

# Phosphatidylinositol 4,5-Bisphosphate Signals Underlie Receptor-Specific $G_{q/11}$ -Mediated Modulation of N-Type $Ca^{2+}$ Channels

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Modulation of voltage-gated  $Ca^{2+}$  channels via G-protein-coupled receptors is a prime mechanism regulating neurotransmitter release and synaptic plasticity. Despite extensive studies, the molecular mechanism underlying  $G_{q/11}$ -mediated modulation remains unclear. We found cloned and native N-type  $Ca^{2+}$  channels to be regulated by phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ). In inside-out oocyte patches,  $PIP_2$  greatly attenuated or reversed the observed rundown of expressed channels. In sympathetic neurons, muscarinic  $M_1$  ACh receptor suppression of the  $Ca^{2+}$  current ( $I_{Ca}$ ) was temporally correlated with  $PIP_2$  hydrolysis, blunted by  $PIP_2$  in whole-cell pipettes, attenuated by expression of  $PIP_2$ -sequestering proteins, and became irreversible when  $PIP_2$  synthesis was blocked. We also probed mechanisms of receptor specificity. Although bradykinin also induced  $PIP_2$  hydrolysis, it did not inhibit  $I_{Ca}$ . However, bradykinin receptors became nearly as effective as  $M_1$  receptors when  $PIP_2$  synthesis,  $IP_3$  receptors, or the activity of neuronal  $Ca^{2+}$  sensor-1 were blocked, suggesting that bradykinin receptor-induced intracellular  $Ca^{2+}$  increases stimulate  $PIP_2$  synthesis, compensating for  $PIP_2$  hydrolysis. We suggest that differential use of  $PIP_2$  signals underlies specificity of  $G_{q/11}$ -coupled receptor actions on the channels.

**Key words:** calcium channel; muscarinic receptor; lipid signaling; bradykinin, G-protein; patch clamp

## Introduction

Modulation of neuronal ion channels by G-protein-coupled receptors occurs via a number of mechanisms, of which two have proven to be very widespread (Ikeda and Dunlap, 1999). In one, which has been called “membrane-delimited,” activated G-proteins directly interact with the channels, either activating or inhibiting them, without the need of any additional intracellular messenger (Hille, 1994). This mechanism usually involves G-protein  $\beta\gamma$  dimers (Herlitze et al., 1996; Ikeda, 1996; Mirshahi et al., 2003), rather than  $G_{\alpha}$ , and can be complete in under 1 sec. Another one, which involves second messengers, is 10–100 times slower and uses the  $G_{q/11}$  class of G-proteins and activation of phospholipase C (PLC), which hydrolyzes the phosphoinositide phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) into products that release  $Ca^{2+}$  from internal stores and activate protein kinase C (Bernheim et al., 1991; Brown et al., 1997). PLC activation can also reduce plasma membrane  $PIP_2$  concentrations, however, and the emerging literature indicating that many ion channels require  $PIP_2$  in their vicinity to function has suggested that such

$PIP_2$  depletion may itself be a physiological signal (Hilgemann et al., 2001).

Muscarinic acetylcholine receptor (mAChR) stimulation inhibits voltage-gated  $Ca^{2+}$  channels via both of these pathways (Catterall, 1997). For both N-type ( $Ca_v2.2$ ) and P/Q-type ( $Ca_v2.1$ ) channels, the mAChR  $M_2$  and  $M_4$  subtypes that couple to pertussis toxin (PTX)-sensitive  $G_{o/i}$  G-proteins use the membrane-delimited mechanism, and the  $M_1$  and  $M_3$  subtypes that couple to  $G_{q/11}$  use the second-messenger pathway (Hille, 1994). Despite much study, the detailed mechanism of this latter pathway has stubbornly resisted elucidation. In the N-type channel-expressing rat superior cervical ganglion (SCG) sympathetic neurons that have served as perhaps the best model system for this phenomena, these two muscarinic actions can be clearly separated on the bases of PTX sensitivity, mAChR subtype, and speed of action (Shapiro et al., 2001). Recently, it has been suggested that  $PIP_2$  depletion is the primary signal mediating muscarinic modulation of the M-type  $K^+$  current (Suh and Hille, 2002; Zhang et al., 2003) and that  $PIP_2$  is required to stabilize the activity of P/Q-type  $Ca^{2+}$  channels (Wu et al., 2002).

A critical question in  $G_{q/11}$ -mediated signaling involves mechanisms for specificity in receptor actions. Thus, whereas  $M_1$  AChR and angiotensin  $AT_1$  stimulation modulate channels in ganglion cells without any accompanying signals of intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) (Beech et al., 1991; Shapiro et al., 1994a), bradykinin  $B_2$ - and purinergic  $P2Y$ -mediated actions are dependent on  $[Ca^{2+}]_i$  signals from  $IP_3$ -sensitive  $Ca^{2+}$  stores (Cruzblanca et al., 1998; Bofill-Cardona et al., 2000; Gamper and Shapiro, 2003).

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Such specificity has been proposed to arise from clustering of certain G<sub>q/11</sub>-coupled receptors together with IP<sub>3</sub> receptors into “microdomains,” enabling certain agonists, but not others, to raise [Ca<sup>2+</sup>]<sub>i</sub> (Delmas et al., 2002). However, a confounding issue has arisen with the understanding of depletion of membrane PIP<sub>2</sub> as a potent intracellular signal. If all these G<sub>q/11</sub>-coupled receptors strongly activate PLC, provoking hydrolysis and depletion of PIP<sub>2</sub>, then why should blockade of [Ca<sup>2+</sup>]<sub>i</sub>-dependent signals prevent modulation of PIP<sub>2</sub>-sensitive channels by receptors that raise [Ca<sup>2+</sup>]<sub>i</sub>?

Here, we perform several different tests for PIP<sub>2</sub> involvement in regulation of N-type Ca<sup>2+</sup> channels using both the oocyte heterologous expression system and SCG neurons, in which the robust Ca<sup>2+</sup> current (*I*<sub>Ca</sub>) is >90% N type (Plummer et al., 1989). We find evidence for PIP<sub>2</sub> involvement in stabilization of channel activity and for its depletion to underlie G<sub>q/11</sub>-mediated muscarinic modulation. We also find striking specificity in G<sub>q/11</sub>-coupled receptor actions on the Ca<sup>2+</sup> channels. We suggest that this differential effect of G<sub>q/11</sub>-coupled receptors arises from their differential ability to induce intracellular Ca<sup>2+</sup> signals, and hypothesize that a Ca<sup>2+</sup>-binding protein acts as a sensor that triggers concurrent PIP<sub>2</sub> synthesis.

## Materials and Methods

**Oocyte expression and electrophysiology.** Rabbit brain Ca<sub>v</sub>2.2, rat brain β<sub>4</sub>, and rabbit skeletal muscle α<sub>2</sub>δ were subcloned into variants of pGEMHE. cRNAs were synthesized *in vitro* and were injected into *Xenopus* oocytes, which were obtained and maintained as described previously (Lu et al., 1999). For inside-out macropatch recordings, the recording pipettes had a diameter of 15–30 μm and were filled with a solution containing (in mM): 45 BaCl<sub>2</sub>, 80 KCl, and 10 HEPES, pH 7.3 with KOH. The control bath solution contained (in mM): 125 KCl, 4 NaCl, 10 HEPES, and 10 EGTA, pH 7.3 with KOH. Macroscopic currents were evoked from a holding potential of –80 mV every 4 sec by 10 msec depolarizations ranging from –40 to 100 mV in 10 mV increments, followed by a 15 msec repolarization to –40 mV. Currents were filtered at 2 kHz, digitized at 10 kHz, and analyzed with pClamp8 (Axon Instruments, Union City, CA). All experiments were performed at ~22°C.

**SCG neuron culture and transfection.** Sympathetic neurons were isolated from the SCGs of 3- to 14-d-old male rats (Sprague Dawley) and cultured for 2–4 d. Rats were anesthetized with halothane and decapitated. Neurons were dissociated using the methods of Bernheim et al. (1991), plated on 4 × 4 mm glass coverslips (coated with poly-L-lysine), and incubated at 37°C (5% CO<sub>2</sub>). Fresh culture medium containing nerve growth factor (50 ng/ml) was added to the cells 3 hr after plating. For exogenous expression of cDNA constructs in the neurons, we used the biolistic particle delivery system (“gene gun”; Bio-Rad, Hercules, CA). We used the PDS-1000/He gene gun (Bio-Rad) according to the instructions of the manufacturer. In brief, cDNA was coated onto 1 μm gold particles, spread onto the supplied macrocarriers in an ethanolic solution, and allowed to dry in a desiccated environment. We used a burst pressure of 650 psi, which we empirically found to give the optimal expression efficiency in SCG neurons. Cells were plated onto glass coverslips at the time of dissociation, cultured overnight in 35 mm dishes, and “shot” in those same dishes, with the cells already adhered to the coverslips that we used for experiments. The culture medium was aspirated from the dishes, bombardment was performed at the top-most slot in the bombardment chamber under 15–17 inches Hg of vacuum, and fresh culture medium was immediately added to the dishes. Transduction efficiency was assumed to be determined by the random distribution of fired gold particles and was up to 10% of cultured neurons.

**Whole-cell and perforated-patch electrophysiology.** Pipettes were pulled from borosilicate glass capillaries (1B150F-4; World Precision Instruments, Sarasota, FL) using a P-97 Flaming–Brown micropipette puller (Sutter Instruments, Novato, CA) and had resistances of 2–3 MΩ when filled with internal solution and measured in standard bath solution.

Membrane current was measured with pipette and membrane capacitance cancellation, sampled at 200 μsec, and filtered at 1–2 kHz by an EPC-9 amplifier and PULSE software (HEKA–Instrutech, Port Washington, NY). The whole-cell access resistance was typically 3–8 MΩ and averaged 5.3 MΩ. Series-resistance compensation was not used. For *I*<sub>Ca</sub> amplitudes that were typically 1 nA, this implies a series-resistance voltage error of ~5 mV. In most experiments on SCG cells, the perforated-patch method of recording was used with amphotericin B (120 ng/ml) in the pipette (Rae et al., 1991). Amphotericin was prepared as a stock solution in 60 mg/ml in DMSO. In these experiments, the access resistance was typically 10 MΩ (mean, 10.7 ± 0.5 MΩ; *n* = 34) 5–10 min after seal formation. Cells were placed in a 500 μl perfusion chamber through which solution flowed at 1–2 ml/min. Inflow to the chamber was by gravity from several reservoirs, selectable by activation of solenoid valves (ValveLink 8; AutoMate Scientific, San Francisco, CA). Bath solution exchange was complete by <30 sec. Experiments were performed at ~22°C.

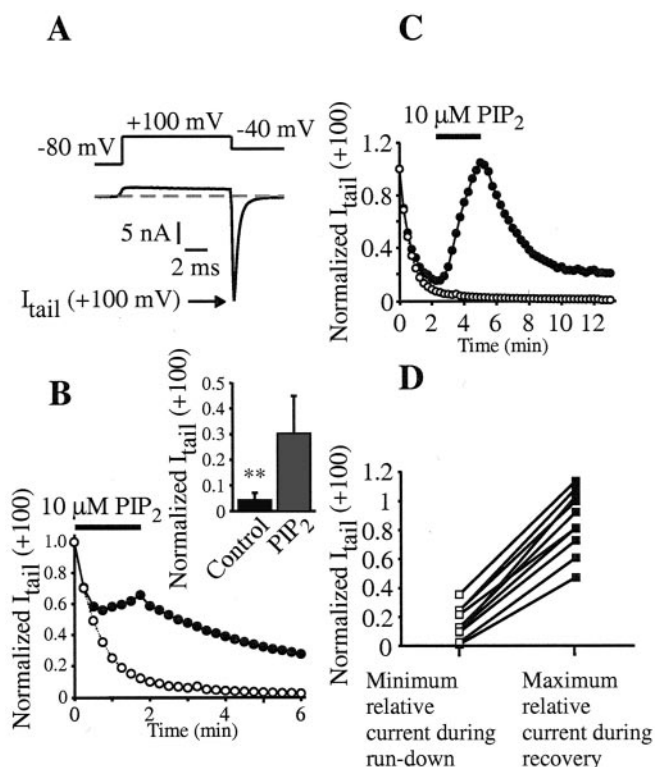
To evaluate the amplitude of *I*<sub>Ca</sub>, cells were held at –80 mV, and 15 msec depolarizing steps to 0 mV were applied every 3 sec. The amplitude of *I*<sub>Ca</sub> was usually defined as the inward current sensitive to Cd<sup>2+</sup> (50 μM). The external solution used to record N-type Ca<sup>2+</sup> currents in SCG cells contained (in mM): 160 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, pH 7.4 with NaOH. The regular pipette solution contained (in mM): 160 CsCl, 5 MgCl<sub>2</sub>, 5 HEPES, 0.1 1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA), 3 K<sub>2</sub>ATP, 0.1 KGTP, pH 7.4 with KOH. In perforated patch experiments, pipette solution contained only 160 mM CsCl and 5 mM MgCl<sub>2</sub>. Data are presented as the mean ± SEM. Statistical tests were performed using paired *t* test or unpaired *t* test, where appropriate. Error bars represent SEM.

**Imaging.** Fluorescent microscopy was performed with an inverted Nikon (Tokyo, Japan) Eclipse TE300 microscope with an oil-immersion 40×, 1.30 numerical aperture objective. A Polychrome IV monochromator (TILL Photonics, Martinsreid, Germany) was used as the excitation light source, and an FITC HQ 96170M filter cube (Chroma Technology, Brattleboro, VT) was used for green fluorescent protein (GFP) imaging. Cells were excited at 470 nm, the fluorescence emission was collected by an IMAGO 12-bit cooled CCD camera, and images were stored with TILLvisION 4.0 software. For the PLCδ-pleckstrin homology (PH) translocation experiments (Fig. 5), two measurements were made, a horizontal scan across the cell for which the fluorescence intensity (*F*) was integrated over the regions indicated by white boxes in the insets and *F* measurements in a region of cytoplasm away from the sides of the cell, indicated by red circles in the insets. The latter measurements are plotted as ratios of fluorescence/initial fluorescence (*F*/*F*<sub>0</sub>).

**Solutions and reagents.** PIP<sub>2</sub> purified from brain (Avanti Polar Lipids, Alabaster, AL) was sonicated for ~12 min before application. 1,2-Dioctanoylglycerol (diC8)-PIP<sub>2</sub> (Echelon Biosciences, Salt Lake City, UT) was prepared as a stock in whole-cell pipette solution at 1 mM and diluted 1:5 for use in experiments. Enhanced GFP (EGFP)-PLCδ-PH, EGFP-Lyn-PH, and EGFP-Akt-PH constructs were a kind gift from Tobias Meyer (Stanford University, Stanford, CA). Dominant-negative (DN) neuronal calcium sensor-1 (NCS-1) was a kind gift from Andreas Jeromin (Mt. Sinai Hospital, Toronto, Ontario, Canada). Reagents were obtained as follows: catalytic subunit of protein kinase A, okadaic acid, Mg-ATP (Sigma, St. Louis, MO); collagenase type I (Worthington Biochemical, Lakewood, NJ); BAPTA (Molecular Probes, Eugene, OR); DMEM, fetal bovine serum, nerve growth factor, penicillin–streptomycin (Invitrogen, San Diego, CA); ATP and GTP (Sigma); amphotericin B (Calbiochem, La Jolla, CA).

## Results

We evaluated the role of PIP<sub>2</sub> in regulation of N-type Ca<sup>2+</sup> channels using two systems. The first is an oocyte expression system in which the channels are heterologously expressed and studied in inside-out macropatches. The second is a preparation of primary rat SCG neurons that display robust N-type Ca<sup>2+</sup> currents in whole-cell or perforated-patch recording and express several different types of mAChRs. In the first system, we focus on the



**Figure 1.** Stabilization and reactivation of N-type Ca<sup>2+</sup> channels by PIP<sub>2</sub> in inside-out membrane patches. *A*, Representative current trace ( $I_{\text{tail}(+100)}$ ) for the experiments shown in this figure, evoked by the indicated voltage protocol. Dashed line indicates zero-current level. *B*, Plotted are the normalized amplitudes of  $I_{\text{tail}(+100)}$  in control bath solution or with a 2 min application of PIP<sub>2</sub> (10  $\mu\text{M}$ ) added to the bath solution immediately after excision (bar). Inset shows summarized normalized amplitudes of  $I_{\text{tail}(+100)}$  5 min after patch excision with or without a 2 min PIP<sub>2</sub> application immediately after excision.  $**p < 0.01$ . *C*, Representative time course of rundown and reactivation of  $I_{\text{tail}(+100)}$  by PIP<sub>2</sub> application during the period shown by the bar. The normalized amplitudes of  $I_{\text{tail}(+100)}$  during the experiment are plotted. *D*, Summary of reactivation of  $I_{\text{tail}(+100)}$  by PIP<sub>2</sub>, applied 2 min after patch excision for 2 min. Minimum current after patch excision and maximum current after PIP<sub>2</sub> application are plotted for 10 patches. In all panels,  $I_{\text{tail}(+100)}$  is normalized by that obtained immediately after patch excision (0 time).

effects of direct application of PIP<sub>2</sub> to the channels, and in the second system, we manipulate membrane [PIP<sub>2</sub>] and focus on the effects on modulation of the channels by G<sub>q/11</sub>-coupled receptor stimulation.

### PIP<sub>2</sub> stabilizes the activity of heterologously expressed Ca<sup>2+</sup> channels in inside-out patches

It has been shown previously that PIP<sub>2</sub> stabilizes the activity of P/Q-type Ca<sup>2+</sup> channels expressed in *Xenopus* oocytes and recorded in inside-out membrane patches, observed as a reduction in channel “rundown” in such patches (Wu et al., 2002). To examine whether PIP<sub>2</sub> exerts the same effects on N-type Ca<sup>2+</sup> channels, we expressed the channels in *Xenopus* oocytes and recorded channel activities in inside-out macropatches. As with P/Q-type channels, we used the tail current after depolarizations to +100 mV ( $I_{\text{tail}(+100)}$ ) as a readout of the functional number of channels in the patch (Fig. 1*A*). We found that N-type channels ran down rapidly after patch excision (Fig. 1*B*), even faster than do P/Q-type channels (Wu et al., 2002). The rundown was significantly attenuated by a 2 min application of PIP<sub>2</sub> (10  $\mu\text{M}$ ) to the intracellular side of the membrane immediately after patch excision (Fig. 1*B*). Such an application of PIP<sub>2</sub> resulted in a per-

sistent stabilization of channel activity even after PIP<sub>2</sub> removal. Thus, the remaining current 5 min after patch excision was only  $4.1 \pm 3.1\%$  (normalized by the current measured immediately after patch excision) in the control solution to  $30 \pm 15\%$  with 2 min PIP<sub>2</sub> treatment (Fig. 1*B*, inset). Furthermore, PIP<sub>2</sub> could reactivate channels that had already run down, sometimes fully recovering the current (Fig. 1*C*). This reactivation effect was more robust than for P/Q-type channels and was observed in all tested patches (Fig. 1*D*). The normalized  $I_{\text{tail}(+100)}$  measured immediately before PIP<sub>2</sub> application was  $15 \pm 11\%$  ( $n = 10$ ), which was increased to  $86 \pm 21\%$  by PIP<sub>2</sub> application. Although this recovery was transient, a fraction of the current remained long after washout of PIP<sub>2</sub> (Fig. 1*C*). Together, these results suggest that PIP<sub>2</sub> stabilizes the activity of N-type Ca<sup>2+</sup> channels in inside-out membrane patches. It has also been suggested that PIP<sub>2</sub> application to inside-out patches containing P/Q-type Ca<sup>2+</sup> channels induces a positive shift in the voltage dependence of channel activation (Wu et al., 2002), reminiscent of the “willing-to-reluctant” mechanism of G-protein-induced channel modulation (Bean, 1989). We found evidence for this for cloned N-type channels as well (supplemental material, available at [www.jneurosci.org](http://www.jneurosci.org)).

### Phosphatidylinositol 4-kinase is required for recovery from PTX-insensitive muscarinic modulation

Having found that N-type Ca<sup>2+</sup> channels are sensitive to the concentration of PIP<sub>2</sub> in the plasma membrane, then hormonal stimulation that hydrolyzes PIP<sub>2</sub> might modulate channel activity via PIP<sub>2</sub> depletion. One such stimulus is activation of M<sub>1</sub> mAChRs, which couple to G<sub>q/11</sub>-class G-proteins, resulting in PLC $\beta$  activation. In this hypothesis, similar to that proposed for M<sub>1</sub> receptor (M<sub>1</sub>R) modulation of M-type (KCNQ) K<sup>+</sup> (Suh and Hille, 2002; Zhang et al., 2003) and certain transient receptor potential (Runnels et al., 2002) channels, the N-type Ca<sup>2+</sup> channel requires some sufficient tonic level of PIP<sub>2</sub> in the membrane to be able to be activated by voltage, and PLC $\beta$  activation by M<sub>1</sub> receptor stimulation is sufficiently strong to significantly decrease membrane [PIP<sub>2</sub>], resulting in channel suppression. This scenario suggests that tonic [PIP<sub>2</sub>] is high enough for appropriate basal channel activity, that muscarinic agonists deplete PIP<sub>2</sub>, inhibiting  $I_{\text{Ca}}$ , and that recovery of the modulation requires resynthesis and/or insertion of PIP<sub>2</sub> in the plasma membrane.

In rat SCG neurons, M<sub>4</sub> receptors act via PTX-sensitive G<sub>o</sub>/G<sub>i</sub> G-proteins to inhibit  $I_{\text{Ca}}$  via the fast, G $\beta\gamma$ -mediated mechanism, whereas M<sub>1</sub> receptors inhibit  $I_{\text{Ca}}$  via G<sub>q/11</sub> and an intracellular second messenger (Wanke et al., 1987; Hille, 1994; Herlitze et al., 1996; Ikeda, 1996; Delmas et al., 1998). The second pathway is >10-fold slower than the first (Zhou et al., 1997). In our experiments on SCG neurons, we focused on this slower second messenger-mediated pathway, and performed several tests to examine whether the second-messenger event involves depletion of membrane PIP<sub>2</sub>. Thus, dissociated neurons were treated overnight with PTX (250 ng/ml) to isolate this pathway (Beech et al., 1992; Shapiro et al., 1994b), and in most experiments, the efficacy of such PTX treatment was verified by stimulation of somatostatin (SS) receptors, which inhibit  $I_{\text{Ca}}$  in these cells solely via the fast pathway (Ikeda and Schofield, 1989; Shapiro and Hille, 1993).

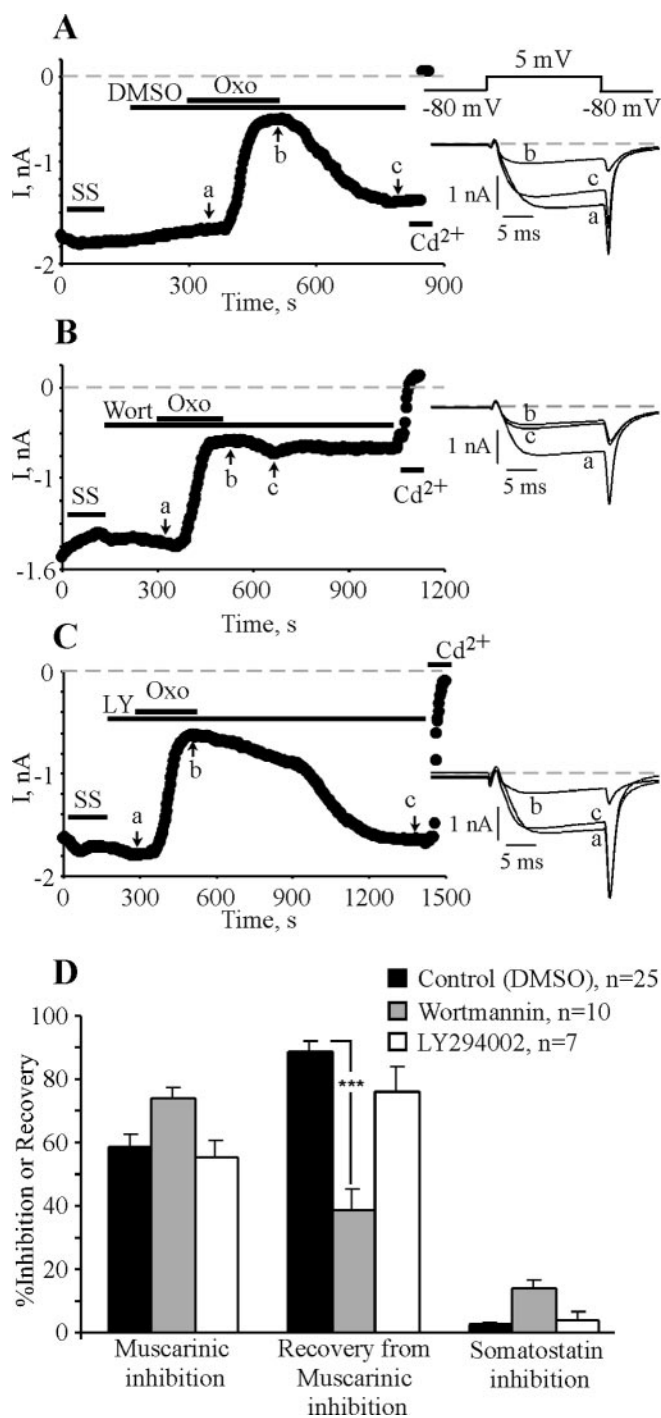
If depletion of PIP<sub>2</sub> mediates the G<sub>q/11</sub>-mediated muscarinic modulation, then blockade of PIP<sub>2</sub> resynthesis should block recovery from modulation in such PTX-treated cells. We tested the effect of wortmannin, which at micromolar concentrations blocks both phosphatidylinositol 3-kinase (PI3-kinase) and phosphatidylinositol 4-kinase (PI4-kinase), on the extent of this



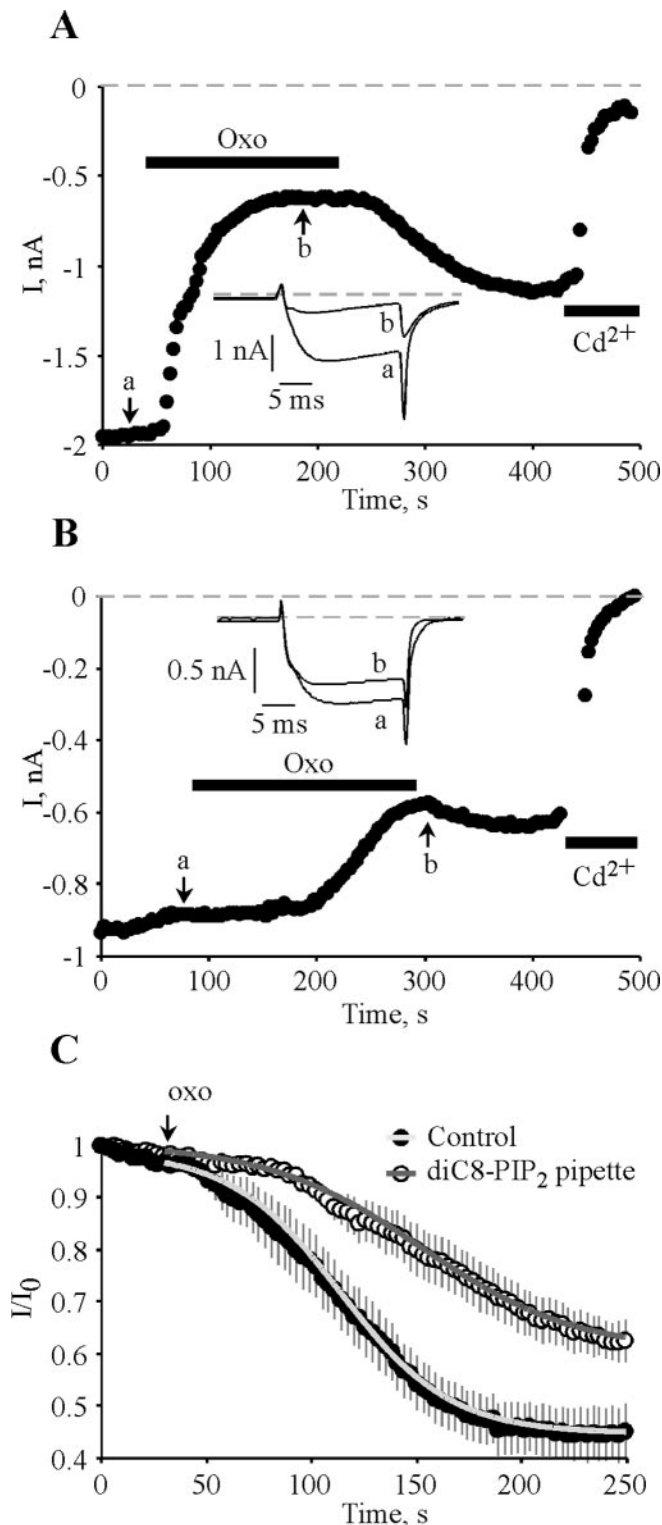
recovery. Experiments were performed in perforated-patch mode, which allows for much fuller recovery in control experiments, compared with whole-cell recording (Brown et al., 1997). In control cells treated only with DMSO vehicle during the recording, the muscarinic agonist oxotremorine-M (oxo; 10 μM) inhibited *I*<sub>Ca</sub> by 58.6 ± 3.9%, and *I*<sub>Ca</sub> recovered to 89.0 ± 3.3% of its initial amplitude (*n* = 25) (Fig. 2A). In cells treated with wortmannin (50 μM) before, during, and after oxo application, the inhibition was 74.3 ± 2.8%, similar to that in the control cells, but the recovery from inhibition was only 38.9 ± 6.5% (*p* ≤ 0.001; *n* = 10) (Fig. 2B). The effect of wortmannin or DMSO vehicle pretreatment on *I*<sub>Ca</sub> was small, only 7.5 ± 2.6% (*n* = 9) and 6.1 ± 1.7% (*n* = 5), respectively. To ask whether the effect of wortmannin on the recovery from inhibition is specific to its blockade of PI4-kinase, versus PI3-kinase, we tested the effect of the specific PI3-kinase inhibitor LY294002 (LY; 50 μM). In cells using LY (Fig. 2C), the inhibition of *I*<sub>Ca</sub> was 55.4 ± 5.3%, and the recovery from inhibition was 75.8 ± 8.5% (*n* = 7), both similar to the values in control. These data are summarized in Figure 2D. In these three groups of PTX-treated cells, the inhibitions by SS (250 nM) were only 2.7 ± 0.9% (*n* = 25), 13.9 ± 3.1% (*n* = 10), and 4.3 ± 2.3% (*n* = 7), respectively, confirming that we have isolated muscarinic modulation mediated by M<sub>1</sub> receptors. We ascertained whether the requirement for PIP<sub>2</sub> synthesis for recovery from modulation is specific for G<sub>q/11</sub>-mediated (second messenger) modulation of *I*<sub>Ca</sub> by testing whether recovery from the PTX-sensitive SS modulation is influenced by wortmannin. As for the experiments shown in Figure 2, wortmannin (50 μM) or only DMSO vehicle were applied to non-PTX-treated neurons before, during, and after application of SS (250 nM). In control neurons, SS inhibition was 52.3 ± 9.8%, and recovery was 95.8 ± 5.7% (*n* = 4), similar to that reported previously (Shapiro and Hille, 1993). SS action on cells treated with wortmannin was not significantly different, for which SS inhibition was 54.2 ± 3.6%, and recovery was 93.0 ± 4.9% (*n* = 5; data not shown). Thus, blockade of PI4-kinase, but not PI3-kinase, strongly (and selectively) attenuates recovery from PTX-insensitive muscarinic modulation, implicating resynthesis of PIP<sub>2</sub> in this process, and suggesting that G<sub>q/11</sub>-mediated depletion of PIP<sub>2</sub> contributes to G<sub>q/11</sub>-mediated muscarinic modulation of N-type Ca<sup>2+</sup> channels.

**Intracellular dialysis with diC8-PIP<sub>2</sub> blunts PTX-insensitive muscarinic modulation**

If PLC activation by muscarinic stimulation suppresses *I*<sub>Ca</sub> by depletion of intracellular PIP<sub>2</sub>, then dialysis of PIP<sub>2</sub> into the cell from a whole-cell recording pipette should blunt the modulation by providing an inexhaustible source of PIP<sub>2</sub>. Because PIP<sub>2</sub> is hydrophobic, we used the short-chain water-soluble analog diC8-PIP<sub>2</sub>. Whole-cell recording was performed on PTX-treated SCG neurons, and the effect of including diC8-PIP<sub>2</sub> (200 μM) in the pipette solution was assayed. The pipette solution was sonicated before each experiment to ensure dispersion of the phosphoinositide. In cells examined using the control pipette solution (Fig. 3A), application of oxo (10 μM) inhibited *I*<sub>Ca</sub> by 54.5 ± 4.6% (*n* = 8), with a half-time of 84.1 ± 0.8 sec. However, using pipettes containing diC8-PIP<sub>2</sub> (Fig. 3B), oxo inhibited *I*<sub>Ca</sub> by only 32.0 ± 4.8% (*p* < 0.01), with a half-time slowed to 123 ± 1 sec (*n* = 10). The effect of oxo on *I*<sub>Ca</sub> for these two groups of cells are summarized in Figure 3C. We also examined whether dialysis of diC8-PIP<sub>2</sub> would shift the voltage dependence of activation of *I*<sub>Ca</sub> but did not see any obvious effect in the neurons (supplemental material, available at www.jneurosci.org). The effects of intracellular dialysis of diC8-PIP<sub>2</sub> on blunting modulation are consistent



**Figure 2.** Blockade of PIP<sub>2</sub> resynthesis with wortmannin prevents recovery of *I*<sub>Ca</sub> from muscarinic modulation. Neurons were treated overnight with PTX (100 ng/ml). Plotted are the amplitudes of inward Ca<sup>2+</sup> currents (black circles) evoked by 15 msec depolarizing voltage pulses given every 3 sec from a holding potential of -80 to 5 mV recorded in the perforated-patch configuration of the patch-clamp technique. A, Control experiment in which the neuron was treated with vehicle only (0.1% DMSO). SS (250 nM), DMSO, oxo (10 μM), and CdCl<sub>2</sub> (Cd<sup>2+</sup>; 100 μM) were applied during the periods indicated by the bars. Insets on the right depict current traces recorded at times indicated by the arrows. B, C, Experiments similar to A, but wortmannin (Wort; 50 μM; B) or LY (50 μM; C) were applied during the periods indicated by the bars. In the cell shown in B, the blockade of recovery by wortmannin was somewhat greater than the mean. D, Bars show mean inhibition by oxotremorine-M and somatostatin as well as recovery from muscarinic inhibition in control (black), wortmannin-treated (gray), and LY294002-treated (white) cells. \*\*\*Significance at the *p* ≤ 0.001 level; Student's *t* test. Error bars represent SEM.



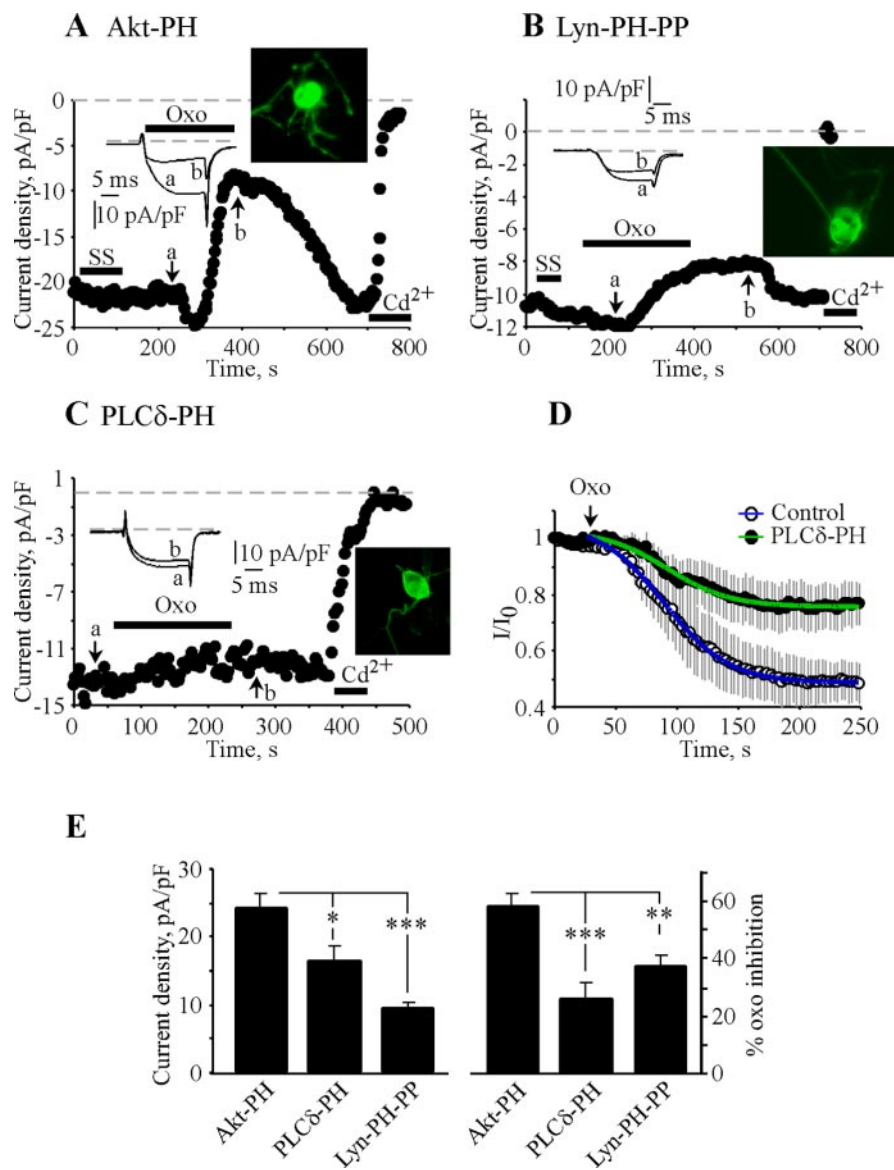
**Figure 3.** Dialysis of diC8-PIP<sub>2</sub> from the patch pipette slows and decreases muscarinic suppression of  $I_{Ca}$ . *A, B*, Voltage protocols are as in Figure 2, but recordings were made in whole-cell configuration. *A*, Control experiment with regular pipette solution. *B*, diC8-PIP<sub>2</sub> (200  $\mu$ M) was added to the pipette solution. The recording started 8–10 min after achieving whole cell to allow for dialysis into the cytoplasm. Oxo (10  $\mu$ M) and CdCl<sub>2</sub> (Cd<sup>2+</sup>; 100  $\mu$ M) were applied during the periods indicated by the bars. Insets on the right depict current traces recorded at the times indicated by the arrows. *C*, Pooled time courses of muscarinic suppression of  $I_{Ca}$  in control (black circles;  $n = 8$ ) and diC8-PIP<sub>2</sub>-dialyzed (white circles;  $n = 10$ ) cells, temporarily aligned to the time of oxtremorine-M application. Data were fitted by the equation  $y = y_0 + a/(1 + \exp(-(t_{1/2} - t)/k))$ , where  $y$  is  $I/I_0$ ,  $a$  is  $y_{max} - y_{min}$ ,  $t_{1/2}$  is a half-time of inhibition, and  $k$  is the slope factor.

with depletion of intracellular PIP<sub>2</sub> having a role in G<sub>q/11</sub>-mediated muscarinic inhibition of  $I_{Ca}$  in SCG cells. With the PIP<sub>2</sub> analog in the pipette dialyzing into the cell and providing an inexhaustible supply, [PIP<sub>2</sub>] cannot fall to a level like that in control cells, and the rate of the decline that does occur is much slower.

### Sequestration or depletion of membrane PIP<sub>2</sub> in SCG neurons reduces tonic current amplitudes and attenuates muscarinic modulation

We further tested for PIP<sub>2</sub> involvement in muscarinic modulation of  $I_{Ca}$  by using several constructs that have been shown to bind and sequester PIP<sub>2</sub> or to decrease its plasma membrane concentration. The N terminus of PLC $\delta$  has a PH domain that binds to PIP<sub>2</sub> to achieve plasma-membrane targeting (Shaw, 1996). Overexpression of the PH domain of PLC $\delta$  in cells has been shown to sequester PIP<sub>2</sub> and thus make it unavailable to act as an intracellular effector (Stauffer et al., 1998; Raucher et al., 2000). Modulation by oxo of  $I_{Ca}$  in SCG neurons treated overnight with PTX was tested in cells transfected with three different cDNA constructs using the biolistic particle delivery system gene gun (Malin and Nerbonne, 2000). The first contains the PH-domain region of PLC $\delta$ , expressed as a fusion protein with EGFP (PLC $\delta$ -PH). The second contains the PH domain of the serine-threonine kinase Akt, also fused with EGFP (Akt-PH). Akt binds to, and is activated by, PI-3 kinase-generated phosphoinositol PI(3,4)P<sub>2</sub>, but not PI(4,5)P<sub>2</sub> (the molecule commonly and in this work abbreviated as PIP<sub>2</sub>) (Franke et al., 1997), and so Akt-PH is a useful control for the PLC $\delta$ -PH construct. The third (Lyn-PH-PP) codes for a PIP<sub>2</sub>-specific phospholipid 5'-phosphatase that selectively reduces plasma membrane PIP<sub>2</sub> concentrations but not other intracellular pools of PIP<sub>2</sub> or other plasma membrane phosphatidylinositol phosphates. The 5'-phosphatase is Inp54p of yeast (Stolz et al., 1998), expressed as a fusion protein to a myristoylation-palmitoylation sequence taken from the Src-family tyrosine kinase Lyn to achieve plasma membrane localization, and to EGFP (Raucher et al., 2000). Because all these PIP<sub>2</sub>-testing constructs are linked to EGFP, we can use single-cell imaging to (1) confirm their presence in the cell and (2) localize their distribution within the cell (i.e., plasma membrane or cytoplasm). In these experiments, we used perforated-patch recordings to maintain the intracellular milieu of the cells tested.

Figure 4*A* shows an example of a perforated patch experiment on a neuron transfected with Akt-PH, which is localized diffusely throughout the cytoplasm (inset), as expected, because plasma-membrane concentrations of PI(3,4)P<sub>2</sub> are very low in the absence of PI3-kinase stimulation (Fruman et al., 1998). In these cells, inhibition of  $I_{Ca}$  by oxo was robust ( $57.8 \pm 4.2\%$ ;  $n = 10$ ) and similar to that found in our control whole-cell experiments (Fig. 3*C*) and in previous work (Hille, 1994; Liu et al., 2004). In contrast, expression of the PIP<sub>2</sub> sequestering-depleting constructs in the neurons had two pronounced effects: (1) tonic  $I_{Ca}$  amplitudes (before application of oxo) were reduced, and (2) inhibition of  $I_{Ca}$  by oxo was attenuated. An example of an experiment on a neuron transfected with PLC $\delta$ -PH is shown in Figure 4*B*. Note the membrane localization of the construct (inset), which was as expected because PLC $\delta$ -PH binds to membrane PIP<sub>2</sub>. In such cells, initial current density was reduced to  $16.3 \pm 2.1$  pA/pF ( $n = 18$ ) compared with the control value of  $24.0 \pm 2.3$  pA/pF ( $n = 18$ ;  $p \leq 0.05$ ), and oxo (10  $\mu$ M) produced only a modest inhibition of  $I_{Ca}$  ( $25.4 \pm 5.7\%$ ;  $n = 10$ ;  $p \leq 0.001$ ). An example of an experiment on a neuron transfected with Lyn-PH-PP is shown in Figure 4*C*. As expected from the binding of



**Figure 4.** Overexpression of PIP<sub>2</sub> depleting–sequestering constructs in SCG neurons decreases *I*<sub>Ca</sub> density and reduces muscarinic suppression. *A–C*, PTX-treated SCG neurons were studied in perforated-patch configuration. Voltage protocols and other experimental conditions and labeling are as in Figure 2. Cells were transfected using the biolistic particle delivery system (gene gun; see Materials and Methods for details) with Akt-PH (*A*), Lyn-PH-PP (*B*), or PLCδ-PH (*C*). Color insets on the right show images of the transfected neurons. Cd<sup>2+</sup>, CdCl<sub>2</sub>. *D*, Pooled time courses of muscarinic suppression of *I*<sub>Ca</sub> in Akt-PH- (white circles; *n* = 8) and PLCδ-PH-transfected (black circles; *n* = 8) cells, temporally aligned to the time of oxo application. Data were fitted by the equation  $y = y_0 + a/(1 + \exp(-(t_{1/2} - t)/k))$ , where  $y$  is  $I/I_0$ ,  $a$  is  $y_{\max} - y_{\min}$ ,  $t_{1/2}$  is a half-time of inhibition, and  $k$  is a slope factor. For Akt-PH-expressing cells,  $y_0$ ,  $a$ ,  $t_{1/2}$ , and  $k$  were 0.01, 1.1, 60.4, and  $-26.3$ , respectively; for PLCδ-PH-expressing cells, they were 0.02, 1.1, 62.2, and  $-25.6$ , respectively. *E*, Bars on the left summarize data of the current density of the Akt-PH-, Lyn-PH-PP-, and PLCδ-PH-expressing neurons (*n* = 18, 23, and 18, respectively), and those on the right summarize their muscarinic modulation (*n* = 10, 12, and 10, respectively). Significance: \**p* ≤ 0.05; \*\**p* ≤ 0.01; \*\*\**p* ≤ 0.001.

the PH domain of Lyn to membrane PIP<sub>2</sub>, this protein is also predominantly membrane localized (inset). In Lyn-PH-PP-overexpressing neurons, the initial current density was especially low,  $9.2 \pm 0.9$  pA/pF (*n* = 23; *p* ≤ 0.001), and muscarinic modulation was reduced to  $36.9 \pm 3.8\%$  (*n* = 12; *p* ≤ 0.05). Pooled time courses of muscarinic inhibition of *I*<sub>Ca</sub> in Akt-PH- and PLCδ-PH-expressing neurons are superimposed in Figure 4*D*, showing a similar time course but reduced inhibition for the latter. Figure 4*E* summarizes the effects of these three fusion constructs on the current density of *I*<sub>Ca</sub> and its modulation by

muscarinic agonists. In neurons expressing either of the constructs that act on PIP<sub>2</sub>, both tonic *I*<sub>Ca</sub> and muscarinic modulation were affected, although to different degrees. Expression of PLCδ-PH more strongly reduces modulation than it does tonic *I*<sub>Ca</sub>, but expression of Lyn-PH-PP reduces *I*<sub>Ca</sub> more than it does modulation. In the model in which the channel requires bound PIP<sub>2</sub> to be openable, and PLC activation suppresses *I*<sub>Ca</sub> by depletion of PIP<sub>2</sub>, the reduction of modulation caused by expression of PLCδ-PH is probably attributable to its sequestering of PIP<sub>2</sub> molecules, making them unavailable to bind to the channels or accessible for PLC cleavage. Only when PLCδ-PH unbinds from PIP<sub>2</sub> in response to IP<sub>3</sub> production are PIP<sub>2</sub> molecules available for either. In other words, changes in [PIP<sub>2</sub>] are buffered by the probe. As PIP<sub>2</sub> begins to be hydrolyzed by PLC and the PLCδ-PH molecules translocate to the IP<sub>3</sub> accumulating in the cytosol, previously sequestered PIP<sub>2</sub> now becomes available to bind to the channels, resulting in blunted modulation. We would also expect that attenuation of modulation to be accompanied by a reduction in tonic *I*<sub>Ca</sub> amplitudes, and this was indeed the case. For the cells expressing PLCδ-PH, we suppose that free [PIP<sub>2</sub>] is tonically reduced, resulting in tonic inhibition of *I*<sub>Ca</sub>. In cells expressing Lyn-PH-PP, the data suggest that *I*<sub>Ca</sub> amplitudes are tonically strongly reduced by dephosphorylation of many PIP<sub>2</sub> molecules by the 5'-phosphatase coded for in the construct. However, the PIP<sub>2</sub> molecules remaining are still fully available for PLC hydrolysis and, calculated as a percentage, muscarinic modulation is only modestly attenuated.

**Translocation of PLCδ-PH by PIP<sub>2</sub> hydrolysis and muscarinic inhibition of *I*<sub>Ca</sub> are correlated**

Because the affinity of the PLCδ-PH construct for IP<sub>3</sub> is some 10-fold higher than for PIP<sub>2</sub>, it serves as a useful reporter for PIP<sub>2</sub> hydrolysis by PLC (but probably not for changes in [PIP<sub>2</sub>] by lipid kinases or phosphatases) (Varnai and Balla, 1998; Hirose et al., 1999; Nash et al., 2001;

Zhang et al., 2003). In unstimulated cells, [IP<sub>3</sub>] is low, and almost all the PLCδ-PH resides in the plasma membrane, bound to PIP<sub>2</sub>. However, after PLC activation, the PLCδ-PH increasingly translocates to the IP<sub>3</sub> molecules accumulating in the cytosol, and this movement of EGFP fluorescence is an optical reporter of PLC activity. In this way, we monitored the time course of PIP<sub>2</sub> hydrolysis in SCG neurons to compare it with that of muscarinic modulation of *I*<sub>Ca</sub> in the neurons also expressing the PLCδ-PH construct. We also similarly assayed the ability of bradykinin stimulation to induce PIP<sub>2</sub> hydrolysis by PLC in the same cells. To

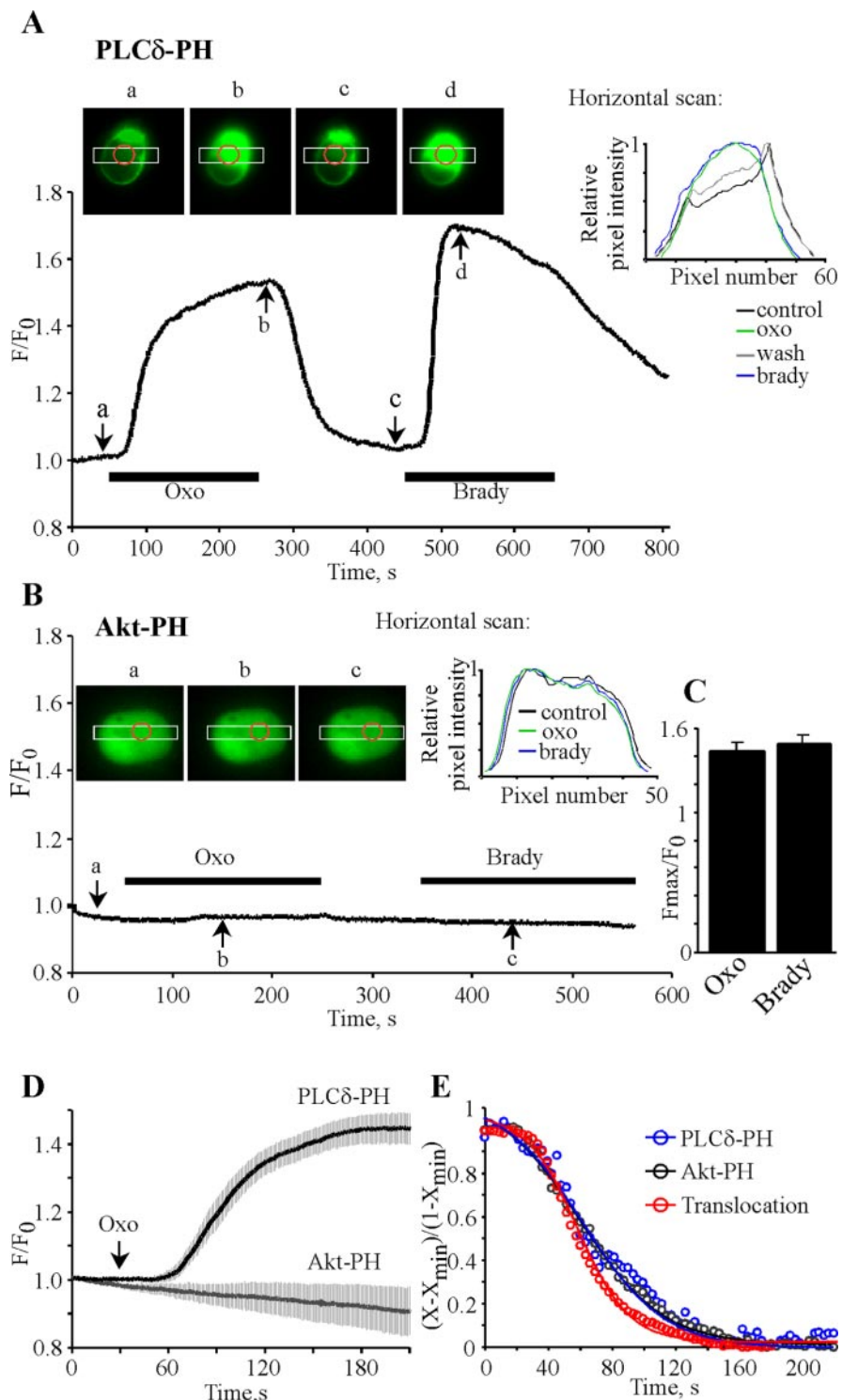


compare the time courses of modulation and translocation, we thought it was important to do the imaging experiments on the same rig as the patch-clamp experiments were done on, using the same (non-rapid) perfusion apparatus and recording chamber. Figure 5A shows such an experiment on an undisturbed (non-patch-clamped) PTX-treated neuron stimulated with oxo (10  $\mu$ M), followed by bradykinin (200 nM). Both agonists caused a robust translocation of the probe from membrane to cytosol that was reversible, as evidenced by the brighter fluorescence in the membrane compared with that in the cytosol before receptor stimulation and the redistribution of the fluorescence afterward (inset). As a control, we also monitored the fluorescence during muscarinic and bradykinin stimulation of a neuron expressing the Akt-PH construct (Fig. 5B). In those cells, neither agonist altered the fluorescence intensity or its distribution in the cell. For neurons transfected with PLC $\delta$ -PH, cytosolic fluorescence during muscarinic stimulation increased by  $45 \pm 4\%$  ( $n = 7$ ), whereas in cells transfected with Akt-PH, cytosolic fluorescence did not increase, but rather, a small decline of the fluorescence intensity by  $9.1 \pm 9\%$  ( $n = 5$ ) during the experiment was observed instead. The pooled time courses of muscarinic-induced translocation in these experiments are shown in Figure 5D. For bradykinin, the increase in cytosolic fluorescence was  $49.3 \pm 6.3\%$  ( $n = 7$ ), equal to that caused by muscarinic agonists (Fig. 5C).

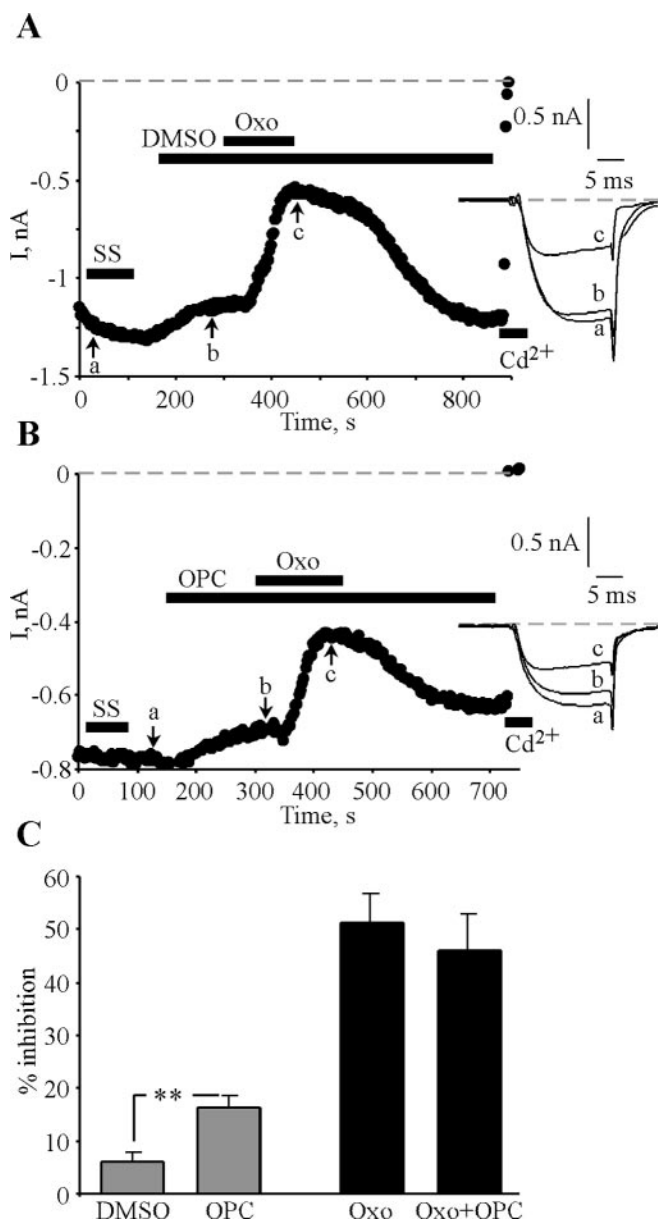
We compared the time courses of  $I_{Ca}$  suppression and PLC $\delta$ -PH translocation by muscarinic stimulation (Fig. 5E). The pooled translocation curve is inverted for comparison and superimposed on the pooled time courses of muscarinic modulation of  $I_{Ca}$  in the PLC $\delta$ -PH- and the Akt-PH-expressing neurons. All data are normalized to maximal effect. It can be seen that the three data sets are temporally well correlated. We calculated half-times for translocation and  $I_{Ca}$  inhibition in cells expressing the Akt-PH or PLC $\delta$ -PH constructs to be  $57.2 \pm 0.5$  sec ( $n = 7$ ),  $60.4 \pm 1.2$  sec ( $n = 8$ ), and  $62.2 \pm 2.3$  sec ( $n = 10$ ), respectively.

### Phospholipase A<sub>2</sub> activity is not required for muscarinic modulation

It has been suggested that arachidonic acid (AA), liberated by PIP<sub>2</sub> hydrolysis and phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity, is the second messenger mediating G<sub>q/11</sub> muscarinic modulation of  $I_{Ca}$  in the rat SCG cells that we are investigating here (Liu and Rit-



**Figure 5.** Translocation of membrane-localized PLC $\delta$ -PH to the cytosol reports PIP<sub>2</sub> hydrolysis by muscarinic and bradykinin (brady) stimulation. Plotted are normalized cytosolic fluorescence of EGFP excited at 470 nm during oxotremorine (10  $\mu$ M) and bradykinin (200 nM) application (indicated by the bars) for PLC $\delta$ -PH- (A) or Akt-PH- (B) transfected cells. Insets on the top depict images of the cells studied taken at the times indicated by the arrows. White boxes define areas where horizontal scans (insets on the right) were performed. Red circles indicate the "regions of interest" in the cytoplasm used for the  $F/F_0$  measurements. Exposures (50 msec, A; 200 msec, B) were acquired every 1 sec. The longer exposures in B led to slight accumulated photobleaching during the experiment. C, Bars summarize the normalized cytosolic fluorescence increase by muscarinic and bradykinin stimulation. D, Pooled data for PLC $\delta$ -PH- and Akt-PH-transfected cells ( $n = 7$  and 5, respectively), temporarily aligned to the time of oxo application. E, Plotted are the data from Figure 4D for muscarinic modulation in PLC $\delta$ -PH- and Akt-PH-expressing neurons, normalized to maximal effect and superimposed with the pooled PLC $\delta$ -PH translocation shown in D, inverted, and normalized to maximal effect. Data were fit by the equation as in Figure 4D, but here,  $y$  represents current inhibition or translocation normalized for maximal effect  $\{(X - X_{min}) / (1 - X_{min})\}$ . Data are shown without error bars for clarity.



**Figure 6.** PLA<sub>2</sub> is not required for muscarinic modulation of *I*<sub>Ca</sub>. SCG neurons were treated overnight with PTX and studied in perforated-patch configuration. Voltage protocols are as in Figure 2, and applications of 0.1% DMSO, OPC (10 μM), oxo (10 μM), or CdCl<sub>2</sub> (Cd<sup>2+</sup>; 100 μM) are indicated by bars. *A*, Vehicle control; *B*, OPC. *C*, Bars show the inhibition by oxo in control cells or in those treated with OPC. \*\**p* ≤ 0.01.

tenhouse, 2003; Liu et al., 2004). In those studies, voltage-independent and PTX-insensitive inhibition by oxo was absent in cells pretreated with the PLA<sub>2</sub> inhibitor oleyloxyethyl phosphocholine (OPC). Thus, we tested whether OPC would block muscarinic inhibition of *I*<sub>Ca</sub> in our PTX-treated neurons. Surprisingly, we found little effect of this compound on the muscarinic action (Fig. 6). Neurons were treated overnight with PTX and studied the following day in perforated-patch configuration. To verify the efficacy of PTX action, SS inhibition was assayed in these cells. A control neuron pretreated with only DMSO vehicle responded strongly to oxo (10 μM) (Fig. 6*A*), and a neuron pretreated with OPC (10 μM) also displayed robust inhibition (Fig. 6*B*). These data are summarized in Figure 6*C*. Application of OPC (10 μM) itself slightly inhibited *I*<sub>Ca</sub> by 16.4 ± 2.1% (*n* = 7),

and subsequent application of oxo in the continued presence of OPC suppressed *I*<sub>Ca</sub> by 46.1 ± 7.0% (*n* = 7), not significantly different from the value in control cells of 51.3 ± 5.5% (*n* = 5). The control experiments were performed during the same days as the OPC experiments. SS inhibited *I*<sub>Ca</sub> by 4.9 ± 1.6 and 1.4 ± 0.9% in OPC-treated and control cells, respectively, indicating that we have isolated G<sub>q/11</sub>-mediated modulation. Although we cannot rule out that AA or PLA<sub>2</sub> activity affects the sensitivity of the Ca<sup>2+</sup> channels to muscarinic stimulation (see Discussion), we conclude that PLA<sub>2</sub> activity is not required for G<sub>q/11</sub>-mediated muscarinic modulation.

**Although bradykinin stimulation causes translocation of PLCδ-PH, it does not inhibit *I*<sub>Ca</sub>**

The experiments presented so far implicate changes in [PIP<sub>2</sub>] in regulation of N-type Ca<sup>2+</sup> channel activity by M<sub>1</sub> receptors. If all such G<sub>q/11</sub>-coupled receptors were to act by depletion of membrane PIP<sub>2</sub>, then they should also induce channel suppression and show the same correlation between PLCδ-PH translocation and modulation of *I*<sub>Ca</sub>. Bradykinin B<sub>2</sub> receptors, like M<sub>1</sub> mAChRs, also inhibit the M-type K<sup>+</sup> current (Jones et al., 1995), but unlike M<sub>1</sub> receptors, do so via IP<sub>3</sub> and increases in [Ca<sup>2+</sup>]<sub>i</sub> (Cruzblanca et al., 1998), acting in concert with calmodulin (Gamper and Shapiro, 2003). The translocation experiments suggest that bradykinin stimulation provokes PIP<sub>2</sub> hydrolysis as well as muscarinic stimulation. However, application of bradykinin did not inhibit *I*<sub>Ca</sub> in the same cells in which oxo robustly did so (Fig. 7*A*). In those cells, the inhibition of *I*<sub>Ca</sub> by oxo was 55.5 ± 2.9% (*n* = 11), but the inhibition of *I*<sub>Ca</sub> by bradykinin was negligible, only 3.0 ± 2.4% (*n* = 12) (performed using the same bradykinin and oxo solutions used in the translocation experiments). Although the translocation of PLCδ-PH, and thus PIP<sub>2</sub> hydrolysis, appeared similar between bradykinin and muscarinic stimulation, there was a dramatic difference in the ability of the two G<sub>q/11</sub>-coupled receptor agonists to modulate the Ca<sup>2+</sup> channels. Muscarinic stimulation does so robustly and with the same time course as the translocation signal, but bradykinin stimulation does not. These experiments suggest that although bradykinin stimulation induces robust PIP<sub>2</sub> catalysis, as evidenced by strong PLCδ-PH translocation, it may not significantly deplete the membrane of PIP<sub>2</sub>. This result agrees with the dependence of bradykinin, but not muscarinic, modulation of the M current (*I*<sub>M</sub>) in these cells on [Ca<sup>2+</sup>]<sub>i</sub> and calmodulin signals (Cruzblanca et al., 1998; Delmas and Brown, 2002; Gamper and Shapiro, 2003). Evidently, M<sub>1</sub>R-induced, but not bradykinin-induced, depletion of PIP<sub>2</sub> is strong enough to suppress both M-channels and Ca<sup>2+</sup> channels. However, the [Ca<sup>2+</sup>]<sub>i</sub> increases induced by bradykinin (but not muscarinic) stimulation (Wanke et al., 1987; Beech et al., 1991; Cruzblanca et al., 1998; Delmas and Brown, 2002) cause M-channel inhibition, but because N-type Ca<sup>2+</sup> channels are not highly sensitive to [Ca<sup>2+</sup>]<sub>i</sub> in the range reachable by release from internal stores (Jones and Marks, 1989), bradykinin does not modulate those channels.

**Blockade of PI4-kinase, IP<sub>3</sub> receptors, or NCS-1 activity bestows modulation of *I*<sub>Ca</sub> to bradykinin**

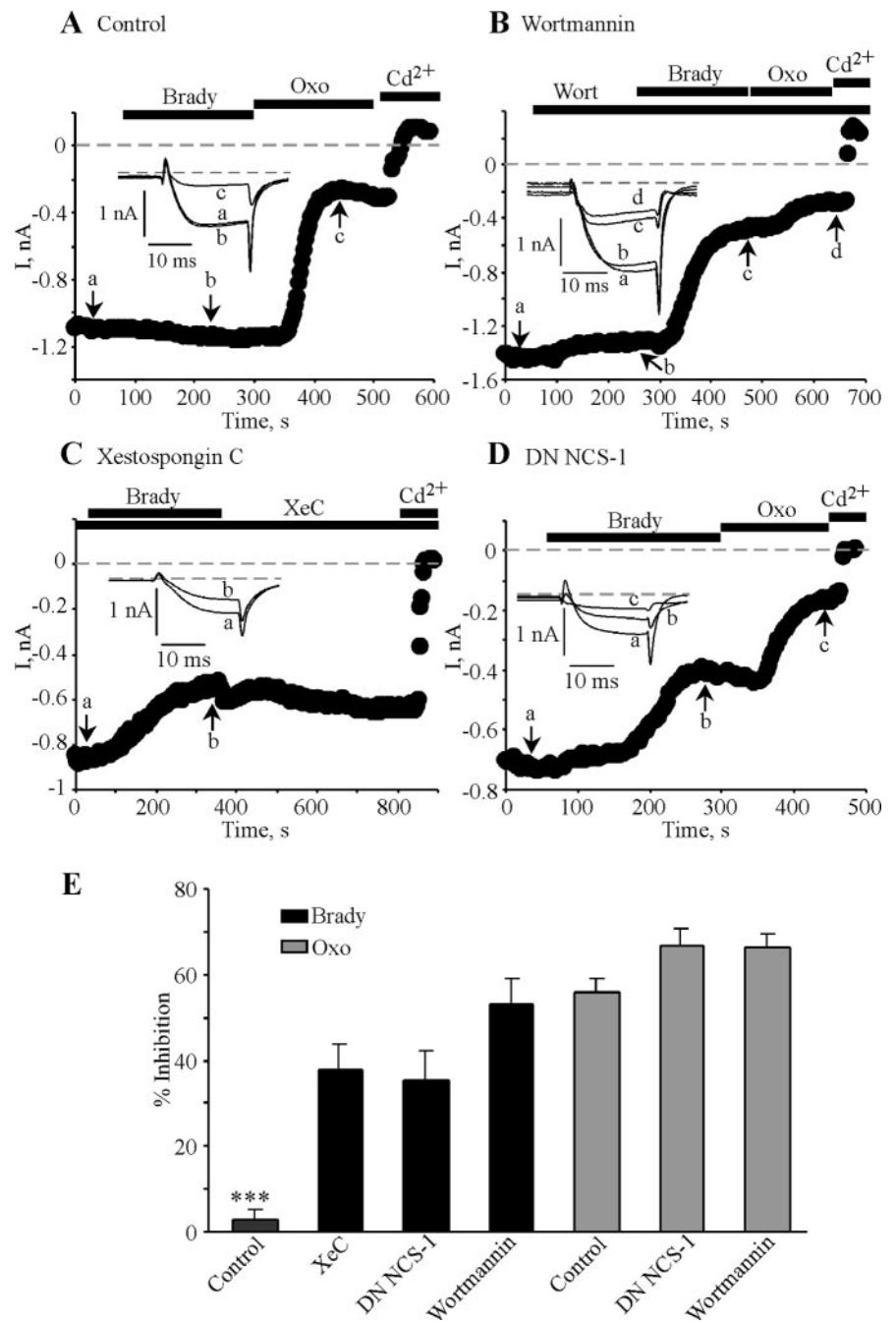
One possible mechanism of maintaining high membrane PIP<sub>2</sub> levels during stimulation of PLC by bradykinin stimulation would be a simultaneous activation of PIP<sub>2</sub> resynthesis. Indeed, Ca<sup>2+</sup>-dependent activation of PI4-kinase by bradykinin stimulation has been suggested as the stimulus for concurrent synthesis of enough PIP<sub>2</sub> to account for the amount of IP<sub>3</sub> produced (Xu et al., 2003). If so, then it should be possible to bestow bradykinin



modulation of N-type Ca<sup>2+</sup> channels in neurons by acute PI4-kinase blockade. To test this scenario, we pretreated neurons with 50 μM wortmannin for 3–5 min and then applied bradykinin (200 nM) in the presence of wortmannin (Fig. 7B). This brief pretreatment by wortmannin should be sufficient to block PI4-kinase but would not be long enough to cause substantial PIP<sub>2</sub> depletion by itself, as evidenced by the only minor rundown of *I*<sub>Ca</sub> during wortmannin application. This maneuver indeed conferred to bradykinin receptors the ability to suppress *I*<sub>Ca</sub> with the same potency as for muscarinic M<sub>1</sub> receptors. Bradykinin suppression of *I*<sub>Ca</sub> in the wortmannin-pretreated cells was 53.1 ± 6.1% (*n* = 9), indistinguishable from the value of muscarinic suppression in control conditions and 16 times greater than the meager bradykinin effect in control conditions. Thus, unlike stimulation of M<sub>1</sub> receptors, stimulation of bradykinin B<sub>2</sub> receptors may not deplete membrane [PIP<sub>2</sub>]<sub>i</sub> as a result of compensatory PIP<sub>2</sub> synthesis; blockade of PI4-kinase bestows modulation of *I*<sub>Ca</sub> to bradykinin receptors by preventing concurrent PIP<sub>2</sub> production.

Is this difference between the ability of M<sub>1</sub> and B<sub>2</sub> receptors to inhibit *I*<sub>Ca</sub> attributable to their differential ability to induce [Ca<sup>2+</sup>]<sub>i</sub> signals? To test this, we assayed bradykinin modulation of Ca<sup>2+</sup> channels in SCG cells in which IP<sub>3</sub> receptors were blocked. If bradykinin-induced [Ca<sup>2+</sup>]<sub>i</sub> signals trigger compensatory PIP<sub>2</sub> synthesis, then preventing those signals should also bestow inhibition of *I*<sub>Ca</sub> to bradykinin. Figure 7C shows a perforated-patch experiment on a PTX-treated SCG neuron pretreated with xestospongine C (5 μM; 10 min), a membrane-permeant blocker of IP<sub>3</sub> receptors (Gafni et al., 1997). This cell responded to bradykinin stimulation with a robust inhibition of *I*<sub>Ca</sub>. In such experiments, bradykinin inhibition of *I*<sub>Ca</sub> was 37.8 ± 6.0% (*n* = 5), nearly as large as that in wortmannin-treated cells. Similar results were obtained using another membrane-permeant blocker of IP<sub>3</sub> receptors, 2-aminoethoxydiphenyl borate (Maruyama et al., 1997) (data not shown).

Much recent work points to potent G<sub>q/11</sub> regulation of PIP<sub>2</sub> synthesis via the Ca<sup>2+</sup>-binding protein NCS-1, with increases of [Ca<sup>2+</sup>]<sub>i</sub> acting as the stimulus (Hendricks et al., 1999; Zhao et al., 2001; Koizumi et al., 2002; Pan et al., 2002; Winks et al., 2002). Thus, we tested whether NCS-1 is the Ca<sup>2+</sup> sensor for PI4-kinase stimulation in SCG cells by asking whether blockade of NCS-1 activity would allow bradykinin modulation of the Ca<sup>2+</sup> channels. PTX-treated neurons were transfected with a DN NCS-1 construct (NCS-1-E120Q) (Weiss et al., 2000) via the gene gun, and perforated-patch re-



**Figure 7.** Bradykinin (Brady) does not inhibit *I*<sub>Ca</sub> unless PI4-kinase, IP<sub>3</sub>-mediated Ca<sup>2+</sup> release or NCS-1 activity is blocked. SCG cells were cultured overnight with PTX and studied in perforated-patch configuration. Voltage protocols are as in Figure 2, and application of bradykinin (200 nM), oxo (10 μM), and CdCl<sub>2</sub> (Cd<sup>2+</sup>; 100 μM) are indicated by bars. *A*, Control. *B*, Wortmannin (Wort; 50 μM; indicated by the bar) was applied 3 min before application of agonists and maintained throughout. *C*, Xestospongine C (XeC; 1 μM) was applied to neurons 10 min before commencing recording. *D*, Neurons were shot with cDNA coding for dominant-negative NCS-1, linked as a fusion protein with EGFP, 1 d before recording with the gene gun. *E*, Bars show pooled data for bradykinin and muscarinic modulation of *I*<sub>Ca</sub> recorded in control and under the experimental conditions indicated (*n* = 11, 9, 5, 8). \*\*\**p* ≤ 0.001.

cordings were made the following day. Figure 7D shows a representative experiment in which bradykinin potently inhibited *I*<sub>Ca</sub> in such a cell, and subsequent inhibition by oxo was only slightly greater. In such DN NCS-1-expressing cells, bradykinin suppression of *I*<sub>Ca</sub> was 35.4 ± 7.1% (*n* = 8). The data in this section are summarized in Figure 7E. In control cells, bradykinin action on *I*<sub>Ca</sub> was nearly absent in the same cells in which the muscarinic

action was strong. Tracing the downstream steps of putative Ca<sup>2+</sup>-dependent stimulation of PIP<sub>2</sub> synthesis, we could bestow modulation of *I*<sub>Ca</sub> by bradykinin by individual blockade of each of them. Thus, prevention of PI4-kinase activity, of IP<sub>3</sub>-mediated Ca<sup>2+</sup> release, or of NCS-1 function, gave to bradykinin the ability to modulate the Ca<sup>2+</sup> channels nearly as well as that by muscarinic agonists. We suggest that, unlike muscarinic M<sub>1</sub> receptors, bradykinin B<sub>2</sub> receptors in SCG neurons do not normally cause substantial decreases in [PIP<sub>2</sub>] in the plasma membrane caused by the concurrent PI4-kinase stimulation resulting from bradykinin-induced [Ca<sup>2+</sup>]<sub>i</sub> increases, and that their physiological actions are mediated instead via IP<sub>3</sub>- and [Ca<sup>2+</sup>]<sub>i</sub>-dependent pathways.

## Discussion

The molecular participants in G<sub>q/11</sub>-mediated signaling endow this system with a rich repertoire of mechanisms available to act on effector proteins. For some time, it has been appreciated that release of Ca<sup>2+</sup> from internal stores and activation of protein kinase C are common motifs. To this has been added direct actions of diacylglycerol and arachidonic acid (Bymaster et al., 1999; Hardie, 2003; Watanabe et al., 2003; Oliver et al., 2004). Another emerging theme involves a critical interaction between channels and the ubiquitous PIP<sub>2</sub> lipid that is the very substrate of the PLC enzyme activated by G<sub>q/11</sub>-coupled receptors. Thus, depletion of plasma-membrane PIP<sub>2</sub>, if strong enough, could itself act as the effector signal. Such conclusions can be complicated, however, because channels can behave differently in excised patches versus intact cells by the differences in how proteins may interact in heterologous systems versus how they do interact in real neurons and by precise receptor specificity in native cells that is often accomplished by clustering of the molecules into signaling microdomains (Delmas et al., 2004). Here, we ask whether Ca<sub>v</sub>2.2 Ca<sup>2+</sup> channels are also sensitive to PIP<sub>2</sub> concentration, and whether G<sub>q/11</sub>-mediated muscarinic suppression of these channels (Bernheim et al., 1991; Beech et al., 1992; Melliti et al., 2001) occurs via depletion of PIP<sub>2</sub>. Using both a heterologous system and primary neurons, we find that the channels are highly sensitive to PIP<sub>2</sub> and that its depletion in neurons does contribute to muscarinic suppression of *I*<sub>Ca</sub>. As for the case of modulation of the M current, however, not all G<sub>q/11</sub>-coupled receptors act in the same way; muscarinic stimulation depletes PIP<sub>2</sub> and suppresses *I*<sub>Ca</sub>, but bradykinin stimulation likely does not, probably because of concurrent Ca<sup>2+</sup>-mediated stimulation of PIP<sub>2</sub> synthesis.

In sympathetic neurons, bradykinin and muscarinic stimulation are equally effective at stimulating PLC (Bofill-Cardona et al., 2000; Delmas et al., 2002) (but see del Rio et al., 1999). However, the subcellular localization of bradykinin receptors (Delmas et al., 2002), which confers bradykinin-induced increases in [Ca<sup>2+</sup>]<sub>i</sub> as well as their rapid desensitization (Prado et al., 2002), likely confer distinctness from M<sub>1</sub> receptors in mechanism of action. In a recent quantitative model of PIP<sub>2</sub> metabolism and hydrolysis and of the distribution–translocation of the PLCδ-PH probe that many laboratories use, Xu et al. (2003) found that the initial response to stimulation of bradykinin receptors in N1E-115 neuroblastoma cells to be an increase in membrane [PIP<sub>2</sub>], attributed to strong stimulation of PI4-kinase, followed by a modest decline. SCG neurons also possess G<sub>q/11</sub>-coupled angiotensin AT<sub>1</sub> and purinergic P2Y receptors. Because the former also do not induce [Ca<sup>2+</sup>]<sub>i</sub> increases and suppress *I*<sub>Ca</sub> and *I*<sub>M</sub> in a manner similar to M<sub>1</sub> receptors (Shapiro et al., 1994a), and the latter do induce [Ca<sup>2+</sup>]<sub>i</sub> increases and suppress *I*<sub>M</sub> similar to bradykinin receptors (Bofill-Cardona et al., 2000), we predict

that angiotensin, but not purinergic, stimulation also depletes PIP<sub>2</sub> and inhibits *I*<sub>Ca</sub> in this manner.

Our results also highlight a caveat in the use of the PLCδ-PH probe as a reporter of [PIP<sub>2</sub>], which we used for two quite distinct purposes: as a PIP<sub>2</sub>-sequestering agent to test the effect of reduced available PIP<sub>2</sub> and as an optical probe of PIP<sub>2</sub> hydrolysis. The sequestering effects of PLCδ-PH that we found here are similar to those seen by others (Raucher et al., 2000; Lei et al., 2001; Varnai et al., 2002), with its degree in different systems probably dependent on the relative concentrations of the probe and of PIP<sub>2</sub> (Nash et al., 2001; Xu et al., 2003). Although it is clear that translocation of PLCδ-PH from membrane to cytosol can report depletion of membrane PIP<sub>2</sub> (Varnai and Balla, 1998; van der Wal et al., 2001; Runnels et al., 2002; Winks et al., 2003; Zhang et al., 2003), the >10-fold higher affinity of the probe for IP<sub>3</sub> over PIP<sub>2</sub> means that translocation can often reflect IP<sub>3</sub> production instead (Hirose et al., 1999), and there seems broad agreement that PLCδ-PH translocation is mostly an accurate reporter of PIP<sub>2</sub> hydrolysis (Nahorski et al., 2003). Quantitative modeling of PIP<sub>2</sub>–PLCδ-PH dynamics indicates that whereas clamping [IP<sub>3</sub>] with concurrent PIP<sub>2</sub> depletion predicts little translocation of PLCδ-PH, a large increase in cytosolic IP<sub>3</sub> while [PIP<sub>2</sub>] is concurrently clamped predicts nearly as large a translocation as when [PIP<sub>2</sub>] is allowed to fall proportionately (Xu et al., 2003). Furthermore, an examination of this model at longer time points than shown by Xu et al. (2003) predicts that repartitioning of the PLCδ probe to the membrane after termination of PLC activity is only <5% faster when [PIP<sub>2</sub>] is not allowed to fall, compared with when [PIP<sub>2</sub>] is allowed to deplete. This is in accord with our results here, for which PLCδ repartitioning was not faster after bradykinin, compared with muscarinic, stimulation. Probably, this process is dominated by the kinetics of IP<sub>3</sub> or PLCδ-bound IP<sub>3</sub> decay, rather than PIP<sub>2</sub> resynthesis. Changes in the abundance of both PIP<sub>2</sub> and IP<sub>3</sub> seem to be reported by the PLCδ probe in this study, in which both muscarinic and bradykinin stimulation induce similar translocations in PLCδ-PH-transfected neurons, but we suggest that only muscarinic stimulation results in PIP<sub>2</sub> depletion significant enough to suppress *I*<sub>Ca</sub>. We hypothesize that bradykinin, because of its induction of [Ca<sup>2+</sup>]<sub>i</sub> increases, stimulates PI4-kinase enough to prevent significant depletions in [PIP<sub>2</sub>]. For the case of M<sub>1</sub>R-induced translocations, we do not attempt to quantify the change in [PIP<sub>2</sub>] from those data but rather attempt to show the temporal correlation between translocation of the probe and M<sub>1</sub>R-induced suppression of *I*<sub>Ca</sub> as further evidence that depletion of PIP<sub>2</sub> molecules, which are buffered by PLCδ-PH in those cells, contributes to inhibition of the channels.

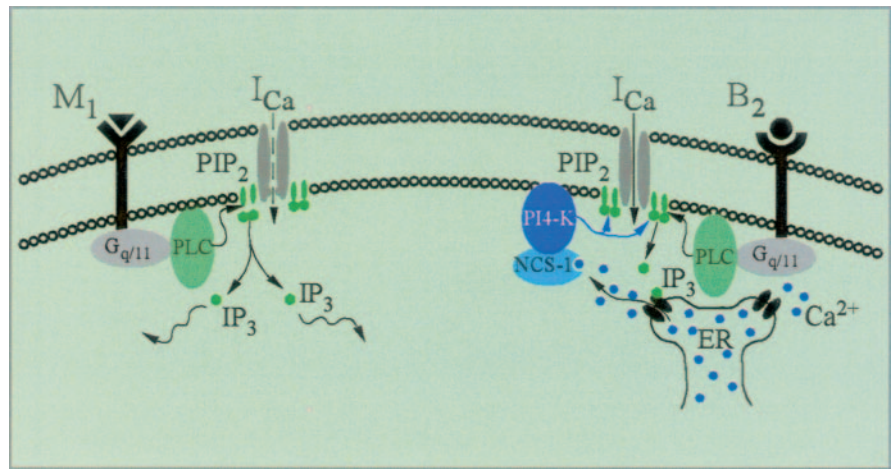
A novel line of investigation from the Rittenhouse laboratory (Liu and Rittenhouse, 2003; Liu et al., 2004) suggests that AA is the second messenger mediating the muscarinic modulation of *I*<sub>Ca</sub> that we study here. Using OPC, the same inhibitor of PLA<sub>2</sub> as was used in those studies, we did not observe a reduction in suppression of *I*<sub>Ca</sub> by a supramaximal concentration of oxo, as was reported. One possible origin for the differing results is that we studied OPC in perforated-patch configuration, whereas the previous studies used whole-cell recording. Additional investigation may reveal other reasons for this discrepancy. AA production may play a role in M<sub>1</sub>-mediated modulation by sensitizing the channels to reductions in membrane [PIP<sub>2</sub>] (i.e., reducing the affinity of the channel–PIP<sub>2</sub> interaction). Such a sensitization mechanism makes sense as a way to ensure fidelity in receptor-induced signals, so that modulation of the channels occurs only on agonist-provoked reduction in [PIP<sub>2</sub>] through PLC action. A

similar scheme may be the case for muscarinic modulation of M channels, in which activation of PKC recruited to the channels by A-kinase anchoring protein scaffolding proteins was suggested to sensitize the channels to concurrent depletion of PIP<sub>2</sub> (Hoshi et al., 2003). A sensitization mechanism seems also compatible with data from the Brown laboratory (Winks et al., 2003) in which an increase in tonic [PIP<sub>2</sub>] in SCG cells by overexpression of PI(4)5-kinase blunted muscarinic modulation but did not increase tonic *I<sub>M</sub>* amplitudes. If M channels (or other PIP<sub>2</sub>-sensitive channels) are to be exquisitely sensitive to PIP<sub>2</sub> depletions, then the affinity of the channels to PIP<sub>2</sub> must be near the range at which tonic [PIP<sub>2</sub>] lies for such sensitivity to be possible, and increasing tonic [PIP<sub>2</sub>] should increase tonic *I<sub>M</sub>* amplitudes (Suh et al., 2004). However, if M-channels or Ca<sup>2+</sup> channels are sensitized (reduced PIP<sub>2</sub> affinity) by concurrent PKC activation or AA production, then the resting affinity could be much higher than tonic [PIP<sub>2</sub>], with agonist-induced depletion of PIP<sub>2</sub> becoming a potent signal.

An exciting result was the dramatic difference in the ability of muscarinic and bradykinin stimulation to suppress *I<sub>Ca</sub>*. Given the similarity in the PLCδ-PH-monitored translocation between the two agonists, one might have supposed that both would be potent modulators of the N-type Ca<sup>2+</sup> channels. However, the basic distinction between these two receptors is that bradykinin stimulation mobilizes Ca<sup>2+</sup> from intracellular stores, but muscarinic stimulation does not, and this appears to be the critical determinant in their use of lipid signals. Given a set pool of plasma-membrane PIP<sub>2</sub>, muscarinic stimulation can significantly deplete PIP<sub>2</sub>, and a recent modeling paper quantifies this explicitly (Suh et al., 2004). However, if a G<sub>q/11</sub>-coupled receptor that raises [Ca<sup>2+</sup>]<sub>i</sub> concurrently stimulates PI kinases, then PIP<sub>2</sub> may be resynthesized nearly as fast as PLC activity would hydrolyze it, preventing significant PIP<sub>2</sub> depletion. This scenario is schematically depicted in Figure 8. Our data suggest that stimulation of PI4-kinase via NCS-1 is required for such compensatory PIP<sub>2</sub> synthesis; however, we do not imply that stimulation of PI(4)5-kinase as well is not also required. It will be interesting to discover what those additional signals may be. Like clustering of signaling proteins in microdomains (Delmas et al., 2004) and the use of scaffolding proteins (Bauman et al., 2004), hormonal regulation of lipid kinases may make it possible for directed signals that accomplish the agenda of the neuron, such that a specific input has its intended consequence for the control of neuronal function.

## References

- Bauman AL, Goehring AS, Scott JD (2004) Orchestration of synaptic plasticity through AKAP signaling complexes. *Neuropharmacology* 46:299–310.
- Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* 340:153–156.
- Beech DJ, Bernheim L, Mathie A, Hille B (1991) Intracellular Ca<sup>2+</sup> buffers



**Figure 8.** Schematic depiction of the signaling pathways using PIP<sub>2</sub> signals suggested in this study. The signals provoked by M<sub>1</sub> mACh (left) or bradykinin B<sub>2</sub> (right) receptors are shown. *I<sub>Ca</sub>* from N-type calcium channels is shown inhibited by muscarinic stimulation (left) but not normally inhibited by bradykinin (right). Both receptor types activate PLC via G<sub>q/11</sub>, resulting in hydrolysis of PIP<sub>2</sub>. In the hypothesis of Delmas et al. (2004), clustering of B<sub>2</sub> but not M<sub>1</sub> receptors in the plasma membrane together with IP<sub>3</sub> receptors in the endoplasmic reticulum (ER) causes the former, but not the latter, to release [Ca<sup>2+</sup>]<sub>i</sub>; downstream of IP<sub>3</sub> production. We suggest that the bradykinin-induced [Ca<sup>2+</sup>]<sub>i</sub> signals induce PIP<sub>2</sub> synthesis, concurrent with its hydrolysis, via stimulation of PI4-kinase (PI4-K) by NCS-1, which acts as the sensor for increases in [Ca<sup>2+</sup>]<sub>i</sub>. Thus, bradykinin does not normally inhibit the N-type Ca<sup>2+</sup> channel as a result of compensatory PIP<sub>2</sub> synthesis, but muscarinic agonists do so robustly from consumption of PIP<sub>2</sub>.

- disrupt muscarinic suppression of Ca<sup>2+</sup> current and M current in rat sympathetic neurons. *Proc Natl Acad Sci USA* 88:652–656.
- Beech DJ, Bernheim L, Hille B (1992) Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. *Neuron* 8:97–106.
- Bernheim L, Beech DJ, Hille B (1991) A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. *Neuron* 6:859–867.
- Bofill-Cardona E, Vartian N, Nanoff C, Freissmuth M, Boehm S (2000) Two different signaling mechanisms involved in the excitation of rat sympathetic neurons by uridine nucleotides. *Mol Pharmacol* 57:1165–1172.
- Brown DA, Abogadie FC, Allen TG, Buckley NJ, Caulfield MP, Delmas P, Haley JE, Lamas JA, Selyanko AA (1997) Muscarinic mechanisms in nerve cells. *Life Sci* 60:1137–1144.
- Bymaster FP, Calligaro DO, Falcone JF (1999) Arachidonic acid release in cell lines transfected with muscarinic receptors: a simple functional assay to determine response of agonists. *Cell Signal* 11:405–413.
- Catterall WA (1997) Modulation of sodium and calcium channels by protein phosphorylation and G proteins. *Adv Second Messenger Phosphoprotein Res* 31:159–181.
- Cruzblanca H, Koh DS, Hille B (1998) Bradykinin inhibits M current via phospholipase C and Ca<sup>2+</sup> release from IP<sub>3</sub>-sensitive Ca<sup>2+</sup> stores in rat sympathetic neurons. *Proc Natl Acad Sci USA* 95:7151–7156.
- Delmas P, Brown DA (2002) Junctional signaling microdomains: bridging the gap between the neuronal cell surface and Ca<sup>2+</sup> stores. *Neuron* 36:787–790.
- Delmas P, Brown DA, Dayrell M, Abogadie FC, Caulfield MP, Buckley NJ (1998) On the role of endogenous G-protein beta gamma subunits in N-type Ca<sup>2+</sup> current inhibition by neurotransmitters in rat sympathetic neurones. *J Physiol (Lond)* 506:319–329.
- Delmas P, Wanaverbecq N, Abogadie FC, Mistry M, Brown DA (2002) Signaling microdomains define the specificity of receptor-mediated IP<sub>3</sub> pathways in neurons. *Neuron* 34:209–220.
- Delmas P, Crest M, Brown DA (2004) Functional organization of PLC signaling microdomains in neurons. *Trends Neurosci* 27:41–47.
- del Rio E, Bevilacqua JA, Marsh SJ, Halley P, Caulfield MP (1999) Muscarinic M<sub>1</sub> receptors activate phosphoinositide turnover and Ca<sup>2+</sup> mobilization in rat sympathetic neurons, but this signaling pathway does not mediate M-current inhibition. *J Physiol (Lond)* 520:101–111.



- Franke TF, Kaplan DR, Cantley LC, Toker A (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 275:665–668.
- Fruman DA, Meyers RE, Cantley LC (1998) Phosphoinositide kinases. *Annu Rev Biochem* 67:481–507.
- Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, Pessah IN (1997) Xestospingins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. *Neuron* 19:723–733.
- Gamper N, Shapiro MS (2003) Calmodulin mediates Ca<sup>2+</sup>-dependent modulation of M-type K<sup>+</sup> channels. *J Gen Physiol* 122:17–31.
- Hardie RC (2003) TRP channels in *Drosophila* photoreceptors: the lipid connection. *Cell Calcium* 33:385–393.
- Hendricks KB, Wang BQ, Schnieders EA, Thorner J (1999) Yeast homologue of neuronal frequenin is a regulator of phosphatidylinositol-4-OH kinase. *Nat Cell Biol* 1:234–241.
- Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca<sup>2+</sup> channels by G-protein beta gamma subunits. *Nature* 380:258–262.
- Hilgemann DW, Feng S, Nasuhoglu C (2001) The complex and intriguing lives of PIP<sub>2</sub> with ion channels and transporters. *Sci STKE* 2001:RE19.
- Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci* 17:531–536.
- Hirose K, Kadowaki S, Tanabe M, Takeshima H, Iino M (1999) Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca<sup>2+</sup> mobilization patterns. *Science* 284:1527–1530.
- Hoshi N, Zhang JS, Omaki M, Takeuchi T, Yokoyama S, Wanaverbecq N, Langeberg LK, Yoneda Y, Scott JD, Brown DA, Higashida H (2003) AKAP150 signaling complex promotes suppression of the M-current by muscarinic agonists. *Nat Neurosci* 6:564–571.
- Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. *Nature* 380:255–258.
- Ikeda SR, Dunlap K (1999) Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. *Adv Second Messenger Phosphoprotein Res* 33:131–151.
- Ikeda SR, Schofield GG (1989) Somatostatin blocks a calcium current in rat sympathetic ganglion neurons. *J Physiol (Lond)* 409:221–240.
- Jones S, Brown DA, Milligan G, Willer E, Buckley NJ, Caulfield MP (1995) Bradykinin excites rat sympathetic neurons by inhibition of M current through a mechanism involving B<sub>2</sub> receptors and G<sub>αq/11</sub>. *Neuron* 14:399–405.
- Jones SW, Marks TN (1989) Calcium currents in bullfrog sympathetic neurons. II. Inactivation. *J Gen Physiol* 94:169–182.
- Koizumi S, Rosa P, Willars GB, Challiss RA, Taverna E, Francolini M, Bootman MD, Lipp P, Inoue K, Roder J, Jeromin A (2002) Mechanisms underlying the neuronal calcium sensor-1-evoked enhancement of exocytosis in PC12 cells. *J Biol Chem* 277:30315–30324.
- Lei Q, Talley EM, Bayliss DA (2001) Receptor-mediated inhibition of G protein-coupled inwardly rectifying potassium channels involves G<sub>αq</sub> family subunits, phospholipase C, and a readily diffusible messenger. *J Biol Chem* 276:16720–16730.
- Liu L, Rittenhouse AR (2003) Arachidonic acid mediates muscarinic inhibition and enhancement of N-type Ca<sup>2+</sup> current in sympathetic neurons. *Proc Natl Acad Sci USA* 100:295–300.
- Liu L, Roberts ML, Rittenhouse AR (2004) Phospholipid metabolism is required for M<sub>1</sub> muscarinic inhibition of N-type calcium current in sympathetic neurons. *Eur Biophys J* 33:255–264.
- Lu T, Nguyen B, Zhang X, Yang J (1999) Architecture of a K<sup>+</sup> channel inner pore revealed by stoichiometric covalent modification. *Neuron* 22:571–580.
- Malin SA, Nerbonne JM (2000) Elimination of the fast transient in superior cervical ganglion neurons with expression of KV4.2W362F: molecular dissection of IA. *J Neurosci* 20:5191–5199.
- Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K (1997) 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P<sub>3</sub>-induced Ca<sup>2+</sup> release. *J Biochem (Tokyo)* 122:498–505.
- Melliti K, Meza U, Adams BA (2001) RGS2 blocks slow muscarinic inhibition of N-type Ca<sup>2+</sup> channels reconstituted in a human cell line. *J Physiol (Lond)* 532:337–347.
- Mirshahi T, Jin T, Logothetis DE (2003) G beta gamma and KACH: old story, new insights. *Sci STKE* 2003:PE32.
- Nahorski SR, Young KW, John Challiss RA, Nash MS (2003) Visualizing phosphoinositide signalling in single neurons gets a green light. *Trends Neurosci* 26:444–452.
- Nash MS, Young KW, Willars GB, Challiss RA, Nahorski SR (2001) Single-cell imaging of graded Ins(1,4,5)P<sub>3</sub> production following G-protein-coupled-receptor activation. *Biochem J* 356:137–142.
- Oliver D, Lien CC, Soom M, Baukowitz T, Jonas P, Fakler B (2004) Functional conversion between A-type and delayed rectifier K<sup>+</sup> channels by membrane lipids. *Science* 304:265–270.
- Pan CY, Jeromin A, Lundstrom K, Yoo SH, Roder J, Fox AP (2002) Alterations in exocytosis induced by neuronal Ca<sup>2+</sup> sensor-1 in bovine chromaffin cells. *J Neurosci* 22:2427–2433.
- Plummer MR, Logothetis DE, Hess P (1989) Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. *Neuron* 2:1453–1463.
- Prado GN, Taylor L, Zhou X, Ricupero D, Mierke DF, Polgar P (2002) Mechanisms regulating the expression, self-maintenance, and signaling-function of the bradykinin B<sub>2</sub> and B<sub>1</sub> receptors. *J Cell Physiol* 193:275–286.
- Rae J, Cooper K, Gates P, Watsky M (1991) Low access resistance perforated patch recordings using amphotericin B. *J Neurosci Methods* 37:15–26.
- Raucher D, Stauffer T, Chen W, Shen K, Guo S, York JD, Sheetz MP, Meyer T (2000) Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell* 100:221–228.
- Runnels LW, Yue L, Clapham DE (2002) The TRPM7 channel is inactivated by PIP<sub>2</sub> hydrolysis. *Nat Cell Biol* 4:329–336.
- Shapiro MS, Hille B (1993) Substance P and somatostatin inhibit calcium channels in rat sympathetic neurons via different G protein pathways. *Neuron* 10:11–20.
- Shapiro MS, Wollmuth LP, Hille B (1994a) Angiotensin II inhibits calcium and M current channels in rat sympathetic neurons via G proteins. *Neuron* 12:1319–1329.
- Shapiro MS, Wollmuth LP, Hille B (1994b) Modulation of Ca<sup>2+</sup> channels by PTX-sensitive G-proteins is blocked by N-ethylmaleimide in rat sympathetic neurons. *J Neurosci* 14:7109–7116.
- Shapiro MS, Gomeza J, Hamilton SE, Hille B, Loose MD, Nathanson NM, Roche JP, Wess J (2001) Identification of subtypes of muscarinic receptors that regulate Ca<sup>2+</sup> and K<sup>+</sup> channel activity in sympathetic neurons. *Life Sci* 68:2481–2487.
- Shaw G (1996) The pleckstrin homology domain: an intriguing multifunctional protein module. *BioEssays* 18:35–46.
- Stauffer TP, Ahn S, Meyer T (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P<sub>2</sub> concentration monitored in living cells. *Curr Biol* 8:343–346.
- Stolz LE, Kuo WJ, Longchamps J, Sekhon MK, York JD (1998) INP51, a yeast inositol polyphosphate 5-phosphatase required for phosphatidylinositol 4,5-bisphosphate homeostasis and whose absence confers a cold-resistant phenotype. *J Biol Chem* 273:11852–11861.
- Suh B, Hille B (2002) Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron* 35:507–520.
- Suh BC, Horowitz LF, Hirdes W, Mackie K, Hille B (2004) Regulation of KCNQ2/KCNQ3 current by G protein cycling: the kinetics of receptor-mediated signaling by G<sub>q</sub>. *J Gen Physiol* 123:663–683.
- van der Wal J, Habets R, Varnai P, Balla T, Jalink K (2001) Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. *J Biol Chem* 276:15337–15344.
- Varnai P, Balla T (1998) Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[<sup>3</sup>H]inositol-labeled phosphoinositide pools. *J Cell Biol* 143:501–510.
- Varnai P, Lin X, Lee SB, Tuymetova G, Bondeva T, Spat A, Rhee SG, Hajnoczky G, Balla T (2002) Inositol lipid binding and membrane localization of isolated pleckstrin homology (PH) domains. Studies on the PH domains of phospholipase C delta 1 and p130. *J Biol Chem* 277:27412–27422.
- Wanke E, Ferroni A, Malgaroli A, Ambrosini A, Pozzan T, Meldolesi J (1987) Activation of a muscarinic receptor selectively inhibits a rapidly inactivated Ca<sup>2+</sup> current in rat sympathetic neurons. *Proc Natl Acad Sci USA* 84:4313–4317.

- Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B (2003) Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature* 424:434–438.
- Weiss JL, Archer DA, Burgoyne RD (2000) Neuronal Ca<sup>2+</sup> sensor-1/frequenin functions in an autocrine pathway regulating Ca<sup>2+</sup> channels in bovine adrenal chromaffin cells. *J Biol Chem* 275:40082–40087.
- Winks JS, Abogadie FC, Delmas P, Weiss JL, Burgoyne RD, Brown DA (2002) Possible role for calcium sensor NCS-1 in M-current modulation. *Soc Neurosci Abstr* 28:438.433.
- Winks JS, Tatulian L, Filippov AK, Abogadie FC, Marsh SJ, Brown DA (2003) Effect of PI4P-5-kinase (PI5-K) over-expression on transmitter-induced inhibition of M- and GIRK currents. *Soc Neurosci Abstr* 29:368.318.
- Wu L, Bauer CS, Zhen XG, Xie C, Yang J (2002) Dual regulation of voltage-gated calcium channels by PtdIns(4,5)P<sub>2</sub>. *Nature* 419:947–952.
- Xu C, Watras J, Loew LM (2003) Kinetic analysis of receptor-activated phosphoinositide turnover. *J Cell Biol* 161:779–791.
- Zhang H, Craciun LC, Mirshahi T, Rohacs T, Lopes CM, Jin T, Logothetis DE (2003) PIP<sub>2</sub> Activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron* 37:963–975.
- Zhao X, Varnai P, Tuymetova G, Balla A, Toth ZE, Oker-Blom C, Roder J, Jeromin A, Balla T (2001) Interaction of neuronal calcium sensor-1 (NCS-1) with phosphatidylinositol 4-kinase beta stimulates lipid kinase activity and affects membrane trafficking in COS-7 cells. *J Biol Chem* 276:40183–40189.
- Zhou J, Shapiro MS, Hille B (1997) Speed of Ca<sup>2+</sup> channel modulation by neurotransmitters in rat sympathetic neurons. *J Neurophysiol* 77:2040–2048.