doi:10.1038/nature05416

LETTERS

Phospholipids and the origin of cationic gating charges in voltage sensors

Daniel Schmidt[1]*, Qiu-Xing Jiang[1]* & Roderick MacKinnon[1]

Cells communicate with their external environment through physical and chemical processes that take place in the cell-surrounding membrane. The membrane serves as a barrier as well as a special environment in which membrane proteins are able to carry out important processes. Certain membrane proteins have the ability to detect the membrane voltage and regulate ion conduction or enzyme activity[1,2]. Such voltage-dependent processes rely on the action of protein domains known as voltage sensors, which are embedded inside the cell membrane and contain an excess of positively charged amino acids, which react to an electric field. How does the membrane create an environment suitable for voltage sensors? Here we show under a variety of conditions that the function of a voltage-dependent K^+ channel is dependent on the negatively charged phosphodiester of phospholipid molecules. A non-voltage-dependent K^+ channel does not exhibit the same dependence. The data lead us to propose that the phospholipid membrane, by providing stabilizing interactions between positively charged voltage-sensor arginine residues and negatively charged lipid phosphodiester groups, provides an appropriate environment for the energetic stability and operation of the voltage-sensing machinery. We suggest that the usage of arginine residues in voltage sensors is an adaptation to the phospholipid composition of cell membranes.

Structural studies on the voltage-dependent K^+ channels KvAP and Kv1.2 show that the voltage sensors are located at the protein–lipid interface and that arginine residues may be exposed to the membrane[3–8]. In the structure of the Kv1.2 K^+ channel, for example, the sensors are arranged as nearly independent domains, with much of their surface surrounded by lipid. The atomic structures have led to the hypothesis that the interaction of voltage sensors with the membrane is important not only to their structure but also to their function[3–8]. This hypothesis is testable through studies on the effect of membrane lipid composition on voltage-dependent channel function.

Figure 1 shows KvAP K^+ channels in three different experimental conditions. In each condition the membrane voltage was held at –100 mV and then stepped to more positive depolarizing voltages to

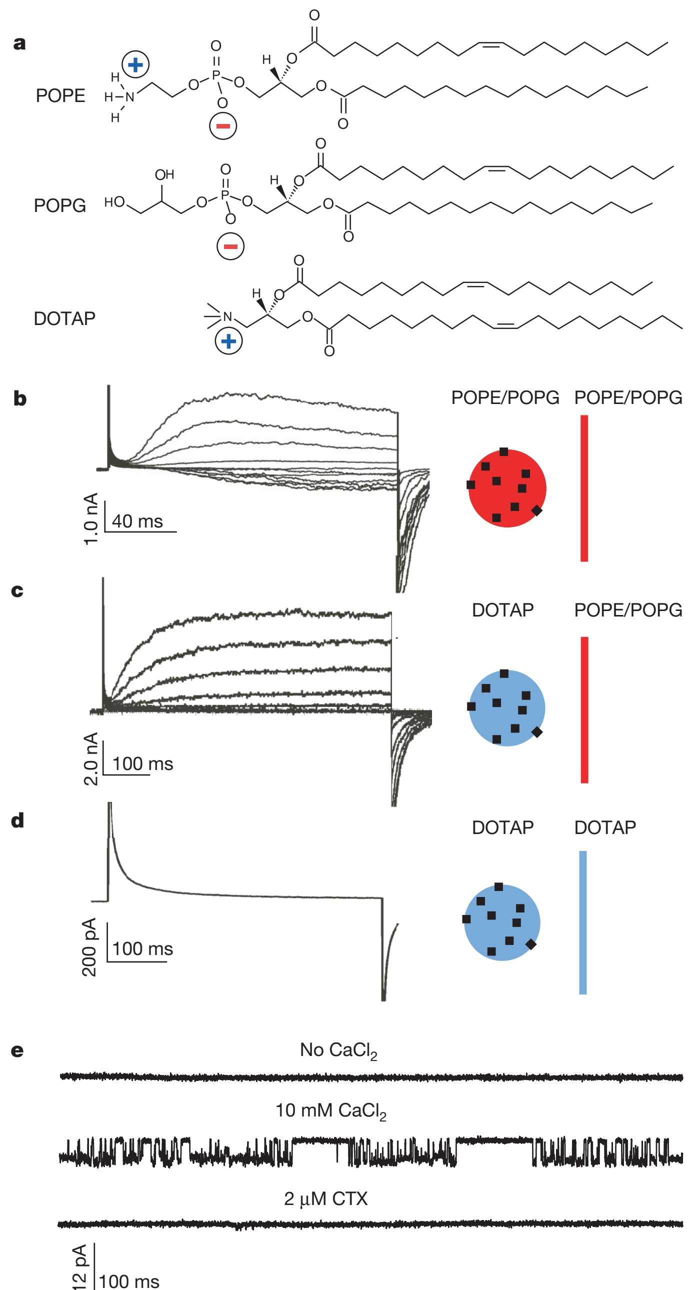

Figure 1 | Assessment of KvAP and MthK function in phospholipid and DOTAP membranes. **a**, Chemical structures of POPE, POPG and DOTAP. **b**, KvAP (black squares in cartoon) in 3:1 POPE:POPG vesicles (red circle) fused into a 3:1 POPE:POPG bilayer (red line) yielded voltage-dependent currents (left traces). **c**, **d**, KvAP in DOTAP vesicles (blue circle) gave functional channels (traces in **c**) after fusion into a POPE:POPG bilayer (red line), but failed to function (trace in **d**) after fusion into a DOTAP bilayer (blue line). Voltage pulses in **b** and **c**: −100 to 40 mV, $\Delta V = 10$ mV, holding potential (h.p.) is −100 mV. The pulse in **d** was from h.p. −100 to 100 mV. **e**, Single-channel recordings of MthK in DOTAP membranes with no $CaCl_2$ (top), 10 mM $CaCl_2$ (middle), and after the addition of 2.0 μM CTX (bottom). h.p. is −150 mV, and channels open downward.

[1]Howard Hughes Medical Institute, Laboratory of Molecular Neurobiology and Biophysics, Rockefeller University, 1230 York Avenue, New York, New York 10021, USA.
*These authors contributed equally to this work.

©2006 Nature Publishing Group

open the channels. When KvAP channels were present in phospholipid membranes made of 1-palmitoyl-2-oleoyl-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-glycero-3-phospho-1-glycerol (POPG) the channels opened in a voltage-dependent manner (Fig. 1b, c). By contrast, when channels were present in membranes consisting of a lipid known as 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)[9], which contains a positively charged trimethylammonium group instead of a negatively charged phosphodiester (Fig. 1a), no channel activity was observed (Fig. 1d). The recording in Fig. 1c shows that KvAP channels are able to reside in a lipid membrane consisting of only DOTAP because the channels were first reconstituted into pure DOTAP lipid vesicles, which were then fused with the planar membrane of phospholipids. The presence of functional channels in the planar phospholipid membrane in Fig. 1c means that KvAP channels had to be present in the DOTAP lipid vesicles that were used to deliver channels to the planar membrane.

Figure 1e shows that a Ca^{2+}-dependent K^+ channel, MthK, is able to function in pure DOTAP membranes. MthK has a pore that is similar to that of KvAP but contains a gating ring structure in the cytoplasm (instead of voltage sensors in the membrane) that enables intracellular Ca^{2+} to open the pore[10]. In DOTAP membranes MthK is activated by Ca^{2+} and inhibited by the scorpion toxin charybdotoxin (CTX) (Fig. 1e). This control experiment shows that DOTAP vesicles can indeed fuse with DOTAP planar membranes and that DOTAP membranes do not prevent the pore of a K^+ channel from functioning. The control also implies that the inability of DOTAP membranes to support KvAP function is related to the voltage-sensing mechanism.

If KvAP channels are present but silent in DOTAP planar membranes then it might be possible to activate them through the subsequent addition of phospholipids to the membranes. Several minutes after fusing channel-containing DOTAP vesicles with DOTAP planar membranes, empty phospholipid vesicles (without KvAP) were fused, resulting in channel activity (Fig. 2a). Because the phospholipid vesicles themselves did not contain KvAP channels, the channels were presumably present in the DOTAP planar membrane but were not functional. In these experiments the number of active channels was always small and in several respects their function was not identical to channels in pure phospholipid membranes. The important point, however, is that channels began to function in a voltage-dependent manner only after phospholipids were delivered to the membrane: that is, in the context of a DOTAP planar membrane KvAP is a 'phospholipid-activated' voltage-dependent K^+ channel.

If DOTAP membranes fail to support channel function then we might expect to observe channels exhibiting 'abnormal' function (that is intermediate behaviour between normal and nonfunctional) in composite membranes containing DOTAP and phospholipids. We therefore studied the effect on voltage-dependent gating of systematically decreasing the molar ratio of phospholipids (Fig. 2b). A dilute solution of phospholipid vesicles containing KvAP channels at

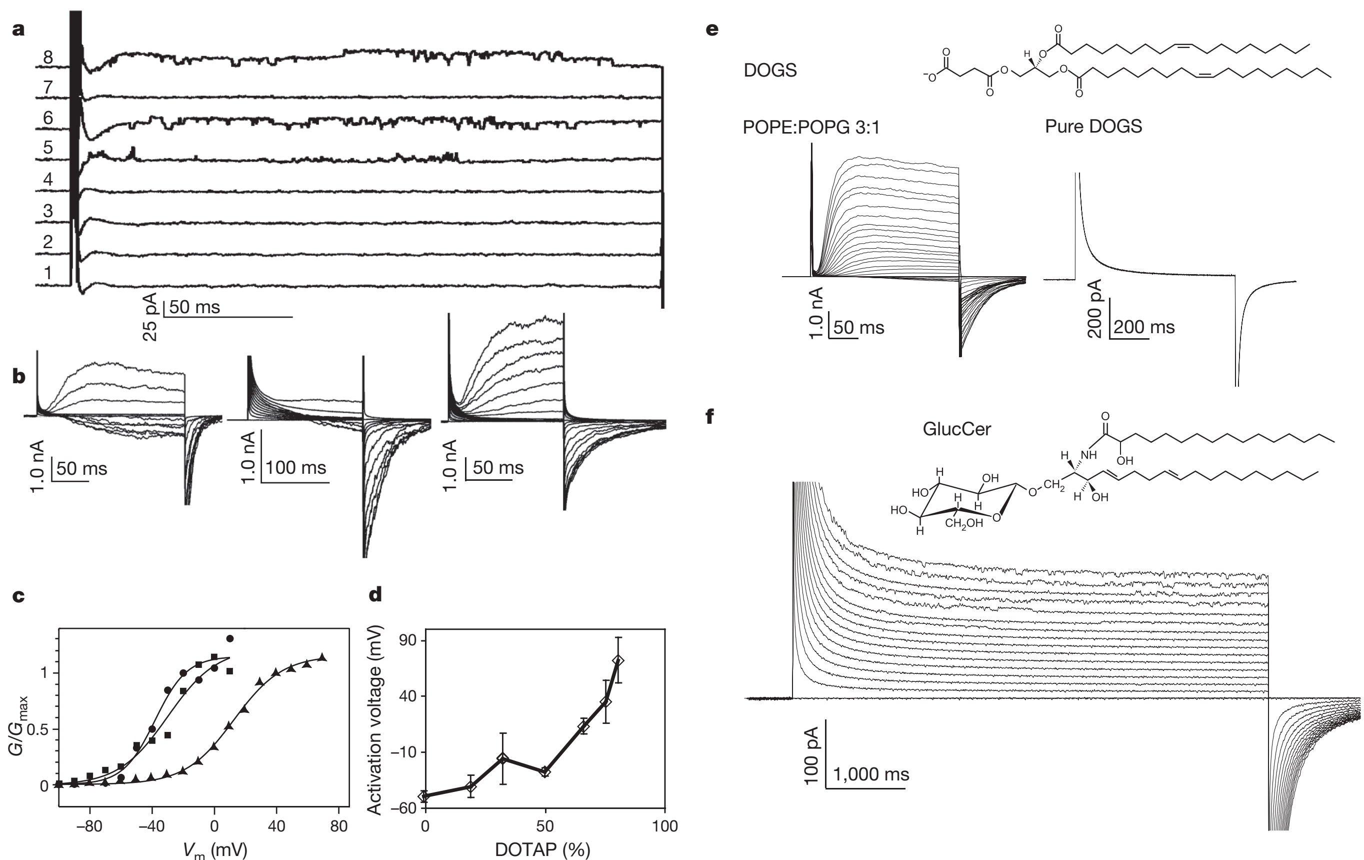

Figure 2 | KvAP function depends on membrane lipid composition. a, KvAP-containing DOTAP vesicles were fused into a DOTAP bilayer. Channel activity was monitored with pulses from −100 to 100 mV every two minutes. Empty POPE:POPG vesicles were introduced between traces 3 and 4. A triple-exponential function was used to subtract capacitance transients. Traces 2–8 were shifted upward successively by 50 pA. **b**, KvAP in POPE:POPG vesicles at a protein-to-lipid ratio (w/w) of 1.0 was fused into bilayers of mixed DOTAP and POPE:POPG with the percentage of DOTAP 0% (left), 50% (middle) and 67% (right). Voltage pulses: h.p. −100 mV to 10 mV (left and middle) and h.p. −100 mV to 70 mV (right), ΔV = 10 mV. **c**, Boltzmann functions (solid lines) fit data from **b** (normalized conductance G/G_{max} as a function of membrane voltage V_m) with the midpoint activation voltage $V_{0.5}$ (mV) and electrical valence Z: −42, 3.1 (0%, circle); −25, 1.9 (50%, square);14, 1.7 (67%, triangle). **d**, $V_{0.5}$ (mean ± s.e.m. or range of mean, n = 2–5) versus percentage of DOTAP. **e**, KvAP in DOGS vesicles fused into bilayers of POPE:POPG (left) and DOGS (right). Voltage pulses: −80 to +180 mV, ΔV = 10 mV, h.p. −100 mV (left); from h.p. −100 to 120 mV (right). **f**, KvAP in DOTAP vesicles fused into a GlucCer bilayer. Voltage pulses: −150 to 150 mV, ΔV = 20 mV, h.p. −150 mV. Structure of GlucCer given above trace.

©2006 Nature Publishing Group

a high protein-to-lipid ratio was used for fusion: this condition best ensured that after fusion the lipid composition of the planar membrane was dominated by the planar membrane lipids (the phospholipid–DOTAP mixture) rather than the vesicle lipids. As the molar ratio of phospholipids was decreased, successively larger depolarization voltages were required to open the channels, and the activation curve became less steep (Fig. 2b, c). At present, we do not know what channel conformations are favoured by the depletion of phospholipids and replacement with DOTAP (that is closed, inactivated, or a non-native state). It is clear, however, that gating becomes progressively more abnormal as the mole fraction of phospholipids is decreased (Fig. 2b, c). The absence of function in pure DOTAP membranes (Fig. 1d) appears to represent the limit of the dilution experiment (Fig. 2b–d), and the restoration of voltage-dependent activity upon addition of phospholipids (Fig. 2a) appears to reflect the fulfilment of a phospholipid requirement for voltage-dependent channel function.

The inability of DOTAP to support KvAP function could be related to the absence of a negatively charged phosphodiester or to the presence of a positively charged trimethylammonium group (Fig. 1a). To distinguish these two possibilities we studied additional non-phospholipid membranes, 1,2-dioleoyl-glycero-3-succinate (DOGS) and soy glucocerebrosides (GlucCer), which do not have a trimethylammonium group (Fig. 2e, f). DOGS is a carboxyl-containing anionic lipid at neutral pH. It forms large unilamellar vesicles in which reconstituted KvAP channels are visible under cryo-electron microscopy (Supplementary Fig. 1) and functional when fused into POPE:POPG planar membranes (Fig. 2e). DOGS also forms stable planar membranes; however, no channel activity is observed. GlucCer is a neutral lipid with a ceramide backbone and sugar head group. Rare (one to three channels) opening events are observed at very positive membrane voltages (Fig. 2f). This lipid is heterogeneous, contains trace phosphate in our phosphate assays, and may exhibit complex phase behaviour. We cannot be certain whether GlucCer or contaminant lipids support the rare openings at very positive voltages. In either case, KvAP function in this membrane is very abnormal. DOTAP, DOGS and GlucCer are chemically very different from each other. None of them appear to create a suitable environment for normal KvAP function.

What special property of phospholipid membranes favours voltage-dependent channel function? We examined the head group requirement by supplementing DOTAP membranes with the zwitterionic phospholipids 1,2-dioleoyl-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-glycero-3-phosphocholine (DMPC) and 1,2-didecyl-glycero-3-phosphocholine (DDecPC), which have successively shorter acyl chains (Fig. 3a). DMPC and DDecPC alone do not form stable planar membranes in the decane bilayer system. Voltage-dependent channel function was supported using phospholipids with acyl chains as short as ten carbons (DDecPC). These short chain lipids apparently plug into the DOTAP membrane and fulfill a head group requirement of the channel. A lipid that supplies only the phosphate group, 1,2-dioleoyl-glycerol-3-phosphate (DOPA), whether mixed with DOTAP or alone, also supports KvAP function (Fig. 3b, Supplementary Fig. 2). Thus, the phosphate group appears to be important for voltage-dependent channel function.

The behaviour of KvAP in mixed phospholipid membranes consisting of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and POPE or POPG is shown (Fig. 3c, d). POPC:POPG membranes (Fig. 3c) are similar to POPE:POPG membranes (Fig. 1b and Fig. 2b) in that both contain a zwitterionic (often referred to as neutral) phospholipid (POPE or POPC) and an anionic phospholipid (POPG). However, these membranes differ in that POPE and POPC have different tendencies to curve[11]. KvAP functions similarly in both membranes, with midpoints of activation of approximately –40 mV for POPE:POPG membranes and –30 mV for POPC:POPG membranes. The POPE:POPC membrane contains only zwitterionic phospholipids: here the midpoint of KvAP activation is shifted to

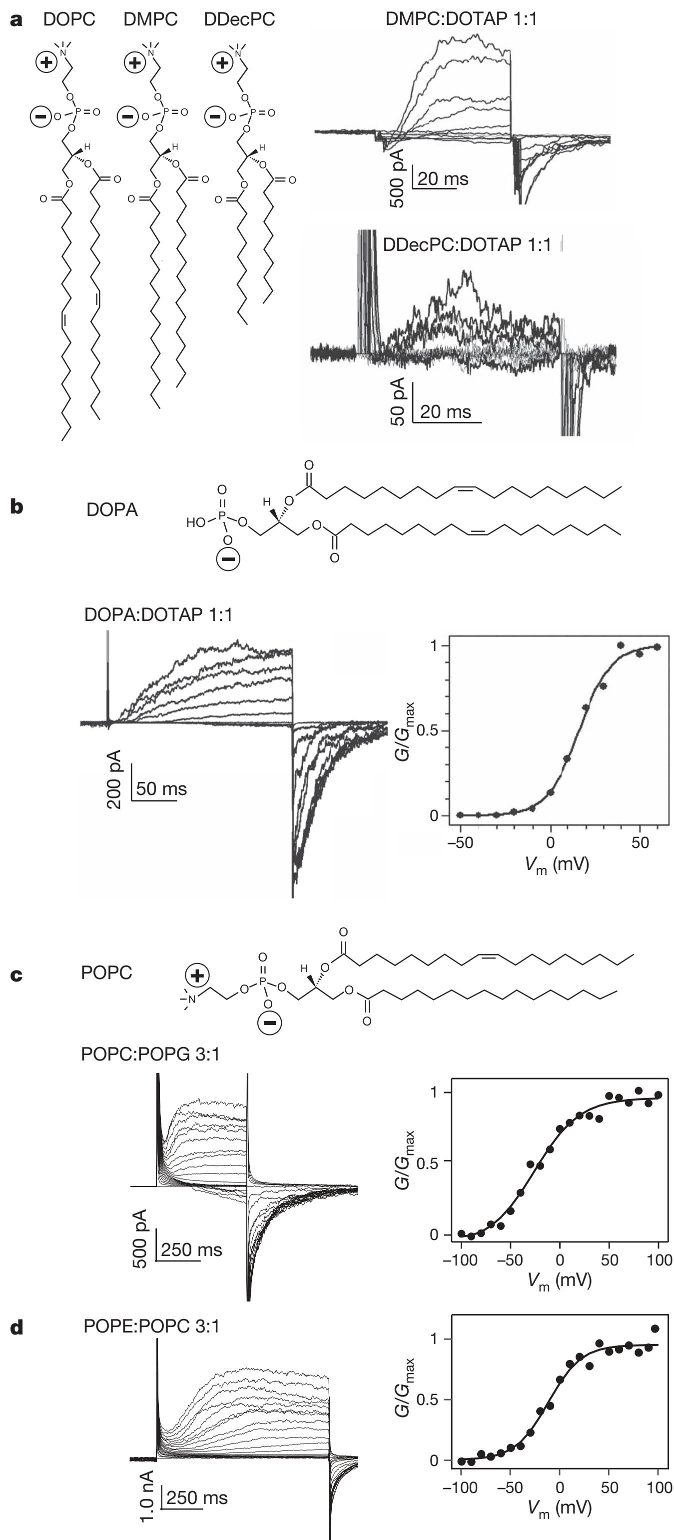

Figure 3 | Phosphate group is important for KvAP voltage-dependent gating. **a**, KvAP in DOTAP vesicles fused into bilayers of 1:1 DOTAP/DMPC (a 14-carbon acyl chain) or DOTAP/DDecPC (a ten-carbon acyl chain). Voltage pulses: −100 to 100 mV, $\Delta V = 20$ mV, h.p. = −100 mV. **b**, KvAP in DOPA vesicles (protein-to-lipid ratio 1.0 w/w) fused into bilayers of 1:1 DOTAP/DOPA. Voltage pulses: h.p. −80 to 60 mV, $\Delta V = 10$ mV. Boltzmann function (continuous line in the right panel) fitted to normalized tail currents (black dots) gave $V_{0.5}$ (mV) and Z: 18, 2.5. **c**, **d**, KvAP in POPC vesicles fused into membranes of 3:1 POPC/POPG (**c**), and POPE/POPC (**d**). Voltage pulses: −100 to 100 mV, $\Delta V = 10$ mV, h.p. −100 mV. Boltzmann functions (continuous lines in right panels) yielded $V_{0.5}$ (mV) and Z: −30.5, 1.8 (**c**), and −9.5, 1.6 (**d**). CTX was used for capacitance transient subtraction in **a** and **b**.

©2006 Nature Publishing Group

approximately –10 mV, but voltage-dependent function is clearly intact (Fig. 3d). Other voltage-dependent channels have also been shown to function in purely zwitterionic phospholipid membranes[12–14]. The comparison of KvAP function in POPE:POPG, POPC:POPG, and POPE:POPC membranes leads us to conclude that membrane curvature tendency and net charge may have modest influences on KvAP function. However, the presence or absence of the phosphate group appears to have a more dramatic influence on voltage-dependent channel function.

In Fig. 4 we begin to investigate the chemical properties of the phosphate group that are important. The phospholipid 1,2-dioleoyl-glycero-3-ethylphosphocholine (EDOPC) is similar to DOPC with respect to many of its physical properties[15]. However, the ethylation means that the resulting phosphotriester is uncharged and it does not participate in intermolecular hydrogen bonding with itself (Fig. 4a)[15]. When normal phospholipids (POPE:POPG) are diluted by increasing the mole fraction of EDOPC, the effects on KvAP gating are qualitatively similar to those observed in DOTAP membranes (Fig. 2b–d and Fig. 4a–c). No KvAP activity is observed in membranes consisting of 95% EDOPC and 5% DOTAP. In control experiments with EDOPC membranes the MthK channel conducts K^+, is Ca^{2+}-activated, and inhibited by CTX (Fig. 4d). Therefore EDOPC membranes support the function of a non-voltage-dependent K^+ channel. It appears that the negative charge on the lipid phosphate group and possibly its hydrogen-bonding potential is specifically important for the function of the KvAP K^+ channel.

KvAP channels function in a variety of phospholipid membranes, including many that have not been presented here. Rates of opening and closing and midpoints of activation vary among membranes of different composition, but voltage-dependent gating is fundamentally intact. Among the many important properties of a lipid bilayer, including its tendency to curve and whether it is net charged or zwitterionic, the presence of the anionic phosphate group appears to be particularly important for KvAP function. This observation suggests that the lipid membrane might provide an environment that is suitable for voltage sensors because phosphate groups can serve as countercharges for arginine residues, as depicted (Fig. 4e). Phosphate groups could be made available through specific binding of lipids to a channel, as appears to be the case for sphingomyelin and certain voltage-dependent channels[16], or through unbound phospholipids in the vicinity of the channel. Arginine–phosphate-group interactions have been described in recent molecular dynamics simulations, which show local membrane distortions that enable the phosphate groups (and water molecules) to approach arginine residues below the surface[17].

Further experiments are needed to confirm the hypothesis that direct interactions between arginine side chains and lipid phosphodiesters stabilize the voltage sensor. The positive charge and multidentate hydrogen-bonding capacity of arginine's guanidinium group makes it an excellent chemical match for favourable electrostatic and hydrogen bonding interactions with the lipid phosphodiester. We suggest that the usage of positively charged amino acids with a preference towards arginine in voltage sensors is an adaptation to the phospholipid composition of the cell membrane.

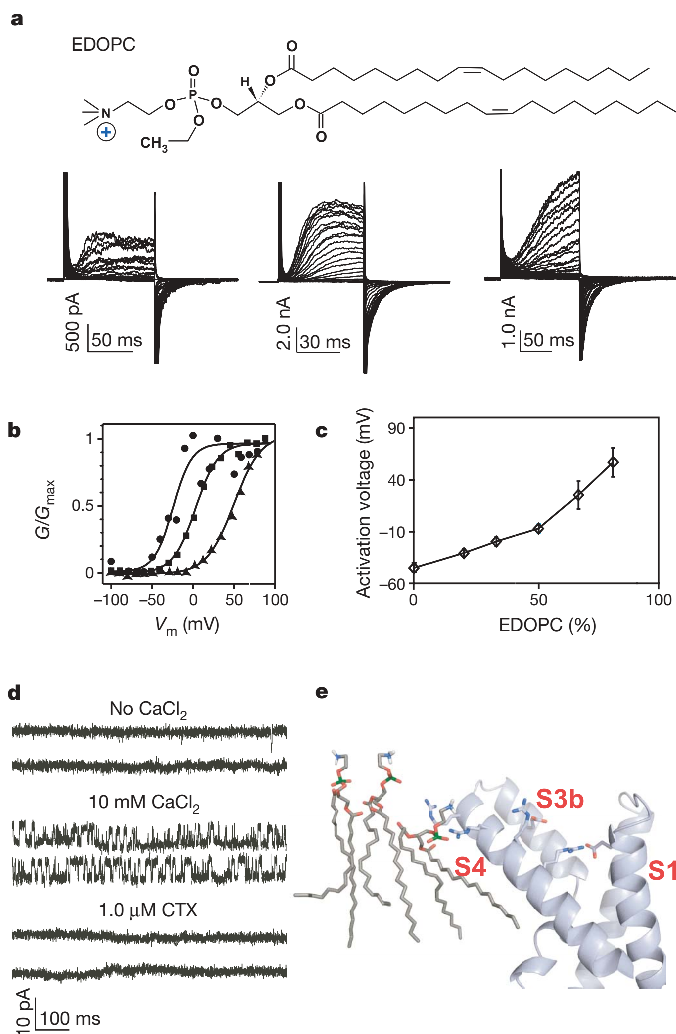

Figure 4 | Role of the negative charge on the phosphate group. a, KvAP in POPE:POPG vesicles (protein-to-lipid ratio 1.0 w/w) fused into bilayers of mixed EDOPC and POPE:POPG with percentage of EDOPC 33% (left), 66% (middle) and 80% (right). Structure of EDOPC shown above. Voltage pulses: −100 to 100 mV, $\Delta V = 5$ or 10 mV, h.p. −100 mV. **b**, Boltzmann functions (continuous lines) from normalized tail currents (black dots) measured from traces in **a** with $V_{0.5}$ (mV) and Z: −25, 2.0 (33%, circle); 19, 1.7 (66%, square); and 70, 1.5 (80%, triangle). **c**, Activation voltage $V_{0.5}$ (mean ± s.e.m., $n = 3$) versus percentage of EDOPC. **d**, Single-channel activity of MthK in EDOPC membranes with no $CaCl_2$ (top), 10 mM $CaCl_2$ (middle), and after addition of 1.0 µM CTX (bottom). h.p. −100 mV. Channels open downward. **e**, The hypothesized interaction between the side chains of Arg residues (first two) in the voltage sensor (Protein Data Bank code 1ORS) and lipid phosphodiester groups (green and red). Voltage-sensor α-helices S1, S3b and S4 are labelled for orientation purposes.

METHODS

Please refer to the Supplementary Information for details.

Preparation and reconstitution of KvAP and MthK channels. KvAP was expressed and purified following published procedures[18], and was concentrated to 5–10 mg ml^{-1} before reconstitution. The reconstitution followed a modification of a published procedure[19]. Lipids of desired compositions were prepared and solubilized (partially or completely) with detergents. The protein and the lipids were mixed at ratios ranging from 0.1 to 1.0. The detergents were then removed by dialysis. Preparation and reconstitution of MthK was based on ref. 10.

Electrophysiological recordings. Bilayer experiments followed published procedures[18]. Bilayers were formed over a 300 µm hole in a polystyrene partition that separated two aqueous chambers[20]. After vesicles with channels were fused into the bilayer, voltage-clamp measurements in whole-cell mode were made. All voltages are reported according to electrophysiological convention, with the extracellular side of the channel taken as ground.

For single-channel recordings of MthK in DOTAP or EDOPC membranes, 5–10 mM $CaCl_2$ was added to the side corresponding to the intracellular side of the channel. Charybdotoxin was added to the side corresponding to the extracellular side of the channel.

Received 5 September; accepted 3 November 2006.
Published online 29 November 2006.

1. Sigworth, F. J. Voltage gating of ion channels. *Q. Rev. Biophys.* **27,** 1–40 (1994).
2. Murata, Y., Iwasaki, H., Sasaki, M., Inaba, K. & Okamura, Y. Phosphoinositide phosphatase activity coupled to an intrinsic voltage sensor. *Nature* **435,** 1239–1243 (2005).
3. Jiang, Y. *et al.* X-ray structure of a voltage-dependent K^+ channel. *Nature* **423,** 33–41 (2003).
4. Jiang, Y., Ruta, V., Chen, J., Lee, A. & MacKinnon, R. The principle of gating charge movement in a voltage-dependent K^+ channel. *Nature* **423,** 42–48 (2003).

©2006 Nature Publishing Group

5. Lee, S. Y., Lee, A., Chen, J. & MacKinnon, R. Structure of the KvAP voltage-dependent K^+ channel and its dependence on the lipid membrane. *Proc. Natl Acad. Sci. USA* **102,** 15441–15446 (2005).
6. Long, S. B., Campbell, E. B. & MacKinnon, R. Crystal structure of a mammalian voltage-dependent *Shaker* family K^+ channel. *Science* **309,** 897–903 (2005).
7. Long, S. B., Campbell, E. B. & MacKinnon, R. Voltage sensor of Kv1.2: structural basis of electromechanical coupling. *Science* **309,** 903–908 (2005).
8. Jiang, Q. X., Wang, D. N. & MacKinnon, R. Electron microscopic analysis of KvAP voltage-dependent K^+ channels in an open conformation. *Nature* **430,** 806–810 (2004).
9. Campbell, R. B., Balasubramanian, S. V. & Straubinger, R. M. Phospholipid–cationic lipid interactions: influences on membrane and vesicle properties. *Biochim. Biophys. Acta* **1512,** 27–39 (2001).
10. Jiang, Y. *et al.* Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* **417,** 515–522 (2002).
11. Israelachvili, J. N. *Intermolecular and Surface Forces* 2nd edn, 366–394 (Academic Press, London, 1991).
12. Hui, K., McIntyre, D. & French, R. J. Conotoxins as sensors of local pH and electrostatic potential in the outer vestibule of the sodium channel. *J. Gen. Physiol.* **122,** 63–79 (2003).
13. MacKinnon, R., Latorre, R. & Miller, C. Role of surface electrostatics in the operation of a high-conductance Ca^{2+}-activated K^+ channel. *Biochemistry* **28,** 8092–8099 (1989).
14. Sun, T., Naini, A. A. & Miller, C. High-level expression and functional reconstitution of *Shaker* K^+ channels. *Biochemistry* **33,** 9992–9999 (1994).
15. MacDonald, R. C. *et al.* Physical and biological properties of cationic triesters of phosphatidylcholine. *Biophys. J.* **77,** 2612–2629 (1999).
16. Ramu, Y., Xu, Y. & Lu, Z. Enzymatic activation of voltage-gated potassium channels. *Nature* **442,** 696–699 (2006).
17. Freites, J. A., Tobias, D. J., von Heijne, G. & White, S. H. Interface connections of a transmembrane voltage sensor. *Proc. Natl Acad. Sci. USA* **102,** 15059–15064 (2005).
18. Ruta, V., Jiang, Y., Lee, A., Chen, J. & MacKinnon, R. Functional analysis of an archeabacterial voltage-dependent K^+ channel. *Nature* **422,** 180–185 (2003).
19. Heginbotham, L., LeMasurier, M., Kolmakova-Partensky, L. & Miller, C. Single *Streptomyces lividans* K^+ channels: functional asymmetries and sidedness of proton activation. *J. Gen. Physiol.* **114,** 551–560 (1999).
20. Miller, C. *Ion Channel Reconstitution* (Plenum Press, New York, 1986).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank Y. Jiang for the MthK channel construct and A. Lee for the CTX. This work was supported by an NIH grant to R.M. R.M. is an investigator in the Howard Hughes Medical Institute.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to R.M. (mackinn@rockefeller.edu).

©2006 Nature Publishing Group