

Regulation of Kv7 (KCNQ) K⁺ Channel Open Probability by Phosphatidylinositol 4,5-Bisphosphate

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Voltage-gated Kv7 (KCNQ) channels underlie important K⁺ currents, including the neuronal M current, and are thought to be sensitive to membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) and PIP₂ depletion to underlie muscarinic receptor inhibition. We studied regulation of Kv7.2–7.4 channels by PIP₂ in Chinese hamster ovary (CHO) cells using single-channel and whole-cell patch clamp and biochemical analysis. Maximal open probabilities (P_o) of Kv7.2–Kv7.4 homomultimers and of Kv7.2/7.3 heteromultimers were found to be strongly dependent on the [diC8-PIP₂] applied to inside-out patches, with differential apparent affinities that correlate with their maximal P_o in on-cell mode. Unitary conductance was not affected by PIP₂. Raising tonic [PIP₂] by coexpression of phosphatidylinositol (4)5-kinase increased the maximal P_o of both Kv7.2 and Kv7.2/7.3 channels studied in on-cell patches and increased whole-cell Kv7.2, but not Kv7.3, current amplitudes. In cells coexpressed with muscarinic M₁ receptors, bath application of muscarinic agonist reduced the maximal P_o of Kv7.2/7.3 channels isolated in on-cell patches. Coexpression of a PIP₂ sequestering construct moderately reduced whole-cell Kv7.2/7.3 currents, and coexpression of a construct containing a PIP₂ phosphatase nearly abolished them. Finally, biochemical analysis of anionic phospholipids in CHO cells stably expressing M₁ receptors shows that PIP₂ and PIP are nearly depleted 1 min after muscarinic stimulation, with an unexpected rebound after 10 min. These results strongly support the direct regulation of Kv7 channels by PIP₂ and its depletion as the mechanism of muscarinic suppression of M channels. Divergent apparent affinities of Kv7.2–7.4 channels for PIP₂ may underlie their highly differential maximal P_o observed in cell-attached patches.

Key words: lipid signaling; potassium channel; M current; phosphatidylinositol 4,5-bisphosphate; single channel; gating; patch clamp

Introduction

Many ion channels and transporters have been shown to be sensitive to the presence of phosphatidylinositol 4,5-bisphosphate (PIP₂) in the membrane (Suh and Hille, 2005). Particularly well studied has been PIP₂ regulation of the family of inward rectifier K⁺ (Kir) channels, for which differential sensitivity to membrane PIP₂ has been suggested to underlie the highly divergent open probabilities of this channel family (Rohacs et al., 2003). In this scenario, PIP₂ has differential apparent affinities for each channel protein, and a given channel with a high apparent affinity for PIP₂ will display a high open probability (P_o) at tonic PIP₂ levels and be relatively insensitive to decreases in PIP₂, whereas channels with a low apparent affinity display a much lower tonic P_o and are very sensitive to depletion of PIP₂, as may happen after strong activation of phospholipase C (PLC).

An emerging literature suggests that a similar permissive requirement for membrane PIP₂ controls the activity of the Kv7

family of M-type voltage-gated K⁺ channels, made by Kv7.1–7.5 (KCNQ1–5) subunits (Delmas and Brown, 2005). Most M currents in neurons are made by homomultimers and heteromultimers of Kv7.2, Kv7.3, and Kv7.5 (Wang et al., 1998; Lerche et al., 2000; Cooper et al., 2001; Roche et al., 2002; Hadley et al., 2003); Kv7.4 makes important K⁺ currents in the inner ear (Kubisch et al., 1999), and Kv7.1 co-assembles with auxiliary KCNE subunits to make the cardiac I_{Ks} current (Sanguinetti et al., 1996). Wide agreement is appearing that M channels are sensitive to the abundance of membrane PIP₂ and that activation of PLC, and subsequent PIP₂ hydrolysis, can sufficiently deplete the membrane of PIP₂ to cause the observed downregulation of M currents by stimulation of G_{q/11}-coupled M₁ muscarinic acetylcholine receptors (mAChRs) (Suh and Hille, 2002; Ford et al., 2003; Zhang et al., 2003; Suh et al., 2004). Although this hypothesis is compelling, single-channel demonstration of the direct gating of M channels by PIP₂ has been lacking.

We recently characterized the gating of Kv7.2–7.5 channels at the single-channel level and found them to have strikingly different open probabilities in cell-attached patches (Li et al., 2004). Whereas Kv7.3 has a maximal P_o near unity, Kv7.2, Kv7.4, and Kv7.5 have much lower maximal P_o values and, in particular, Kv7.4 has a maximal P_o over 10-fold lower than Kv7.3. Using a chimeric strategy, the highly divergent P_o values for Kv7.3 and Kv7.4 were found to be conferred by the cytoplasmic C terminal of the channel. Here, we sought to more directly test the putative

Received June 23, 2005; revised Aug. 26, 2005; accepted Sept. 9, 2005.

This work was supported by National Institutes of Health Grants R01 NS043394 (M.S.S.) and R01 HL067942 (D.W.H.) and American Heart Association Postdoctoral Fellowship 0325120Y (N.G.). We thank Pamela Martin, Kathryn Boyd, and Chengcheng Shen for expert technical assistance and Brad Rothberg and James Stockand for helpful discussions.

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DOI:10.1523/JNEUROSCI.2597-05.2005

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PIP₂ regulation of Kv7 channel gating at the single-channel and whole-cell level and by biochemical analysis. We find that Kv7 channels are indeed highly sensitive to the PIP₂ concentration at the cytoplasmic face of the patch and that the different Kv7 channels display a highly differential apparent affinity for PIP₂ that may underlie their differential P_o in intact cells. Furthermore, changes in [PIP₂] have the expected effect on channel activity consistent with the PIP₂-regulation hypothesis. Finally, PIP₂ and PIP levels are shown to dramatically decrease and then rebound partially in parallel with a continuing fall of phosphatidylinositol (PI) and rise of phosphatidic acid (PA).

Materials and Methods

cDNA constructs. Plasmids encoding human Kv7.2, rat Kv7.3, human Kv7.4, and human Kv7.5 (GenBank accession numbers AF110020, AF091247, AF105202, and AF249278, respectively) were kindly given to us by D. McKinnon (State University of New York, Stony Brook, NY; Kv7.2 and Kv7.3), T. Jentsch (Zentrum für Molekulare Neurobiologie, Hamburg, Germany; Kv7.4), and K. Steinmeyer (Aventis Pharma, Frankfurt am Main, Germany; Kv7.5). Kv7.2 and Kv7.3 were subcloned into pcDNA3 (Invitrogen, San Diego, CA) as described previously (Shapiro et al., 2000). Kv7.4 and Kv7.5 were subcloned into pcDNA3.1zeo+ and pcDNA3.1zeo- (Invitrogen) using *XhoI/HindIII* and *XbaI/EcoRI*, respectively. EGFP-PLCδ-PH, EGFP-Lyn-PH-PP, and EGFP-Akt-PH constructs were kind gifts from T. Meyer (Stanford University, Stanford, CA). Type 1α PI(4)P5-kinase [PI(4)5-kinase] was kindly given to us by L. Pott (Ruhr-University, Bochum, Germany). The Chinese hamster ovary (CHO) cell line stably expressing M₁ muscarinic receptors was kindly given to us by Prof. D. A. Brown (University College London, London, UK). The human mAChR M₁ clone was purchased from the Guthrie Foundation (Sayre, PA).

Cell culture and transfections. CHO cells were used for electrophysiological analysis as described recently (Gamper et al., 2005a). Cells were grown in 100 mm tissue culture dishes (Falcon; Becton Dickinson, Mountain View, CA) in DMEM with 10% heat-inactivated fetal bovine serum and 0.1% penicillin and streptomycin in a humidified incubator at 37°C (5% CO₂) and passaged every 3–4 d. Cells were discarded after ~30 passages. For transfection, cells were plated onto poly-L-lysine-coated coverslip chips and transfected 24 h later with Polyfect reagent (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. For electrophysiological experiments, cells were used 48–96 h after transfection. As a marker for successfully transfected cells, cDNA encoding green fluorescent protein (GFP) was cotransfected together with the cDNAs of the genes of interest. We found that >95% of green-fluorescing cells expressed KCNQ currents in control experiments.

Cell-attached/inside-out patch/single-channel electrophysiology. The methods of recording and analysis were similar to those previously used for studying unitary Kv7 channels (Li et al., 2004). Channel activity was recorded 48–72 h after transfection in the cell-attached configuration. Pipettes had resistances of 7–15 MΩ when filled with a solution of the following composition (in mM): 150 NaCl, 5 KCl, 1 MgCl₂, and 10 HEPES, pH 7.4 with NaOH. Cells were bath perfused with a solution of the following composition (in mM): 175 KCl, 4 MgCl₂, and 10 HEPES, pH 7.4 with KOH. There was no Ca²⁺ nor Ca²⁺-chealator added to these solutions, and we assume free Ca²⁺ to be in the range of ~50 μM. The resting membrane voltage was assumed to be 0 mV. Currents were recorded using an Axopatch 1-D amplifier (Molecular Devices, Union City, CA), sampled at 5 kHz, and filtered at 200–500 Hz (Kv7.4) or at 0.5–1 kHz (all others). The short-chain, water-soluble PIP₂ analog diC8-PIP₂ (Echelon Biosciences, Salt Lake City, UT) was dissolved in an aqueous stock solution at 10 mM and sonicated immediately before each use.

Single-channel data were analyzed using PulseFit and TAC (Bruxton, Seattle, WA) software. Open and closed events were analyzed by using the “50% threshold criterion.” All events were carefully checked visually before being accepted. P_o histograms were generated using TACFit (Bruxton). The presence of only one channel in a patch was assumed if the P_o was >0.25 for >1 min without superimposed openings, especially at strongly depolarized potentials at which P_o is highest. At a given po-

tential, the single-channel amplitude (i) was calculated by fitting all-point histograms with single or multi-Gaussian curves. The difference between the fitted “closed” and “open” peaks was taken as i . When superimposed openings were observed, the number of channels in the patch was estimated from the maximal number of superimposed openings. The apparent NP_o was estimated as follows:

$$NP_o = \left(\sum_{j=1}^N t_j \right) / T \quad (1)$$

where t_j is the time spent at each current level corresponding to $j = 0, 1, 2, \dots, N$, T is the duration of the recording, and N is the number of current levels (minimum number of active channels).

Dose–response curves of channel P_o versus [diC8-PIP₂] were fit using IgorPro software (WaveMetrics, Lake Oswego, OR) by the following Hill equation:

$$P_o = P_{o,max} / \left[1 + \left(\frac{x_{half}}{x} \right)^n \right] \quad (2)$$

where $P_{o,max}$ is set to 1, x is [diC8-PIP₂], x_{half} is the [diC8-PIP₂] at which $P_o = 0.5$, and n is the Hill coefficient.

Whole-cell electrophysiology. Whole-cell and perforated-patch experiments were performed as described previously (Gamper et al., 2003). To evaluate the amplitude of macroscopic Kv7.2/7.3 currents, CHO cells were held at 0 mV, and 800 ms hyperpolarizing steps to –60 mV, followed by 1 s pulses back to 0 mV, were applied. The amplitude of the current was usually defined as the difference between the holding current at 0 mV and the current at the beginning (after any capacity current as subsided) of the 1 s pulse back to 0 mV. In some cells, a more precise measurement was the XE991- or linopirdine-sensitive current at the holding potential of 0 mV. CHO cells have negligible endogenous macroscopic K⁺ currents under our experimental conditions, and 50 μM XE991 or linopirdine completely blocked the K⁺ current in Kv7-transfected CHO cells but had no effect on currents in nontransfected cells (Gamper et al., 2005a). All results are reported as mean ± SEM.

Phosphoinositide analysis. Anionic phospholipids were extracted from cells, deacylated, and quantified from the glycerol head groups by suppressed conductivity as described previously (Nasuhoglu et al., 2002).

Results

PIP₂ stabilizes opening of unitary Kv7.2/7.3 channels and reactivates “run-down” channels

We first examined the sensitivity of Kv7.2/7.3 (KCNQ2/3) heteromultimers to PIP₂, because these heteromeric channels comprise most “M-type” channels in sympathetic ganglia cells (Wang et al., 1998; Roche et al., 2002). Cloned Kv7.2+7.3 subunits were expressed in CHO cells and recordings from channels isolated in cell-attached patches, followed by excised inside-out patches. Heterologously expressed Kv7.2/7.3 channels produce voltage-gated K⁺ currents very similar to native M currents in neurons (Wang et al., 1998; Selyanko et al., 2000; Shapiro et al., 2000). Because the muscarinic inhibition of M-type channels, thought to be attributable to depletion of PIP₂ (Delmas and Brown, 2005), is voltage independent (Shapiro et al., 2000), we focus here on the P_o of the channels at saturating voltages, which we refer to as their maximal P_o . This value was measured usually at 0 mV, which is a saturating voltage for these channels (Li et al., 2004).

For channels sensitive to membrane PIP₂ abundance, their activity in cell-attached patches should reflect the tonic level of PIP₂, channel run-down during excision the loss of PIP₂ in the patch by membrane-associated phosphatases, and channel activity in inside-out patches should be governed by the PIP₂ concentration ([PIP₂]) in the cytoplasmically facing bath solution. Because full-length PIP₂ itself is water insoluble, making quantification of its concentration in aqueous solution uncer-

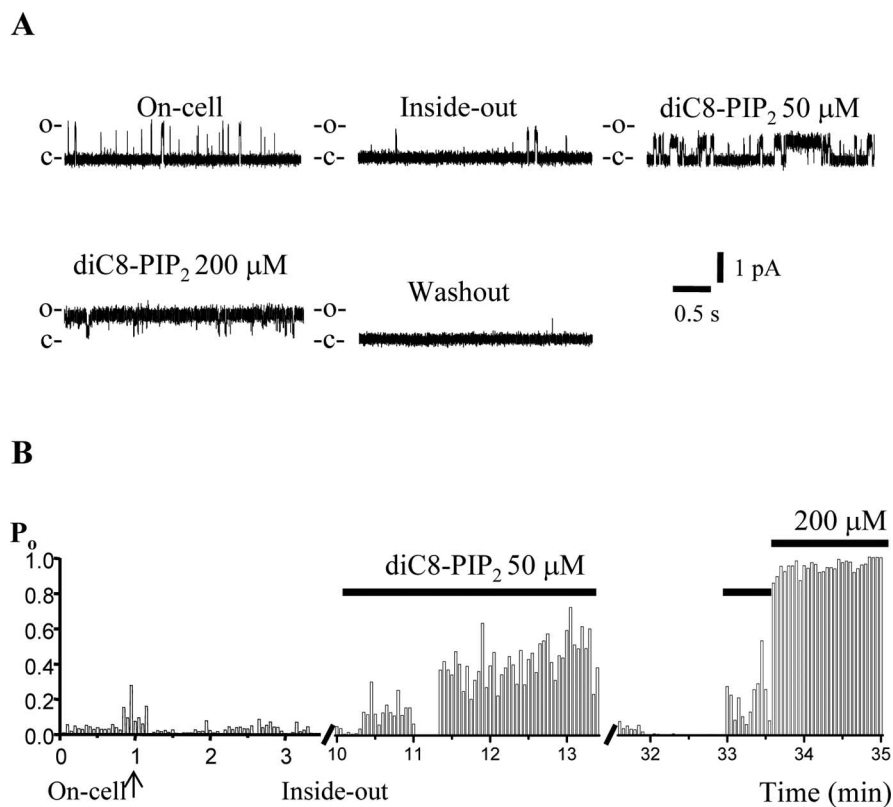


Figure 1. DiC8-PIP₂ stabilizes the opening of Kv7.2/7.3 channels. **A**, Current records from a patch containing a single Kv7.2/7.3 channel in cell-attached mode (On-cell) and in inside-out mode with various concentrations of diC8-PIP₂ in the cytoplasmically facing bath solution. **B**, Each 3 s sweep during the experiment was analyzed for channel P_o, and the time course of the P_o during the experiment is plotted. The transition from on-cell to inside-out mode is indicated by the arrow, and the applications of different diC8-PIP₂ concentrations are indicated by the bars.

tain, we used the short-chain, water-soluble analog dioctanoyl (diC8)-PIP₂, as have other investigators studying the PIP₂ sensitivity of these channels (Zhang et al., 2003). Figure 1 shows a representative experiment on a patch containing a single Kv7.2/7.3 channel examined at a saturating voltage of 0 mV, which is sufficiently positive to induce maximal P_o (Li et al., 2004). In on-cell mode, the activity of the channel was present, but modest. After excision, the channel ran down considerably, displaying only rare open events. The channel activity, however, was strongly reactivated by the addition of diC8-PIP₂ to the bath, resulting in channel activity much greater than that exhibited in on-cell mode. The modest channel activity in on-cell mode in this patch probably reflects a low tonic PIP₂ abundance in this particular cell. At 50 μM diC8-PIP₂, the channel P_o was almost 0.5, and application of 200 μM diC8-PIP₂ caused the channel to remain open almost all of the time. For Kv7.2/7.3 channels isolated in patches, the maximal P_o in on-cell mode was 0.31 ± 0.04 ($n = 41$); after excision, the channels ran down to a P_o of 0.14 ± 0.04 ($n = 18$), and the addition of 50 μM diC8-PIP₂ reactivated the channels to a P_o of 0.65 ± 0.09 ($n = 8$). An additional increase in diC8-PIP₂ to 200 μM increased the P_o at 0 mV to near unity (0.95 ; $n = 2$).

Kv7.2–7.4 channels are sensitive to PIP₂ with different apparent affinities

We then similarly investigated the PIP₂ sensitivity of Kv7.2–7.4 homomultimers. We have previously showed Kv7.2–7.4 homomultimers to have a very different maximal P_o (Li et al., 2004), raising the possibility that a differential apparent affinity for PIP₂

may underlie these differences. Figure 2 shows representative experiments at a patch potential of 0 mV, which is a saturating voltage for these channels. A patch containing a single Kv7.2 channel (Fig. 2A) was excised and ran down to the marginal P_o value of 0.01. Application of 3 μM diC8-PIP₂ failed to significantly increase channel activity, but application of 100 μM had a robust effect, increasing channel P_o to 0.35. Records from a patch containing a unitary Kv7.3 channel are shown in Figure 2B. In on-cell mode, channel activity was near unity, as is usual for this channel (Li et al., 2004). After excision, channel activity somewhat ran down, but remained substantial, with a P_o value of 0.24. Application of only 3 μM diC8-PIP₂ increased the P_o to 0.72, and 30 μM diC8-PIP₂ sufficed to restore channel P_o to near its value in on-cell mode. A patch containing a single Kv7.4 channel, on the other hand, exhibited a negligible P_o after excision to inside-out mode (0.001), and this value was only marginally increased by application of 20 μM diC8-PIP₂ (P_o = 0.005). However, the increased concentration in diC8-PIP₂ to 150 μM resulted in a dramatic increase in channel P_o, to 0.43.

These types of experiments on homomeric Kv7.2–7.4 channels were performed over a range of diC8-PIP₂ concentrations, and the data were summarized as a dose–response relationship as P_o versus diC8-PIP₂ concentration (Fig. 3A). For Kv7.2–7.4 homomultimers and Kv7.2/7.3 heteromultimers, channel P_o was well described by such a dose–response relationship with diC8-PIP₂ concentration, but with dramatically differential apparent affinities. For all of the channels, channel P_o near unity could be achieved by application of diC8-PIP₂, but at very different concentrations. For Kv7.2/7.3, Kv7.2, Kv7.3, and Kv7.4, the EC₅₀ values were 40 ± 6 μM ($n = 1–4$ patches), 205 ± 36 μM ($n = 1–8$ patches), 2.6 ± 0.5 μM ($n = 1–7$ patches), and 215 ± 19 μM ($n = 1–3$ patches), respectively. Thus, there is a 100-fold difference in the apparent affinity of Kv7.3 versus Kv7.2 or Kv7.4 to diC8-PIP₂, and we suppose also to full-length PIP₂, and heteromeric Kv7.2/7.3 channels have an intermediate apparent affinity, as one would expect for a heteromeric channel composed of Kv7.2 and Kv7.3 subunits that both contribute to PIP₂ binding, and to gating. For the four types of channels, the Hill coefficients were 1.24 ± 0.24 , 1.33 ± 0.15 , 0.97 ± 0.17 , and 1.74 ± 0.15 , respectively, very near that reported previously for diC8-PIP₂ action on Kv7.2/7.3 (Zhang et al., 2003) and slightly lower than that modeled for full-length PIP₂ action on Kv7.2/7.3 channels (Suh et al., 2004).

The differential apparent affinities of diC8-PIP₂ for activation of Kv channels parallels their maximal P_o in cell-attached patches, for which the rank order is Kv7.3 > Kv7.2/7.3 > Kv7.2 > Kv7.4 (Li et al., 2004), suggesting that the differential P_o of channels in on-cell mode may be governed by their differential apparent affinity for plasma-membrane PIP₂. By correlating the maximal P_o of Kv7 channels in on-cell mode with their values as a function of diC8-PIP₂ concentration, we can estimate what diC8-PIP₂ concentration correlates with tonic [PIP₂] in intact cells. In cell-

attached patches, the maximal P_o of Kv7.2/7.3, Kv7.2, Kv7.3, and Kv7.4 are 0.30–0.31, 0.04–0.17, 0.59–0.89, and 0.07 (Selyanko et al., 2001; Li et al., 2004; present study), respectively. From these values, we can estimate that an applied diC8-PIP₂ concentration of $\sim 23 \mu\text{M}$ in inside-out patches corresponds to the tonic [PIP₂] seen by channels in intact cells, because this concentration gives P_o values similar to that seen in on-cell patches for all of the channels. We presume that the affinity of the channels to PIP₂ differs from that for the shorter-chain diC8-PIP₂ analog but that the relative apparent affinities for the different channels are likely similar for the two phosphoinositides. We also investigated whether PIP₂ changes the single-channel conductance of Kv7 channels. Kv7.2/7.3 channels were recorded in inside-out patches over a range of potentials, both in the presence or absence of $30 \mu\text{M}$ diC8-PIP₂. The presence of the phosphoinositide, although having a dramatic effect on channel P_o , did not affect their unitary conductance, which was $6.1 \pm 0.4 \text{ pS}$ ($n = 5$) and $6.8 \pm 0.4 \text{ pS}$ ($n = 5$) in the absence or presence of $30 \mu\text{M}$ diC8-PIP₂, respectively (Fig. 3B).

The molecule we call PIP₂ is not the only doubly phosphorylated phosphoinositide used as a membrane signaling molecule; the other is phosphatidylinositol 3,4-bisphosphate [PI(3,4)P₂]. The latter is produced by activation of phosphatidylinositol 3'-kinase (PI3-kinase), typically as a result of growth factor receptor activation, and is well known for activation of the kinase Akt/PKB. Although plasma-membrane PI(3,4)P₂ concentrations are typically only a small fraction of [PIP₂], even after strong PI3-kinase activation, the sensitivity of other PIP₂-sensitive channels to [PI(3,4)P₂] has been investigated as a probe of phosphoinositide binding (Rohacs et al., 1999). We tested whether bath application of PI(3,4)P₂ to inside-out patches containing Kv7.2/7.3 channels would also stabilize channel activity. We found that at $10 \mu\text{M}$, PI(3,4)P₂ reactivated channels to a similar maximal P_o observed in cell-attached patches, and at $20 \mu\text{M}$, channel activity was further increased (data not shown). Thus, like certain types of Kir3 channels (Rohacs et al., 1999), Kv7.2/7.3 channels may have a nonspecific phosphoinositide activation profile, at least in terms of these two distinct moieties of doubly phosphorylated phosphoinositides.

Increasing tonic [PIP₂] by overexpression of PI(4)5-kinase increases on-cell channel activity

Having ascertained that Kv7 channels are sensitive to plasma-membrane PIP₂, we then manipulated [PIP₂] in intact cells in several different ways and examined the effects on maximal P_o . PIP₂ is produced in the plasma membrane by sequential phosphorylation of PI by PI4-kinase and PI(4)5-kinase. Overexpression of PI(4)5-kinase in atrial myocytes or in sympathetic neurons has been shown to tonically increase membrane PIP₂ and to dramatically reduce desensitization of Kir3 channels in the former case and muscarinic modulation of endogenous M current in the latter case (Bender et al., 2002; Winks et al., 2005). We

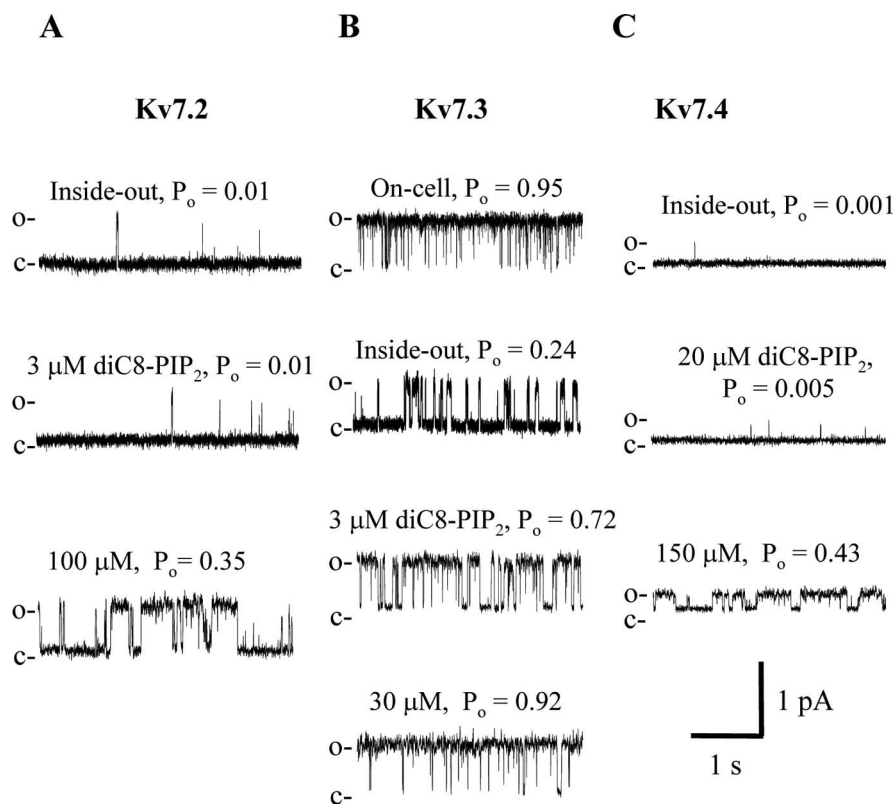


Figure 2. A–C, Individual sweeps from patches containing a single Kv7.2 (A), Kv7.3 (B), or Kv7.4 (C) channel in the presence of the indicated concentration of diC8-PIP₂. The records were filtered at 500 Hz (A, B) and 200 Hz (C).

thus reasoned that if Kv7 channels are indeed sensitive to membrane [PIP₂], then its tonic increase by overexpression of PI(4)5-kinase should increase channel P_o recorded in cell-attached patches. CHO cells were coexpressed with Kv7.2+Kv7.3 or only Kv7.2, either alone or together with PI(4)5-kinase. For both Kv7.2/7.3 and Kv7.2 channels, there was a dramatic increase in maximal P_o in cells coexpressed with PI(4)5-kinase. In addition, the profound run-down in channel activity observed after excision of patches from control cells was sharply reduced in patches from PI(4)5-kinase-transfected cells (Fig. 4A). These data are summarized in Figure 4B. In on-cell patches containing Kv7.2 channels only, the P_o at 0 mV was 0.035 ± 0.013 ($n = 12$), but in patches from cells coexpressing PI(4)5-kinase, the P_o was 0.74 ± 0.09 ($n = 10$). In on-cell patches containing Kv7.2/7.3 channels only, the P_o at 0 mV was 0.31 ± 0.04 ($n = 41$), but in patches from cells coexpressing PI(4)5-kinase, the P_o was 0.95 ± 0.03 ($n = 13$). These results are in accord with recent work from the Brown laboratory, which showed that overexpression of PI(4)5-kinase increased [PIP₂] nearly threefold in sympathetic neurons, resulting in strongly blunted muscarinic modulation of M currents (Winks et al., 2005). Although we cannot quantify the increase produced here, we estimate it to be at least that much, judging from the robust increase in on-cell channel P_o and from the strongly reduced run-down of the channels after patch excision.

Heterologously expressed homomeric Kv7.2 and 7.3 channels display whole-cell current amplitudes some 10-fold smaller than do Kv7.2/7.3 heteromultimers (Wang et al., 1998; Gamper et al., 2003; Maljevic et al., 2003; Schwake et al., 2003). Although it has been suggested that this difference is attributable to differential surface expression of the homomeric and heteromeric channels (Schwake et al., 2000), a number of factors are likely involved (Etxeberria et al., 2004). The dramatic increase in maximal P_o of

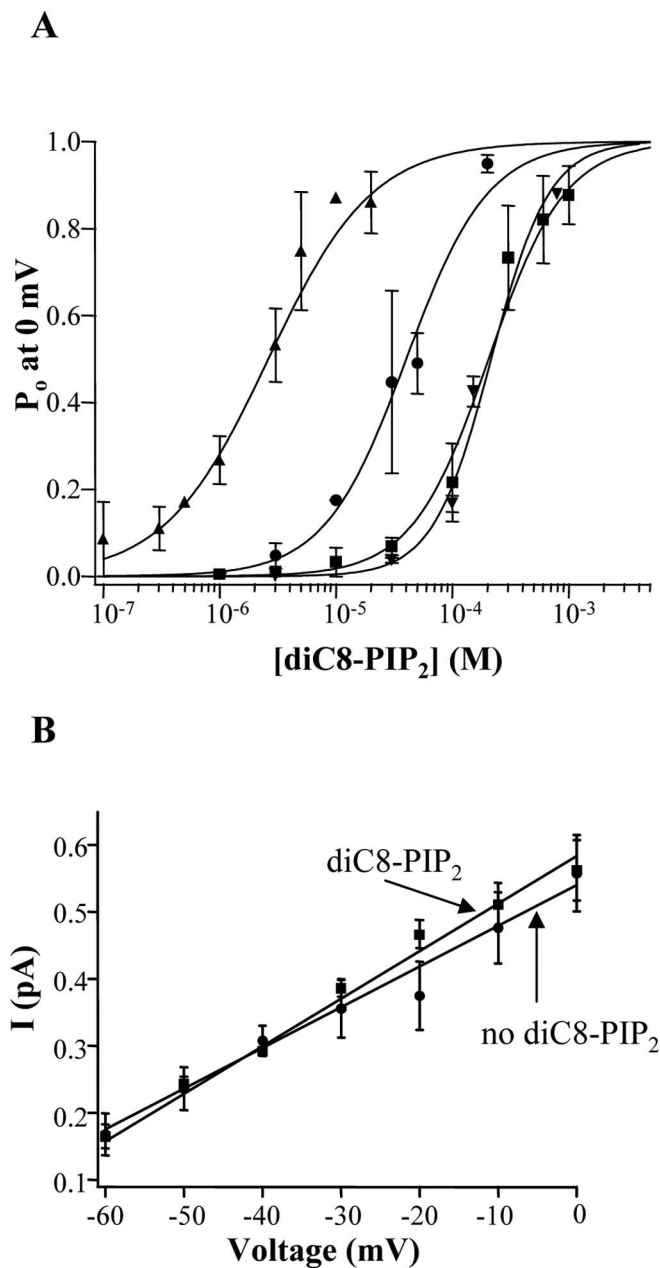


Figure 3. *A*, Dose–response relationship of the P_o for Kv7.2 (squares), Kv7.3 (triangles), Kv7.4 (inverted triangles), and Kv7.2/7.3 (circles) channels as a function of diC8-PIP₂ concentration in inside-out patches. The data were fit by a Hill equation (see Materials and Methods), with data errors taken into account by the fitting routine for all channels, except Kv7.2/7.3. The parameters of the fits are given in the text. *B*, The unitary current amplitudes for Kv7.2/7.3 channels over a range of voltages, either in the absence or presence of diC8-PIP₂ (30 μM) in the bath, are plotted. The data were fit linearly, yielding single-channel conductances given in the text.

Kv7.2 seen in cell-attached patches from PI(4)5-kinase-expressing cells suggested that part of the reason for the low macroscopic expression of Kv7.2 channels might be as a result of its low apparent affinity for PIP₂ and that such expression might be much larger in cells with higher tonic [PIP₂] produced by overexpression of PI(4)5-kinase. Indeed, this turned out to be the case (Fig. 4C). Control cells displayed very low Kv7.2 current densities (4.5 ± 1.1 pA/pF; $n = 10$), but those coexpressed with PI(4)5-kinase displayed current densities some eightfold larger (34.7 ± 5.8 pA; $n = 10$; $p < 0.001$). On the other hand, the high maximal

P_o of Kv7.3 homomultimers (Li et al., 2004) and its high apparent affinity for diC8-PIP₂ (present study) predicts that overexpression of PI(4)5-kinase should not increase macroscopic Kv7.3 currents, and this was indeed the case as well (Fig. 4C). In control cells, Kv7.3 current densities were 7.0 ± 1.5 pA/pF ($n = 10$), and in cells coexpressed with PI(4)5-kinase, they were still low, at 4.9 ± 1.0 pA/pF ($n = 10$). These data reinforce the conclusion that Kv7.2 and Kv7.3 channels have highly differential apparent affinities for PIP₂ and furthermore suggest that part of the difference between Kv7.2 and Kv7.2/7.3 current amplitudes may be attributable to the lower apparent affinity of Kv7.2 for PIP₂. However, the high affinity of Kv7.3 for diC8-PIP₂ found in this study, reinforced by the lack of increase in whole-cell currents in cells overexpressing PI(4)5-kinase, suggests that the low macroscopic expression of Kv7.3 is not attributable to weak binding to PIP₂ and indicates a further difference between Kv7.2 and Kv7.3.

Cell-attached channel P_o is suppressed by bath application of muscarinic agonists

To further explore PIP₂ regulation of Kv7 channels, we reduced membrane PIP₂ abundance in intact CHO cells by receptor stimulation and by tonic PIP₂ depletion. The separation of types of G-protein-mediated modulation of channels between “direct” and “second-messenger-mediated” mechanisms is best observed using cell-attached patch experiments. In direct mechanisms involving interactions between G-protein and channels, bath application of receptor agonists does not modulate the activity of channels isolated in the patch membrane, presumably because activated G-protein subunits cannot diffuse in the plane of the membrane past its interface with the glass of the pipette tip. However, when second-messenger molecules underlie the modulation, as is the case for G_{q/11} actions, channels in the patch can be modulated by bath-applied agonists. For the case of muscarinic modulation of N-type Ca²⁺ channels in SCG neurons, the rapid inhibition by G-protein βγ dimers, thought to underlie modulation by G_{o/i}-coupled M_{2/4} receptors (Herlitze et al., 1996), is not seen in such cell-attached patch experiments, but the slower inhibition by G_{q/11}-coupled M₁ receptors does affect channels in the patch, implicating an intracellular second messenger in the action (Hille, 1994). The latter pathway has been shown to be attributable to depletion of PIP₂ in the membrane (Gamper et al., 2004). Likewise, the M₁ receptor-mediated modulation of M channels that gives M current its name also reaches past the glass/membrane interface, suppressing M channels in the cell-attached patch (Selyanko et al., 1992). Finally, M₁ receptor-mediated desensitization of GIRK (G-protein-activated inwardly rectifying K⁺; Kir3) channels, thought also to be attributable to depletion of membrane PIP₂, is also evident on channels in the patch when M₁ receptors in the rest of the cell are stimulated (Zhang et al., 2003). Thus, it seems likely that, unlike membrane proteins, membrane PIP₂ can diffuse into and out of the membrane in the tips of patch pipettes and is the exemplar case of a type signal recently termed “diffusible membrane delimited” (Delmas et al., 2005).

We wanted to analyze the muscarinic suppression of Kv7.2/7.3 channels, mediated by depletion of PIP₂, at the single-channel level, to compare it to regulation of the channels by diC8-PIP₂ in inside-out patches. CHO cells transfected with Kv7.2, Kv7.3, and the human M₁ receptor were studied in cell-attached patches, and unitary currents recorded before, during, and after application of the muscarinic agonist oxotremorine-M (oxo-M) (Fig. 5). Although such stimulation of heterologously expressed M₁ receptors likely raises [Ca²⁺]_i (Shapiro et al., 2000), we have shown

Kv7.2/7.3 channels heterologously expressed in CHO cells to be mostly insensitive to rises in [Ca²⁺]_i in the absence of coexpressed calmodulin (Gamper and Shapiro, 2003). In the experiment shown in Figure 5A, P_o was 0.40 in control, slightly higher than that typically seen for Kv7.2/7.3 channels. Bath application of oxo-M (10 μM) strongly reduced channel P_o within several minutes to 0.07, and channel activity was reversible to a P_o of 0.24 several minutes after washout of the agonist. In all such patches tested, the P_o in control was 0.22 ± 0.06; after application of oxo-M, the P_o was sharply reduced by 73% to 0.060 ± 0.018, and after washout, it mostly recovered, to 0.19 ± 0.03 (n = 4). This action of bath-applied muscarinic agonists on unitary Kv7.2/7.3 channels isolated in the cell-attached patch corresponds very closely with the action on whole-cell currents seen previously (Selyanko et al., 2000; Shapiro et al., 2000). These data suggest that, although there are known differences in this signaling pathway between a heterologous expression system and real neurons, muscarinic modulation of neuronal M currents can be wholly accounted for by a modulation of channel P_o. It also reinforces the conclusion that PIP₂ can diffuse into and out of patches of membrane in the tips of patch pipettes. From similarly transfected CHO cells, we also used “macropatches,” containing many Kv7.2/7.3 channels to observe such inhibition from bath-applied agonist. These experiments also included the membrane-permeant IP₃-receptor blocker 2-aminoethoxydiphenyl borate (Maruyama et al., 1997) to exclude the possibility that the inhibition was attributable to rises in [Ca²⁺]_i. Bath application of oxo-M (10 μM) strongly and reversibly inhibited the Kv7.2/7.3 currents in the patches (data not shown), similar to previous results using such macropatch experiments (Selyanko et al., 2000).

Coexpression of PIP₂ sequestering or degrading constructs reduces whole-cell Kv7.2/7.3 currents in CHO cells

We also assayed the PIP₂ sensitivity of Kv7.2/7.3 channels by testing several enhanced GFP (EGFP)-tagged constructs that have been shown to bind and sequester PIP₂ or to decrease its plasma membrane concentration. The first, PLCδ-PH, contains the “pleckstrin homology” (PH) domain of PLCδ that binds to PIP₂ to achieve its plasma-membrane targeting (Shaw, 1996). Overexpression of PLCδ-PH has been shown to sequester PIP₂ and thus make it unavailable to act as an intracellular effector (Stauffer et al., 1998; Raucher et al., 2000; Gamper et al., 2004). The second, Akt-PH, contains the PH domain of the serine/threonine kinase Akt. Akt binds to, and is

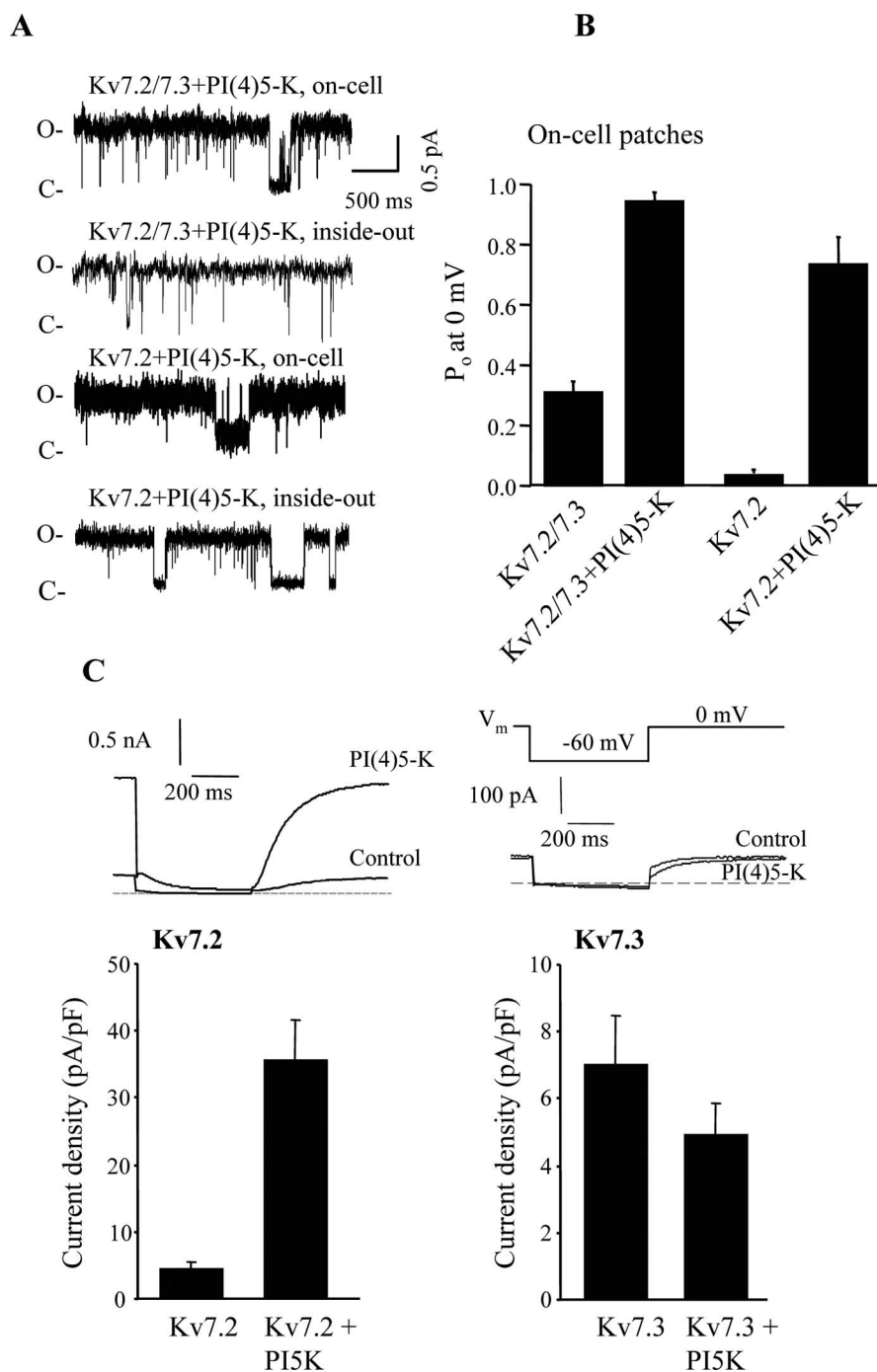


Figure 4. Overexpression of PI(4)5-kinase increases the P_o of Kv7 channels in patches and increases whole-cell Kv7.2 but not Kv7.3, currents. **A**, Individual sweeps from a patch containing either a single Kv7.2/7.3 or a Kv7.2 channel from control CHO cells or those cotransfected with PI(4)5-kinase are shown. Recordings were first made in on-cell modes, followed by excision to inside-out modes. O, Open channel levels; C, closed channel levels. **B**, Bars show the mean P_o at 0 mV for all of the on-cell patches. **C**, Bars show whole-cell current amplitudes from CHO cells transfected with Kv7.2 or Kv7.3 alone or together with PI(4)5-kinase. Traces from representative experiments are displayed above. The voltage protocol used is as shown.

activated by, PI3-kinase-generated phosphoinositol PI(3,4)P₂ but not PI(4,5)P₂ (the molecule in this work abbreviated as PIP₂) (Franke et al., 1997), and so Akt-PH is a useful control for PLCδ-PH. The third, Lyn-PH-PP, codes for a PIP₂-specific phospholipid, 5'-phosphatase (Stolz et al., 1998), that selectively reduces plasma membrane PIP₂ concentrations but not other intracellular pools of PIP₂ or other plasma membrane PI phosphates, expressed as a fusion protein to a myristoylation/palmitoylation

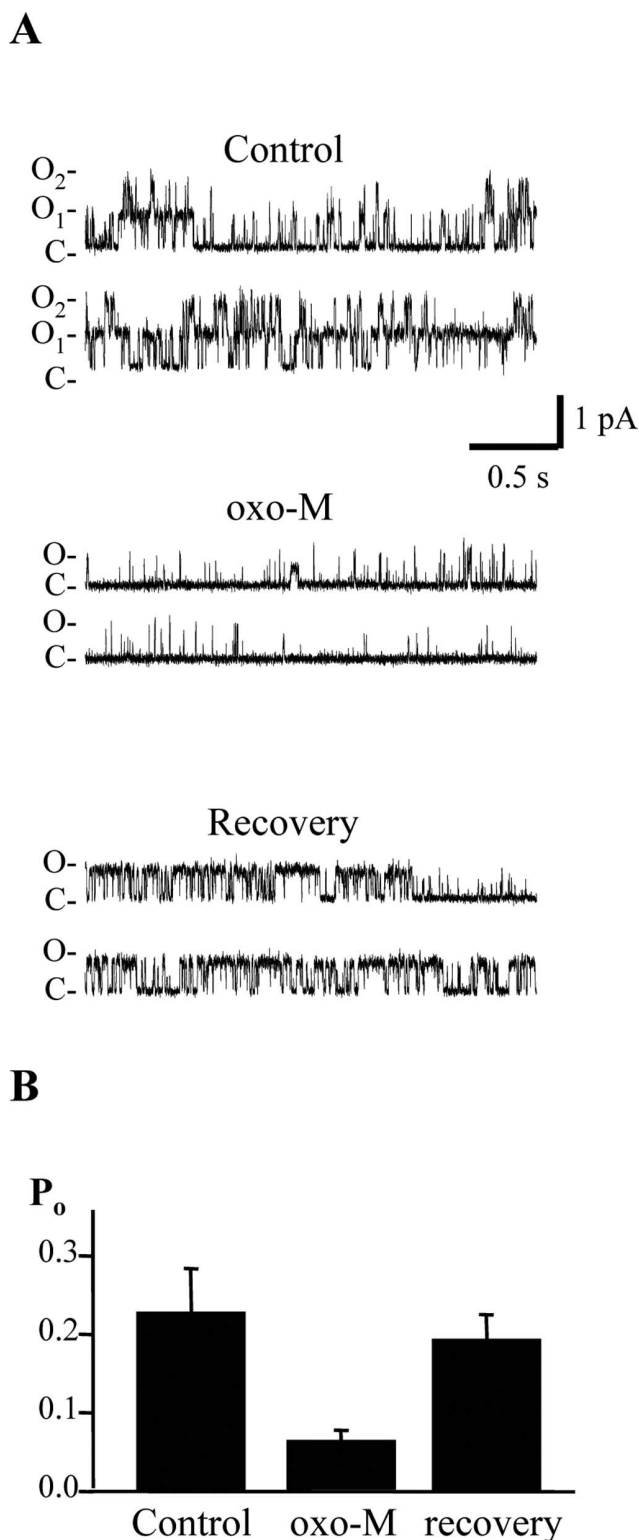


Figure 5. Stimulation of M₁ receptors by bath application of agonist reduces the P_o of channels in cell-attached patches. CHO cells were cotransfected with Kv7.2+7.3 channels and M₁ receptors. **A**, Sweeps show channel activities from a patch containing two Kv7.2/3 channels before, during, and after bath application of oxo-M (10 μ M). **B**, Bars show the mean unitary P_o from four experiments such as in **A**. O, Open channel levels; C, closed channel levels.

sequence of the tyrosine kinase Lyn that achieves its plasma membrane localization (Raucher et al., 2000). Because all of these PIP₂-testing constructs are linked to EGFP, we can use single-cell imaging to (1) confirm their presence in the cell and (2) localize

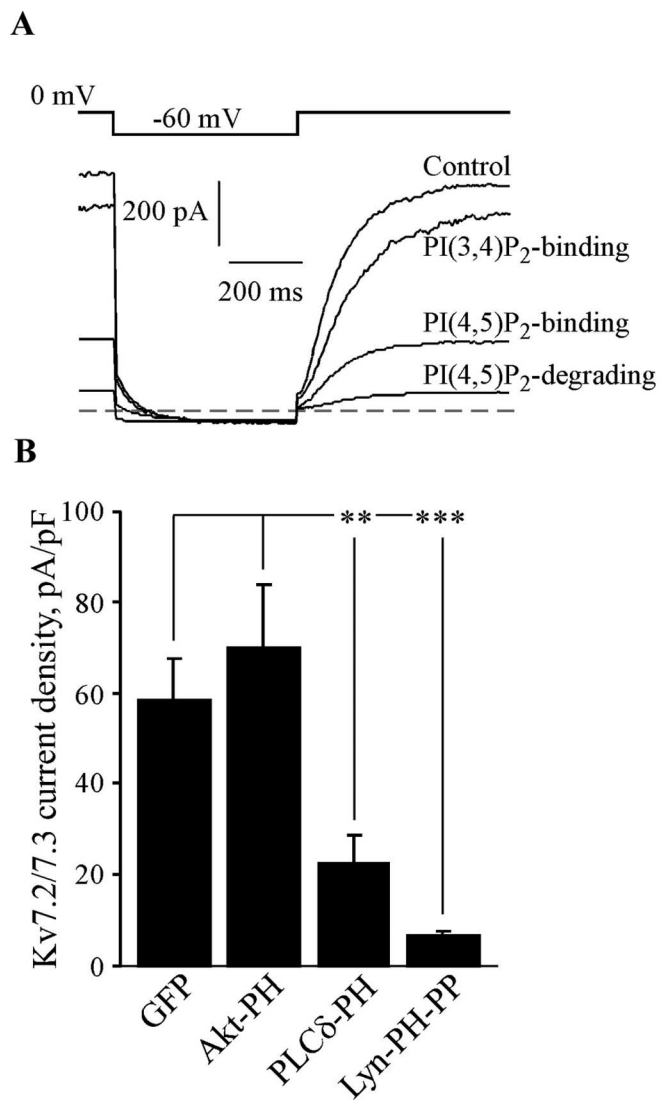


Figure 6. Expression of PIP₂ sequestering or degrading constructs reduces whole-cell Kv7.2/7.3 current amplitudes. CHO cells were cotransfected with Kv7.2+Kv7.3 together with either GFP only (control) or the PLC δ PH, Lyn-PH-PP, or Akt-PH constructs. Currents were elicited using the indicated voltage protocol in whole-cell configuration. **A**, Plotted are superimposed current traces from four different cells transfected with each different construct. **B**, Bars show the mean whole-cell current amplitudes for these four groups of cells. ** $p < 0.01$; *** $p < 0.001$.

their distribution within the cell (i.e., plasma membrane or cytoplasm).

CHO cells were cotransfected with Kv7.2/7.3 channels and only GFP, Akt-PH, PLC δ -PH, or Lyn-PH-PP. For all four, we observed robust fluorescence that was strongly membrane localized for the cases of PLC δ -PH and Lyn-PH-PP or diffusely cytoplasmic for the case of GFP and Akt-PH. We found that cotransfection of PLC δ -PH strongly reduced the amplitude of the Kv7.2/7.3 currents and Lyn-PH-PP nearly abolished the currents, but cotransfection of Akt-PH had little effect (Fig. 6). For these four groups of cells, the mean current densities were 58.3 \pm 9.4 pA/pF ($n = 12$), 69.9 \pm 14 pA/pF ($n = 9$), 22.4 \pm 6.5 pA/pF ($p < 0.05$; $n = 14$), and 6.5 \pm 1.0 pA/pF ($p < 0.001$; $n = 19$). We interpret the reduced currents in the PLC δ -PH cells as being attributable to the reduced abundance of free PIP₂, resulting in a reduced tonic maximal P_o of the channels, consistent with the inability of PLC δ PH to bind to channels and stabilize their opening. The Lyn-PH-PP-expressing cells are predicted to have a se-

verely depleted PIP₂ abundance from the strongly augmented PIP₂ phosphatase activity and the strongly reduced current density to arise from a much lower P_o of the channels. We verified that expression of the PIP₂-binding constructs does not affect expression of channel protein using immunoblots of whole-cell lysates from cells expressed with myc-tagged Kv7.2+Kv7.3 together with each construct (data not shown). Assuming that the effects on current amplitudes are as a result of reduction in the P_o , we can use the dose–response curve of unitary Kv7.2/7.3 P_o versus [diC8-PIP₂] (Fig. 3) to estimate changes in tonic membrane PIP₂ caused by expression of the two PIP₂ sequestering/degrading constructs. Assuming a normal maximal Kv7.2/7.3 P_o of 0.31 from our data, that corresponds to a [diC8-PIP₂] of 23 μ M in our inside-out patch experiments. In cells expressing PLC δ -PH, the whole-cell currents were reduced by 69%, corresponding to a P_o of 0.096 and a [diC8-PIP₂] of 8.2 μ M; in cells expressing Lyn-PH-PP, whole-cell currents were reduced by 91%, corresponding to a P_o of 0.026 and a [diC8-PIP₂] of 3.0 μ M. Thus, under the assumption that changes in [diC8-PIP₂] and in [PIP₂] have similar effects, we estimate that expression of PLC δ -PH and Lyn-PH-PP reduced free PIP₂ in CHO cells by 64 and 87%, respectively.

Stimulation of G_{q/11}-coupled M₁ receptors depletes PIP₂ in CHO cells

As discussed previously, muscarinic suppression of M-type currents is thought to primarily involve depletion of membrane PIP₂ by activation of PLC (Suh and Hille, 2002; Zhang et al., 2003; Suh et al., 2004). Our findings here suggest that the P_o of these channels are highly sensitive to PIP₂ abundance but that a strong depletion of PIP₂ would be required to cause the profound (>70%) suppression of the currents that is seen. Because this hypothesis of how muscarinic stimulation suppresses M channels predicts a strong reduction in PIP₂, we took advantage of a CHO cell line stably expressing M₁ receptors to analytically measure changes in phosphoinositide abundance induced by muscarinic stimulation of these cells. Using HPLC analysis, we assayed the abundance of several different phosphoinositides, including PIP₂, phosphatidylinositol 4-phosphate (PIP), PI, and PA, before and after exposure of the cells to the cholinergic agonist carbachol (100 μ M) for 1 or 10 min (Fig. 7).

We found that muscarinic stimulation for 1 min indeed reduced PIP₂ abundance. Results are given as percentages of total anionic phospholipids, a pool that includes the phosphatidylserine and cardiolipin moieties that are not involved in PLC activity. The %PIP₂ was strongly reduced from $0.80 \pm 0.02\%$ ($n = 7$) before to $0.10 \pm 0.04\%$ ($n = 5$) after 1 min of carbachol exposure. Receptor stimulation also caused a strong decline in %PIP, from $0.99 \pm 0.06\%$ ($n = 7$) before to $0.19 \pm 0.04\%$ ($n = 5$) after 1 min of carbachol exposure. This parallel decline in %PIP₂ and %PIP is similar to that measured in human neuroblastoma cells (Willars et al., 1998). Unexpectedly, the %PI also displayed a significant decline, falling from $49.5 \pm 0.7\%$ ($n = 7$) before to $46.5 \pm 0.3\%$ ($n = 5$) after 1 min of carbachol exposure. Because diacylglycerol (DAG), the membrane-bound byproduct of PIP₂ hydrolysis, is rapidly converted to PA by DAG-kinase, the %PA increased after receptor stimulation, as expected. The %PA rose from $15.8 \pm 1.1\%$ ($n = 7$) before to $17.6 \pm 0.9\%$ ($n = 5$) after 1 min of carbachol exposure. Although muscarinic suppression of M/Kv7 channels does not noticeably desensitize in patch-clamp experiments, we found the abundance of PIP₂, and to a lesser extent PIP, to slowly recover during maintained muscarinic stimulation. Thus, the %PIP₂ and the %PIP recovered to $0.57 \pm 0.15\%$ ($n = 5$) and $0.40 \pm 0.06\%$ ($n = 5$) after 10 min of carbachol

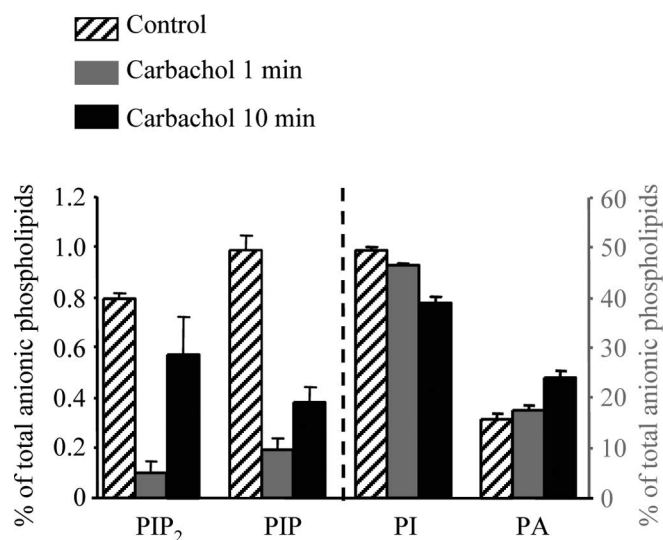


Figure 7. Effects of muscarinic stimulation on anionic phospholipids in CHO cells expressing M₁ receptors. Anionic phospholipids were determined from their deacylation products by anion exchange HPLC with suppressed conductivity detection. Data are compiled for application of the cholinergic agonist carbachol (100 μ M) for 1 or 10 min. Bars show the mean PIP₂, PIP, PI, or PA, expressed as a percentage of total membrane phosphoinositides. The left axis refers to %PIP₂ and %PIP, and the right axis refers to %PI and %PA.

exposure, respectively. The %PI, however, continued to fall during maintained receptor stimulation, falling to $39.0 \pm 1.1\%$ ($n = 5$) after 10 min of carbachol exposure. The %PA also continued to rise during the maintained stimulation, rising further to $24.1 \pm 1.4\%$ ($n = 5$) after 10 min of carbachol exposure. Both the continued fall in %PI and the continued rise in %PA suggest that PIP₂ hydrolysis continued unabated during the longer carbachol exposure, arguing against receptor desensitization as the reason for the partial recovery of %PIP₂ and %PIP during the 10 min treatment. Perhaps this partial recovery is attributable to the agonist-mediated stimulation of PIP and PIP₂ synthesis by PI4-kinase and PI(4)5-kinase, as has been shown recently for G_{q/11}-coupled bradykinin receptors in neuroblastoma cells (Xu et al., 2003) and suggested in sympathetic neurons (Gamper et al., 2004).

Discussion

The importance of phosphoinositides in cellular signals triggered by plasma-membrane receptors coupled to the G_{q/11} family of G-proteins has been appreciated for decades. In this pathway, hormonal stimulation results in activation of PLC, which hydrolyzes PIP₂ into membrane-bound DAG and soluble inositol trisphosphate, both potent intracellular signaling molecules. However, in the past decade, it has become clear that the PIP₂ molecule functions as a second messenger in its own right. Much evidence has accumulated over the past several years suggesting that Kv7 channels are sensitive to the abundance of PIP₂ in the plasma membrane (Delmas and Brown, 2005). Although Kv7 channels are voltage-gated K⁺ channels (unlike PIP₂-sensitive Kir channels), the presence of PIP₂ would act to stabilize opening by favoring the allosteric conformational change that opens the channel, such that P_o is increased at all voltages. The physiological role of such PIP₂ sensitivity is perhaps most clear for the case of M-type channels, which are thus regulated by depletions of membrane PIP₂ produced by PLC activation downstream of G_{q/11}-coupled receptor stimulation. This mechanism predicts the maximal P_o of Kv7 channels in intact cells to be governed by the tonic

abundance of PIP₂ in the membrane and increases or decreases in tonic PIP₂ levels to increase or decrease maximal channel P_o , respectively. Stimulation of other G_{q/11}-coupled receptors in sympathetic neurons also suppress M currents, however not by large changes in membrane PIP₂ (Gamper et al., 2004) but by generation of intracellular Ca²⁺ signals (Cruzblanca et al., 1998; Bofill-Cardona et al., 2000), which may act in concert with calmodulin (Gamper and Shapiro, 2003; Gamper et al., 2005b) to alter the affinity of the channels for PIP₂, such that tonic PIP₂ abundance is insufficient to keep most channels open (Winks et al., 2005).

Here, we directly tested the PIP₂-gating hypothesis by a variety of methods. The first was inside-out patch recording and a range of water-soluble diC8-PIP₂ concentrations applied to the cytoplasmic face of the patch. We found that the maximal single-channel P_o of Kv7.2–7.4 and Kv7.2/7.3 channels is highly governed by [diC8-PIP₂], with a strongly differential apparent affinity of the channels for diC8-PIP₂ that parallels the differential maximal P_o in cell-attached patches (Li et al., 2004). In this study, we did not posit that the affinity of the channels for diC8-PIP₂ is the same as for (full-length) PIP₂, but did assume that relative differences in the apparent affinities of the different channels for diC8-PIP₂ and full-length PIP₂ do correspond. For all of the channels studied in inside-out patches, the P_o could be increased well beyond their values normally seen in cell-attached patches by increasing the diC8-PIP₂ concentration. Consistent with this result is the strong increase in maximal channel P_o of Kv7.2/7.3 and Kv7.2 observed in cell-attached patches from cells overexpressing PI(4)5-kinase, resulting presumably from greater tonic PIP₂ abundance in the membrane. The increase in macroscopic Kv7.2, but not Kv7.3, currents by overexpression of PI(4)5-kinase was particularly dramatic and reinforces both the conclusions of low apparent affinity of Kv7.2 for PIP₂ and the highly differential maximal P_o of Kv7.2 and Kv7.3 in cell-attached patches reported here and previously (Li et al., 2004). Conversely, maneuvers that tonically decreased the abundance of free membrane PIP₂ strongly lowered tonic current amplitudes, almost certainly by lowering channel P_o . A strong reduction in unitary channel P_o by muscarinic stimulation was observed that correlates well with the suppression of macroscopic currents, and that resembles the changes in channel P_o seen in the direct diC8-PIP₂ applications. Finally, HPLC analysis confirms the necessary profound PIP₂ depletion in cells heterologously expressing M₁ receptors. There is a discrepancy at later times, in that PIP₂ and PIP levels recover in part over a few minutes during receptor stimulation, whereas the inhibition of channel activity is usually stable over a similar time course. One explanation is that PIP₂ is being generated in membrane domains from which PIP₂ diffusion to M channels is in some way restricted. Despite this discrepancy, our work is strongly supportive of PIP₂ regulation of Kv7 channels as being physiologically integral to G-protein channel signaling.

Although our data suggest that the highly divergent P_o values of Kv7 channels are associated with very differential apparent affinities for PIP₂, we have no biochemical data on their binding affinities. Indeed, although several investigators assume that PIP₂-sensitive channels are regulated by direct binding of PIP₂ molecules to the channel proteins in the membrane, clear evidence for this remains sparse. However, if there is an intermediate protein that mediates PIP₂ regulation of Kv7 channels, it would have to be membrane associated because the regulation happens in excised inside-out patches and must be present both in the plasma membrane of mammalian cells (present study) and of oocytes (Zhang et al., 2003). For the case of inward rectifier K⁺

channels, the large effects on their PIP₂ regulation by neutralizations of positively charged amino acids lends support to the idea of direct binding, as does a recent crystal structure (Pegan et al., 2005). Thus, direct binding to Kv7 channels is our working hypothesis. For both families of channels that contain members with divergent apparent affinities for PIP₂, it seems as yet unknown whether their biochemical binding affinities are so divergent, or whether their intrinsic energy of opening diverges, resulting in a differential amount of stabilization energy coming from PIP₂ binding being required to achieve the same level of channel opening. Because K⁺ channels are tetramers, and presumably each subunit can bind PIP₂, it will be critical to learn the stoichiometry between PIP₂ molecules and channel and the relationship between bound PIP₂ molecules/channel and opening. Work from the Logothetis laboratory has also demonstrated direct gating of Kv7 channels by PIP₂ in oocyte-excised macropatches (Zhang et al., 2003). Their EC₅₀ value for activation of Kv7.2/7.3 channels by diC8-PIP₂ was 87 μM, with a Hill slope of 1.35, in the range of that seen here for regulation of unitary channel P_o . They also showed direct regulation of Kv7.1, Kv7.4, and Kv7.5 channels by PIP₂, but they did not investigate any differential apparent affinities among the channels. The mild cooperativity for the binding of multiple PIP₂ molecules to the channel modeled by Suh et al. (2004) seems in the correct range both for the slope of the dose-response curves and of muscarinic agonist versus M current seen by many laboratories and for diC8-PIP₂ versus single-channel P_o seen here ($n_{Hill} = 1-1.8$).

The maximal on-cell P_o for Kv7.2/7.3 heteromultimers is seen here to be ~0.3, an intermediate value between low- P_o Kv7.2 and high- P_o Kv7.3 (Li et al., 2004), and to be in the range of 0.13–0.3 by the Brown laboratory (Selyanko et al., 2001; Tatulian and Brown, 2003). Our inside-patch data show that the maximal P_o continues to rise past this range of values as the applied [diC8-PIP₂] is increased, and in on-cell patch, overexpression of PI(4)5-kinase likewise strongly raises maximal P_o . Together, these data suggest that the tonic [PIP₂] in intact cells is well under that necessary to saturate PIP₂ binding to the channels. However, in SCG neurons, the M channels of which heavily consist of Kv7.2/7.3 heteromultimers, overexpression of PI(4)5-kinase did not increase tonic M-current amplitudes (Winks et al., 2005), and we found that its overexpression likewise did not significantly increase whole-cell Kv7.2/Kv7.3 currents expressed in CHO cells (our unpublished observations). Perhaps this discrepancy between single-channel and macroscopic effects is attributable to a secondary effect of increased [PIP₂] in promoting endocytosis of membrane proteins, as has been described recently (Huang et al., 2004). Thus, an increase in current amplitudes by higher tonic [PIP₂] increasing channel P_o might be balanced by a decrease in tonic surface abundance of the channel proteins.

Left unanswered is the molecular mechanism by which PIP₂ binding to the channel proteins stabilizes the allosteric conformational change that is opening. Because the “kink” in the sixth transmembrane segment (S6) thought to be the swivel point of the main gate of Kv channels (Jiang et al., 2002) is located in the middle of the membrane, and because the S6 helix is thought to end just after it emerges from the inner leaflet of the membrane, PIP₂ should bind to the channel downstream of S6 (i.e., to the cytoplasmic tail). Indeed, mutagenesis (Zhang et al., 2003) and single-channel recording of Kv7.3/Kv7.4 chimeras (Li et al., 2004; Gamper et al., 2005b) indicate that this is where PIP₂-mediated regulation of channel opening occurs. From that point, the PIP₂/C-terminal interaction could result in some force that pulls the S6 helix away from the pore, opening it. Because Kv7 channels, like

other Kv channels, are voltage gated, this must occur after the movement of the voltage sensors of the channels permits gating. Perhaps the PIP₂ action is to anchor the post-S6 end of the channel in place, causing the voltage-induced flexing of the channel to splay the pore open. Alternatively, the hydrophobic part of the PIP₂ molecule may cause it to favor being surrounded by lipid, generating a force away from the channel axis as the carboxy-tail is pulled from the rest of the protein. Such structural questions await more detailed knowledge of the molecular architecture of Kv7 channels and of how PIP₂ binding to the protein alters its configuration in the plasma membrane.

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