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Published in:
Biochemical and Biophysical Research Communications

DOI:
10.1016/j.bbrc.2012.06.038

Publication date:
2012

Document version
Early version, also known as pre-print

Citation for published version (APA):
PIP2 modulation of Slick and Slack K⁺ channels

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**Article info**

Article history:
Received 3 June 2012
Available online 21 June 2012

Keywords:
Slick (Slo2.1)
Slack (Slo2.2)
PI(4,5)P2
Cell volume regulation
Phosphoinositides

**Abstract**

Slick and Slack are members of the Slo family of high-conductance potassium channels. These channels are activated by Na⁺ and Cl⁻ and are highly expressed in the CNS, where they are believed to contribute to the resting membrane potential of neurons and the control of excitability. Herein, we provide evidence that Slick and Slack channels are regulated by the phosphoinositide PIP2. Two stereoisomers of PIP2 were able to exogenously activate Slick and Slack channels expressed in Xenopus oocytes, and in addition, it is shown that Slick and Slack channels are modulated by endogenous PIP2. The activating effect of PIP2 appears to occur by direct interaction with lysine 306 in Slick and lysine 339 in Slack, located at the proximal C-terminus of both channels. Overall, our data suggest that PIP2 is an important regulator of Slick and Slack channels, yet it is not involved in the recently described cell volume sensitivity of Slick channels, since mutated PI(2)-insensitive Slick channels retained their sensitivity to cell volume.

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**1. Introduction**

Na⁺-activated K⁺ channels (KNa) are relatively newly characterized channels which were first identified in cardiac myocytes and CNS [1–3]. KNa channels are activated by Cl⁻ as well, and they have been associated with tuning the resting membrane potential and basal excitability of neurons [4]. Two members of the KNa channel family have been cloned within the last decade, namely Slick (Slo2.1) and Slack (Slo2.2) high-conductance potassium channels [4,5]. It has been suggested that they may play an important role for neuronal slow after-hyperpolarization [6]; however neither their physiological importance nor their regulation is at present entirely understood. We have recently shown that Slick channels, but not Slack channels, are precisely regulated by changes in cell volume [7], and this is consistent with a possible role for Slick channels in cell volume regulation, e.g. during ischemia [4]. The mechanism for regulation of ion channels by changes in cell volume is unknown, but it has recently been suggested that phosphatidylinositol biphosphate (PIP2) interaction may confer sensitivity to cell volume changes to ion channels [8].

PIP2 is a common signalling phospholipid present in eukaryotic cells. This phosphoinositide, localized at the inner leaflet of the plasma membrane, exists under different isoforms which are characterized by phosphorylation at distinct positions of the inositol ring; namely phosphatidylinositol 3,4-biphosphate (PI(3,4)P2), phosphatidylinositol 4,5-biphosphate (PI(4,5)P2), and phosphatidylinositol 3,5-biphosphate (PI(3,5)P2). The most abundant isoform is PI(4,5)P2, which is often referred to as PIP2. PIP2 is a substrate for phospholipase C (PLC) for production of inositol triphosphate (IP3) and diacylglycerol (DAG), which in turn, trigger Ca2⁺ release from intracellular stores and activate Ca2⁺/calmodulin-dependent protein kinases II (CamKII), Protein kinase C (PKC) and other major signalling pathways. PIP2 is now also recognized as a signalling molecule per se and it has been suggested to modulate the activity of a number of ion channels, either by direct interaction or indirectly through one of the above mentioned signalling pathways [9]. At present, certain members of most ion channel families, i.e. Ca2⁺ channels, Cl⁻ channels, Na⁺ channels, TRP channels and K⁺ channels have been shown to be regulated by PIP2 [9]. Recently, also two members of the family of high conductance K⁺ channels, namely Slo1 (BK channels) and Slo3 channels have been shown to be activated by PIP2 [10,11].

The current study presents evidence for phosphoinositide mediated activation of Slick and Slack K⁺ channels as a novel regulatory mechanism for these channels. We show that two isomers of PIP2, namely PI(3,4)P2 and PI(4,5)P2, are able to increase currents through Slick and Slack channels expressed in Xenopus laevis oocytes. We also identify a lysine residue at the C-terminus of both channels, which probably interacts directly with PIP2. Finally, our results show that the strict regulation of Slick by small, fast changes in cell volume is independent of PIP2.

**2. Methods**

2.1. Heterologous expression and Molecular Biology:

Xenopus laevis oocytes were prepared as previously described by Grunnet [12]. All procedures were approved by the Danish
National Committee for Animal Studies. Stage V and VI oocytes were chosen for injections and cultured in Kulori medium at 19°C (Kulori: 90 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH 7.4). Site-directed mutagenesis was performed using QuikChange (Stratagene) and mutations were verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany). Wild-type hSlick (Slo2.1) and rSlack (Slo2.2) in the pOX vector were kindly provided by L. Salkoff and Aquaporin-1 (AQP1) in pBlue-script was kindly provided by P. Agre. Vectors were linearized with NotI for Slick and Slack or PstI for AQP1. mRNA was produced using the mMessage mMachine kit and purified with MEGAclear (Ambion). For expression 10 ng (50 nl) of mRNA of Slick, Slack or Slick + AQP1 (3:1 ratio) was injected into oocytes.

2.2. Electrophysiology

Total currents were measured 4–5 days after injection by two-electrode voltage clamp (TEVC) as described before [12]. Currents through expressed channels were elicited using either a step protocol (500 ms depolarizations from 100 to +80 mV, holding potential −80 mV for 4 s) or a pulse protocol (500 ms depolarizations to +80 mV from −80 mV, holding the membrane potential at −80 mV for 3 s). Changes in cell volume were achieved by exposure of the oocytes to isotonic, hypotonic (−50 mOsm/l) or hypertonic (+50 mOsm/l) media as described by Grunnet [12]. To evaluate the effect of phosphoinositides, their water soluble analogs diC8 PI(3,4)P₂ (Kemtec) and diC8 PI(4,5)P₂ (Cayman), were directly dissolved in Kulori at a final concentration of 10 μM. Currents through expressed Slick or Slack channels were carefully measured by TEVC and oocytes were allowed to rest for 2 h in Kulori. Subsequently oocytes were incubated for 2 h with added phosphoinositides before the currents were measured a second time by TEVC. In order to chelate the effect of PI₄,5P₂, oocytes were incubated with neomycin or wortmannin dissolved in Kulori; pH 7.4 to final concentrations. All chemicals were from Sigma (unless otherwise stated).

2.3. Data analysis and Statistics

Data acquisition and analysis were performed using pClamp (Molecular Devices), GraphPad Prism 5 and Excel (Microsoft) packages. Sequences were analyzed with CLC Main Workbench 6.5 (Cambridge, MA, USA). Data are presented means ± S.E.M. (unless otherwise stated). Statistical comparisons were evaluated by paired Student’s t-tests (for two means), one-way ANOVA with Tukey’s post-test (for more than two means), or two-way ANOVA with Bonferroni post-test for grouped analysis. Statistical significance of p-values: * (p < 0.05), ** (p < 0.005), *** (p < 0.0005).

3. Results

3.1. PI₄,5P₂ effect on Slick and Slack K⁺ channels

In order to analyze the sensitivity of Slick and Slack channels to phosphoinositides, DiC8 PI₄,5P₂ and DiC8 PI₃,4P₂ were applied at 10 μM to Xenopus laevis oocytes expressing either of the channels. Whole cell currents were measured by TEVC before and after a 2 h incubation period using a step protocol. Fig. 1 shows that both phosphoinositide isoforms were able to activate currents through Slick and Slack channels. PI₄,5P₂, which is the most abundant isoform naturally occurring in eukaryotic cells, activated Slick channels (225% ± 22% of control) more potently than Slack channels (168% ± 6% of control) (Fig. 1; A1, A2). PI₃,4P₂ stimulated currents Fig. 1. Phosphoinositide-mediated activation of Slick and Slack K⁺ channels. Xenopus laevis oocytes expressing Slick or Slack channels were stimulated by a step protocol (see Methods) and currents were measured at the end of the +80 mV step for Slick (red) and Slack (blue) channels before (control) and after a 2 h incubation with 10 μM PI₄,5P₂ (A1) or PI₃,4P₂ (B1). Currents for control oocytes were normalized to 100% and the columns show the relative effects of incubation with the phosphoinositides (means ± SEM for 5–7 independent experiments). Panels A2 and B2 represent current traces (+80 mV) for single, representative oocytes expressing Slick (red) or Slack (blue) channels before (black) or after PI₄,5P₂ treatment (Slick, red and Slack, blue). Non-injected oocytes showed currents less than 100 nA and did not respond to treatment with PI₄,5P₂ (data not shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
through Slick channels by 160% ± 24%, while Slack currents were enhanced by 125% ± 6% (Fig. 1, B1, B2). Thus, Slick channels seemed more sensitive to PIP2 than Slack channels.

3.2. Manipulation of endogenous PIP2 on Slick and Slack channels

The effect of the PIP2 scavenger neomycin was examined to evaluate the role of endogenous PIP2 on the function of Slick and Slack channels. The channels were initially stimulated with PIP2 as before (10 μM for 2 h), and subsequent addition of neomycin was able to reverse the activating effect of this phosphoinositide for both channels (Fig. 2A). For Slack channels neomycin significantly reduced the PIP2 stimulation in a concentration-dependent manner (from 10 μM to 10 mM); however, the PIP2-induced activation could not be completely reversed, whereas for Slick channels, 10 μM neomycin was sufficient to completely reverse the activation induced by PIP2. Higher concentrations of neomycin resulted in a further decrease in Slick channel currents, suggesting that neomycin was in addition sequestering endogenous PIP2.

As a second approach for exploring the role of endogenous PIP2 on Slick and Slack channel function, wortmannin was employed. Wortmannin affects the concentration of PIP2 in cells by selectively blocking certain phosphatidylinositol (PI) kinases. Nanomolar concentrations of wortmannin block PI3 kinases, which are involved in the conversion of PIP2 to PIP3, and thereby induces accumulation of PIP2 in the plasma membrane [13]. Micromolar concentrations of wortmannin inhibit PI4 kinases, responsible for the re-synthesis of PIP2, leading to decreased PIP2 levels [14]. Fig. 2B, shows the effect of increasing concentrations of wortmannin on the function of Slick and Slack channels. Slack channels were significantly stimulated (119% ± 3%) by treatment with 10 nM wortmannin consistent with an accumulation of PIP2 in the cell membrane and inhibited (72% ± 2% of control levels) by 10 μM wortmannin probably reflecting a decrease of PIP2. Slick channel currents showed a mild, hardly significant, stimulation after treatment with 10 nM wortmannin, but at 1 and 10 μM wortmannin Slick channel currents were reduced to 62% ± 6% and 25% ± 3% of the control value, respectively.

3.3. Effect of mutations at the C-termini of Slick and Slack channels in their PIP2 sensitivity

The next series of experiments were designed to analyze if the stimulating effect of PIP2 on Slick and Slack channels is mediated through direct interaction with the channels. It was previously shown that certain residues, arginines and lysines, located at the proximal C-termini of Slo3 and Slo1 (BK) are necessary for the interaction of these channels with PIP2 [10,11]. Given this information, we made a sequence alignment and found potential candidates that could interact with PIP2 (Fig. 3A). The highly conserved residues, arginine at position 304 and lysine 306 in Slick channels, as well as arginine 337 and lysine 339 in Slack appeared as attractive candidates for PIP2 interaction. Therefore, we performed site-directed mutagenesis in order to substitute these basic residues for alanines. All mutated channels (Slick R304A, Slack K306A, Slack R337A and Slack K339A) could be successfully expressed in Xenopus oocytes and were subsequently exposed to PIP2 exactly as described for wild type (WT) Slick and Slack channels (Fig. 1). The results of these experiments are shown in Fig. 3. In Fig. 3B and E, PIP2 activation of WT Slick and Slack channels has been repeated as a reference to compare the effect of the mutations on the PIP2 sensitivity of the channels. For Slick channels, R304A was still activated to 143% ± 18% by PIP2 (Fig. 3C), whereas neutralization of lysine 306 gave rise to a totally PIP2 insensitive channel (Slick K306A, Fig. 3D). Similarly, Slack R337A was activated to 127% ± 9% of control after treatment with PIP2 (Fig. 3F), whereas Slack K339A completely lost its sensitivity to this phosphoinositide (Fig. 3G). Thus, our results show that specific lysines, K306 for Slick and K339 for Slack, are required for PIP2 interaction and activation of the channels.

3.4. PIP2 effect on the volume sensitivity of Slick channels

It has previously been suggested that PIP2 interaction could confer volume sensitivity to ion channels, such as the KCNQ1 channel [8]. Since Slick channels, but not Slack channels, were previously found to be strongly affected by small and fast changes in cell volume [7], we sought to determine if the sensitivity of Slick...
channels to cell volume changes is dependent on interaction with PI(4,5)P2. For that purpose we co-expressed Slick channels together with AQP1 and Slick currents were recorded by TEVC during cell volume changes induced by exposure with hypotonic and hypertonic buffers (for methodological details see [12]). Measurements were performed before and after treatment with PI(4,5)P2, and, although the current through the channels was activated as before, Fig. 4A and B show that the volume sensitivity of Slick channels was not affected by exogenous PI(4,5)P2. Furthermore, we tested if the volume sensitivity of mutated Slick channels (R304A (Fig. 4C) and K306A (Fig. 4D)) had been affected as a result of their altered ability to interact with PI(4,5)P2. Both mutants were still strongly activated by cell swelling and inhibited by cell shrinking, in a similar manner as WT Slick channels. In addition, manipulation of endogenous PI(4,5)P2 levels with neomycin and wortmannin did not affect the volume sensitivity of WT and mutated Slick channels (data not shown). Taken together, we suggest that PI(4,5)P2 is not critically involved in the volume sensitivity of Slick channels.

4. Discussion

4.1. PIP2 regulates Slick and Slack channels

It has been shown that PIP2 is a regulatory co-factor for a number ion channels [9], including KCNQ1 [15], BK (Slo1) [11] and Slo3 [10], and the present study provides evidence that PIP2 is in addition a novel regulator of the activity of Slick and Slack channels. We have not only assessed the effect of the most common form of PIP2 in mammalian cells, PI(4,5)P2, but also the effect of PI(3,4)P2. Slick and Slack channels were heterologously expressed in Xenopus laevis oocytes, and for stimulation with PI(3,4)P2 and PI(4,5)P2, we used water soluble dioctanoyl analogs, which have short acyl chains and a high partition coefficient, which allows diffusion into the plasma membrane [16]. Both phosphoinositides were able to stimulate Slick and Slack channels in our expression system at a concentration of 10 μM, which is the estimated concentration in mammalian cells [17]. We suggest that PIP2 is an effective modulator of Slick and Slack channels in its more common and physiologically relevant form, PI(4,5)P2, which in our hands induced the strongest stimulation. However, other interconverting isomers, such as PI(3,4)P2 may also modulate the channel activities to a lesser extent. The apparent activating effect of PIP2 (10 μM) was stronger on Slick than Slack channels. One interpretation could be that Slick channels have lower affinity for PIP2 and therefore are more susceptible to regulation by exogenous PIP2 than Slack, since Slack channels may be stimulated to a higher degree by endogenous PIP2.

4.2. Manipulation of endogenous PIP2 levels modulates the activity of Slick and Slack

We evaluated the consequences of manoeuvres to alter PIP2 levels in the oocytes by applying a polycation, neomycin, of which the positives charges interact with the negatively charged head-group...
of PIP2, thereby preventing it from interacting with the channels [18]. Neomycin not only acted as a scavenger, removing the activating effect of exogenously applied PIP2 on Slick and Slack, but at high concentrations (>10 \mu M) it also reduced the currents through Slick channels below control levels, which is likely to be caused by suppression of the endogenous PIP2 stimulation of Slick channels. Therefore, we provide evidence that PIP2 is a co-factor for the normal function of Slick and Slack channels in agreement with previous studies on other ion channels [9–11,15]. The effect of neomycin was more pronounced on Slick than Slack channels, which is consistent with the idea that PIP2 may be more easily removed from Slick than from Slack by the scavenger.

A second approach to modify the PIP2 levels at the plasma membrane was done by application of wortmannin to selectively block PI kinases involved in the synthesis and metabolism of PI(4,5)P2. Our data shows a weak, hardly significant stimulation of Slick channels upon pre-incubation of oocytes with low concentrations (10 nM) of wortmannin, which increases the PI(4,5)P2 concentration in the membrane. In contrast, Slack channels were significantly activated by the same treatment. Thus, elevated PI(4,5)P2 levels obtained through blockade of PI3 kinase by nanomolar concentrations of wortmannin seems to activate at least one of the channels. Application of wortmannin at micromolar concentrations resulted in decreased currents through Slick as well as Slack channels, as would be expected if wortmannin decreases the levels of PI(4,5)P2 at the membrane by blocking PI4 kinase. 10 \mu M wortmannin was sufficient to affect the function of Slick channels, and higher concentrations (100 \mu M), which have previously been shown to regulate other PIP2-sensitive K⁺ channels, [10,11,15], significantly decreased the activities of Slick as well as Slack channels. The effect of a high concentration of wortmannin was more pronounced on Slick than Slack channels, which again could reflect a lower affinity of Slick than of Slack for PI(4,5)P2.

4.3. PI(4,5)P2 modulation of Slick and Slack channels occurs by direct interaction

The activating effect of PI(4,5)P2 on ion channels can be either indirect (Cf. Introduction) or by direct interaction with the channels [9]. Given our previous experiments, we anticipated that the activating effect of PI(4,5)P2 was produced by direct interaction and identified residues in Slick and Slack C-termini that could be potential interaction sites. Of the four mutants, the ones that had the lysine neutralized with alanine entirely lost their PI(4,5)P2 sensitivity, and these experiments indeed seem to prove that PIP2 interacts directly with Slick and Slack channels. Mutations of the arginines resulted in an apparent lower activation by exogenously added PIP2, an observation which could be consistent with a lower affinity for PIP2 binding (Cf. Fig. 3). The head groups of PIP2 may be recognized by a protein through five to
ten atomic contacts and each basic residue provides only one or two interactions [19]. Therefore it seems reasonable to conclude that the mutated lysines are one of the key residues involved in PIP₂ binding to Slick and Slack channels, whereas the mutated arginines, given their close proximity with these lysines probably affect the proper interaction of PIP₄₅P₂ with its binding pocket. However, a detailed mapping of the PIP₂ binding sites will demand more extensive studies.

4.4. PI₄₅P₂ is not involved in the volume sensitivity of Slick channels

It has recently been suggested that PIP₂ is involved in the volume sensitivity of KCNQ1 channels [8], and in addition, it was suggested that PIP₂-interaction in general mediate the volume-sensitivity of K⁺ channels [8]. According to this hypothesis, the intracellular concentrations of Mg²⁺ and polyamines are diluted during cell swelling resulting in a reduced interaction of these cations with PIP₂, which in turn leads to an enhanced stimulatory interaction of PIP₂ with the apparently volume sensitive K⁺ channels [8]. This may, a priori, seem unlikely, since we have earlier shown that only minor changes in cell volume (5%) may lead to a change in channel activity of more than 100% [7,12]. In the present paper we show that although the absolute current through Slick channels is indeed modulated by exogenously added PIP₂, the relative sensitivity to changes in cell volume is absolutely unaffected. In addition, we show that the mutated Slick channel K306A has full sensitivity to changes in cell volume, although it is completely insensitive to modulation by PIP₂. Thus, although PIP₂ does in a number of cases regulate cell volume sensitive K⁺ channels, PIP₂ is not involved in this particular regulatory mechanism, at least not for Slick channels.

In conclusion, we have shown that Slick and Slack channels are activated by two isomers of PIP₂, namely PI₄₅P₂ and PI₃₄P₂, and of these the first seems to be the more potent. In a series of experiments (stimulation by exogenous PIP₂, neomycin and wortmannin) we have shown that Slick and Slack channels are regulated by endogenous PIP₂ after expression in Xenopus laevis oocytes as well as by exogenously added PIP₂. This phosphoinositide regulates the channels through direct interaction with basic residues in their C-termini as evidenced by construction of PIP₂ insensitive mutants. Finally, we show that the sensitivity of Slick channels to cell volume changes is not mediated through interaction with PIP₂. The mechanism underlying cell volume sensitivity of Slick and other K⁺ channels therefore still awaits clarification.

Acknowledgments

Ms. Z. Rasmussen is thanked for expert technical assistance. This work was supported by grants from The Danish Medical Research Council (FSS), the Novo Nordisk Foundation, the Carlsberg Foundation, the Lundbeck Foundation (Lucens) and the Fougé-Hartmann Foundation.

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