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Combined Phosphoinositide and Ca^{2+} Signals Mediating Receptor Specificity toward Neuronal Ca^{2+} Channels*

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Phosphatidylinositol 4,5-bisphosphate (PIP_2) regulates Ca^{2+} (I_{Ca}) and M-type K^+ currents in superior cervical ganglion sympathetic neurons. In those cells, M_1 muscarinic and AT_1 angiotensin types do not elicit $Ca^{2+}{}_i$ signals and suppress both currents via depletion of PIP_2, whereas the B_2 bradykinin and P2Y purinergic types elicit robust IP_3-mediated $[Ca^{2+}]_i$ rises and neither deplete PIP_2 nor inhibit I_{Ca}. We have suggested that this specificity arises from differential $Ca^{2+}{}_i$ signals underlying receptor-specific stimulation of PIP_2 synthesis by phosphatidylinositol (PI) 4-kinase. Here, we investigate which PI 4-kinase isoform underlies this signal, whether stimulation of PI 4-phosphate 5-kinase is also required, and the origin of receptor-specific $Ca^{2+}{}_i$ signals. Recordings of I_{Ca} were used as a PIP_2 "biosensor." In control, stimulation of M_1, but not B_2 or P2Y, receptors robustly suppressed I_{Ca}. However, when PI 4-kinase IIIβ, diacylglycerol kinase, Rho, or Rho-kinase was blocked, agonists of all three receptors robustly suppressed I_{Ca}. Overexpression of exogenous M_1 receptors yielded large $[Ca^{2+}]_i$ rises by muscarinic agonist, and transfection of wild-type IRBIT decreased $Ca^{2+}{}_i$ signals, whereas dominant negative IRBIT-S68A had little effect on B_2 or P2Y responses but greatly increased muscarinic responses. We conclude that overlaid on microdomain organization is IRBIT, setting a "threshold" for $[IP_3]$, assisting in fidelity of receptor specificity.

Phosphoinositides comprise a diverse array of plasma membrane signaling molecules with temporal effects ranging from 1 ms to the lifetime of the organism. Most intensively studied are the polyphosphorylated phosphatidylinositols (PIs).[2] The species phosphorylated at the 4′- and 5′-positions of the inositol ring by PI 4-kinase and PI 4-phosphate (PI(4)P) 5-kinase, called PI 4,5-bisphosphate (PIP_2), have been implicated as a critical player in myriad cellular activities. These include cytoskeletal remodeling, vesicular/protein trafficking among intracellular membranes, transcriptional control, and regulation of ion channels and transporters. Regarding the latter role, a large and widening array of such membrane transport proteins have been shown to be regulated by plasma membrane PIP_2, presumably due to direct binding (1, 2). The action is thought to arise from either PIP_2 depletion by stimulation of phospholipase C (PLC)-coupled receptors or from alterations in the affinity of PIP_2 for the channels caused by generation of other signaling molecules (3). Using sympathetic neurons of the superior cervical ganglion (SCG) as a model, we have focused on PIP_2-mediated regulation of voltage-gated "M-type" (KCNQ) K^+ and "N-type" Ca^{2+} currents. The former is so named for its depression by muscarinic stimulation, whose mechanism has been established as being due to depletion of PIP_2 (4–9). The latter is also PIP_2-sensitive (10–12) as well as sensitive to arachidonic acid (13), and stimulation of PLC-linked muscarinic receptors likewise depresses I_{Ca} in the same neurons (10, 14). However, stimulation of other PLC-linked receptors (bradykinin B_2 and purinergic P2Y) in those neurons does not deplete PIP_2 and displays a pattern of M-current depression via intracellular Ca^{2+} ($Ca^{2+}{}_i$) signals (15, 16), acting on KCNQ channels via calmodulin (CaM) (17, 18), and little action on the N-type I_{Ca} (10, 14) (although many Ca_V channels are modulated by CaM, the N-type channels are probably insensitive to $[Ca^{2+}]_i$ rises in the range ($<2\ \mu M$) reachable by release from stores).

This receptor specificity parallels stark receptor specificity in the induction of IP_3-mediated $[Ca^{2+}]_i$ signals. Although stimulation of PLC-coupled M_1 receptors in SCG neurons produces robust PIP_2 hydrolysis and the downstream products, IP_3 and diacylglycerol (DAG) (19), little IP_3-mediated $[Ca^{2+}]_i$ rises are detected, whereas stimulation of PLC-coupled bradykinin B_2 or purinergic P2Y receptors produce reliable $Ca^{2+}{}_i$ signals (14, 16, 20–23). What accounts for the pronounced receptor specificity in $[Ca^{2+}]_i$ signals? One hypothesis involves subcellular clustering of certain plasma membrane PLC-linked receptors into microdomains together with endoplasmic reticulum membrane IP_3 receptors. Thus, B_2, but not M_1, receptors have been shown to physically interact with IP_3 receptors, and the two proteins have been shown to strongly co-localize under confocal microscopy (22). Recently, however, several regulators of IP_3 receptors have been characterized that modify the efficacy of IP_3 to open its receptor (24); among those, IRBIT (IP_3 receptor-binding protein released with IP_3) (25, 26) has seemed a likely candidate to be involved in tuning the extent of receptor-induced $[Ca^{2+}]_i$ rises. In this work, we perform several tests of these mechanisms.

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[2] The abbreviations used are: PI, phosphatidylinositol; PI(4)P, PI 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; SCG, superior cervical ganglion; CaM, calmodulin; PM, plasma membrane; EGFP, enhanced green fluorescent protein; DN, dominant negative; BK, bradykinin; oxo-M, oxotremorine methiodide; KD, kinase-dead; DAG, diacylglycerol; PA, phosphatidic acid; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor.

The receptor specificity in $Ca^{2+}{}_i$ signaling parallels the receptor specificity in which receptors deplete PIP_2, whose origin is suggested to at least partly lie with $Ca^{2+}{}_i$-mediated stimulation of PIP_2 synthesis, via NCS-1 (neuronal Ca^{2+} sensor-1) action on PI 4-kinase (9, 10, 14). Indeed, dramatic hormonal stimulation of PIP_2 synthesis concurrent with PLC activation is well known in the literature (*e.g.* stimulation of PLC-linked muscarinic receptors accelerates PIP_2 synthesis many-fold in smooth muscle and platelets (27, 28), and careful measurements coupled with cellular modeling indicate that strong stimulation of PIP_2 synthesis in neuroblastoma cells and cerebellar spines in the brain is required to account for the mass of IP_3 produced (29–32)). However, is acceleration of PI 4-kinase sufficient to maintain PIP_2 levels in the face of PLC activation, or is concurrent acceleration of PI(4)P 5-kinase required as well? In addition, among four mammalian PI 4-kinase isoforms, only PI 4-kinase IIIβ has been shown to be stimulated by calcified NCS-1 (33), and this isoform has been localized within cells to the Golgi (34, 35), not to the plasma membrane (PM), where PIP_2 synthesis would seem to be relevant for regulation of ion channel activity. Thus, we here also test the involvement of phosphatidic acid and the Rho monomeric GTPase, two types of signaling molecules reported to stimulate PI(4)P 5-kinase activity, either downstream of receptors or in a receptor-independent fashion (36, 37). We also test whether PI 4-kinase IIIβ is a critical player in stimulation of PIP_2 synthesis by B_2 and P2Y receptors in sympathetic neurons. Our work highlights the central role of intracellular Ca^{2+} signals in conferring receptor specificity toward ion channel targets.

EXPERIMENTAL PROCEDURES

cDNA Constructs, Antibodies, and Drugs—The plasmids for wild-type and dominant negative (S68A) IRBIT, GST-tagged IRBIT(1–104), and the anti-IRBIT antibody were kindly given to us by the laboratory of Humbert De Smedt (Laboratory of Molecular and Cellular Signaling, University of Leuven, Belgium). The cDNAs for wild-type and D656A bovine PI 4-kinase IIIβ and PIK93 were kindly given to us by Tamas Balla (National Institutes of Health, Bethesda, MD).

SCG Sympathetic Neuron Culture and cDNA Transfections—Sympathetic neurons were isolated from the superior cervical ganglia of 7–14-day-old rats of both genders (Sprague-Dawley) and cultured for 2–4 days. Rats were given a lethal overdose of halothane and decapitated. Neurons were dissociated using methods of Bernheim *et al.* (38), plated on 4 × 4-mm glass coverslips (coated with poly-L-lysine) and incubated at 37 °C (5% CO_2). Fresh culture medium containing nerve growth factor (50 ng/ml) and pertussis toxin (100 ng/ml) were added to the cells 3 h after plating. For exogenous expression of cDNA constructs, we used the PDS-1000/He biolistic particle delivery system ("gene gun," Bio-Rad), as described previously (39). Transfection efficiency was assumed to be determined by the random distribution of fired gold particles and was up to 10% of cultured neurons.

Immunostaining—Cells grown on poly-L-lysine-coated coverslips were fixed in 4% paraformaldehyde, washed twice with 100 mM sodium phosphate (PB, pH 7.4), three times with PB + 150 mM NaCl (PBS), and blocked with 5% goat serum and 0.1% saponin in PBS (PBS + GS). The cells were incubated for 3 h at room temperature with primary affinity-purified rabbit anti-IRBIT (40) (diluted 1:500 in PBS + GS) and mouse anti-tyrosine hydroxylase (1:5000) antibodies. In the blocking controls, the anti-IRBIT antibody was pre-adsorbed with a 10-fold molar excess of affinity-purified recombinant GST-tagged IRBIT(1–104) peptide used to raise the antibody. Cells were washed six times with PBS and then incubated with goat rhodamine red-conjugated anti-rabbit and FITC-conjugated anti-mouse secondary antibodies (1:500, Jackson Immunoresearch) in PBS + GS for 1 h. Cells were then washed three times with PBS, twice with PB, and three times with water. Air-dried slides were mounted on a drop of Vectashield (Vector Laboratory) and sealed with nail polish. Stained cells were viewed with a Nikon TE2000 inverted microscope, using excitation/emission filters appropriate for rhodamine red and FITC, and images were taken with a Cascade 512F enhanced-CCD camera controlled by Metamorph software running on a PC. The purification of the GST-tagged IRBIT(1–104) peptide was performed by us as previously described by others (40), except that we did not cleave the GST moiety from the peptide.

Perforated Patch Voltage Clamp Electrophysiology—Pipettes were pulled from borosilicate glass capillaries (1B150F-4, World Precision Instruments, Sarasota, FL) using a Flaming/Brown micropipette puller P-97 (Sutter Instruments) and had resistances of 1–2 megaohms when filled with internal solution and measured in standard bath solution. Membrane current was measured with pipette and membrane capacitance cancellation, sampled at 200 μs, and filtered at 1 kHz by an EPC-9 amplifier and PULSE software (HEKA/Instrutech, Port Washington, NY). In all electrophysiological experiments, the perforated patch method of recording was used with amphotericin B (600 ng/ml) in the pipette (41). Amphotericin was prepared as a stock solution as 60 mg/ml in DMSO. In these experiments, the access resistance was <10 megaohms 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 (Warner Scientific). Bath solution exchange was essentially complete by <30 s. Experiments were performed at room temperature. To evaluate the amplitude of I_{Ca}, cells were held at −80 mV, and 20-ms depolarizing steps to 5 mV were applied every 5 s. The amplitude of I_{Ca} was usually defined as the inward current sensitive to Cd^{2+} (100 μM). UTP, bradykinin (BK), oxotremorine methiodide (oxo-M), and Cd^{2+} were used at concentrations of 10 μM, 250 nM, 10 μM, and 100 μM, respectively. The external solution used to record Ca^{2+} currents contained 150 mM NaCl, 2.5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 500 nM tetrodotoxin, pH 7.4, with NaOH. The perforated patch pipette solution for voltage clamp experiments contained 150 mM CsCl, 5 mM $MgCl_2$, and 10 mM HEPES. Data are presented as mean ± S.E. Statistical tests were performed using a paired *t* test or an unpaired *t* test where appropriate.

Imaging—Fluorescent microscopy was performed with an inverted Nikon Eclipse TE300 microscope with an oil immersion ×40/1.30 numerical aperture objective. A Polychrome IV monochromator (T.I.L.L. Photonics, Martinsreid, Germany) was used as the excitation light source, and a FURA2 71000 filter cube (Chroma) was used for fura-2 imaging. SCG neurons were bath-loaded with fura-2/AM (2 μM) for 30 min at 37 °C in the presence of pluronic acid (0.01%). Cells were excited alternatively at 340 and 380 nm (50–200 ms every 2 s), the fluorescence emission was collected by an IMAGO 12-bit cooled CCD camera, and images were stored/analyzed with TILLvisION 4.0 software. These fura-2 signals were not calibrated due to inherent difficulties in calibrating esterified indicator dyes (54). Because the EGFP used in our SCG transfection experiments is very poorly excited in the UV (at 340 or 380 nm), it negligibly contaminates the fura-2 emission signals. In control experiments, the combined contamination from EGFP emission and from autofluorescence of the cell was measured to alter the 340/380 fura-2 emission ratio by <0.5%.

Reagents—Reagents used were as follows: oxotremorine methiodide and UTP (Sigma); DMEM, fetal bovine serum, and penicillin/streptomycin (Invitrogen); and amphotericin B, bradykinin, and Y27632 (Calbiochem).

RESULTS

To assay the extent of PIP_2 depletion upon receptor stimulation in living cells, a convenient paradigm is to use the activity of an ion channel known to be PIP_2-sensitive as a read-out of PIP_2 levels in the membrane. Thus, we turned to our measurements of the inhibition in SCG neurons of N-type voltage-gated Ca^{2+} channels, which have been shown to be very sensitive to PIP_2 abundance. SCG neurons were studied under perforated patch whole-cell voltage clamp and were pretreated with the $G_{o/i}$ blocker, pertussis toxin, to isolate $G_{q/11}$-mediated actions (42). Our previous work reported suppression of I_{Ca} by muscarinic M_1 receptors, but not bradykinin B_2 or purinergic P2Y receptors, via depletion of PIP_2 molecules in the membrane, which are necessary for Ca^{2+} channel activity (10, 11, 14). Thus, the Ca^{2+} channels act as a PIP_2 biosensor, and depression of I_{Ca} reports depletion of PIP_2.

Compensatory, Receptor-specific Stimulation of PIP_2 Synthesis Uses PI 4-Kinase IIIβ—Our previous work has demonstrated receptor-specific stimulation of PI 4-kinases via $Ca^{2+}{}_i$ signals and NCS-1 to be involved in compensatory PIP_2 synthesis concurrent with PLC hydrolysis. Thus, acute treatment of cells by the broad-spectrum PI 3- and PI 4-kinase blocker, wortmannin, or transfection into the neurons of a dominant negative (DN) NCS-1 mutant that cannot bind Ca^{2+} bestowed upon P2Y and B_2 receptors the ability to depress I_{Ca} in SCG neurons (10, 14). However, precisely which molecule might NCS-1 be targeting? Mammalian cells express at least four distinct isoforms of PI 4-kinases with varying functions and pharmacological profiles (43). Although types IIα and IIβ are found at the PM, neither is wortmannin-sensitive (44, 45), ruling out these isoforms in this process. PI 4-kinase IIIα has been localized to the PM of vertebrate cells, where it would be well placed to underlie rapid synthesis of PI(4)P, whereas type IIIβ has been localized primarily to the Golgi (35, 46). Thus, the literature presents us with something of a conundrum because the type IIIβ isoform implicated in regulation by Ca^{2+}/NCS-1 (33) would appear to not be in the correct subcellular location.

To test whether IIIβ is actually the relevant isoform in this system, we used two approaches. The first exploited the development of a new isoform-specific PI 4-kinase blocker, PIK93, with high potency against the type IIIβ but little effect on the type IIIα isoforms (35). We compared the suppression of I_{Ca} in control neurons with those pretreated with PIK93. In Fig. 1*A* are plotted the amplitudes of inward I_{Ca}, elicited by brief depolarizations to 5 mV in a control neuron, with representative current traces during different times in the experiment shown in the *insets*. We tested the responses of I_{Ca} to bath application of supramaximal concentrations of UTP, BK, and oxo-M, agonists of $G_{q/11}$-coupled purinergic P2Y, bradykinin B_2, and muscarinic M_1 receptors, respectively. As seen in our previous work, neither UTP nor BK caused significant suppressions of I_{Ca}, although oxo-M induced a strong suppression of the current in the same cell. However, when PI 4-kinase IIIβ was blocked by pretreatment of a neuron with PIK93 (0.3 μM, 7-min pretreatment), all three agonists caused significant suppressions of I_{Ca} (Fig. 1*B*), suggesting that PI 4-kinase IIIβ activity is required to maintain PIP_2 levels during stimulation of B_2 or P2Y receptors.

Our second approach involved transfection into the neurons of the PI 4-kinase IIIβ kinase-dead (KD) D656A mutant that acts as a DN (47). We again used I_{Ca} amplitudes as a PIP_2 biosensor and compared the responses to UTP, BK, and oxo-M in neurons transfected with WT PI 4-kinase IIIβ or the D656A mutant. In a SCG neuron transfected with the WT kinase, the responses to the three agonists were similar to those seen in control neurons, with only oxo-M, but not BK or UTP, inducing a robust depression of I_{Ca} (Fig. 1*C*). However, in the cell transfected with the KD mutant, both UTP and BK now induced a significant depression of I_{Ca} (Fig. 1*D*). These data are summarized in Fig. 1*E*. For control neurons, the suppressions of I_{Ca} by UTP, BK, and oxo-M were 3.3 ± 0.5, 5.5 ± 0.8, and 57 ± 3% ($n = 12$), respectively. For PIK93-treated neurons, UTP, BK, and oxo-M suppressed I_{Ca} by 23 ± 6% ($p < 0.01$), 37 ± 7% ($p < 0.01$), and 59 ± 6% ($n = 7$), respectively. For neurons transfected with WT PI 4-kinase IIIβ, the suppressions of I_{Ca} by UTP, BK, and oxo-M were 5.0 ± 1.0, 3.4 ± 1.3, and 60 ± 5% ($n = 5$), respectively. For neurons transfected with the KD D656A mutant, the suppressions of I_{Ca} were 31 ± 3 ($p < 0.001$), 38 ± 4 ($p < 0.001$), and 56 ± 5% ($n = 7$), respectively. Taken together, these data suggest that heightened PI 4-kinase IIIβ isoform activity is required to prevent PIP_2 levels from falling upon stimulation of P2Y and B_2 receptors in SCG neurons. We also quantified tonic I_{Ca} amplitudes in these two groups of cells to see what effect expression of WT or KD PI 4-kinase IIIβ might have on tonic PIP_2 levels. Indeed, those values were 60 ± 5 and 37 ± 6 pA/picofarad ($p < 0.05$), respectively (Fig. 2*G*), indicating that not only is PI 4-kinase IIIβ necessary for receptor stimulation of PIP_2 synthesis, but it is also needed for homeostatic mainte-

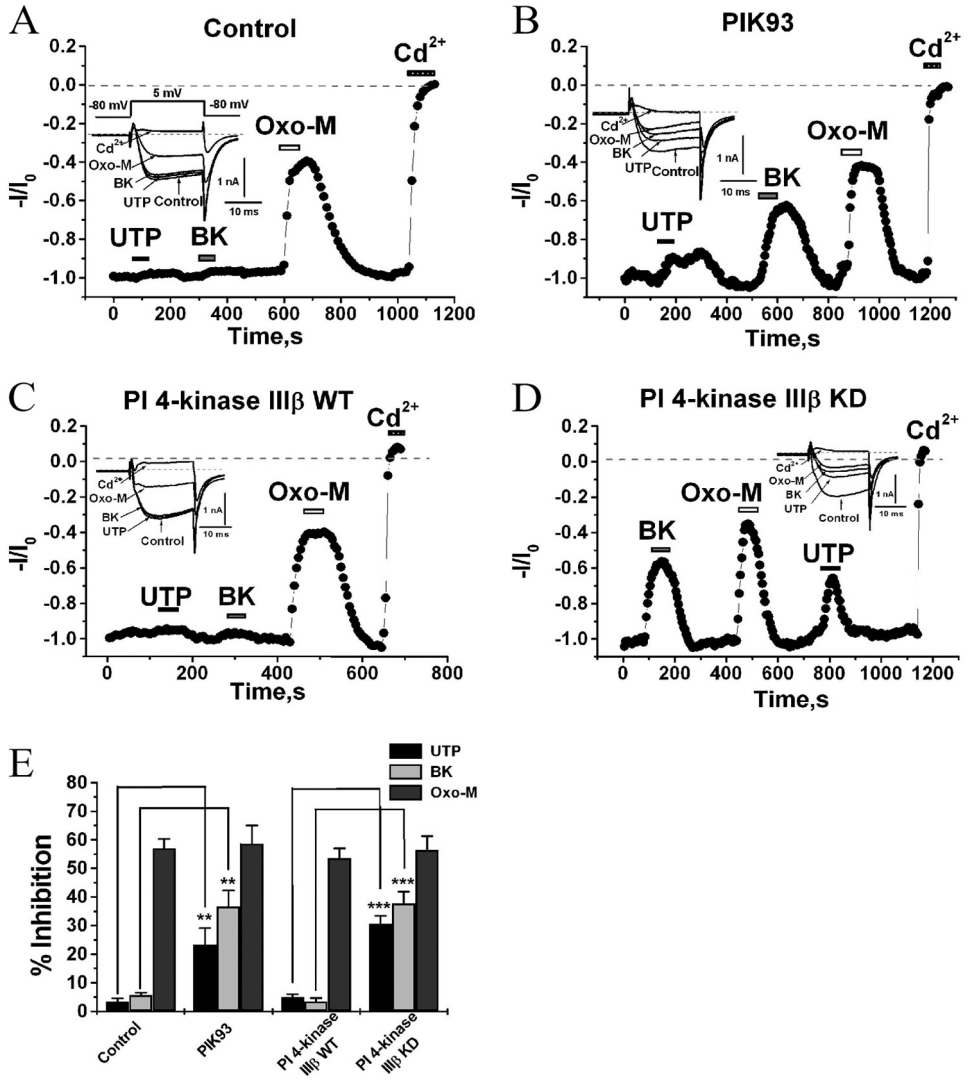

FIGURE 1. **Blockade of PI 4-kinase IIIβ allows PIP_2 depletion by stimulation of P2Y or B_2 receptors.** Shown are perforated patch voltage clamp experiments on cultured SCG neurons. *A–D*, plotted are normalized amplitudes of inward Ca^{2+} currents elicited by brief depolarizations to 5 mV, during the experiments in which UTP (10 μM), BK (250 nM), oxo-M (10 μM), and Cd^{2+} (100 μM) were bath-applied during the times indicated by the *bars*. In the *insets* are shown representative currents at the indicated times during the experiments and the voltage protocol used. Cells were treated with PIK93 (300 nM; *B*) or transfected with wild-type (*C*) or KD (*D*) PI 4-kinase IIIβ. *E*, *bars* are summarized suppressions of I_{Ca} by each agonist for the four groups of experiments. **, $p < 0.01$; ***, $p < 0.001$. *Error bars*, S.E.

nance of PIP_2 abundance (although overexpression of the WT kinase did not augment tonic I_{Ca} amplitudes).

RhoA, Rho-kinase, and DAG-kinase Are Required for PIP_2 Synthesis—Given our hypothesis of receptor stimulation of PI(4)P 5-kinase, we considered two putative signaling mechanisms found in the literature. A number of laboratories have shown PI(4)P 5-kinases to be regulated by Rho family GTPases, either directly or via its effector, Rho-kinase (48–51). Thus, we performed two tests of the involvement of these signaling molecules in PIP_2 synthesis. To test the role of RhoA action in this process, we evaluated the effect of transfection into the neurons of the RhoA T19N mutant that acts as a DN (50). SCG neurons were transfected with EGFP either alone or together with DN RhoA, using the biolistic method. Again, we used I_{Ca} to assay the extent of PIP_2 depletion by receptor agonists in pertussis toxin-treated SCG neurons. In the cell transfected with only EGFP, neither UTP nor BK induced a significant depression of I_{Ca}, but oxo-M caused a robust effect (Fig. 2*A*). However, in the neuron transfected with DN RhoA (Fig. 2*B*), all three agonists caused a significant depression of I_{Ca}, although to a somewhat lesser degree than in control cells. Such data are summarized in Fig. 2*F*. In cells transfected with only EGFP, the suppressions of I_{Ca} by UTP, BK, and oxo-M were 3.4 ± 0.9, 4.2 ± 1.1, and 55 ± 4% ($n = 8$), respectively. However, in the cells transfected with RhoA T19N, they were 16 ± 3% ($p < 0.01$), 25 ± 3% ($p < 0.01$), and 52 ± 3% ($n = 6$), respectively. In the experiment shown in Fig. 2*B*, the current amplitudes appear smaller than usual; indeed, that was the case in neurons transfected with DN RhoA. In cells transfected with EGFP either alone or together with RhoA T19N, the current densities were 50 ± 4 pA/picofarad ($n = 8$) and 25 ± 4 pA/picofarad ($n = 6$, $p < 0.051$), respectively (Fig. 2*G*). Thus, blockade of endogenous RhoA activity prevents compensatory PIP_2 synthesis during B_2 or P2Y receptor stimulation. We also conclude that RhoA activity is required to maintain normal PIP_2 levels because

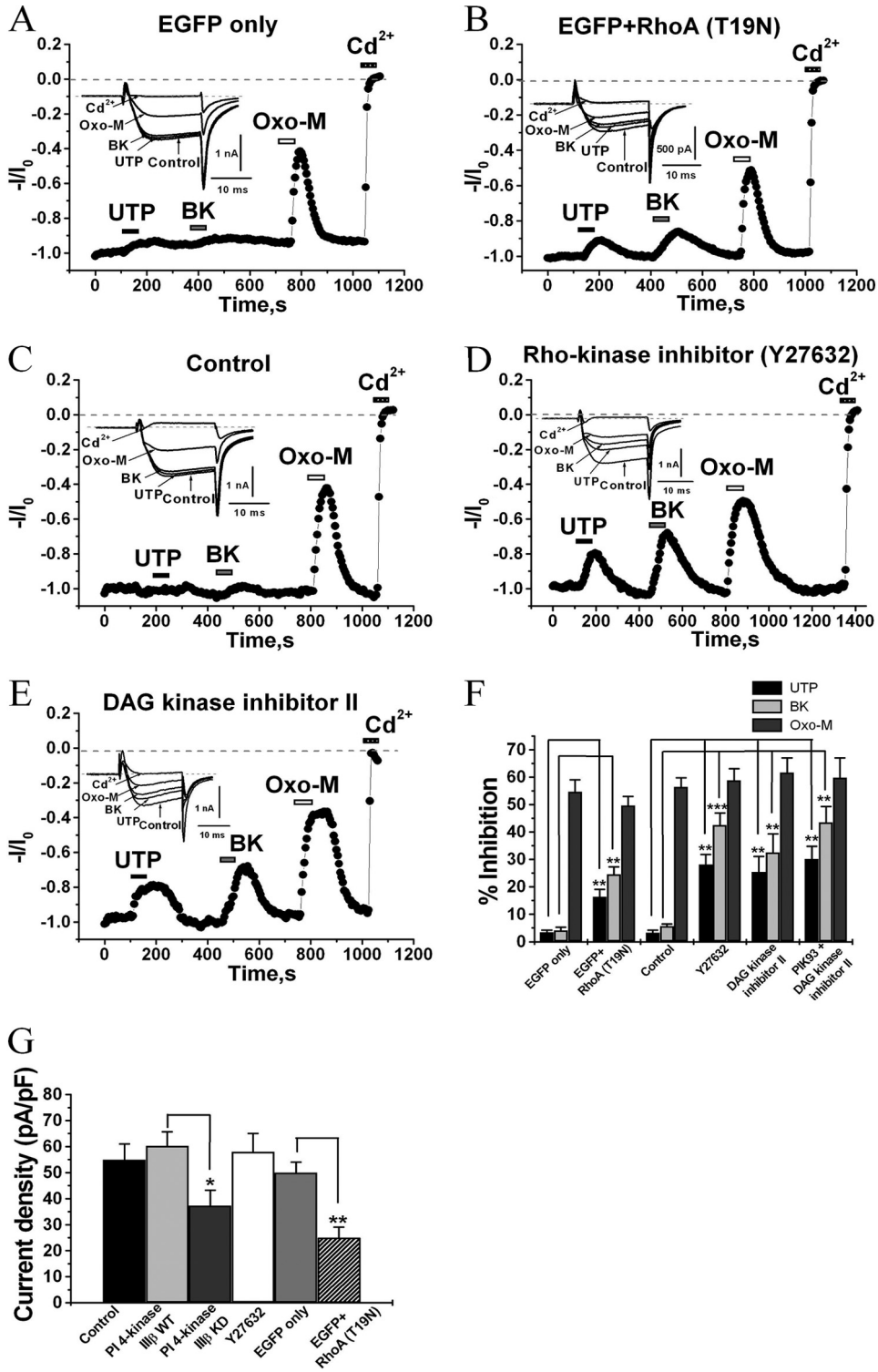

FIGURE 2. **Expression of dominant negative RhoA or blockade of Rho-kinase or DAG-kinase allows PIP_2 depletion by P2Y or B_2 receptors.** Shown are perforated patch voltage clamp experiments on cultured SCG neurons. *A–E*, plotted are normalized amplitudes of inward Ca^{2+} currents elicited by brief depolarizations to 5 mV, during the experiments in which UTP (10 μM), BK (250 nM), oxo-M (10 μM), and Cd^{2+} (100 μM) were bath-applied during the times indicated by the *bars*. In the *insets* are shown representative currents at the indicated times during the experiments. Cells were transfected with EGFP only, transfected with EGFP plus RhoA (T19N) (*B*), treated with vehicle only (*C*), treated with an inhibitor of Rho-kinase (Y27632, 1 μM, 40 min) (*D*), or treated with DAG-kinase inhibitor II (20 μM) (*E*). *Bars* are summarized suppressions of I_{Ca} by each agonist (*F*) or initial I_{Ca} amplitudes (*G*) for the six groups of experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *pF*, picofarads. *Error bars*, S.E.

I_{Ca} amplitudes were tonically reduced in DN RhoA-expressing cells.

The actions of RhoA are often through its effector, Rho-kinase. As a probe for Rho-kinase involvement, we tested the effect of a Rho-kinase inhibitor, Y27632 (52). As before, neither BK nor UTP induced a significant suppression of I_{Ca} in the vehicle-treated cell (Fig. 2*C*), whereas oxo-M had a robust effect. However, in a cell treated with Y27632 (1 μM, 40-min

pretreatment) (Fig. 2*D*), I_{Ca} was significantly depressed by UTP, BK, and oxo-M, an effect similar to that seen by blockade of PI 4-kinase IIIβ. These data are summarized in Fig. 2*F*. In control cells, the suppressions of I_{Ca} by UTP, BK, and oxo-M were 3.3 ± 1, 5.7 ± 0.8, and 56 ± 3% ($n = 7$), respectively. In cells pretreated with the Rho-kinase inhibitor, however, they were 28 ± 4% ($p < 0.01$), 43 ± 4% ($p < 0.001$), and 59 ± 4% ($n = 10$), respectively. Application of Y27632 itself did not lower PIP_2 levels during the 40-min pretreatment period, as indicated by the lack of reduced I_{Ca} amplitudes before the addition of any agonist. In control cells or those treated with the drug, the current densities were 55 ± 6 ($n = 7$) and 58 ± 7 ($n = 10$), respectively (Fig. 2*G*). Thus, Rho-kinase activity is necessary for receptor-specific stimulation of PIP_2 synthesis, but its blockade does not result in PIP_2 depletion during the lifetime of a patch clamp experiment.

Finally, we investigated the part played in this system by phosphatidic acid (PA), which has been shown to stimulate the type I PI(4)P 5-kinase isoform (37). PA is produced downstream of DAG production by DAG-kinase, and thus PA levels would be expected to increase with activation of PLC. To probe this notion, we tested the effect of the drug DAG-kinase inhibitor II (R59949) on the suppression of I_{Ca} by UTP, BK, and oxo-M. Indeed, when DAG-kinase was blocked by pretreatment of a neuron with DAG-kinase inhibitor II (20 μM, 1-h pretreatment) (53), all three agonists caused significant suppressions of I_{Ca} (Fig. 2*E*). For neurons treated with DAG-kinase inhibitor II, the suppressions by UTP, BK, and oxo-M were 25 ± 6 ($p < 0.01$), 33 ± 7 ($p < 0.01$), and 62 ± 5% ($n = 8$), respectively (Fig. 2*F*). Thus, the maintenance of PIP_2 levels in the face of PLC activity requires functional DAG-kinase activity, presumably in order for PA to participate in stimulation of PI(4)P 5-kinase. We also asked whether simultaneous blockade of PI 4-kinase IIIβ and DAG-kinase would result in greater depression of I_{Ca} by UTP or BK than blockade of either alone. However, we found neurons treated with both PIK93 and DAG-kinase inhibitor II to display responses similar to those treated with only one inhibitor. For those cells, the suppressions of I_{Ca} were 30 ± 5, 44 ± 6, and 60 ± 7%, respectively (Fig. 2*F*). To conclude this section, our working hypothesis is that stimulation of PIP_2 synthesis from PI(4)P via PI(4)P 5-kinase is controlled synergistically by RhoA, Rho-kinase, and phosphatidic acid.

Overexpression of M_1 Receptors in SCG Neurons Confers Muscarinic Agonist-induced $Ca^{2+}{}_i$ Signals—The data presented so far suggest that stimulation of $G_{q/11}$-coupled receptors, which induce $Ca^{2+}{}_i$ signals, does not deplete PIP_2 due to concurrent stimulation of PIP_2 synthesis. However, for the case of M_1 receptors, which do not provoke $[Ca^{2+}]_i$ rises, a wealth of data indicate that PIP_2 is strongly depleted. But what mechanism underlies this receptor specificity in generation of $Ca^{2+}{}_i$ signals in the first place? The "microdomain" hypothesis suggests co-localization or physical association between plasma membrane G protein-coupled receptors and endoplasmic reticulum membrane IP_3 receptors to be the key determinant (22). We thought a test of this hypothesis would be to overexpress exogenous M_1 receptors in SCG neurons, reasoning that such overexpression should override any native subcellular localization of endogenous receptors. Neurons were transfected either with EGFP only or with EGFP plus M_1 receptors, and $Ca^{2+}{}_i$ signals induced by P2Y, BK, and M_1 receptor stimulation were assayed using fura-2 imaging, with the dye loaded into the cells as the AM-ester. We did not attempt to calibrate these measurements, due to its inherent difficulty in such AM-ester-loaded experiments (54).

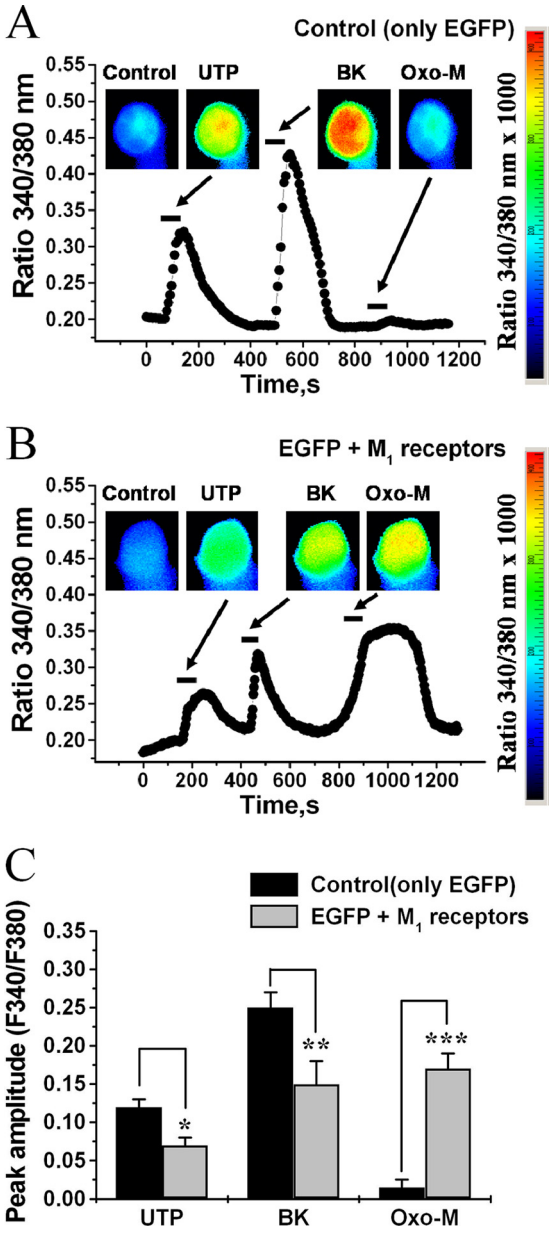

FIGURE 3. **Overexpression of M_1 receptors results in robust muscarinic agonist-induced $Ca^{2+}{}_i$ signals.** Plotted are ratio measurements of fura-2 emission from excitation at 340 or 380 nm of cells transfected with cDNA coding for EGFP only (*A*) or EGFP plus M_1 receptors (*B*) using biolistic delivery. UTP (10 μM), BK (250 nM), and oxo-M (10 μM) were bath-applied during the times indicated by the *bars*. In the *insets* are 340/380 nm ratiometric emission images of the cells studied, *pseudocolored* in *rainbow*, using the indicated *scale bar*. *C*, *bars* shown summarized rises in the 340/380 nm ratio induced by each agonist for both groups of cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Error bars*, S.E.

Fig. 3*A* shows the results from a control neuron transfected with EGFP only, with the ratio of emission from 340- and 380-nm light, which reports Ca^{2+}, plotted during the experi-

ment. Shown in the *inset* are *pseudocolor* 340/380 images of the neuron at various time points. Both UTP and BK induced robust rises in $[Ca^{2+}]_i$, whereas the $[Ca^{2+}]_i$ rises from muscarinic stimulation were insignificant, consistent with the literature (14, 16, 18, 20, 55). However, in the cell overexpressing M_1 receptors, muscarinic stimulation now induced a very significant rise in $[Ca^{2+}]_i$, even larger than that produced by UTP or BK (Fig. 3*B*). These data are summarized in Fig. 3*C*. For neurons transfected with only EGFP, the increases in the 340/380 nm ratio induced by UTP, BK, and oxo-M were 0.12 ± 0.01, 0.25 ± 0.02, and 0.02 ± 0.01 ($n = 20$), respectively, whereas for those transfected with EGFP plus M_1 receptors, they were 0.07 ± 0.01 ($p < 0.05$), 0.15 ± 0.03 ($p < 0.01$), and 0.17 ± 0.02 ($p < 0.001$) ($n = 24$), respectively. Thus, overexpression of exogenous M_1 receptors into the neurons results in muscarinic agonists now inducing large $Ca^{2+}{}_i$ signals, which we presume to be due to more widespread expression of M_1 receptors, including in microdomains containing IP_3 receptors, although we cannot exclude the possibility that the difference lies partly in enhanced production of IP_3. We do argue, however, that stimulation of endogenous muscarinic receptors in SCG neurons does result already in very large IP_3 production, as evidenced from multiple biochemical (9, 19) and PIP_2 hydrolysis probe imaging (8–10) experiments. We note that the $[Ca^{2+}]_i$ rises by UTP and BK were slightly, but significantly, reduced by M_1 receptor overexpression. This result might be due to displacement of some endogenous P2Y and B_2 receptors from their preferred position in association with IP_3 receptors by the heterologously expressed M_1 receptors, but we did not investigate this further.

The Protein IRBIT Underlies a "Threshold" Mechanism for IP_3-induced Ca^{2+} Release—One aspect on this topic that has puzzled us is the seeming "all-or-none" nature of the $Ca^{2+}{}_i$ response to receptor agonists (*i.e.* the $Ca^{2+}{}_i$ signals emanating from M_1 (and angiotensin AT_1 (56)) receptor stimulation are not just *less* than from P2Y and B_2 receptors but nearly wholly *absent*). Thus, we wondered if another signaling molecule were involved, one that might inhibit opening of IP_3 receptors in either a receptor-dependent or receptor-independent way. IRBIT is one such candidate inhibitory molecule because it has been shown to be a competitive antagonist of IP_3 for its binding site on IP_3 receptors (25). We first asked if IRBIT is expressed in SCG neurons by immunostaining and fluorescence microscopy. Cultured neurons were fixed and immunolabeled with anti-IRBIT antibodies (kindly given to us by Herbert De Smedt (Leuven, Belgium)) and an antibody against tyrosine hydroxylase as a sympathetic neuron marker. Fig. 4 shows images of the neurons showing clear expression of IRBIT. As controls, there was no significant labeling when the anti-IRBIT antibody was preadsorbed with a 10-fold molar excess of the immunizing peptide used to raise the antibody or when the primary antibody was omitted. The interaction between IRBIT and the IP_3 receptor is regulated by phosphorylation of IRBIT at Ser^{68}, such that the S68A mutant binds only weakly to the IP_3 receptor, does not compete with IP_3, and inhibits binding of endogenous IRBIT by formation

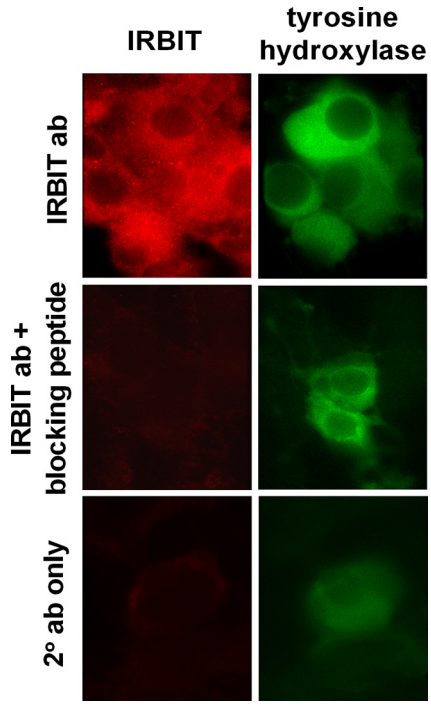

FIGURE 4. **IRBIT is expressed in SCG neurons.** Shown are fluorescence images of fixed SCG neurons immunostained for IRBIT and tyrosine hydroxylase, visualized using rhodamine red and FITC-conjugated secondary antibodies, respectively. Cells were stained either with the anti-IRBIT antibody (*ab*), the antibody preadsorbed with a 10-fold excess of the peptide used to raise the antibody, or with the secondary (*2°*) antibody only.

of an inactive heteromer (25). Thus, we utilized the IRBIT-S68A mutant as a DN test.

To examine the role of IRBIT in regulating $Ca^{2+}{}_i$ signals in SCG neurons, we compared the $[Ca^{2+}]_i$ responses to stimulation of P2Y, B_2, and M_1 receptors in neurons transfected with EGFP only, with WT IRBIT, or with IRBIT-S68A. In a cell transfected with WT IRBIT (Fig. 5*A*), the responses to UTP or BK were present but modest, and there was no response to oxo-M. In a cell transfected with IRBIT-S68A (Fig. 5*B*), the responses to UTP and BK were large, but now there was a modest but significant response to oxo-M. We quantified the data in two ways: by calculating the fraction of cells that responded to each agonist in all three groups of cells (Fig. 5*C*) or by quantification of the mean rise in the 340/380 nm ratio (Fig. 5*D*). For the case of UTP, for cells transfected with only EGFP, with WT IRBIT or with IRBIT-S68A, the fractions of cells with significant rises in $[Ca^{2+}]_i$ were 86, 67, and 93%, respectively. For BK, the fractions were 86, 58, and 93%, respectively, and for oxo-M, they were 15, 9, and 64%, respectively. Thus, for UTP and BK, overexpression of WT IRBIT reduced the number of responding cells, and for oxo-M, expression of IRBIT-S68A greatly increased it. We then analyzed the changes in the 340/380 nm ratio for the cells that did respond, to see if the $[Ca^{2+}]_i$ rises for such cells were affected by WT or S68A IRBIT. For UTP, there were no effects on the amplitude of the responses that reached the $p = 0.05$ level of significance, which were 0.12 ± 0.01, 0.08 ± 0.02, and 0.16 ± 0.02 ($n = 17$, 17, and 15) for cells transfected with EGFP only, WT IRBIT, and IRBIT-S68A, respectively. For BK, there was a reduction in the 340/380 nm ratio in cells trans-

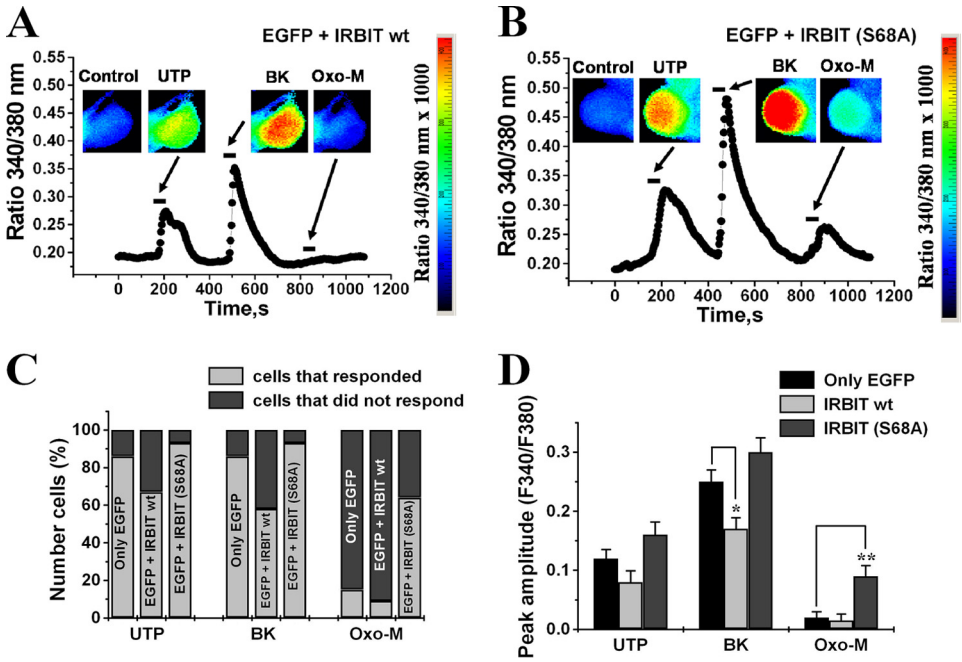

FIGURE 5. **IRBIT regulates receptor-induced $Ca^{2+}{}_i$ signals from IP_3-gated stores.** *A* and *B*, plotted are ratio measurements of fura-2 emission from excitation at 340 or 380 nm of cells transfected with cDNA coding for EGFP plus WT IRBIT (*A*) or EGFP plus IRBIT S68A (*B*) using biolistic delivery. UTP (10 μM), BK (250 nM), and oxo-M (10 μM) were bath-applied during the times indicated by the *bars*. In the *insets* are 340/380 nm ratiometric emission images of the cells studied, *pseudocolored* in *rainbow*, using the indicated *scale bar*. *C*, *bars* summarize the percentages of cells transfected with EGFP only, EGFP plus WT IRBIT, or EGFP plus IRBIT-S68A that responded with measurable rises in $[Ca^{2+}]_i$. *D*, *bars* quantify the rises in $[Ca^{2+}]_i$ elicited by UTP (10 μM), BK (250 nM), or oxo-M (10 μM) in the three groups of cells, for cells in which there were measurable $[Ca^{2+}]_i$ rises. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.E.

fected with WT IRBIT. The values were 0.24 ± 0.02, 0.17 ± 0.02 ($p < 0.05$), and 0.31 ± 0.02 ($n = 17$, 17, and 15), respectively. For oxo-M, transfection of IRBIT-S68A significantly increased the amplitude of the $[Ca^{2+}]_i$ rises in responding cells. For the three groups of cells, the increases in the values of the 340/380 nm ratio were 0.02 ± 0.01, 0.01 ± 0.01, and 0.09 ± 0.02 ($p < 0.01$) ($n = 17$, 17, 15), respectively.

Thus, IRBIT plays a role in tuning IP_3-mediating $Ca^{2+}{}_i$ signaling but probably not in a receptor-specific manner. Notably, the actions of IRBIT were seen here at saturating concentrations of agonist, although they were most manifest at subsaturating agonist concentrations in previous work in HeLa cells (24). The difference is likely to be the paucity of spatiotemporal mechanisms of specificity in tissue culture cells as opposed to primary neurons. Indeed, when one tests cloned $G_{q/11}$-coupled receptors expressed in tissue cells, they all respond with similar $[Ca^{2+}]_i$ rises or similar depletion of PIP_2, etc. In SCG neurons, we think $[IP_3]$ at the IP_3 receptor, even after B_2 or P2Y receptor stimulation, to be comparably modest, compared with that seen in tissue culture cells. Thus, even at saturating [agonist], local $[IP_3]$ at IP_3 receptors in SCG neurons perhaps corresponds to $[IP_3]$ at the IP_3 receptor produced by subsaturating [agonist] in the HeLa cell experiments.

DISCUSSION

Our work in sympathetic neurons has highlighted the mechanistic underpinnings of receptor-specific actions toward voltage-gated Ca^{2+} and K^+ channels. In SCG neurons, both N-type Ca^{2+} (10) and Kv7 (M-type) K^+ (2, 57) channels are highly sensitive to PIP_2 abundance, and PIP_2 binding/unbinding almost certainly underlies their PIP_2 sensitivity. Another factor in the literature has been complex regulation of N-type channels by arachidonic acid (58–60), but we do not delve into that subject here. SCG neurons express two groups of PLC-linked receptors, which we have called "mode 1" and "mode 2" (14), the former consisting of the M_1 and AT_1 types that elicit sparse $Ca^{2+}{}_i$ signals, deplete PIP_2, and suppress both M current and I_{Ca} and the latter consisting of the B_2 and P2Y types that induce reliable $Ca^{2+}{}_i$ signals, do not deplete PIP_2 or suppress I_{Ca}, and depress M current via Ca^{2+}/calmodulin binding (61). Thus, the I_{Ca} in these cells makes an ideal biosensor of PIP_2 depletion by receptors, similar to the reporting by highly PIP_2-sensitive GIRK K^+ channels when expressed in the same neurons (9). It is important to note that such dramatic receptor specificity is only seen in native cells (23, 62–67) and is wholly absent in reconstituted systems in which channels and receptors are heterologously expressed, in which all PLC-linked receptors elicit large $Ca^{2+}{}_i$ signals and all probably work similarly (8, 11, 68, 69).

We have suggested at least part of such receptor-specific actions to be mediated by Ca^{2+}/NCS-1-mediated acceleration of PI 4-kinase activity. However, the apparent mismatch between the proposed PI 4-kinase isoform (IIIβ) and subcellular location (Golgi) provoked us to test if the IIIβ isoform was actually required. Our positive result suggests that 1) either PI 4-kinase IIIβ is localized to the plasma membrane in neurons, *versus* its localization in cell lines (35, 46), or 2) PI(4)P is produced in the Golgi but is then rapidly (<1 min) transported to the membrane, where it is converted to PIP_2 by PI(4)P 5-kinase. Indeed, the latter possibility is interesting and is worth

testing, given the established ability of phosphoinositide transfer proteins (70, 71) or other proteins (see Ref. 72 for a review) to rapidly shuttle phosphoinositides between subcellular compartments. As recently discussed (73), it could then be the phosphoinositide transfer protein transport rate that is stimulated by the "mode 2" receptors rather than the PI 4-kinase rate itself. The recent work using voltage-sensitive PIP_2 phosphatases makes clear that PI(4)P replenishment at the PM is rate-limiting for PIP_2 synthesis (73–75). However, along with stimulation of plasma membrane PI(4)P levels by receptor stimulation, several lines of evidence indicate that acceleration of PI(4)P 5-kinase activity must also be required to maintain [PIP_2] upon activation of PLC. Using the Virtual Cell environment, the Loew laboratory has demonstrated the requirement for ~10-fold stimulation of both PI 4- and PI(4)P 5-kinases in order to fit their biochemical measurements of PI(4)P and PIP_2 levels during bradykinin stimulation of neuroblastoma cells (30) and stimulation of metabotropic glutamate receptors in cerebellar spines (32). Using their model, we find that PI(4)P must be allowed to rise to levels >30-fold over that present tonically in order to produce enough PIP_2 by mass action, without increasing the rate of PI(4)P 5-kinase activity,[3] and biochemical measurements indicate that such a massive increase in PI(4)P abundance does not occur (30, 69, 76, 77). Likewise, Falkenburger *et al.* (73) examined the kinetics and dose-response relationship of muscarinic modulation of cloned M-type channels by cloned M_1 receptors heterologously expressed in tsA-201 cells, and using the Virtual Cell, showed 10-fold stimulation of both PI 4-kinase and PI(4)P 5-kinase by agonist to be required to adequately fit their data. Strong acceleration of PIP_2 synthesis by agonists is documented in platelets, in which thrombin stimulation of $G_{q/11}$-coupled PAR1 receptors increased PI(4)P 5-kinase activity nearly 10-fold and DAG-kinase activity 4.5-fold (78). In heterologous systems, stimulation of these same PAR1 receptors resulted in a translocation of type I PI(4)P 5-kinases from the Golgi to the PM that was Rho-dependent, representing a possible mechanism (48), although it is unclear if such translocation could occur on the time scale of a patch clamp experiment. Finally, overexpression of PI(4)P 5-kinase strongly increases the single-channel open probabilities and tonic current amplitudes of PIP_2-sensitive Kv7 (M-type) channels (5, 7, 9), indicating the sensitivity of PIP_2 levels to PI(4) 5-kinase activity. Our data here conform to the thinking that stimulation of both PI 4-kinase and PI(4)P 5-kinase is required to explain our data and that of others.

In a variety of cells, Rho family GTPases have been shown to physically associate with type I PI(4)P 5-kinases (79–81) and, together with Rho-kinase and PA, to stimulate production of PIP_2 by PI(4)P 5-kinase (for reviews, see Refs. 82 and 83). At least some of this activity seems to be constitutive, and the reduction in I_{Ca} that we observed in DN RhoA-transfected neurons is consistent with that idea. But what could underlie receptor-mediated stimulation of PI(4)P 5-kinase activity? Clearly, PA action provides one such mechanism because PA is produced downstream of DAG production, whose levels are expected to be elevated by PLC hydrolysis of PIP_2 (see Refs. 84 and 85 for reviews). In addition, PA, via CDP-DAG, is the precursor to PI production, and blockade of PA production might eventually reduce tonic PI levels. We must also consider the subcellular localization of PI(4)P 5-kinase molecules. Indeed, as for PI 4-kinases, the subcellular localization of mammalian PI(4)P 5-kinases is controversial. Whereas several laboratories report the latter to localize to the Golgi of resting cells (48, 86, 87), the general literature supports the subcellular localization of PI(4)P 5-kinases to be regulated, with the regulatory molecules including Rho (via Rho-kinase), Rac and Arf GTPases, and PA. Slow (<1 h) regulation of ion channels by small GTPases, comprising trafficking steps and tuning of functional channels, has been documented (50). However, as for the discussion on the site of PI(4)P synthesis, receptor-induced translocation of PI(4)P 5-kinases to the PM as the mechanism underlying $G_{q/11}$-coupled receptor stimulation of PIP_2 synthesis suffers from a time constraint. Not only would this event have to transpire during a patch clamp measurement of current amplitudes (as we perform here), but measurements of the time course of PI(4)P phosphorylation following PIP_2 dephosphorylation by a voltage-sensitive PIP_2 phosphatase indicate a time scale of <30 s (73). Thus, careful measurements of how fast PI(4)P 5-kinases can translocate to the membrane and phosphorylate PI(4)P are required to determine if this is a plausible mechanism.

The lack of $Ca^{2+}{}_i$ signals elicited by stimulation of M_1 receptors in SCG neurons has been a persistent enigma, given that such stimulation causes intense PIP_2 hydrolysis, as measured by biochemical (19) and optical (88) methods. Although the microdomain hypothesis seemed a logical explanation, observations from these neurons transfected with the PLCδ-PH probe that binds to PIP_2 and to IP_3 (89), in which the probe appears to flood the entire cytoplasm upon muscarinic agonist (8–10), made us intuitively wonder if subcellular localization is the entire story. There seems nothing special about M_1 receptors in SCG neurons that prevents their stimulation from causing $[Ca^{2+}]_i$ rises because when we heterologously overexpressed them here, oxo-M produced large $Ca^{2+}{}_i$ signals. We acknowledge that overexpression of M_1 receptors might result in much greater numbers of receptors expressed in the membrane of the neurons, resulting in greater or faster turn-on of $G\alpha_{q/11}$ and PLC for a given concentration of agonist. Quantification of the increase over endogenous levels of membrane proteins by their expression suggests that this factor might be as large as 100-fold, although most expressed proteins would probably not be in the plasma membrane (90). However, that same paper concludes that the steps of agonist binding to receptors and receptor activation of $G_{q/11}$ and PLC are much faster than PLC-mediated hydrolysis of PIP_2, and thus it is the number of PLC molecules that largely determines the speed and amount of IP_3 production, at least at the supramaximal agonist concentrations used here. Thus, we take our results as a validation of microdomain organization being a major part of the story.

The other part lies in IRBIT as an inhibitory "gatekeeper" of IP_3 receptor activation. Although [IP_3] may rise in all domains

[3] O. Zaika, J. Zhang, and M. S. Shapiro, unpublished observations.

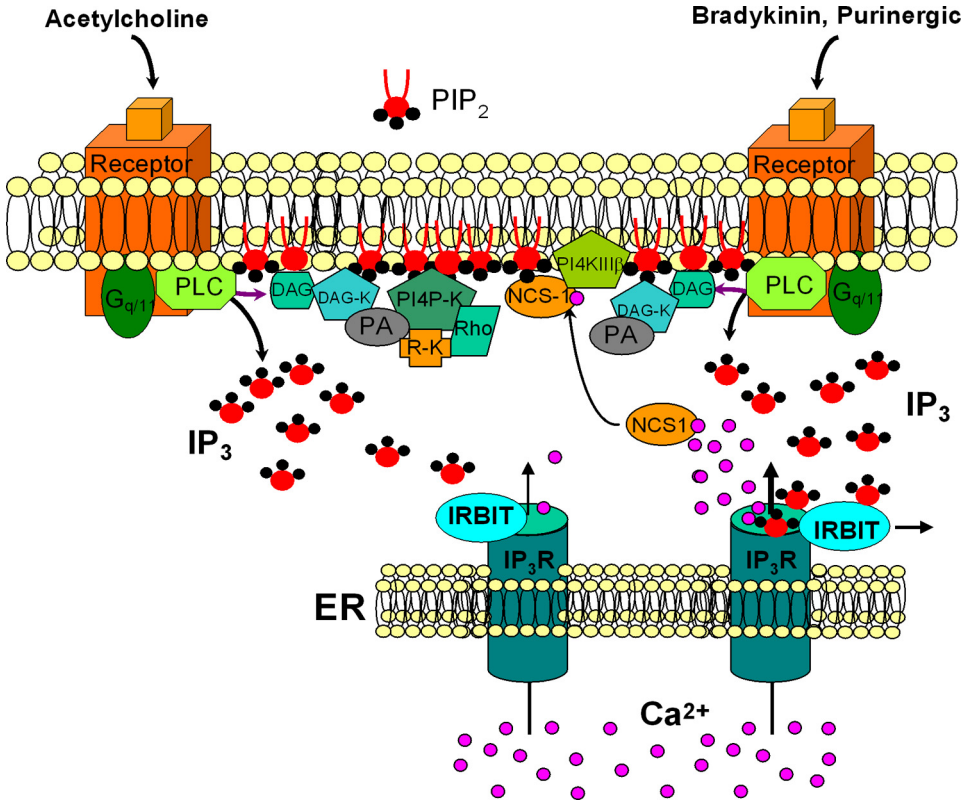

FIGURE 6. **Model accounting for receptor-specific phosphoinositide and Ca^{2+} signals in SCG neurons.** All receptors activate PLCβ, which in turn hydrolyzes PIP_2 to IP_3 and DAG. The released Ca^{2+} is regulated by the IP_3R-binding protein, IRBIT, which sets a threshold for $[IP_3]$ sufficient to open IP_3Rs. Stimulation of M_1 muscarinic acetylcholine receptors (*left*) is ineffective in producing cytoplasmic Ca^{2+} signals because the IP_3 produced is too far away from IP_3Rs; thus, $[IP_3]$ at the IP_3R is too low to overcome the IRBIT threshold. Bradykinin B_2 and purinergic P2Y receptors (*right*) produce robust cytoplasmic Ca^{2+} signals due to their spatial co-localization with IP_3Rs, where $[IP_3]$ is sufficiently high. Via NCS-1, bradykinin and purinergic, but not muscarinic, stimulation accelerates PI 4IIIβ-kinase activity. Via DAG-kinase conversion to PA, the produced DAG increases PI(4)P 5-kinase (*PI4P-K*) activity, in concert with Rho family proteins and Rho-kinase (*R-K*). PI(4)P 5-kinase activity is also increased by bradykinin and purinergic stimulation, but for clarity this is only shown for muscarinic stimulation. Acceleration of both PI 4IIIβ- and PI(4)P 5-kinases is required to increase PIP_2 synthesis that compensates for consumption of PIP_2 by PLCβ.

of the cytoplasm, it rises most near its site of production by PLC. At low or only modestly raised $[IP_3]$, IRBIT remains bound to IP_3 receptors, antagonizing their activation by IP_3. This may be the case for M_1 receptor stimulation. When $[IP_3]$ rises to a certain point in the microdomain around the IP_3 receptor (between 0.3 and 1 μM (26)), IRBIT is released from the IP_3 receptor, allowing unhindered binding of IP_3, maximal opening of the IP_3 receptor, and evident rises in $[Ca^{2+}]_i$. This is likely to be the case for B_2 and P2Y-receptor stimulation. Thus, IRBIT sets a threshold for $[IP_3]$ for Ca^{2+} release, conferring specificity to cellular signals that depend on co-localization of IP_3 receptor and the site of IP_3 production. A similar threshold mechanism has been proposed for CaM via Ca^{2+}-dependent feedback inhibition of IP_3Rs, and expression in SCG cells of DN CaM likewise increased $[Ca^{2+}]$; rises induced by oxo-M (22). We found that IRBIT affected the fraction of neurons that responded to each agonist and also affected the amplitude of the resultant $[Ca^{2+}]_i$ rise, although this latter effect was most evident for the case of oxo-M. Overexpression of IRBIT-S68A had little effect on the responses to UTP or BK, suggesting that $[IP_3]$ at IP_3 receptors is usually high enough to overcome endogenous IRBIT (although this might not be the case at subsaturating concentrations of those agonists) but had large effects for oxo-M, for which the fraction of responding cells was much higher and the amplitude of the $[Ca^{2+}]_i$ rises in responding cells was larger. Finally, although there was a reduction in BK-induced $[Ca^{2+}]_i$ rises in neurons overexpressing WT IRBIT, the biggest effects were on the muscarinic responses. Thus, one possibility is that the physical association between IP_3 receptors and B_2, but not M_1, receptors (21) in some way physically or allosterically occludes the action of IRBIT on the IP_3 receptors.

Our model encompassing $Ca^{2+}{}_i$ and phosphoinositide signals endowing receptor specificity is summarized in Fig. 6. Shown are two classes of receptors, the M_1 muscarinic and the B_2/P2Y bradykinin/purinergic. The former are localized at some distance from IP_3 receptors, resulting in local $[IP_3]$ being too low to overcome inhibition by bound IRBIT. Thus, the $[Ca^{2+}]_i$ rise observed is normally negligible. The B_2/P2Y class is co-localized in microdomains with IP_3 receptors, as postulated previously (91). Consequently, local $[IP_3]$ is high enough to overcome IRBIT action, causing release of IRBIT from IP_3 receptors, amplification of IP_3 action, and reliable $[Ca^{2+}]_i$ rises. The released Ca^{2+} ions bind to and activate NCS-1, which accelerates activity of PI 4-kinase IIIβ, which may or may not need to reside in the PM. Because the B_2 receptors physically associate with IP_3 receptors (55), NCS-1 need not translocate to the PM during this event but is probably already preassembled in a complex with PI 4-kinase IIIβ. Our

results indicate that stimulation of PI(4)P 5-kinases is also required to explain the lack of B_2/P2Y action on I_{Ca}. This signaling pathway requires Rho, Rho-kinase, and DAG-kinase activities because disruption of any one of these prevents compensatory PIP_2 synthesis. We depict these molecules as clustered together, associated with PI(4)P 5-kinase, but we have little information about which of them are constitutively pretethered and which associate together only upon receptor stimulation. We have no reason to think that this signal is receptor-specific. Importantly, this stimulation must not be tonic but rather receptor-stimulated. Because tonic rates of PI(4)P 5-kinase activity must be low enough to allow the PIP_2 depletion part of receptor action (30, 73, 92), and tonic increases in PI(4)P 5-kinase activity prevent PLC-mediated modulation of K^+ channels (5, 7, 9, 93), the molecules clustered around PI(4)P 5-kinases that we identify here cannot be all constitutively active, but rather one or more must be turned on by receptor agonists. Interestingly, IRBIT is only functional if phosphorylated at multiple amino-terminal serines, including Ser^{68} (25), meaning that its cognate kinase could regulate IRBIT action and $Ca^{2+}{}_i$ signaling in neurons. Although IRBIT was shown to be phosphorylated *in vitro* by casein kinase I and is known to be dephosphorylated by PP1 protein phosphatases (25), its physiological kinase *in vivo* is unknown; also, it is unknown whether such a kinase could be activated downstream of stimulation of G protein-coupled receptors. If so, this could underlie another mechanism of $G_{q/11}$-coupled receptor specificity.

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