depolarization of the holding potential to +80 mV reversibly inhibited recombinant $K_v7.2$ and $K_v7.3^T$ channels. In contrast, $K_v12.1$-mediated current amplitudes were largely insensitive to voltage-dependent activation of Ci-VSP. **(A)** shows representative recordings of $K_v7.2$, $K_v7.3^T$ or $K_v12.1$ currents before (*black*) and at the end of Ci-VSP activation for 30 s (*red*) (voltage steps to activate K^+ channel were applied every 5 s; Ci-VSP was activated by depolarization to + 80 mV between these voltage steps). **(B)** displays averaged time course of recordings as shown in (A). **(C)** In only three out of five cells, we found slight acceleration of $K_v12.1$ activating kinetics upon voltage-dependent activation of Ci-VSP. **(C)** shows exemplary normalized $K_v12.1$ currents activating upon a voltage step from −80 mV to −20 mV before (*black*) and after activation of Ci-VSP at +80 mV for 30 s (*red*).

In summary, $K_v12.1$ channels were not affected by Ci-VSP-dependent depletion of $PI(4,5)P_2$ in CHO cells. Thus, we conclude that $K_v12.1$ channels are not relevantly modulated by membrane $PI(4,5)P_2$ in this cell line. As Ci-VSP stoichiometrically converts $PI(4,5)P_2$ into PI(4)P thereby substantially increasing PI(4)P levels in the membrane [39], these data also demonstrated that $K_v12.1$ channels are not modulated by membrane-associated PI(4)P.

Discussion

Activation of PLCβ through $G_{q/11}$-coupled receptors is an important intercellular signaling pathway that induces PI dynamics in neurons [17,18,21]. Although PLCβ possibly also hydrolyses PI(4)P [20,40,41], it is well known that PI $(4,5)P_2$ is the prime substrate of its enzymatic activity in living cells [21]. Whereas actual changes in membrane-associated $PI(4,5)P_2$ might vary significantly depending on the receptor type, as well as on the activity of PLCβ and $PI(4,5)P_2$ resynthesis pathways, it is generally believed that the signaling cascade affects cellular physiology also through depletion of $PI(4,5)P_2$ [20,21,32]. Thus, as $PI(4,5)P_2$ is an important cofactor for many ion channels, activation of PLCβ is directly linked to neuronal excitability through $PI(4,5)P_2$ depletion and $PI(4,5)P_2$-dependent modulation of ion channel activity. As one prominent example, stimulation of endogenous G_q-coupled muscarinic receptors induces significant depolarization of neuronal membrane potentials through $PI(4,5)P_2$-dependent inhibition of K_v7 potassium channels [22,23,42].

Here, we evaluated whether human $K_v12.1$ channels were sensitive to physiological changes of $PI(4,5)P_2$ for two reasons: (i) As no physiological function could be assigned to $K_v12.1$ channels yet, novel properties of these channels might be useful to identify native $K_v12.1$-mediated currents in neurons [c.f. 10]. We thus considered PLCβ sensitivity of $K_v12.1$ channels a potentially interesting feature fostering future attribution of neuronal K^+ currents to $K_v12.1$ channels. And (ii), recently it was shown for $K_v12.1$ channels that excision of membrane patches from *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 $PI(4,5)P_2$ (and possibly other PI species) through irreversible activation of phosphatases [19,24,28,31,43,44]. As application of a water-soluble $PI(4,5)P_2$ analogue restored $K_v12.1$ channel properties in the excised inside-out patches, these findings demonstrated $PI(4,5)P_2$ sensitivity of $K_v12.1$ channels [7]. We considered this $PI(4,5)P_2$ sensitivity especially interesting, as by attenuating mode shift and by affecting voltage dependence of these channels, impact of PLCβ activation on the excitability of $K_v12.1$ 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 PLCβ in an expression system, we studied sensitivity of human $K_v12.1$ channels to G_qPCR signaling. It has been shown in many studies that overexpression of a G_qPCR (such as m1R) adequately reconstitutes PLCβ signaling in expression systems (including CHO cells used in our report). Through activation of endogenous PLCβ, stimulation of recombinant m1R substantially depletes $PI(4,5)P_2$ [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 $PI(4,5)P_2$ depletion [20,22,29,30,47], but also the modulation of ion channels through second messengers downstream of PLCβ activation [48–51]. Importantly, heterologous systems can even be utilized to dissect $PI(4,5)P_2$ dependence of ion channels from their sensitivity to second messengers produced during activation of PLCβ [47,49,52]. Heterologous expression systems accordingly constitute a well-accepted model to study the G_qPCR pathway and $PI(4,5)P_2$ dependence of ion channels [17,18]. The stimulation of PLCβ as well as voltage-dependent activation of Ci-VSP strongly inhibited *bona-fide* $PI(4,5)P_2$-dependent K_v7 channels (c.f. Figures 2 and 3). Especially, the significant inhibition of $K_v7.3^T$ channels that exhibit considerably higher $PI(4,5)P_2$ affinity than $K_v7.2$ or $K_v7.4$ channels [29,30,38] demonstrated substantial $PI(4,5)P_2$ depletion under our experimental conditions. Translocation of the optical $PI(4,5)P_2$ biosensor $PLC_{\delta1}$-PH-RFP from the membrane during stimulation of m1R additionally showed strong reduction of $PI(4,5)P_2$ levels in these experiments. However, $K_v12.1$ channels were not sensitive to this PLCβ-mediated $PI(4,5)P_2$ depletion at all. Theoretically, a rise in DAG, $I(1,4,5)P_3$ and intracellular Ca^{2+} or activation of PKC could stimulate $K_v12.1$, which might counterbalance PI depletion thereby masking $PI(4,5)P_2$ sensitivity of these channels. However, although we consider such a mechanism rather unlikely, we want to point out that we cannot exclude sensitivity of $K_v12.1$ to these second messengers completely at present. K_v7-mediated currents are indeed reduced by PKC-dependent phosphorylation [48] and Ca^{2+}/calmodulin signaling [53], but importantly G_qPCR-dependent inhibition of these channels almost exclusively depends on $PI(4,5)P_2$ depletion and not on messengers produced by PLCβ [23,29]. Based on these considerations, we conclude that $K_v12.1$ channels are resistant to stimulation of G_qPCR and importantly insensitive to physiologically-relevant $PI(4,5)P_2$ dynamics. This insensitivity of $K_v12.1$ channels to stimulation of PLCβ is not surprising, as several other K^+ channels, including members of the K_v1, K_v2, K_v3, and K_v4 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 K_v channels (K_v1.1, K_v1.4, K_v1.5, K_v3.4), just as for K_v12.1 channels, PI(4,5)P_2 sensitivity was reported using excised patches from *Xenopus laevis* oocytes as experimental model [54,55]. Importantly however, like K_v12.1 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)P_2 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)P_2 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)P_2 depletion through patch excision and subsequent application of exogenous PI(4,5)P_2. On the other hand, the degree of PLCβ-dependent PI(4,5)P_2 depletion might be just too low to induce relevant modulation of K_v12.1 channel activity. And second, as comprehensively discussed and pointed-out by Li and colleagues [7], K_v12.1 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)P_2 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 K_v12.1 channels was sensitive to application of PI(4)P, PI(4,5)P_2 and PI(3,4,5)P_3 [7]. This strongly indicated that K_v12.1 channels (in contrast to e.g. K_v7 channels) do not exhibit highly selective binding to a certain PI species, but rather general electrostatic interactions with several PI [7]. Completely in line, K_v12.1 channels were resistant to activation of Ci-VSP in CHO cells, which stoichiometrically converts PI(4,5)P_2 and PI(3,4,5)P_3 into PI(4)P and PI(3,4)P_2, respectively [35–37,39]. Whilst depleting PI(4,5)P_2 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 K_v12.1 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)P_2 thereby indicating a rather general PI sensitivity of K_v12.1 channels. In line, EC_{50} values of K_v12.1 channels for application of exogenous PI(4,5)P_2 expressed in *Xenopus* oocytes (approx. 10 μM [7]) were well in the range previously demonstrated for K_v7.3 channels [28]. However, most probably due to high PI(4,5)P_2 specificity and selectivity, K_v7.3 channels are readily inhibited by activation of m1R and Ci-VSP, whereas K_v12.1 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 K_v12.1 channels.

Thus, in summary, human K_v12.1 channels most probably exhibit unselective PI sensitivity thereby adding to the growing list of K_v channels resistant to stimulation of G_qPCR/PLCβ-signaling and Ci-VSP-dependent PI(4,5)P_2 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% CO_2 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: K_v7.2-pBK-CMV (gene: human KCNQ2; UniProt accession number: O43526), K_v7.3(A315T)-pBK-CMV (human KCNQ3(A315T); O43525), K_v7.4-pBK-CMV (human KCNQ4; P56696), K_v12.1(Elk1)-pcDNA3.1-IRES-eGFP (human KCNH8; Q96L42), human muscarinic receptor 1 (human M1R)-pSGHV0 (Q96RH1), Ci-VSP-mRFP-C1 (Q4W8A1), $PLC_{\delta1}$-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 $CaCl_2$ (100 nM free Ca^{2+}), 3.5 $MgCl_2$, 5 HEPES, 5 EGTA, 2.5 Na_2ATP, 0.1 Na_3GTP, pH 7.3 (with KOH), 290–295 mOsm/kg. Series resistance (R_s) 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/I_0). The extracellular solution contained (in mM) 144 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 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). K_v 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, K_v7.4 or K_v12.1 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 $I = I_{min} + (I_{max}\text{-}I_{min})/(1 + exp((V\text{-}V_h)/s))$, 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_{max}-I_{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 measured with confocal microscopy with Zen2009 (Zeiss) and IgorPro (Wavemetrics). $PLC_{\delta 1}$-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(J)), *n* represents the number of individual cells and *e* 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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