Dual Effect of Phosphatidyl (4,5)-Bisphosphate PIP2 on Shaker K+ Channels

Phosphatidylinositol (4,5)-bisphosphate (PIP2) is a phospholipid of the plasma membrane that has been shown to be a key regulator of several ion channels. Functional studies and more recently structural studies of Kir channels have revealed the major impact of PIP2 on the open state stabilization. A similar effect of PIP2 on the delayed rectifiers Kv7.1 and Kv11.1, two voltage-gated K+ channels, has been suggested, but the molecular mechanism remains elusive. Indirect evidence suggests that such open state stabilization by PIP2 might also be true for Kv channels, as indicated for Kv7.1 (17) and Kv11.1 (8).

By combining giant-patch ionic and gating current recordings in COS-7 cells, and voltage-clamp fluorimetry in Xenopus oocytes, both heterologously expressing the voltage-dependent Shaker channel, we show that PIP2 exerts 1) a gain-of-function effect on the maximal current amplitude, consistent with a stabilization of the open state and 2) a loss-of-function effect by positive-shifting the activation voltage dependence, most likely through a direct effect on the voltage sensor movement, as illustrated by molecular dynamics simulations.

It has been demonstrated to be involved in the production of the second messengers inositol trisphosphate and diacylglycerol, in cytoskeletal organization, membrane trafficking, and regulation of ion channels and transporters activities (1, 2). Examples of PIP2-dependent ion channels and receptors include inwardly rectifying K+ channels (Kir) (3, 4), voltage-gated K+ channels (Kv) (5–8), voltage-gated Ca2+ channels (9), TRP channels (10), and NMDA receptors (11, 12).

To address this issue, we compared the effect of PIP2 on ionic and gating currents of Shaker channel (24), which was used as a model in this study, as it allows recording of large gating currents. We observed non-concomitant and opposite effects on current amplitude and channel voltage dependence, suggesting a dual effect of PIP2 on channel activity. Gating current measurement and voltage-clamp fluorimetry suggest that a direct effect of PIP2 on the voltage-sensor movement underlies the PIP2-induced modification of the channel voltage dependence, but not of the current amplitude, suggesting two binding sites.
EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

The COS-7 cell line, derived from the African green monkey kidney, was obtained from the American Type Culture Collection (CRL-1651, Rockville, MD) and cultured in DMEM supplemented with 10% serum and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin), all from Gibco, (Paisley, Scotland). Cells were transiently transfected with the plasmids using FuGene-6 (Roche Molecular Biochemical, Indianapolis, IN) according to the standard protocol recommended by the manufacturer.

The WT Shaker (clone E, kind gift from Toshinori Hoshi), the N terminus-deleted Shaker Δ6–46 (Shaker-IR) in which fast inactivation is removed (25), and the non-conducting Shaker-W434F and Shaker-IR-W434F mutants were expressed in COS-7 cells using a pGW1 expression vector. The W434F mutation was used to permanently inactivate channels (26, 27) to record gating currents. The plasmid coding for the green fluorescent protein (pEGFP) used to identify transfected cells was purchased from Clontech (Palo Alto, CA).

For giant-patch experiments on Shaker and Shaker-IR, a total of 1 μg of DNA was used. For giant-patch experiments on Shaker-W434F and Shaker-IR-W434F, a total of 8 μg of DNA was used. In both cases, 20% pEGFP combined with 80% of DNA of interest were used for transfection in a well of a 12-well plate.

Electrophysiology

From 24 to 72 h after transfection, COS-7 cells were performed on the stage of an inverted microscope and constantly superfused at a rate of ~2 ml/min. Experiments were performed at room temperature (23 ± 2 °C). Acquisition and analysis were performed using pCLAMP 10.2 software (Molecular Devices). Electrodes were connected to an Axopatch 200A (Molecular Devices). For giant-patch experiments, the procedure described by Hilgemann (Hilgemann, 1989) was adapted to excise giant patches from COS-7 cells. Pipettes were pulled from borosilicate glass capillaries (glass type 8250, King Precision Glass, Claremont, CA) on a vertical puller (P30, Sutter Instruments, Novato, CA) and fire-polished using a microforge (MF-83, Narishige, Tokyo, Japan) to obtain tip diameters of ~10 μm for patch pipettes and ~20 μm for excision pipettes. The excision pipette, filled with the standard bath solution (see below), was connected to a 20-ml syringe to apply suction for excision. A micropipette system allowed local application and rapid change of the different experimental solutions (28). In this giant-patch configuration, series resistances (around 0.5 MOhm) were not compensated, leading to a maximal error of 2 mV in the recordings with the current of the highest amplitude during the depolarizing pulse.

Protocols for Shaker and Shaker-IR Ion Currents

Activation Protocol—Plasma membranes were held at −80 mV, depolarized to various potentials during 40 ms, from −10 to −75 mV, with 2.5 mV decrements, then repolarized to −80 mV, every 5 s. This protocol allowed several measurements:

Maximal current, measured as the maximal activation current amplitudes at −10 mV obtained by fitting both activation and inactivation phases with Equation 1,

\[
l(t) = I_{\text{max}} \times \left(1 - \exp\left(-\frac{t}{\tau_{\text{act}}}\right)^4 \times (r - (r - 1)) \times \exp\left(-\frac{t}{\tau_{\text{inact}}}\right)\right)
\]

(Eq. 1)

where \(I_{\text{max}}\) is the maximal current amplitude, \(\tau_{\text{act}}\) the time constant of activation, \(\tau_{\text{inact}}\) the time constant of inactivation, and \(r\) the residual percentage of current upon full inactivation. This equation allowed determining both activation and inactivation time constants at different potentials.

Half-activation potential and slope factor of activation curve were obtained by fitting the maximal current at each step divided by the electromotive force, with a Boltzmann function (Equation 2),

\[
I_{\text{rel}} = \frac{1}{1 + \exp\left(-\frac{(V_m - V_{1/2})}{k}\right)}
\]

(Eq. 2)

where \(V_m\) is the membrane potential, \(V_{1/2}\) is the half-activation potential, and \(k\) is the slope factor.

Tail Protocol—Plasma membranes were held at −80 mV, depolarized to −10 mV during 50 ms, then repolarized to various potentials during 10 ms, from −20 to −110 mV, with 5-mV decrements, and finally repolarized to −80 mV, every 5 s. This protocol allowed determining the deactivation time constants at different potentials by fitting relaxation of ion current upon the first repolarization with a single exponential.

Inactivation Protocol—Membrane holding potential was −80 mV. Plasma membranes were depolarized (pre-pulse) during 4 s to various potentials, from −95 to −15 mV, with 5 mV increments, then depolarized (pulse) to +50 mV during 250 ms, before being repolarized to −80 mV, every 5 s. Inactivation curves, obtained by normalizing maximum amplitude upon second pulse, were fitted with the following Boltzmann Equation 3,

\[
I_{\text{rel}} = \frac{1}{1 - \exp\left(+\frac{(V_m - V_{1/2})}{k}\right)}
\]

(Eq. 3)

where \(V_m\) is the membrane potential, \(V_{1/2}\) is the half-activation potential, and \(k\) is the slope factor.

This inactivation curve allowed characterizing of half-inactivation potential and slope factor of inactivation curve.

Recovery from Inactivation Protocol—Plasma membranes were held at −80 mV or −100 mV. An initial 200 ms pulse (prepulse) to +20 mV was followed by a second similar pulse (pulse) after an interval from 0 to 2 s, with 0.1 s increments. Pulse/prepulse relative amplitude was plotted against time interval. Recovery from inactivation time constants were obtained from the single exponential fit of recovery from inactivation curves.

Protocol for Shaker-W434F and Shaker-IR-W434F “ON” Gating Currents (\(I_{\text{CON}}\))—Activating ON gating currents (\(I_{\text{CON}}\)) were elicited during 30 ms depolarizing potentials between −100 and +60 mV (with +10 mV increments) starting from a −80 mV holding potential. Subsequently, deactivating OFF gating currents (\(I_{\text{COFF}}\)) were recorded during a 30 ms repolarizing step to −100 mV. Capacitive and leak currents were subtracted.
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using a -P/4 protocol starting from −100 mV or holding potential. Interpulse interval was 5 s long.

This protocol allowed to determine: (i) the total amount of gating charges moved, measured by integrating the ON gating current at +60 mV and (ii) the voltage dependence of charge movement (half-activation potential and slope factor) by plotting the amount of gating charge as a function of depolarizing potential (Q-V curve) and fitting the relation with a Boltzmann function (Equation 2).

Protocol for Shaker-IR-A359C-C445V Ion Currents and Voltage-clamp Fluorimetry—Oocytes were prepared as previously reported (29). Oocytes were placed in a bath chamber that was perfused with control ND96 bath solution containing (in mmol/liter), 96 NaCl, 3 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, and 5 HEPES, titrated to pH 7.4 with NaOH. Micropipettes were filled with 3 mol/liter KCl and had resistances of 1 to 5 MΩ. Voltage control and data acquisition was achieved with a Warner Instruments OC-725C amplifier (Hamden, CT), and Axon Digidata 1322 A/D converter (Axon Instruments, Foster City, CA), connected to a personal computer running pClamp9 software (Molecular Devices Corp.).

Fluorimetry was performed on the Shaker-IR-A359C-C445V (30, 31) simultaneously with two-electrode voltage clamp. Labeling of the oocytes with tetramethylrhodamine-5-maleimide (TMRM; Invitrogen, Carlsbad, CA) dye was performed at 10 °C in a depolarizing solution containing (in mmol/liter), 100 KCl, 1.5 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, and 5 HEPES, titrated to pH 7.4 using KOH, with 5 μmol/liter TMRM. After 30 min of labeling, oocytes were stored in ND96 solution in the dark until voltage-clamped. Fluorimetry was performed using a Nikon TE300 inverted microscope with Epi-Fluorescence attachment and a 9124b Electron Tubes photomultiplier tube (PMT) module (Cairn Research, Kent, UK) as was described previously (Es-Salah-Lamoureux et al., 29). To minimize fluorophore bleaching, a Uniblitz computer-controlled shutter (Vincent Associates, Ottawa, ON, Canada) was used, and opened shortly prior to application of voltage clamp pulses. Fluorescence signal sampling frequency was 6.67 kHz; signal traces were filtered offline at 300–1000 Hz. To correct for photobleaching of fluorophore that occurred during shutter opening during activation protocol and single sweep experiments, control fluorescence data were recorded in the absence of any change in voltage, and subtracted from the voltage-dependent signal.

Membrane holding potential was −80 mV. Membranes were depolarized during 100 ms to various potentials, from −120 to 60 mV, with 10 mV increments, then repolarized to −80 mV, every 2 s.

Solutions and Drugs

For giant-patch experiments, cells were superfused with a standard solution containing (in mm) 145 KCl, 10 HEPES, and 1 EGTA, pH 7.3 with KOH. A solution of (in mm) 145 K-glucosucinate, 10 HEPES, and 1 EGTA, pH 7.3 with KOH, was used to superfuse the cell during measurements and to fill the tip of the patch pipettes. Polysine (Sigma-Aldrich) was diluted to 25 μg/ml before use. PIP<sub>2</sub> (Calbiochem, Villeneuve d’Ascq, France) was diluted to 5 μM and sonicated on ice for 30 min before application to inside-out patches.

Molecular Dynamics Simulations

We used all-atom models of the equilibrated open and closed conformations of the Kv1.2 embedded in fully hydrated POPC lipid bilayer (22). The first ring of the bottom leaflet of POPC molecules around the Kv1.2 was replaced by PIP<sub>2</sub> molecules (54 molecules for the closed conformation and 65 molecules for the open conformation) (supplemental Fig. S2). The system was then solvated in 150 mM KCl solution (A total of ~350,000 atoms) and gradually relaxed using a standard procedure: the entire protein was fixed for 6 ns, enabling reorganization of the lipid and solution, then the backbone only for 2 ns, enabling relaxation of the side chains before finally letting the system relax freely for over 50 ns until reaching equilibrium.

The MD simulations were carried out using the program NAMD2 (32). Langevin dynamics was applied to keep the temperature (300 K) fixed. The equations of motion were integrated using a multiple time-step algorithm. Short- and long-range forces were calculated every 1 and 2 time steps, respectively, with a time step of 2.0 fs. Chemical bonds between hydrogen and heavy atoms were constrained to their equilibrium value. Full three-dimensional periodic boundary conditions were used and long-range electrostatic forces were taken into account using the particle mesh Ewald (PME) approach. The water molecules were described using the TIP3P model (33). The simulation used the CHARMM22-CMAP force field with torsional cross-terms for the protein (34), CHARMM36 for the POPC phospholipids (35), and the CHARMM-compatible parameters for PIP<sub>2</sub> developed in the group of Pr. Osman (36). The simulations were performed on the SGI ALTIX ICE Machine JADE at the CINES supercomputer center (Montpellier, France).

Statistical significance of the observed effects was assessed by Student’s t test or two-way ANOVA test, using SigmaStat 3.1 software. p < 0.05 was considered significant.

RESULTS

The Function of Shaker Channels is PIP<sub>2</sub>-dependent—The effect of PIP<sub>2</sub> on Shaker potassium channels was studied using the inside-out configuration of the patch-clamp technique. Patch excision of the membrane of a COS-7 cell expressing Shaker-IR mutant, in which the amino terminus responsible for the fast (N-type) inactivation was removed (25), led to a current rundown, potentially attributable to a decrease in membrane PIP<sub>2</sub> levels. Consistent with that, addition on the inner side of the membrane of 25 μg/ml of a PIP<sub>2</sub>-scavenger, polysine (Fig. 1, lower panel), accelerated the observed rundown, and eventually led to a complete loss of the channel activity (data not shown). Thus, polysine was only transiently added (~25 s) to reduce the current to approximately one-half of its initial value in order to maintain enough current to measure the biophysical parameters (Fig. 1, left lower panel). Polysine removal did not lead to recovery of the current, suggesting that polysine was not blocking the pore. Most importantly, intracellular addition of 5 μM PIP<sub>2</sub> restored the current amplitude (Fig. 1, middle lower panel) confirming that the observed rundown is PIP<sub>2</sub>-dependent as previously reported for Kv11.1 and Kv7.1 channels (5, 8, 17).
PIP₂ Induces an Increase in Maximal Current Amplitude: a Gain of Function—To fully characterize the effect of PIP₂ on channel function, biophysical parameters were recorded at three distinct periods: just after patch excision (“ctrl”), after a 25-s application of polylysine (“ctrl post-poly-K”), and at steady state after ∼15 min (14.3 ± 3.3 min) of 5 μM PIP₂ application (“PIP₂”). Of note, since excision is associated with the dilution of many cytosolic components, the variation of the biophysical parameters observed between “ctrl” and “ctrl post-poly-K” may be due to other factors in addition to PIP₂ decrease (8). For this reason, we rather focused on the comparison between “ctrl post-poly-K” and “PIP₂” conditions.

Ionic current recordings from a representative patch are shown in Fig. 1, upper panel. Membrane depolarization led to fast activation of the channels, followed by the expected remaining slow C-type inactivation (25). From the activation protocol (Fig. 1, inset) fitting both activation and inactivation with Equation 1 allowed determining accurately the maximal current at −10 mV obtained from 15 patches (***, p < 0.001).


definition of activation and the activation and deactivation kinetics (Fig. 1, upper panel) were used to construct the activation curves and to determine the activation kinetics using Equation 1 (see “Experimental Procedures.”). After ∼15 min of PIP₂ application, the activation curves were shifted by about +15 mV (Fig. 2, A and C), and the slope factor was slightly decreased (Fig. 2D). The decrease in the slope factor may be due to an incomplete decrease in PIP₂ leading to a combination of channels with two profiles (PIP₂-bound and PIP₂-free), resulting in a shallower activation curve at a macroscopic level. In addition, PIP₂ application significantly slowed the activation kinetics measured at potentials from −42.5 to −10 mV (Fig. 2B), and accelerated the deactivation kinetics at different potentials (Fig. 2, E and F). All the parameters (activation curve, activation, and deactivation kinetics) showed a similar 15-mV shift in voltage dependence (Fig. 2, A, B, and F), suggesting that the PIP₂-induced loss-of-function might be acting on the closed to open equilibrium with no structural change in the activation mechanism.

One could argue that open pore stabilization may interfere with voltage sensor movement. In other words, PIP₂ may not

FIGURE 1. Rundown of Shaker-IR currents and reversal by PIP₂ application. Upper panel, representative Shaker-IR currents obtained from a giant patch of a transfected COS-7 cell in response to an activation protocol (inset) after excision (left panel, ctrl, empty circle), after a 25-s application of 25 μg/ml polylysine (middle panel, ctrl post-poly-K, gray triangle), and at steady state, after ∼15 min of PIP₂ (5 μM) application (right panel, PIP₂, dark square). Lower panel, left, kinetics of relative peak tail-current amplitude from a representative cell, showing a slight rundown that is increased by addition of poly-K. Middle, time course of ion current from a representative cell, showing an increase in the current upon addition of PIP₂. Right, maximal full activation current amplitudes at −10 mV obtained by fitting both activation and inactivation phases with Equation 1 (see “Experimental Procedures”). Mean of maximal current at −10 mV obtained from 15 patches (***, p < 0.001).

FIGURE 2. Modification of Shaker-IR activation properties in presence of PIP₂. A, mean relative conductance-voltage (G/G_max − V) relationships fitted with a Boltzmann function and obtained using the same protocol as in Fig. 1, upper panel (n = 15). B, mean activation time constants at different potentials, obtained from 15 patches (***, p < 0.001 PIP₂ versus ctrl post-poly-K). C, mean half-activation potentials and D, slope factors of G/G_max − V curves (n = 15, *p < 0.05). E, currents obtained from a giant patch of a representative transfected COS-7 cell with a tail protocol (shown as inset). F, deactivation time constants obtained by fitting relaxation of ion current upon repolarization with a single exponential (n = 8).
Effect of PIP$_2$ on nonconducting Shaker gating current. 

A, representative Shaker-W434F “On” gating currents obtained from a giant patch of a transfected COS-7 cell in response to an activation protocol (inset) after excision (left panel, ctrl, empty circle), after a 25-s application of 25 µg/ml polylysine (middle panel, ctrl post-poly-K, gray triangle) and at steady state, after ~3 min of PIP$_2$ (5 µM) application (right panel, PIP$_2$, dark square). 

B, mean total amount of gating charges moved at 60 mV (n = 8), C, relative “ON” charge movement-voltage (Q-V) relationships fitted with a Boltzmann function (n = 8) and obtained using the same protocol as in A, D, half-charge movement potential (**, p < 0.01) and E, slope factor of activation curve (mV).

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directly interact with the voltage sensor, but allosterically acts on its movement by stabilizing the open state. Measuring PIP$_2$ effect as early as ~3 min (2.8 min ± 0.8) after PIP$_2$ application allowed us to notice that the PIP$_2$ impact on the voltage-dependence of activation was preceding the impact on current amplitude (supplemental Fig. S1). This rather suggests a direct effect of PIP$_2$ on the voltage sensor.

To test if the modification of the voltage-dependence of activation originates from an effect of PIP$_2$ on the voltage sensor, we recorded gating currents from the non-conducting Shaker-IR mutant W434F, using the giant-patch technique. To our knowledge, this is the first report of recording the PIP$_2$ effect on ion channel gating currents. Data from a representative patch are shown on Fig. 3A. The protocol shown in the inset was used to determine the total amount of gating charges moved and the relative charge-voltage (Q-V) curve (Fig. 3, B and C) from integrating the activating “On” gating current (Q$_{ON}$) (the “Off” gating current was too slow and too small to be accurately measured). The results show that the total amount of gating charges moved and the slope factor k of Q-V curve are similar for the three conditions (Fig. 3, B and E). Most interestingly, following ~3 min (3.1 min ± 0.6) PIP$_2$ addition, the voltage dependence of the “On” charge movement was shifted by about 10 mV toward positive potentials (Fig. 3, C and D), reminiscent of what was observed at the ion current level (Fig. 2A). The effect of PIP$_2$ on the activation curve is therefore correlated with an effect of the phospholipid on the voltage sensor movement.

To test if the interaction of PIP$_2$ with the voltage sensor is affected when the N-type inactivation module is intact, we also observed the effect of PIP$_2$ on the gating current in the full-length channel, Shaker W434F (Fig. 4). After ~3 min of PIP$_2$ application, we observed, as for Shaker-IR W434F, a positive shift of about 10 mV in the voltage dependence of Shaker W434F “On” charge movement-voltage (Q-V) relationships fitted with a Boltzmann function (n = 9) and obtained using the same protocol as in A, (D) half-charge movement potential (**, p < 0.01) and (E) slope factor of activation curve.

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To confirm PIP$_2$ influence on gating and ion currents, we used the voltage-clamp fluorimetry technique, that allows monitoring in real time voltage-dependent and time-dependent conformational changes related to the S4 segments (37), while recording ion currents simultaneously. We studied the effects of a phosphatidylinositol kinase inhibitor, wortmannin, using the Shaker-IR-A359C-C445V mutant (30, 31). Wortmannin inhibits, at micromolar concentrations, PI(4) kinase (PI4K) (38), a critical enzyme in the synthetic cascade of PIP$_2$. Hence, wortmannin application leads to a decrease in membrane PIP$_2$ (5, 38, 39). In this study, cytosolic PIP$_2$ level of oocytes was reduced by a 40-min incubation with 15 µM wortmannin. Reduction of PIP$_2$ stocks in treated oocytes resulted in comparable 14 and 11-mV negative shifts of both ionic current...
(Fig. 5, A–C) and fluorescence voltage-dependences (Fig. 5, D–F). These data mirror the 15 and 10-mV positive shifts of ionic and gating currents when PIP2 was increased in COS-7 cells. Of note, there was no effect of PIP2 reduction on Shaker half-activation potential in COS-7 cells (Fig. 2, A and C). As said earlier in this study, but also for hERG channels (8), it is more difficult to compare the conditions before and after poly-lysine application, the variation of the biophysical parameters observed may be due to the dilution of cytosolic factors in addition to PIP2 decrease. In summary, the data obtained in oocytes are in accordance with what was observed on COS-7 cells, and further suggest that the loss-of-function effects of PIP2 on the activation voltage-dependence are a consequence of the effect on the voltage sensor itself.

**PIP2 Induces a Gain of Function on the Inactivation Gate**—Shaker channels show two distinct inactivation processes, N-type and C-type (25). Using the Shaker-IR mutant in which there is no N-type inactivation, we evaluated whether PIP2 affects C-type inactivation voltage dependence and the kinetics of C-type inactivation and of its recovery. All these biophysical parameters were recorded in the ctrl, ctrl post-poly-K, and PIP2 conditions (Fig. 6). An inactivation protocol (Fig. 6A, inset) was applied in the three experimental conditions (Fig. 6A) and used to determine inactivation curves. The protocol shown in Fig. 1 was used to determine inactivation kinetics (Fig. 6C). We observed that, in presence of PIP2, the voltage dependence of inactivation was shifted by about 26 mV toward positive potentials, the slope factor of inactivation curve was reduced, and the inactivation kinetics were slowed. In addition, recovery from inactivation was accelerated in presence of PIP2 (Fig. 6, F–H). The recovery from inactivation at –80 mV in “PIP2” condition was similar to the one at –100 mV in ctrl post-poly-K condition, suggesting a
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FIGURE 7. Effect of PIP₂ on N-type inactivation of Shaker channels. A, representative ion currents from a giant patch of a Shaker-transfected COS-7 cell, obtained with an inactivation protocol (inset, holding potential was −80 mV, pre-pulse during 4 s at various potentials, with 5-mV increments and pulse at 50 mV during 250 ms, every 5 s). B, inactivation curves, obtained by normalizing the test pulse maximum amplitudes. C, N-type inactivation time constants, calculated by using equation 1 to fit Shaker recordings, obtained with an activation protocol (Fig. 7, inset), were plotted against voltage (n = 7, **, p < 0.01, *, p < 0.05). D, half-inactivation potentials and E, slope factors of inactivation curves (***, p < 0.001).

+20 mV-shift in the recovery from inactivation kinetics. All the parameters (inactivation curve, inactivation, and recovery from inactivation kinetics) showed a similar shift in voltage dependence (Fig. 6, B, C, and H), reminiscent of the PIP₂ effect on activation. Since C-type inactivation is coupled to activation (40), PIP₂ effect on channel inactivation may occur through the activation process.

On the full-length Shaker, we evaluated the PIP₂ effect on N-type inactivation, which develops around 10 times faster than C-type inactivation, as shown on the representative trace (Fig. 7A). Interestingly, we observed that PIP₂ induces a slowing of inactivation kinetics (Fig. 7C) and a positive shift of about 10 mV in the voltage dependence of inactivation (Fig. 7, B and D). A slowing of N-type inactivation in presence of PIP₂ was already demonstrated in Kv1.1 (Shaker-like) and Kv3.4 channels (41).

DISCUSSION

We used Shaker and a combination of electrophysiology and voltage-clamp fluorimetry to better understand the molecular mechanism underlying the effect of PIP₂ on the Kv channel activity. We show that PIP₂ 1) exerts a gain-of-function effect on the channel by increasing the maximal current amplitude and 2) exerts also a loss-of-function effect by right-shifting the voltage dependence of channel activation, most likely through a direct effect on the voltage sensor movement. This study demonstrates for the first time the influence of PIP₂ on both ion and gating currents of a voltage-dependent channel.

Two Opposite PIP₂ Effects on Shaker Channels—Until now, only a gain-of-function effect of PIP₂ was described for several potassium channels. For instance, analysis of single channel recordings of the voltage-independent potassium K₅ ATP channel showed that PIP₂ stabilizes the open state by acting on the final concerted opening transition (13). Simulation of the macroscopic current of Kv7.1 (17) and Kv11.1 (8) Kv channels by kinetic models were consistent with PIP₂ stabilizing the open state, by acting on the final concerted opening, as in the case of the K₅ ATP channel. In Shaker channel, PIP₂ up-regulates the channel activity with (i) no concomitant change in the voltage-dependence (any change in voltage dependence occurs before the current increase, and is associated with a loss of function) and (ii) no concomitant change in total charge movement. These observation suggest that PIP₂ activates the channel through a direct stabilization of the activation gate, similar as Kir6.2, Kv7.1, and Kv11.1 (8, 13, 17).

Here we show that PIP₂ application leads, in addition to the gain of function, to changes in several biophysical parameters of Shaker activation that are all associated with a loss of function: accelerated deactivation, slowed activation, and positive shift in the activation curve. Such a dual effect is a new concept in potassium channels, although it has been reported for two voltage-gated calcium channels (9) and more recently for a sea urchin HCN channel (42). Interestingly enough, in both cases, an effect on maximal current amplitude was observed in addition to an action on the voltage-dependence of the channels, as in our study.

While an effect of PIP₂ on the activation gate has been described for several channels, this study suggests an additional effect of PIP₂ on the voltage sensor. Of note, the effects of PIP₂ on the activation and C-type inactivation properties of the ionic current were comparable (10–20 mV shift toward positive potentials). In Shaker, the C-type inactivation gate seems voltage-independent per se (25) and coupled to activation (40). The fact that the PIP₂ effects on activation and inactivation are similar suggests that PIP₂ modulates the early process of voltage sensor movement rather than the coupling between inactivation and activation gates. This idea is consistent with the following model.

A Molecular Model of Kv1.2 Suggests that PIP₂ Interacts Both with S4 Lower Residues and with S6 C Terminus—To gain a molecular insight into the mechanism of PIP₂ effect on Shaker function, molecular dynamics simulations of an open- and closed-state Shaker-like (Kv1.2) channel embedded in a membrane containing a ring of PIP₂ molecules in the inner leaflet were conducted (supplemental Fig. 52). The analyses of the equilibrated conformations of the channel suggest that the PIP₂ molecules interact electrostatically with positively charged residues of the channel in a state-dependent manner.

In particular, PIP₂ lipids may interact with two different regions that bear an excess of positive charges (Fig. 8). The first encloses the S4–S5 linker outermost residues (Lys-312, Arg-
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326) and is occupied by the same residue in both open and closed forms of the channel. The second region is located in the middle of the S4–S5 linker (close to Lys-322) and is of particular interest as the channel positively charged residues occupy it in a state-dependent manner: In the closed state, aside from the linker residue Lys-322, this site encloses the S4 lower basic residues Arg-303, Lys-306, and Arg-309, consistent with PIP₂ stabilization of the resting position of the voltage sensor and the slowed activation. In the open state, as was found for Kv1.2 embedded in a zwitterionic POPC bilayer (22), these S4 residues are in interaction with negative residues of the VSD, and are not in direct interaction with the PIP₂ molecules. Nonethe-

less they remain close enough to their headgroups to feel the electric field generated by their high negative charge. Such an observation could account for the accelerated deactivation kinetics reported above. Besides, in this open state, the Arg-419 of the S6 C-terminal part come close to this interaction site to interact with PIP₂, consistent with the fact that this negatively charged lipid PIP₂ stabilizes the open gate conformation, possibly through a mechanism similar to the one described for Kir channels (15).

In summary, the molecular model seems to rationalize the electrophysiology and fluorimetry data presented above and provides an insight into the molecular mechanisms involved in the seemingly contradictory gain- and loss-of-function effects of intracellular PIP₂. Of course further experiments will be necessary to further validate this model.

Physiological Relevance of the PIP₂ Effect—In many channels, the presence of PIP₂ and/or related phosphoinositides is required for channel function. The experiments presented here suggest that this also applies to the Shaker channel. Even if exogenous PIP₂ impact the activity of many channels, modulation of channel activity in response to physiological variation of PIP₂ does not need to apply to every PIP₂-dependent channel (44). The Kv channels, Kv7 (5, 45), and Kv11.1 (7, 18) do indeed respond to physiological changes of PIP₂ levels.

Shaker-like Kv1.2 channels play fundamental roles in generating electrical pulses and regulating membrane potential in brain (46), and this physiological importance of the Kv1.2 channel has been confirmed by the fact that Kv1.2 knock-out mice exhibit enhanced seizure susceptibility and die in the third postnatal week (47). In that context, it would be interesting to evaluate if a decrease in PIP₂ induced by a PLC-coupled receptor (5) will either increase the Kv1.2 current, via a shift in the voltage-dependence, or decrease the Kv1.2 current, via a decrease in maximal current amplitude. Recently, Kruse et al. studied the effect of PIP₂ depletion on some Shaker-like channels (Kv1.1, Kv1.3, Kv1.4, and Kv1.5) using physiological enzymes such as G protein-coupled receptor, voltage-sensitive lipid 5-phosphatase or an engineered fusion protein carrying both lipid 4-phosphatase and 5-phosphatase activity (pseudo-janin). Their results suggested that a physiological PIP₂ depletion does not affect the activity of these Shaker-like channels (48). Similar experiments should be done on native tissues to clearly establish (or not) a role of PIP₂ in receptor activated transduction pathways. But it is indeed important to keep in mind that polylysine, wortmannin, and PIP₂ application (used here) may lead to variation in PIP₂ beyond physiological range (44). Nevertheless, the complete rundown of Shaker current and complete recovery in presence of PIP₂ suggest a role of PIP₂ as a permissive cofactor, necessary for channel activation in the membrane, as discussed previously (49). Of note, comparison of Kv2.1 gating and ion currents indicates that only 2% of the surface channels conduct (50). It is possible that the inactive channel are not interacting with PIP₂, resulting in detectable gating currents with no ion currents.

To summarize, the experiments presented here suggest that PIP₂ is necessary for Shaker channels activity. Our results also suggest that there are at least two modes of action of PIP₂, one potentially acting on the final concerted opening and stabilizing
the open state (like for kir6.2, Kv7.1, Kv11.1) and another, as evidenced here, on the voltage dependence of the channel, through a direct effect on voltage sensor movement. Consistently, while this paper was under revision, Rodriguez-Menchaca et al. showed a similar dual effect of PIP2 on Kv1.2 channels and highlighted the interaction of PIP2 with two residues in S4-S5, partially consistent with the molecular model presented here (43).

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