Structural Requirements of Membrane Phospholipids for M-type Potassium Channel Activation and Binding.

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Running title: Phospholipid activation of M-type potassium channels

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[Capsule]

Background. M-channels are potassium channels that are activated by phosphatidylinositol-4,5-bisphosphate but their response to other phospholipids is unknown.

Results. M-channel proteins were activated by phosphoinositides and lipid phosphates but not by inositol phosphates.

Conclusion. Minimal activation requirements are an acyl chain and one or more phosphate groups.

Significance. M-channels control cell excitability, so their regulation by membrane constituents is important for biology.

SUMMARY

M-channels are voltage-gated potassium channels that regulate cell excitability. They are heterotrameric assemblies of Kv7.2 and Kv7.3 subunits. Channels were activated to similar extents (maximum open probability ~0.8 at 0 mV) by 0.1 to 300 µM of dioctanoyl (diC₈) homologs of the three endogenous phosphoinositides PI(4)P, PI(4,5)P₂, and PI(3,4,5)P₃, sensitivity increasing with increasing number of phosphates. Non-acylated inositol phosphates had no effect up to 100 µM. Channels were also activated with increasing efficacy by 1-300 µM of the monoacyl monophosphates fingolimod phosphate, sphingosine-1-phosphate and lysophosphatidic acid, but not by phosphate-free fingolimod or sphingosine, nor by phosphate-masked phosphatidylcholine or phosphatidylglycerol. An overlay assay confirmed that a fusion protein containing the full-length C-terminus of Kv7.2 could bind to a broad range of phosphoinositides and phospholipids. A mutated Kv7.2 C-terminal construct with reduced sensitivity to PI(4,5)P₂ showed significantly less binding to most polyphosphoinositides. It is concluded that M-channels bind to, and are activated by, a wide range of lipid phosphates, with a minimal requirement for an acyl chain and a phosphate head-group. In this, they more closely resemble Kir6.2 inward-rectifier potassium channels than the more PI(4,5)P₂-specific Kir2 channels. Notwithstanding, the data also support the view that the main...
endogenous activator of M-channels is PI(4,5)P₂. [244 words]

[Introduction]
The activity of many membrane ion channels is regulated by the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) (1-3). One such channel is the M-type potassium channel. This is a voltage-gated potassium channel composed of Kv7 family subunits, primarily Kv7.2 and Kv7.3 (4). Though gated by membrane voltage, the channels require PI(4,5)P₂ to enter in, and stabilize, the open state (5-7). They are particularly interesting because their sensitivity to PI(4,5)P₂ is set at such a level that they rapidly close when the endogenous membrane content of PI(4,5)P₂ is reduced by a neurotransmitter such as acetylcholine that activates phospholipase C and so stimulates PI(4,5)P₂ hydrolysis (2, 3, 8). The loss of outward potassium current then initiates a marked increase in the excitability of many neurons, with profound physiological consequences (9).

Ion channels (and other proteins) vary in the specificity of their interaction with PI(4,5)P₂ vis-à-vis other phosphoinositides or other membrane phospholipids (3). Kv7.2/7.3 channels appear to show a rather specific dependence on PI(4,5)P₂ in that activity is rapidly and substantially reduced when the 5’-phosphate is cleaved by an inositol-5-phosphatase (10). Further, in experiments where the individual channel subunits were expressed and then isolated membrane patches exposed to the water-soluble PI(4,5)P₂ analog diC₈PI(4,5)P₂, the two subunits showed a very large (~100-fold) difference in the concentrations of diC₈PI(4,5)P₂ required to activate them (7). This was attributable to variations within a small cluster of basic amino acids in a region of the C-terminus of the subunit protein, suggesting a specific PI(4,5)P₂ interaction site (11).

However, there are also some indications that Kv7.2/7.3 channels may be activated by other phosphoinositides (6, 7). Further, a C-terminal fusion protein of the homologous cardiac Kv7.1 channel was noted to bind to a variety of phosphoinositides and phospholipids in a protein-lipid overlay assay (12). This raised the question: how broad might be the range of phosphoinositides and lipid phosphates that can interact with Kv7.2/7.3 channels?

In the present experiments we have tried to answer this question by (a) testing a range of phosphoinositides, their analogs and derivatives, for their ability to activate Kv7.2/7.3 channels when applied to an isolated membrane patch in which they are expressed, and (b) using a lipid overlay assay to observe the binding of a Kv7.2 C-terminus fusion protein to a number of membrane phospholipids. From this, we have been able to draw some conclusions regarding the minimal requirements for M-channel activation by membrane phospholipids and lipid phosphates.

[Experimental Procedures]

Kv7.2 and Kv7.3 constructs.
Electrophysiological experiments were undertaken using Chinese hamster Ovary (CHO) cells stably co-transfected with the full-length human Kv7.2 and Kv7.3 M-channel subunits (designated Kv7.2/7.3 cells) (13). For in vitro binding tests, the full-length C-terminus of Kv7.2 (Kv7.2C; amino acids 318 to 845, accession number NM_004518) was cloned into the pmalc2x vector (NEB) to create a C-terminal fusion with maltose-binding protein (MBP). MBP-Kv7.2C was expressed and purified as previously described (12). Experiments were repeated using a full-length C-terminus of the mutant channel Kv7.2 (K452E/R459E/R461E), Kv7.2C-EEE, which is about two-fold less sensitive to PI(4,5)P₂ than the wild-type when expressed as a homomer (11).

Cell culture. Kv7.2/7.3-CHO cells were incubated in Gibco Alpha-MEM minimal essential medium supplemented with 10 % fetal calf serum, 1 % L-glutamine, 1 % penicillin/streptomycin, 0.2 mg/ml hygromycin and neomycin 0.4 mg/ml (K2/3-CHO media). The cell line was maintained in a humidified incubator gassed with 5% CO₂/95% air. Cells were passaged every 2–3 days in a ratio of 1:10. Ca²⁺- and Mg²⁺-free phosphate-buffered Hanks’ balanced saline solution was used to detach the cells. This was subsequently followed by centrifugation at 800 G and re-suspension in the supplemented medium described above. For subsequent electrophysiological experimentation cells were settled in specially-designed siliconized chambers (volume ~200 μl) within plastic Petri dishes. They were incubated for at...
least 24 h before being mounted on the stage of an inverted microscope equipped with phase-contrast optics and continuously superfused at ~ 5 mL/min⁻¹.

Electrophysiological recordings. Single M-channel activity was recorded using patch electrodes in membrane patches excised from Kv7.2/7.3 CHO cells in inside-out configuration at a controlled room temperature (22 ± 0.5 °C). Pipette voltage was set at 0 mV. For current recording, we used an Axopatch 200A amplifier and Digidata 1440 A/D interface (Axon Instruments, Forster City, CA, USA) and pipette holder optimized for low-noise recordings (G3 Instruments, UCL). All recordings were filtered with an 8-pole Bessel filter at 2 kHz and digitized at 5 kHz. The pipette resistance when filled with the pipette solution was ~ 5-10 MΩ. Recorded channel currents were judged to be through a single class of heteromeric Kv7.2/7.3 channels because they had a constant single current amplitude of 0.52 ± 0.01 pA at -20 mV (n = 27 patches). In parallel studies on these cells, a channel slope conductance of 9.2 ± 0.1 pS (n = 6) was determined in cell-attached mode, in agreement with previous data (7, 14).

Bath and pipette solutions contained (in mM): 144 NaCl, 2.5 KCl, 0.5 MgCl₂, 2 CaCl₂, 10 D-Glucose, and 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); pH was adjusted to 7.4 with Trizma base (TRIS). Bath solutions during the inside-out studies contained (in mM): 165 KCl, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 10 ethylene-glycol-tetra-acetic acid (EGTA), pH was adjusted at 7.2 by NaOH. Non-hydrolysable ATPγS (0.1 µM) was constantly present in the bath solutions during inside-out studies in order to inhibit endogenous production of PI(4,5)P₂ by phosphatidylinositolphosphate kinases, which possibly remained associated with the patch after excision. All the studied compounds were applied to the isolated inside-out membrane patches in incremental concentrations using a fast microperfusion system (delivery time < 1 s). All applied phospholipids were reversible. Effects of water soluble phosphoinositides recovered within 100-500 ms on washout. A longer recovery time (up to 10 s) was needed to wash out compounds dissolved in methanol, which itself slightly depressed M-channel activity.

Data analysis. Single channel current amplitudes were determined individually in each patch using a Gaussian all-point amplitude distribution. Since in these experiments we were only concerned with \( P_{\text{open}} \) values, not open-shut time distributions, we included patches with several channels in the analysis. The number of channels \( N \) in multi-channel patches was determined in two ways: first by observing the number of incremental current steps attained as \( P_{\text{open}} \) rose to 0.6 – 0.8; and second using variance analysis. In the latter, current variance \( \text{Var}(I_n) \) was fitted to the mean current \( \langle I_n \rangle \) using the parabolic function:

\[
\text{Var}(x) = \text{Var}_{\text{Background}} + I_{\text{unit}} \langle I_n \rangle - \frac{I_n^2}{N}
\]

with the unit current \( I_{\text{unit}} \) constrained to the value estimated from the point amplitude distribution. Values for \( N \) were accepted if the two estimates agreed to the nearest whole number. Patch \( NP_{\text{open}} \) was measured over the last 20 s of each concentration of phospholipid, when activity was stable as judged from stability plots and then corrected for the estimated number of channels in each patch to give channel \( P_{\text{open}} \) as a function of phospholipid concentration.

The concentration values of half-activation (EC₅₀), were calculated from concentration-response curves of the open probability \( P_{\text{open}} \) using two-component Hill equations (15):

\[
P_{\text{open}} = \frac{P_{\text{open Max1}}}{1 + \left( \frac{\text{EC}_{501}}{[\text{Phospholipid}]} \right)^{nH1}} + \frac{P_{\text{open Max2}}}{1 + \left( \frac{\text{EC}_{502}}{[\text{Phospholipid}]} \right)^{nH2}}
\]

When fitting, the Hill coefficient, \( nH \) was constrained to be the same for both components. Two component curves were found to fit the \( P_{\text{open}}/[\text{phospholipid}] \) concentration-response curves of heteromeric M-channels significantly better than single component curves. Statistical comparisons were performed using one way ANOVA and the difference was considered as significant at the level of \( P < 0.05 \). Data were analyzed using Clampfit 10.2, WinEDR, Microsoft Office Excel 2003 and Microcal Origin 6.0 software.

Protein-lipid overlay assay. Purified MBP-Kv7.2C was incubated with PIP Strips (Invitrogen) at a concentration of 5 µg/mL as
described previously (16, 17). Rabbit anti-MBP antiserum (NEB) and ECL detection was used in order to detect binding of MBP-Kv7.2C to the relevant phospholipids. Densitometric analysis was carried out using Scion Image. Experiments were repeated using a full-length C-terminus of the mutant channel Kv7.2 (K452E/R459E/R461E), Kv7.2C-EEE, which is about two-fold less sensitive to PI(4,5)P$_2$ than the wild-type when expressed as a homomer (11).

**Chemicals.** All compounds for solutions, ATP$_8$S, dihydroxylacetone phosphate (DHAP) were purchased from Sigma-Aldrich Company. Phosphatidylinositol mono-, bis-, and trisphosphates (diC$_8$PI(4)P, diC$_8$PI(4,5)P$_2$ and diC$_8$PI(3,4,5)P$_3$), inositol-1,4,5-trisphosphate (I(1,4,5)P$_3$), sphingosine-1-phosphate (S-1-P), D-erythro-sphingosine (S) and biotinylated phosphatidylglycerol (PG) and phosphatidylcholine (PC) were purchased from Echelon Inc. Fingolimod phosphate (FTY720-P), fingolimod (FTY720) and 1-oleoyl lysophosphatidic acid (LPA) were purchased from Cayman Chemical Company. Inositol-4,5-bisphosphate (I(4,5)P$_2$)-TEA salt was provided by Prof. B. V.L. Potter (University of Bath). Stock solutions of S-1-P, S, FTY720-P, FTY720, LPA, PG and PC were dissolved in methanol (maximum concentration applied to patch = 12 %).

**RESULTS**

**Electrophysiological assays.**

**Phosphoinositides.** PI(4,5)P$_2$ is normally synthesized from PI(5)P by PI(5)-kinase, then can be further phosphorylated to PI(3,4,5)P$_3$ by PI(3)-kinase (Figure 1; (18)). We therefore tested the effect of incremental concentrations of the dioctanoyl (diC$_8$) analogues of these three phosphoinositides on the activity of Kv7.2/7.3 channels in excised membrane patches held at a constant pipette voltage of 0 mV. All three produced a strikingly similar activation of the channels (Figure 2 A-C). As previously noted for diC$_8$PI(4)P$_2$ (15), incremental concentrations between 0.1 and 300 µM generated a biphasic concentration - $P_{\text{open}}$ curve, resolvable into ‘high-affinity’ and ‘low-affinity’ components; the former maximized at $P_{\text{open}} = 0.19$ (0.10 for diC$_8$PI(4)P), while the latter carried $P_{\text{open}}$ up to an extrapolated value approaching ~0.8 (Figure 2 D). The three phosphoinositides had very similar EC$_{50}$s (EC$_{50}$1: 1.1-1.7 µM) for the high-affinity component, while the low-affinity EC$_{50}$ (EC$_{50}$2) varied inversely with the number of phosphates (Table 1). As a result, diC$_8$PI(4)P produced a slightly lower observed $P_{\text{open}}$ (0.61 ± 0.10, n = 9) at the maximum applied concentration of 300 µM than diC$_8$PI(4,5)P$_2$ (0.79 ± 0.05, n = 11) or diC$_8$PI(3,4,5)P$_3$ (0.80 ± 0.04, n = 11) (Table 1). Thus diC$_8$ homologs of all three endogenous phosphoinositides strongly activated the M-channels, with a potency that increased with increasing number of phosphates.

**[Figures 1 and 2 near here]**

**Table 1 near here**

**Inositol phosphates.** Studies on other potassium channels indicate that the inositol phosphate head-group of PI(4,5)P$_2$ projects from the inner leaflet of the lipid bilayer into the cytoplasm where it docks onto a receptive site (or sites) in the cytoplasmic domain of the channel (19, 20). The above results suggest that the charges on the head group are one factor governing the ability of phosphoinositides to activate M-channels. We therefore wondered whether the inositol phosphates themselves might activate the Kv7.2/7.3 channels, or conversely, compete with PI(4,5)P$_2$ to inhibit its action. These are not unreasonable possibilities because (a) the PI(4,5)P$_2$-binding pleckstrin homology domain of membrane enzyme phospholipase C also binds inositol-1,4,5-trisphosphate (I(1,4,5)P$_3$) with high affinity (21, 22), and (b) inositol-1,4,5-trisphosphate (I(1,4,5)P$_3$) has been reported to antagonize the effect of PI(4,5)P$_2$ on some TRPC channels (23). Accordingly, we applied incremental concentrations of I(1,4,5)P$_3$ to excised Kv7.2/7.3-containing patches as illustrated in Figure 3 A. No channel activation occurred up to 300 µM in any of 6 experiments, whereas in each case 100 µM PI(4,5)P$_2$ increased $P_{\text{open}}$ to an average value of 0.54 ± 0.09 (n = 6). Further, 100 µM I(1,4,5)P$_3$ did not inhibit the effect of PI(4,5)P$_2$ when applied either before or during the application of 100 µM PI(4,5)P$_2$, so that mean $P_{\text{open}}$ remained unaffected (0.58 ± 0.08, n = 5) (Figure 3 B). The inositol bisphosphate I(4,5)P$_2$ (n = 5) or a small water-soluble monophosphate, dihydroxylacetone phosphate (n = 8) also had no effect at concentrations of 100 µM (data not shown).
Thus, water-soluble analogs of the phosphoinositide head-groups did not activate M-channels.

[Fig. 3 near here]

Other phospholipids. The above results indicate that, although the docking of the inositol head-groups of the phosphoinositides onto the cytoplasmic domain of the channel may be responsible for channel opening, attachment of the phosphate to a lipophilic moiety is necessary for (or facilitates) this effect. This accords with recent structural information regarding inward rectifier Kir channels, in which the acyl chain of PI(4,5)P₂ interacts non-selectivity with the transmembrane domain of Kir2 (20). To assess what the minimal lipophilic acyl phosphate requirement for M-channel activation might be, we tested the effect of several other phospholipids and their congeners (Figure 4):
sphingosine-1-phosphate (S-1-P); its analogue fingolimod phosphate (FTY720-P); and 1-oleoyl lysophosphatidic acid (LPA), whose aliphatic structure corresponds in principle to PI(4,5)P₂.

[Figure 4 near here]

S-1-P activated the channels at concentrations from 3 µM upwards, to a mean $P_{\text{open}}$ of 0.16 ± 0.03 (n = 9) at 100 µM (Figure 5 A, B). FTY720-P also activated the channels over the same concentration range, but only to a lower mean $P_{\text{open}}$ of 0.02 ± 0.006 (n = 8) (Figure 5 C). Concentration - $P_{\text{open}}$ curves (Figure 5 D) could be resolved into two, high- and low-affinity, components like those generated by the phosphoinositides (Table 2). In both cases incorporating two-components gave a significantly better fit than a single component. This suggests that they acted mechanistically rather like the phosphoinositides.

[Figure 5 near here]

[Table 2 near here]

Somewhat surprisingly, LPA proved as effective as PI(4,5)P₂ as a channel activator, driving $P_{\text{open}}$ to a value of 0.68 ± 0.09 (n = 6) at 100 µM (Figure 6 A, B). Again, the concentration - $P_{\text{open}}$ curve could be resolved into two components (though less clearly demarcated than with PI(4,5)P₂ (Figure 6 C, Table 2).

Hence, the property of activating M-channels is not totally restricted to diacylphosphoinositides, but extends to some monoacyl phosphates.

[Figure 6 near here]

Phosphate-free acyl compounds. As noted, the acyl groups of phosphoinositides interact with the transmembrane domain of the Kir2 channels (19). The question then arises whether such an interaction might itself be capable of activating the M-channel in the absence of a phosphate head-group. To answer this, we tested phosphate-free D-erythro-sphingosine (S), its analogue fingolimod (FTY720), and the biotinylated phospholipids phosphatidylinositol (PG) and phosphatidylcholine (PC), in which the phosphates are masked with glycerol and choline groups, respectively. However, neither of the non-phosphorylated precursors of S-1-P and FTY720-P, S and FTY720, nor the biotinylated phosphatidylglycerol (PG) or phosphatidylcholine (PC), were able to activate the Kv7.2/7.3 channels when tested at 100 µM (n = 6 to 13) (Figure 7). In each test, channels were strongly activated by 100 µM PI(4,5)P₂. Thus, the charged phosphate head-group seems an absolute requirement for phospholipid activation of M-channels.

[Figure 7 near here]

Protein-Lipid Overlay assays.

A fusion protein between maltose binding protein and the full-length C-terminus of the Kv7.2 channel proteins (MBP-Kv7.2C, 100 kDa) was expressed and purified (Figure 8 A). The full length protein is visible at 100 kDa, in addition there is a clear degradation product of around 43 kDa which most likely corresponds to MBP alone. When the purified protein was incubated with the PIP strips, the overlay assay revealed a broad association with all phosphoinositides including phosphatidylinositol mono-, bis- and trisphosphates (Figure 8 B, C). There was also some association with PS (12, 24, 25). Binding to PI, LPA and S-1-P was low or non-significant when compared with background. As previously reported (12), the MBP when expressed alone did not show any binding to the PIP strips, indicating that the observed binding came directly from the Kv7.2C portion of the protein. These results are similar to those obtained with a fusion protein...
containing the C-terminus of Kv7.1 (12) and confirm a wide range of potential phospholipid modulators of Kv7 channel function.

[Fig. 8 near here]

A Kv7.2 C-terminal fusion protein containing the mutation K452E/R459E/R461E (Kv7.2C-EEE) showed significantly reduced binding to most of the phosphoinositides and also to PA (Figure 8 B, C). No significant change in the low-level binding to LPA and S-1-P could be detected.

DISCUSSION

The first point emerging from these experiments is that M-channel subunits show a rather broad spectrum of interactions with phosphoinositides (and some other phospholipids). This is evident both from the activation of Kv7.2/Kv7.3 heteromers by intracellularly-applied phospholipids, and from phospholipid binding of the purified, solubilised Kv7.2 C-terminus fusion protein. The apparent order of interactions revealed by these two approaches appears to diverge quite appreciably but this is not surprising. The fusion protein only contains the C-terminus (albeit full-length) so is devoid of other cytoplasmic domains of the channel to which the phospholipids might potentially bind (26), and also of the transmembrane domains with which the lipophilic moieties of the phospholipids might interact (20); nor do the overlay assays provide any information about relative affinities. Conversely, binding does not necessarily lead to channel activation; even where it does, the link between agonist binding and channel opening may be complex (27).

The second point concerns the activation by the phosphoinositides. We find that the channels can be equally-well activated (i.e., to near-comparable maximum \( P_{\text{open}} \) values) by the mono-, di- and tri-phosphates diC\(_4\)P(4)\(_5\), diC\(_4\)P(4,5)\(_2\) and diC\(_4\)P(3,4,5)\(_3\). This accords with previous observations that diC\(_4\)P(4,5)\(_2\) and diC\(_6\)P(3,4,5)\(_3\) are equally efficacious (6), and that the channels are also activated by diC\(_6\)P(3,4)\(_2\) (6, 7). It seems likely that they all interact with the same domain of the channel since a Kv7.2 C-terminus fusion protein that contained a group of mutations which reduce the sensitivity of the functional channel to P(4,5)\(_2\) by twofold (11) showed less binding to all of the polyphosphoinositides. Notwithstanding, the channels show a quantitative difference in their response to the different phosphoinositides, the sensitivity increasing with increasing numbers of phosphates. Interestingly, this only applied to the ‘low-affinity’ component of the concentration-response curve, with no significant differences between the EC\(_{50}\)s for the ‘high-affinity’ component; instead, diC\(_6\)P(4)\(_5\) appeared to show a lower efficacy on this component of channel response. One possibility is that these two components relate to the contributions made by binding to the two subunits, Kv7.3 and Kv7.2, the former having a higher ‘affinity’ than the latter (7). If this is the case, then the differences in the amino acid sequences in the C-terminal that are responsible for the different sensitivities of the two subunits to diC\(_6\)P(4,5)\(_2\) (11) also affect the relative activities of the different phosphoinositides at the two sites.

In contrast to the phosphoinositides, neither of the free inositol phosphates, I(1,4,5)\(_3\) and I(4,5)\(_2\), activated the M-channels, nor inhibited their response to P(4,5)\(_2\) at concentrations up to 300 \( \mu \text{M} \). Thus, the presence of the lipophilic diacyl chain appears essential to orient the polar head group in position with respect to the channel in order to activate it. It has recently been shown that this lipophilic chain forms a non-specific association with the transmembrane domains of the Kir channel (20), to facilitate the interaction of the polar head group with the binding sites in the cytoplasmic domain. The natural P(4,5)\(_2\) contains 16-20 carbon diacyl chains (28, 29). The 8-carbon derivative is less lipophilic, and hence more convenient experimentally since it more rapidly enters and leaves the membrane, so is more rapid in both onset and offset (30). A 4-carbon analog proved ineffective on Kir channels (30), presumably because it does not insert into the membrane so readily.

In this study we also found that the M-channels could be activated by the monoacyl lipid monophosphates 1-oleoyl-lyso phosphatidic acid (LPA), sphingosine-1-phosphate (S-1-P) and fingolimid phosphate (FTY720-P). Although in other circumstances lysophospholipids are known to act on specific G protein-coupled transmembrane receptors when released into the extracellular fluid (31), in our experiments they presumably interacted directly with the inner
monophosphates (16). In contrast, Kir6.2 (K)
of interactions with phosphatylinositol
binding studies show evidence of a wider range
of these, plus PI(3,4)P
PI(3,4,5)P
Kir2.2 and Kir2.3 are also activated by
activated most strongly by PI(4,5)P.
Kir2 channels are
phosphoinositide selectivity are the Kir inward-
rectifier channels (26). Kir2 channels are
activated most strongly by PI(4,5)P,
though Kir2.2 and Kir2.3 are also activated by
PI(3,4,5)P;
Kir3 channels are activated by both of
these, plus PI(3,4)P,
and in fusion protein
binding studies show evidence of a wider range
of interactions with phosphatidylinositol
monophosphates (16). In contrast, Kir6.2 (KATP)
channels do not discriminate between PI(4,5)P,
PI(3,4)P and PI(3,4,5)P,
and can also be
activated by other negatively-charged lipids such
as long-chain acetyl coenzyme A (32, 33). The
M-channel appears to more closely resemble the
Kir6.2 channel in this respect, rather than the
more PI(4,5)P-specific Kir2 channels.

Other phospholipid-sensitive membrane proteins
show very variable ligand selectivities. The PH
domain of PLCδ is selective for PI(4,5)P but
binds I(1,4,5)P3 even more tightly (21). In
contrast, the membrane-located transcription
factor tubby binds PI(3,4)P,
PI(4,5)P
and
PI(3,4,5)P but not the inositol phosphates (34).
Very many proteins bind phospholipids, with a
wide range of substrate specificities that are
associated with distinct differences in the
structures and flexibility of their binding sites
(35). Indeed, interaction of basic proteins or
regions thereof with abundant membrane
phospholipids may occur simply through
electrostatic attraction, rather than specific
binding (36).

A plausible binding site in the Kv7.2 and Kv7.3
subunits of the M-channel, in which electrostatic
interactions are reinforced by strong hydrogen
bonding, has been derived from previous
mutational studies coupled with homology
modelling (11); it would be interesting to know
whether this has sufficient flexibility to
accommodate the range of phospholipids that we
have studied.

A further question arises what might be the
physiological significance of these observations?
The first major consideration is how the
concentrations of phospholipid applied to the
inner face of the membrane relate to those the
channel may encounter in the normal cell
membrane. PI(4,5)P comprise about 1 % of the
phospholipids in the cell membrane (37). It has
been estimated that the concentration of
membrane PI(4,5)P,
if dissolved in the
cytoplasm of a cell with a radius of 10 µm
would yield a cytoplasmic concentration of 10
µM (29). Thus the lower concentrations of
PI(4,5)P,
giving a Popen up to ~0.2, are likely to
encompass this physiological range. PI(4)P is
also present in cell membranes at ~78 % of
PI(4,5)P (38). However, its lower potency and
considerably weaker effect at low (< 10 µM)
concentrations (Popen = 0.1 for PI(4)P, versus
0.2 for PI(4,5)P; Figure 2) might explain why it
is unable to maintain channel activity when the
5-phosphate is selectively cleaved from PI(4,5)P
(10). Further, the high potency of PI(3,4,5)P
cannot compensate for the fact that its maximal
concentration is only about one-hundredth of that
of PI(4,5)P (39); the same applies to PI(3,4)P,
which also activates M-channels (6, 7). Thus,
within the phosphoinositides, our results accord
with the conclusions of others in suggesting that
PI(4,5)P is the specific physiological regulator
(2, 40). The insensitivity of the channels to the
inositol 1,4,5-trisphosphate at up to 300 µM also implies that this will not affect M-channel activity at the cytoplasmic concentrations likely to be generated following receptor-mediated hydrolysis of PI(4,5)P$_2$ (up to around 16 µM in neuroblastoma cells (41)).

Activation by lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S-1-P) raises new possibilities. Though derived from abundant membrane lipids (phosphatidic acid and sphingosine), they are not retained in the membrane at high concentrations, but instead are released into the extracellular solution where they act primarily on specific G protein-coupled receptors (31). However, their ability to activate M-channels when applied to their inside face raises the interesting question whether they might have additional, more direct, effects on these and other PI(4,5)P$_2$-regulated membrane proteins when present in the cytoplasm.

In conclusion, our experiments reveal that the M-channel can be activated by a wide range of lipid phosphates, with a minimal requirement for an acyl chain of sufficient length and one or more phosphate head-groups; and, within the phosphoinositides, that potency increases with increasing number of phosphates. Notwithstanding, they also accord with the view that the phosphoinositol PI(4,5)P$_2$ is the primary endogenous phospholipid regulator.

REFERENCES

1. Hilgemann, D. W., Feng, S., and Nasuhoglu, C. (2001) *Sci STKE* **2001**, re19
2. Gamper, N., and Shapiro, M. S. (2007) *Nat.Revs.Neurosci.* **8**, 921-934
3. Suh, B. C., and Hille, B. (2008) *Annu. Rev. Biophys.* **37**, 175-195
4. Wang, H. S., Fan, Z., Shi, W., Brown, B. S., Wymore, R. S., Cohen, I. S., Dixon, J. E., and McKinnon, D. (1998) *Science* **282**, 1890-1893
5. Suh, B. C., and Hille, B. (2002) *Neuron* **35**, 507-520
6. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2003) *Neuron* **37**, 963-975
7. Li, Y., Gamper, N., Hilgemann, D. W., and Shapiro, M. S. (2005) *J Neurosci.* **25**, 9825-9835
8. Delmas, P., and Brown, D. A. (2005) *Nat.Revs.Neurosci.* **6**, 850-862
9. Brown, D. A., and Passmore, G. M. (2009) *Br. J. Pharmacol.* **156**, 1185-1195
10. Suh, B. C., Inoue, T., Meyer, T., and Hille, B. (2006) *Science* **314**, 1454-1457
11. Hernandez, C. C., Zaika, O., and Shapiro, M. S. (2008) *J. Gen. Physiol.* **132**, 361-381
12. Thomas, A. M., Harmer, S. C., Khambra, T., and Tinker, A. (2011) *J. Biol.Chem.* **286**, 2088-2100
13. Main, M. J., Cryan, J. E., Dupere, J. R., Cox, B., Clare, J. J., and Burbidge, S. A. (2000) *Mol. Pharmacol.* **58**, 253-262
14. Selyanko, A. A., Hadley, J. K., and Brown, D. A. (2001) *J. Physiol.* **534**, 15-24
15. Telezhkin, V., Brown, D. A., and Gibb, A. J. (2010) *Soc. Neurosci. Program# F20*(Poster# 340.1), Online
16. Thomas, A. M., Brown, S. G., Leaney, J. L., and Tinker, A. (2006) *J. Membr.Biol.* **211**, 43-53
17. Thomas, A. M., and Tinker, A. (2008) *Meth. Mol.Biol. (Clifton, N.J)* **491**, 103-111
18. Osborne, S. L., Meunier, F. A., and Schiavo, G. (2001) *Neuron* **32**, 9-12
19. Stansfeld, P. J., Hopkinson, R., Ashcroft, F. M., and Sansom, M. S. (2009) *Biochemistry* **48**, 10926-10933
20. Hanssen, S. B., Tao, X., and Mackinnon, R. (2011) *Nature* **477**, 495-498
21. Garcia, P., Gupta, R., Shah, S., Morris, A. J., Rudge, S. A., Scarlata, S., Petrova, V., McLaughlin, S., and Rebecchi, M. J. (1995) *Biochemistry* **34**, 16228-16234
22. Lemmon, M. A., Ferguson, K. M., O’Brien, R., Sigler, P. B., and Schlessinger, J. (1995) *Proc.Natl.Acad.Sci.,USA*, **92**, 10472-10476
23. Ju, M., Shi, J., Saleh, S. N., Albert, A. P., and Large, W. A. (2010) *J. Physiol.* **588**, 1419-1433
24. Manna, D., Bhardwaj, N., Vora, M. S., Stahelin, R. V., Lu, H., and Cho, W. (2008) *J. Biol. Chem.* **283**, 26047-26058
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FOOTNOTES

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Abbreviations used are: I(4,5)P$_2$ = inositol-4,5-bisphosphate; I(1,4,5)P$_3$ = inositol-1,4,5-trisphosphate; PI = phosphatidylinositol; PI(n)P = phosphatidylinositol (n) phosphate; PI(n)P$_2$ = phosphatidylinositol (n) bisphosphate; PI(n)P$_3$ = phosphatidylinositol (n) trisphosphate; PA = phosphatic acid; PS = phosphatidyl serine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; LPA, lysophosphatic acid; LPC, lysophosphatidyl choline; S-1-P = sphingosine-1-phosphate; PLC = phospholipase C.

FIGURE LEGENDS

FIGURE 1. Structures and phosphorylation of phosphatidylinositols.

Sequential reactions for the endogenous phosphorylation of phosphatidylinositol-4-mono-phosphate (PI(4)P) to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$) and phosphatidylinositol-3,4,5-
trisphosphates (PI(3,4,5)P₃) by phosphatidylinositol-5-kinase (PI5K) and phosphatidylinositol-3-kinase (PI3K).

FIGURE 2. M-type channel activity stimulated by dioctanoyl (diC₈) phosphatidylinositolides.

A, B, C – Exemplar traces of M-channel recordings showing effects of sequentially-increasing concentrations of diC₈Pl(4)P, diC₈Pl(4,5)P₂ and diC₈Pl(3,4,5)P₃ applied to the inner leaflet of excised inside-out patches from CHO cells stably expressing the M-channel subunits Kv7.2 and 7.3. Holding potential 0 mV. C = closed state; O₁-O₅ = open states current for the channels.

D – Mean (± SEM.) for P_open of M-channels plotted against 0.1 – 300 µM concentration range of phosphotidylinositols. Solid line and filled circles (●): diC₈Pl(4)P (n = 6-24); dashed line and open circles (○): diC₈Pl(4,5)P₂ (n = 10-27); solid line and filled squares (■): diC₈Pl(3,4,5)P₃ (n = 7-18). The data points were fitted to a two-component Hill equation (see Methods) with the parameters listed in Table 1.

FIGURE 3. I(1,4,5)P₃ does not stimulate or inhibit M-channel activity.

A – Exemplar trace of sequential increase of I(1,4,5)P₃ concentrations showing no stimulation of M-channel activity applied to the inner leaflet of excised inside-out patches from CHO cells stably expressing M-type Kv7.2/7.3 channels. Holding potential 0 mV. C = closed state; O = open state current level.

B – Superimposition of 100 µM PI(4,5)P₂ does not reduce channel activity stimulated by 100 µM diC₈Pl(4,5)P₂. Holding potential 0 mV. C = closed state; O = open state current level.

FIGURE 4. Structures of some lipid phosphates tested.

FIGURE 5. M-channel activity stimulated by sphingosine-1-phosphate (S-1-P) and fingolimod phosphate (FTY720-P).

A, B, C – Exemplar traces of M-channel recordings showing effects of sequential increase of (A,B) S-1-P and (C) FTY720-P concentrations applied to the inner leaflet of excised inside-out patches from K2/3-CHO cells stably expressing M-type Kv7.2/7.3 channels. Holding potential 0 mV. C = closed state; O₁-Oₙ = open state current levels for 1 to n channels.

D – Mean (± SEM.) for M-channel P_open plotted against concentration of phosphatidylinositides. Dashed line: diC₈Pl(4,5)P₂; filled upward triangles (▲): S-1-P; filled downward triangles (▼): FTY720-P (including inset); open upward and downward triangles: diC₈Pl(4,5)P₂ 100 µM, reference points for S-1-P and FTY720-P, respectively. The data points were fitted to a two-component Hill equation (see Methods) with the parameters listed in Table 2.

FIGURE 6. M-type channel activity stimulated by lyso-phosphatidic acid (LPA).

A and B – Exemplar traces of M-channel recordings showing effects of sequential increase of LPA concentrations applied to the inner leaflet of excised inside-out patches from CHO cells stably expressing M-type Kv7.2/7.3 channels. Holding potential 0 mV. C = closed state; O₁-On = open states current for the channels.
D – Mean (± SEM.) for $P_{\text{open}}$ of M-channels plotted against of LPA. Dashed line: diC$_8$P(4,5)P$_2$ (from Figure 2; filled diamonds (♦): LPA, open diamond: diC$_8$P(4,5)P$_2$, 100 µM (reference point for LPA). The data points were fitted to a two-component Hill equation (see Methods) with the parameters listed in Table 2.

**FIGURE 7.** D-erythro-sphingosine (S), fingolimod (FTY720), and biotinilized phosphatidyglycerol (PG) and phosphatidylycholine (PC) are unable to stimulate M-type channel activity.

A, B, C – applications of 100 µM (A) D-erythro-sphingosine (S), (B) fingolimod (FTY720) and (C) biotinylated phosphatidyglycerol (PG) and phosphatidylycholine (PC) to three separate patches did not activate M-channels. In each patch channels were activated 10 µM diC$_8$P(4,5)P$_2$. Holding potential 0 mV. C = closed state; O = open state current level.

**FIGURE 8.** Purification and lipid binding of MBP-Kv7.2C.

A – 10 % SDS-PAGE of typical MBP-Kv7.2C protein purification. The soluble fraction (S) and 2µg of the eluted (E) protein were loaded as indicated. The protein size was estimated using the BioRad prestained markers (M) of known molecular weight (kDa). The band representing the expected size of the protein is highlighted by a box.

B – PIP strips were incubated overnight with 5µg/ml of MBP, MBP-Kv7.2C or MBP-Kv7.2C-EEE protein. (Kv7.2C-EEE contained the mutations K452E/R459E/R461E (11)). Recognition of binding was obtained by incubation with rabbit anti-MBP antibody (1/1000) followed by anti rabbit antibody as contained in the ECL kit (GE healthcare).

C – Densitometric measurements (A.U. = arbitrary units) of the binding of MBP- Kv7.2C (WT, dark blocks) and MBP-Kv7.2C-EEE (EEE, light blocks) to phospholipids. Bars are SEM for the number of strips shown in brackets. *, **: MBP-Kv7.2C-EEE binding significantly less than that of MBP-Kv7.2C at $P < 0.05$ (*) and $P < 0.01$ (**), two-tailed $t$-test for unequal numbers.

**Abbreviations:** PI = phosphatidylinositol; PI(n)P = phosphatidylinositol (n) phosphate; PI(n)P$_2$ = phosphatidylinositol (n) bisphosphate; PI(n)P$_3$ = phosphatidylinositol (n) trisphosphate; PA = phosphatidic acid; PS = phosphatidyl serine; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; LPA = lyso phosphatidic acid; LPC = lyso phosphatidyl choline; S-1-P = sphingosine-1-phosphate.
Table 1

Activation of Kv7.2/Kv7.3 M-channels by phosphoinositides

Data from least-squares curve fits (see Experimental Procedures and Figure 2)

|          | PI(4)P   | PI(4,5)P₂ | PI(3,4,5)P₃ |
|----------|----------|-----------|-------------|
| 'High-affinity' component |          |           |             |
| EC₅₀(1)  µM | 1.7 ± 0.3 | 1.1 ± 0.1 | 1.1 ± 0.2   |
| P_open(max)(1) | 0.10 ± 0.007** | 0.19 ± 0.007 | 0.19 ± 0.017 |
| 'Low-affinity' component |          |           |             |
| EC₅₀(2)  µM | 98.6 ± 4.5 | 49.6 ± 1.9 | 35.4 ± 2.4  |
| P_open(max)(2) | 0.57 ± 0.022 | 0.61 ± 0.012 | 0.64 ± 0.025 |
| Combined |          |           |             |
| P_open(max) | 0.61 ± 0.10 | 0.79 ± 0.05 | 0.80 ± 0.04 |
| nH       | 1.9 ± 0.2 | 2.0 ± 0.1 | 1.5 ± 0.1   |
| *n       | 6-24     | 10-27     | 7-18        |

*Number of patches at each concentration.

**Value significantly differ from P_open(max)(1) of PI(4,5)P₂ (P < 0.03).
Table 2

Activation of Kv7.2/Kv7.3 M-channels by non-inositol lipid monophosphates

Data from least-squares curve fits (see Figures 5 and 6)

|        | S-1-P | FTY720-P | LPA |
|--------|-------|----------|-----|
| **High-affinity' component** | | | |
| EC₅₀(1) µM | 3.2 ± 0.6 | 0.5 ± 0.001 | 1.5 ± 0.4 |
| P_{open(max)}(1) | 0.072 ± 0.006** | 0.007 ± 0.000001** | 0.204 ± 0.027 |
| **Low-affinity' component** | | | |
| EC₅₀(2) µM | 157.3 ± 40.8 | 63.2 ± 0.2 | 40.0 ± 9.2 |
| P_{open(max)}(2) | 0.26 ± 0.043*** | 0.021 ± 0.000006*** | 0.626 ± 0.075 |
| **Combined** | | | |
| P_{open(max)} | 0.26 ± 0.05*** | 0.02 ± 0.006*** | 0.68 ± 0.09 |
| nH | 1.5 ± 0.2 | 1.8 ± 0.0042 | 1.3 ± 0.2 |
| n | 6-15 | 8-11 | 4-12 |

*Number of patches at each concentration.

**Values significantly differ from P_{open(max)}(1) of PI(4,5)P₂ (P < 0.02)

***Values significantly differ from P_{open(max)}(2) and combined P_{open(max)} of PI(4,5)P₂ (P < 0.000006, and P < 0.0000000001, for S-1-P and FTY720-P, respectively)
Figure 2

DiC8-PI(4)P

DiC8-PI(4,5)P2

DiC8-PI(3,4,5)P3

Po
Figure 3

A

| I(1,4,5)P₃ | DIC8-PI(4,5)P₂ |
|-------------|----------------|
| 3 μM        | 100 μM         |
| 10 μM       | 100 μM         |
| 30 μM       | 100 μM         |
| 100 μM      | 300 μM         |
| 300 μM      |                |

0.5 pA
30 s

B

| I (1,4,5)P₃ (100 μM) | DiC8-PI(4,5)P₂ (100 μM) |
|----------------------|--------------------------|

0.5 pA
30 s

100 ms
1 s

O →
C →
Figure 4

A

$\text{PI(4,5)P}_2$

1-Oleoyl lysophosphatidic acid (LPA)

Sphingosine-1-phosphate (S-1-P)

Fingolimod phosphate (FTY720-P)

B

Phosphatidylglycerol (PG)

Phosphatidylcholine (PC)

D-erythro-Sphingosine (S)

Fingolimod (FTY720)
A

DiC8-PI(4,5)P₂
100 µM

S-1-P

0.5 pA
30 s

1 pA
30 s

0.5 pA 1 10 100
0.0 0.2 0.4 0.6 0.8

DiC8-PI(4,5)P₂
100 µM

FTY720-P

0.5 pA
30 s

0.5 pA 1 10 100
0.005 0.010 0.015 0.020 0.025 0.030

B

S-1-P

0.5 pA
30 s

100 ms

S-1-P

FTY720-P

D

Po

Po

FTY720-P (µM)

FTY720-P

S-1-P

Po

FTY720-P (µM)

0.1 1 10 100

S-1-P

FTY720-P

Po

FTY720-P (µM)

0.1 1 10 100

Figure 5
Figure 8

A

M S E

100 - 75 - 50 - 37 -

B

MBP-Kv7.2C MBP-Kv7.2C-EEE

LPA LPC PI PI(3)P PI(4)P PI(5)P PE PC

S-1-P PI(3,4)P_2 PI(3,5)P_2 PI(4,5)P_2 PI(3,4, 5)P_3 PA PS BLANK

C

Spot Intensity (A.U.)

|               | WT (n=10) | EEE (n=5) |
|---------------|-----------|-----------|
| LPA          |           |           |
| LPC          |           |           |
| PI           |           |           |
| PI(3)P       |           |           |
| PI(4)P       |           |           |
| PI(5)P       |           |           |
| PE           |           |           |
| PC           |           |           |
| S1P          |           |           |
| PI(3,4)P_2   |           |           |
| PI(3,5)P_2   |           |           |
| PI(4,5)P_2   |           |           |
| PI(3,4, 5)P_3|           |           |
| PA           |           |           |
| PS           |           |           |

* **
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