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Activation and Inhibition of Neuronal G Protein-Gated Inwardly Rectifying K^+ Channels by P2Y Nucleotide Receptors

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ABSTRACT

Neuronal signaling by G protein-coupled P2Y nucleotide receptors is not well characterized. We studied here the coupling of different molecularly defined P2Y receptors to neuronal G protein-gated inward rectifier K^+ (GIRK) channels. Individual P2Y receptors were coexpressed with GIRK1+GIRK2 (Kir3.1 + 3.2) channels by intranuclear plasmid injections into cultured rat sympathetic neurons. Currents were recorded using perforated-patch or whole-cell (disrupted patch) techniques, with similar results. $P2Y_1$ receptor stimulation with 2-methylthio ADP (2-MeSADP) induced activation of GIRK current (I_{GIRK}) followed by inhibition. In contrast, stimulation of endogenous α_2-adrenoceptors by norepinephrine produced stable activation without inhibition. $P2Y_1$-mediated inhibition was also seen when 2-MeSADP was applied after I_{GIRK} preactivation by norepinephrine or by expression of $G\beta_1\gamma_2$ subunits. In contrast, stimulation of $P2Y_4$ receptors with UTP or $P2Y_6$ receptors with UDP produced very little I_{GIRK} activation but significantly inhibited preactivated currents. Current activation was prevented by pertussis toxin (PTX) or after coexpression of the $\beta\gamma$-scavenger transducin-Gα. I_{GIRK} inhibition by all three nucleotide receptors was insensitive to PTX and was significantly reduced after coexpression of RGS2 protein, known to inhibit $G_q\alpha$ signaling. Inhibition was not affected 1) after coexpression of RGS11, which interferes with $G_q\beta\gamma$ action; 2) after coexpression of phospholipase C (PLC) δ-Pleckstrin homology domain, which sequesters the membrane phospholipid phosphatidylinositol 4,5-bisphosphate; (3) after buffering intracellular Ca^{2+} with 1,2-bis(2-aminiphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM); and (4) after pretreatment with the protein kinase C inhibitor 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride (GF 109203X). We conclude that activation of I_{GIRK} by P2Y receptors is mediated by $G_{i/o}\beta\gamma$, whereas I_{GIRK} inhibition is mediated by $G_q\alpha$. These effects may provide a mechanism for P2Y-modulation of neuronal excitability.

The family of G protein-coupled P2Y nucleotide receptors comprises, in mammals, at least eight members—$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (reviewed in Abbracchio et al., 2003). All of these are expressed in brain (Barnard et al., 1997; Moore et al., 2000; Communi et al., 2001; Hollopeter et al., 2001). However, unlike the ligand-gated P2X ion channel receptors, which are involved in fast synaptic transmission (Robertson et al., 2001), the neural function of P2Y receptors is not yet clear.

In previous experiments, we have sought to assess the potential neural effects of these receptors by expressing them in primary sympathetic neurons, which possess a variety of neuron-specific ion channels. We found that activating $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, or $P2Y_{12}$ receptors could (to varying degrees) inhibit voltage-activated M-type (KCNQ2/3) K^+ channels and/or N-type voltage-gated Ca^{2+} channels (reviewed in Brown et al., 2000; Filippov et al., 2000, 2003).

In other systems, subtypes $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ signal through a $G_{q/11}$/PLC/IP_3/Ca^{2+} release pathway (reviewed by Communi et al., 2000; von Kugelgen and Wetter, 2000), whereas $P2Y_{12}$, acts as a $G_{i/o}$-linked receptor (Hollopeter et al., 2001; Simon et al., 2002). Likewise, closure of M-type K^+ channels by $P2Y_1$, $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptors was insensitive to pertussis (PTX) toxin (see also Bofill-Cardona et al., 2000), whereas inhibition of N-type Ca^{2+} channels by the $P2Y_{12}$ receptor was fully prevented by PTX (Simon et al., 2002). However, Ca^{2+} current inhibition by the other four subtypes in our

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ABBREVIATIONS: PTX, pertussis toxin; RGS, regulator of G protein signaling; PLC, phospholipase C; PH, Pleckstrin homology; SCG, superior cervical ganglia ; BAPTA, 2-bis(2-aminiphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; 2-MeSADP, 2-methylthio-ADP; U-73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U-73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5- pyrrolidine-dione; GF 109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride; PIP_2, phosphatidylinositol 4,5-bisphosphate; GFP, green fluorescent protein; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; GIRK, G protein-gated inward rectifier K^+.

studies showed varying degrees of PTX sensitivity, suggesting parallel involvement of a non-$G_i/_o$ protein,

Other $G_{i/o}$-linked receptors that inhibit N-type Ca^{2+} channels usually also activate G protein-gated inward rectifier (GIRK or Kir3) channels. In keeping with this, $P2Y_2$ receptors have been reported to activate GIRK channels in oocytes (Mosbacher et al., 1998; Mark et al., 2000), and stimulation of either $P2Y_1$ or $P2Y_{12}$ receptors activated GIRK (Kir3.1/3.2) channels when these were coexpressed in sympathetic neurons (Simon et al., 2002). However, in the latter experiments, whereas the $P2Y_{12}$ receptors produced a stable activation of GIRK current (I_{GIRK}), the $P2Y_1$ receptors produced a transient activation followed by deactivation in the continued presence of agonist. This seemed to correlate with their effects on M currents because, although both inhibited N-type Ca^{2+} currents, only the $P2Y_1$ receptor inhibited M currents (Simon et al., 2002). Likewise, in oocytes, the $P2Y_2$ receptor [which inhibits M currents (for review, see Brown et al., 2000)] also produced a transient I_{GIRK} activation followed by a slow decline in the continued presence of agonist (Mark et al., 2000). Thus, P2Y receptors might be capable of both activating and inhibiting I_{GIRK}.

In the present experiments, we have explored this possibility further by coexpressing $P2Y_1$, $P2Y_4$, or $P2Y_6$ receptors with brain-specific Kir3.1/3.2 (GIRK 1/ 2) channels in sympathetic neurons. We identify two separate effects of P2Y receptors on GIRK channels, mediated by two different G proteins and transduction pathways.

Materials and Methods

DNA Plasmids. The plasmids expressing rat $P2Y_1$, human $P2Y_4$, rat $P2Y_6$, the enhanced green fluorescent (mutant S65T) protein, $G\beta_1$, $G\gamma_2$, transducin $G\alpha$, GIRK1, and GIRK2 were the same as those used previously (Filippov et al., 2000, 2003; Fernandez-Fernandez et al., 2001). HA-tagged human RGS11 in pcDNA3.1 was a gift from Dr. Richard Miller (University of Chicago), RGS2 in pC1 vector was a gift from Dr. Stephen Ikeda (Guthrie Research Institute), GFP-tagged Pleckstrin homology domain of PLCδ (PLCδ-PH) in pEGFP-C1 vector was a gift from Dr. Tobias Meyer (Stanford University). The presence of the inserts was confirmed by digestion analysis. Plasmids were stored at −20°C for injection in sterile 10 mM Tris and 1 mM EDTA, pH 8.

Cell Culture and DNA Injection. Sympathetic neurons were dissociated from superior cervical ganglia (SCG) of 17- to 19-day-old Sprague-Dawley rats killed by CO_2 asphyxiation using an approved schedule 1 procedure. Isolation and injection procedures were identical to those described previously (Brown et al., 2000). Cells were plated on glass coverslips coated with laminin and incubated at 37°C for 4 to 5 h before DNA injection. The plasmids prepared in sterile Tris/EDTA solution were microinjected into the nucleus. The concentration of plasmids for GIRK1, GIRK2, $P2Y_1$, $P2Y_4$, $P2Y_6$, and PLCδ-PH was 100 ng/μl. cDNA for RGS11 was at 300 ng/μl, and cDNA for $G\beta_1$ and $G\gamma_2$ and for transducin $G\alpha$ were at 200 ng/μl each. The enhanced green fluorescent protein cDNA in pcDNA3 (BD Biosciences Clontech) was coinjected (20–25 ng/μl) where necessary to enable later identification of the cells with successful expression. After injections, cells were incubated at 37°C for 14 to 24 h before electrophysiological recording.

Electrophysiological Recording of I_{GIRK}. I_{GIRK} was recorded from GFP-labeled neurons at room temperature (22–24°C) as described previously (Simon et al., 2002). Most of the experiments have been done using the perforated-patch technique, so no correction has been made for the junction potential between pipette and bath solutions. In some experiments, we also used the whole-cell (disrupted patch) technique (indicated in figure legends); the results were qualitatively indistinguishable from those obtained with the perforated patch technique. For perforated patch recordings, borosilicate glass electrodes (2–4 MΩ) were filled with a solution containing 90 mM potassium acetate, 20 mM KCl, 3 mM $MgCl_2$, 40 mM HEPES, 0.1 mM BAPTA, and 0.125 mg/ml amphotericin B (pH adjusted to 7.4 with KOH). For whole-cell recordings, similar glass electrodes were filled with a solution containing 60 mM potassium acetate, 60 mM KCl, 2.5 mM $MgCl_2$, 30 mM HEPES, 10 mM BAPTA, 2 mM Na_2ATP, and 0.1 mM Na_3GTP (pH adjusted to 7.2 with KOH). Cells were superfused initially with a bath solution continuously flowing at 25 to 30 ml/min and containing 120 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, and 11.1 mM glucose (pH adjusted to 7.4 with NaOH). Before measurements, the KCl concentration in a bath solution was increased to 6 mM (KCl substituted for NaCl), and tetrodotoxin (0.5 μM) was added to bath solution to block the fast sodium current. Membrane currents were recorded using a discontinuous ('switching') amplifier (Axoclamp 2B) sampling voltage at 6 to 8 kHz. Drugs were applied via the same perfusing solution (bath exchange rate ≤1 s); this avoids any change in nucleotide composition caused by enzymic action at the surface of the cells. Voltage commands were generated and currents digitized and analyzed using 'pClamp 8' software (Axon Instruments, Foster City, CA). I_{GIRK} was typically recorded using a 200-ms voltage ramp between −140 mV and −40 mV from a holding potential of −60 mV (Ruiz-Velasco and Ikeda, 1998) applied every 5 s. The peak amplitude of the current was acquired by averaging currents between −125 and −130 mV. Activated current was measured as the current after agonist application less the current before agonist application. Values are shown as the mean ± S.E.M., for recordings from n cells. Differences were taken as significant at $p < 0.05$ (Student's test).

Fluorescence Measurements. Fluorescence images of GFP-PLCδ-PH (excitation and emission wavelengths: 475 and 530 nm, respectively) were subjected to a digital deconvolution procedure ('Openlab'; Improvision UK, Coventry, UK) to remove image 'blur' and enhance special definition. Five images were taken at 1-μm intervals using a 12-bit gray-scale camera (C4880-80; Hamamatsu Photonics UK Ltd., Welwyn Garden City, UK) both above and below the optic section of interest, and these were subjected to a nearest-neighbor algorithm to remove 65% of the predicted 'blur'.

Chemicals. UTP was from Pharmacia Biotech, molecular biology grade; it was 99.5% pure and free from adenine nucleotides, UDP was from Roche Applied Science (Mannheim, Germany). GTP, Na_2ATP, (−)-norepinephrine bitartrate, EGTA, BAPTA, and amphotericin B were all from Sigma (St. Louis, MO). Oxotremorine-M and 2-methylthio-ADP (2-MeSADP) were from RBI-Sigma (Natick, MA); the latter was freed from triphosphates as before (Simon et al., 2002). Tetrodotoxin, U73122, U73343, and GF 109203X were from Tocris Cookson Inc. (Bristol, UK), PTX from Porton Products (U.K.) and $BaCl_2$ was from Aldrich (Milwaukee, WI).

Results

Activation and Inhibition of Neuronal GIRK Channels by $P2Y_1$ Receptors. Fig. 1 illustrates the effects of activating expressed $P2Y_1$ receptors in ganglion cells previously injected with Kir3.1 + Kir3.2 cDNA plasmids. Application of the $P2Y_1$ agonist 2-MeSADP produced a rapid activation of the I_{GIRK} (identifiable by its inhibition by 1 mM Ba^{2+}) but activation was transient (Fig. 1A) and subsided within 30 s by 66.4 ± 6.7% from its peak value (n = 12). No effect of 2-MeSADP was seen in cells not injected with $P2Y_1$ or Kir cDNAs. In contrast, activation of endogenous α_2-adrenoceptors by norepinephrine produced stable activation of I_{GIRK} lasting for several minutes; superimposed application of 2-MeSADP then produced very little, if any, additional

activation but instead strongly reduced the norepinephrine-induced current (Fig. 1B).

This inhibitory action of 2-MeSADP might have been caused by an interaction with the α_2-adrenoceptors. To bypass the receptors, we therefore preactivated the I_{GIRK} by coexpressing $G\beta_1$ and $G\gamma_2$ G protein subunits (Stanfield et al., 2002). Under these conditions, a substantial resting I_{GIRK} can be recorded from these neurons that is inhibited by 1 mM Ba^{2+} (Fernandez-Fernandez et al., 2001). Application of 2-MeSADP to such neurons coexpressing $P2Y_1$ receptors again produced very little, if any, additional activation but instead strongly inhibited the preactivated I_{GIRK} (Fig. 2). Hence, the inhibitory effect of 2-MeSADP was directed at the GIRK channels themselves, or the process of $G\beta\gamma$-activation, and not the α_2-receptors.

Activation and inhibition of the GIRK current by $P2Y_1$ receptor stimulation could be separated by the use of PTX. Thus, overnight incubation with 0.5 μg/ml PTX fully prevented activation of I_{GIRK} by norepinephrine and very substantially reduced activation by 2-MeSADP (Fig. 3A). In contrast, the inhibition of $G\beta\gamma$-preactivated currents was not significantly reduced (Fig. 3B). This indicates that activation and inhibition are separate events, mediated through the independent coupling of the $P2Y_1$ receptor to two different G proteins—activation by G_i/G_o (most likely G_I; see Fernandez-Fernandez et al., 2001) and inhibition by a PTX-insensitive G protein.

To estimate the potency of 2-MeSADP as a $P2Y_1$ receptor-mediated inhibitor of I_{GIRK}, we used cells in which I_{GIRK} was preactivated by $G\beta\gamma$ expression (Fig. 4). In these cells, 2-MeSADP inhibited I_{GIRK} with an extremely low IC_{50} of 1.02 $\pm$ 0.06 nM ($n = 4$). A higher value (~150 nM) has been reported for 2-MeSADP stimulation of $P2Y_1/G_q$-mediated GTPase activity (Waldo and Harden, 2004): this difference may be explained by functional amplification through the downstream pathway that subsequently leads to I_{GIRK} inhibition (see *Discussion*).

On the Mechanism of P2Y-Mediated Activation of I_{GIRK}. We tested further whether endogenous $G\beta\gamma$ subunits are responsible for $P2Y_1$-mediated activation of GIRK channels. For this, we coexpressed in SCG neurons transducin

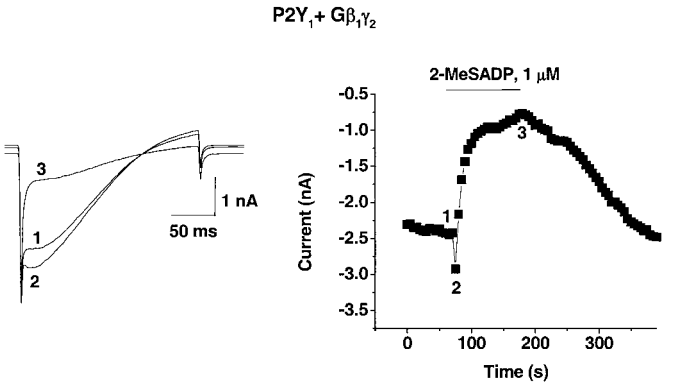

Fig. 2. Inhibition by a $P2Y_1$ receptor agonist of I_{GIRK} preactivated by coexpression of G protein $G\beta_1$ and $G\gamma_2$ subunits. The recordings (left) and the plot (right) show ramp-currents and time-plots of peak I_{GIRK}, respectively, as described for Fig. 1. The current was preactivated (record 1) by injecting cDNA plasmids encoding $G\beta_1$ and $G\gamma_2$ subunits 24 h previously. Note that the $P2Y_1$ agonist 2-MeSADP (1 μM) induced only a small additional activation of I_{GIRK} (record 2) followed by a considerable, but reversible, inhibition of the preactivated current (record 3).

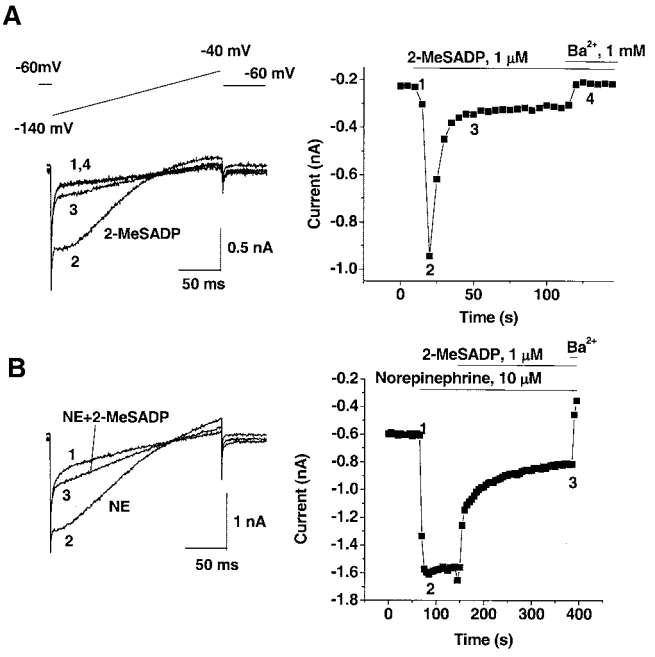

Fig. 1. Activation and inhibition of G protein-gated inward rectifier K^+ current (I_{GIRK}) via the $P2Y_1$ nucleotide receptor coexpressed in SCG neurons. Records show I_{GIRK} evoked by a ramp voltage protocol from −140 to −40 mV (trace above). Graphs show absolute current values measured every 5 s. Measurements were made 20 ms after starting the ramp (near the maximal induced current). Points numbered on the graphs correspond to numbers on the records. Ba^{2+} (1 mM) was added at the end to block all of the I_{GIRK}. A, application of the $P2Y_1$ agonist, 2-MeSADP (1 μM), produced a transient activation of the I_{GIRK} (record 2) followed by inhibition of the activated current (record 3). B, application of the α_2-adrenoceptor agonist, norepinephrine (NE), produced stable activation of I_{GIRK} (record 2), which was then inhibited by subsequent application of the $P2Y_1$ receptor agonist (record 3).

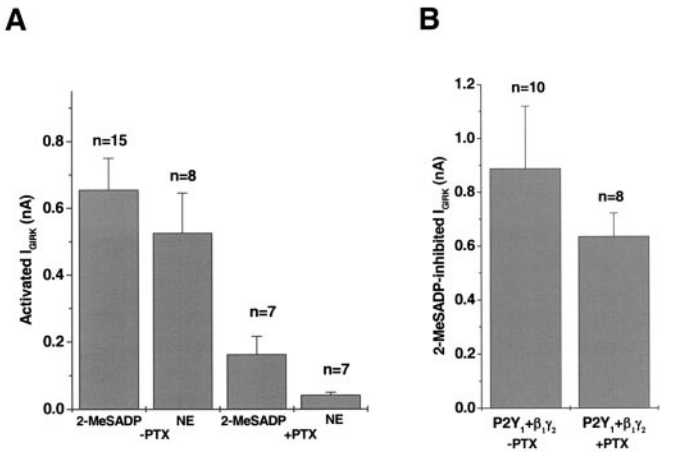

Fig. 3. Inactivation of $G_{i/o}$ protein by PTX pretreatment prevents I_{GIRK} activation but not I_{GIRK} inhibition. Columns show I_{GIRK} activated by 2-MeSADP (1 μM) or norepinephrine (NE, 10 μM) (A) or inhibited by 2-MeSADP (1 μM) (B) before or after overnight pretreatment of the neurons with PTX (500 ng/ml). Activated current was measured as maximal current after agonist application minus current before agonist application. Inhibited current was measured as maximal activated current (induced by coexpression of $G\beta_{1\gamma 2}$ subunits; see Fig. 2) minus current at the end of agonist application (but before Ba^{2+} was added to the solution). Note that PTX pretreatment strongly reduced the activation of I_{GIRK} by the exogenous $P2Y_1$ receptor or by the endogenous α_2-adrenoceptor but did not change $P2Y_1$-mediated inhibition of I_{GIRK} (Whole-cell mode recording in B).

Gα, which sequesters Gβγ subunits. This we have previously shown to suppress activation of I_{GIRK} mediated by M_2-muscarinic and α_2-adrenergic receptors (Fernandez-Fernandez et al., 2001) or by $P2Y_{12}$ receptors (Simon et al., 2002) in these neurons. Coexpression of transducin Gα dramatically reduced activation of I_{GIRK} mediated by $P2Y_1$ receptors (Fig. 5). Transducin Gα expression also prevented $P2Y_1$-mediated inhibition of the N-type Ca^{2+} current in these cells (data not shown). Thus, both effects involved Gβγ subunits.

Inhibition of GIRK Channels by $P2Y_4$ and $P2Y_6$ Receptors. In contrast to the response to $P2Y_1$ receptor activation, stimulation of expressed $P2Y_4$ receptors with UTP failed to activate I_{GIRK}, even under conditions in which norepinephrine produced strong and stable activation (Fig. 6, A and C). Nevertheless, UTP still produced strong inhibition of currents preactivated by norepinephrine (Fig. 6, B and D) or by Gβγ-expression (Fig. 6D). This inhibition (like that produced by $P2Y_1$ receptor activation) was insensitive to PTX (Fig. 6D). Thus, $P2Y_4$ receptors were purely inhibitory. These effects were the same when recording was in the perforated patch or the whole cell mode, despite a difference shown previously between these two recording modes with respect to $P2Y_4$-mediated Ca^{2+} current inhibition (Filippov et al., 2003). Results virtually identical to those obtained with $P2Y_4$ receptors were produced by stimulating expressed $P2Y_6$ receptors with their preferred agonist, UDP (Table 1).

On the Mechanism of P2Y-Mediated Inhibition of I_{GIRK}. The above results clearly indicate that P2Y receptors can exert an inhibitory action on I_{GIRK} and that (unlike theactivation) this is mediated by a PTX-insensitive G protein. Because all three P2Y receptors can couple to $G_{q/11}$ protein to stimulate phospholipase C, we tested the involvement of $G_{q/11}$ and its downstream pathways in this inhibitory action. Furthermore, and notwithstanding its initial stimulatory action, we concentrated on the inhibitory action of the $P2Y_1$ receptors, because this is prominently expressed in neurons in major regions of the brain (Moore et al., 2000).

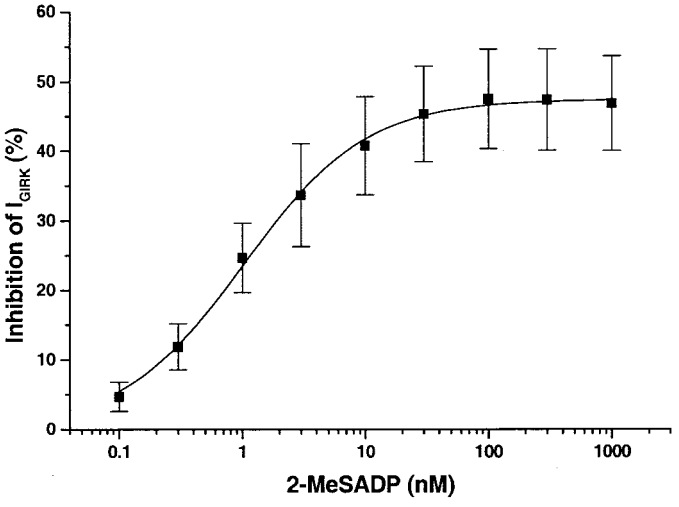

Fig. 4. $P2Y_1$ receptors couple to GIRK channels with very high potency. Dose-response curve for inhibition of I_{GIRK} by 2-MeSADP. I_{GIRK} was preactivated by prior expression of Gβγ as in Fig. 2. Currents were evoked and measured as in Fig. 1. Points show mean ± S.E.M. percentage of inhibition (n = 4) of preactivated I_{GIRK} The total amplitude of the current available for inhibition was determined by adding 1 mM Ba^{2+} at the end of the experiment. Concentrations were added cumulatively after steady-state inhibition was reached at each concentration. Curves were fitted to pooled data points using Origin 5 software to the Hill equation $y = y_{max} \times x^{nH}/(x^{nH} + K^{nH})$ where y = observed percentage inhibition, y_{max} = extrapolated maximal percentage inhibition, x = 2-MeSADP concentration (nanomolar), $K = IC_{50}$ (nanomolar), and n_H = Hill coefficient. Values of Hill constants (mean ± S.E.M.) were as follows: y_{max} = 47.5 ± 0.5%; K = 1.02 ± 0.06 nM; n_H = 0.87 ± 0.04. Cells were pretreated with PTX to prevent the initial activation (see Fig. 2).

G Proteins. We tried first to inhibit $G_q\alpha$ signaling using coexpression of RGS2 protein. RGS2 protein has been shown to interact preferentially with $G_{q/11}\alpha$ (Heximer et al., 1997) and to inhibit modulation of ion currents via receptors coupled to $G_{q/11}\alpha$ (Kammermeier and Ikeda, 1999; Melliti et al., 2001). For example, RGS2 prevented inhibition of M-current by the metabotropic glutamate receptor, mGluR1, when exogenously expressed in sympathetic neurons (Kammermeier and Ikeda, 1999) and prevented the slow inhibition of N-type Ca^{2+}-current by M1-muscarinic receptors coexpressed in HEK293 cells (Melliti et al., 2001). Coexpression of RGS2 also prevented inhibition of GIRK1/4 channel current evoked by TRH-R1 receptor stimulation in HEK293 cells (Lei et al., 2001). In our experiments, $P2Y_1$-mediated inhibition of I_{GIRK} was significantly reduced after coexpression of RGS2, such that 2-MeSADP now produced a more prolonged activation of the I_{GIRK} (Fig. 7A; compare with Fig. 1A) with only a 16% decline after 30 s (Fig. 8). In contrast, RGS2 did not change $P2Y_1$-mediated GIRK activation: currents activated by 1 μM 2-MeSADP were 0.65 + 0.09 nA (n = 15) without RGS2 and 0.93 + 0.26 nA (n = 9) with RGS2; the difference was statistically insignificant

We next tried to inhibit $G_q\beta\gamma$ signaling using coexpression of RGS11 protein. Most of the Gβγ subunits have been shown to activate GIRK channels, whereas only $\beta_5\gamma_2$ or $\beta_5\gamma_{11}$ subunits, which interact selectively with $G_q\alpha$ via β_5 (Fletcher et al., 1998), can inhibit GIRK channels (Lei et al., 2000, 2001). RGS11 contains a Gγ-like domain that binds specifically to $G\beta_5$ and prevents $\beta_5\gamma_2$-mediated voltage-dependent inhibition of the N-type Ca^{2+}-current (Zhou et al., 2000). In contrast, RGS2 and RGS4 do not have this domain and do not prevent $\beta_5\gamma_2$-mediated inhibition (Zhou et al., 2000). However, inhibition of I_{GIRK} was not affected after coexpression of RGS11 in our experiments (Fig. 8). Expression of HA-tagged RGS11 was verified by specific HA-tag antibody labeling (data not shown). Thus, these experiments indicate that $G_q\alpha$ but not βγ is responsible for $P2Y_1$-mediated inhibition of I_{GIRK}.

Phospholipase C. Pretreatment with a phospholipase C inhibitor, U73122 (1 μM for 15–30 min), strongly reduced $P2Y_1$-mediated inhibition of GIRK current (Figs. 7B and 8). However, it also dramatically reduced the preceding activation, from 0.65 ± 0.09 nA without U73122 (n = 15) to 0.11 ± 0.04 nA after pretreatment with U73122 (n = 8). This latter action seemed to be nonspecific because similar pretreatment with an isomer, U73343 (1 μM), that lacks inhibitory effect on phospholipase C totally eliminated the $P2Y_1$-mediated activation of I_{GIRK} (current amplitudes, 0 and 0.08 nA, 2 experiments). Similar nonspecific suppression of acetylcholine-induced GIRK current activation by both U73122 and U73343, which was not attributable to PLC inhibition, has been reported in atrial cells (Cho et al., 2001b).

PIP_2. It is now clearly recognized that the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is a necessary cofactor for GIRK activation and that a decrease of $PI(4,5)P_2$ levels may be responsible for inhibition of GIRK mediated by several receptors (Kobrinsky et al., 2000; Meyer

et al., 2001; for review, see (Stanfield et al., 2002). Lei et al. (2001) reported that coexpression of GFP-tagged PLCδ-PH, a Pleckstrin homology domain of PLCδ that binds with high affinity to PIP_2 and consequently sequesters PIP_2, reduces inhibition of GIRK1/4 current induced by the TRH-R1 receptor in human embryonic kidney 293 cells. In our experiments, however, coexpression of GFP-PLCδ-PH did not change the inhibition of I_{GIRK} mediated by the $P2Y_1$ receptor (Fig. 8). In contrast, GFP-PLCδ-PH significantly reduced the amplitude of I_{GIRK}, activated via $P2Y_1$ receptors, from 0.65 ± 0.09 (n = 15) to 0.19 ± 0.1 nA (n = 9). Increasing GFP-PLCδ-PH expression (by injecting 300 ng/μl cDNA instead of 100 ng/μl) completely suppressed activation of I_{GIRK} via $P2Y_1$ receptor (n = 3). These results demonstrate the importance of PIP_2 for I_{GIRK} activation but do not indicate to what extent PIP_2 hydrolysis might be responsible for its inhibition.

GFP-tagged PLCδ-PH has also been used as a sensor of PIP_2 hydrolysis and IP_3 generation (Stauffer et al., 1998; Hirose et al., 1999). Using confocal microscopy, we observed that stimulation of $P2Y_1$ receptors induced a rapid translocation of GFP-PLCδ-PH from the membrane into the cytosol (Fig. 9, A and B). The time course of translocation was similar to, or even faster than, that of I_{GIRK} inhibition (Fig. 9C), indicating that $P2Y_1$-mediated inhibition of I_{GIRK} is correlated with PIP_2 hydrolysis.

Calcium. Activation of phospholipase C causes hydrolysis of PIP_2 to IP_3 and subsequent release of intracellular Ca^{2+}. This is thought to be responsible for the inhibition of the M current in sympathetic neurons after activation of endogenous P2Y receptors, because—among other lines of evidence—inhibition was attenuated by buffering intracellular Ca^{2+} with the membrane-permeable Ca^{2+}-chelator BAPTA-AM (3 μM, Bofill-Cardona et al., 2000). However, preincubation for 1 to 4 h with 10 μM BAPTA-AM did not decrease the inhibition of I_{GIRK} measured in perforated patch mode (Fig. 8). In contrast, a similar incubation with BAPTA-AM completely prevented the increase of intracellular Ca^{2+} produced by activating endogenous nicotinic receptors (data not shown) indicating effective buffering of intracellular Ca^{2+} (see also Trouslard et al., 1993). Furthermore, $P2Y_1$-mediated inhibition of I_{GIRK} was clearly seen also in whole-cell mode with no added Ca^{2+} and 10 mM BAPTA in the pipette solution, which should effectively buffer intracellular Ca^{2+}.

Protein Kinase C. We tested the effect of the selective PKC inhibitor GF 109203X (1 μM, for 10–15 min). This treatment reduces the PKC-mediated Cl^- current in these cells (Leaney et al., 1997) but it had no significant effect on the inhibition of I_{GIRK} (Fig. 8). It is interesting to note here that GF 109203X also failed to affect TRH receptor-mediated inhibition of GIRK1/4 channels expressed in HEK 293 cells (Lei et al., 2001) or M_1 muscarinic receptor-mediated inhibition of GIRK1/4 channels expressed in *Xenopus laevis* oocytes (Hill and Peralta, 2001).

Discussion

Sympathetic neurons are useful for studying the coupling of receptors to neural ion channels because 1) they express a number of endogenous neuron-specific ion channels, 2) they also harbor a substantial complement of neuron-associated G proteins (Caulfield et al., 1994) and intracellular signaling pathways, and 3) they rapidly and readily express large cDNA constructs (see, e.g., Delmas et al., 2002). Furthermore, they show aspects of coupling selectivity through endogenous pathways that are not readily apparent from experiments on non-neural cells but that may be important for neuronal function (Fernandez-Fernandez et al., 1999).

The present experiments clearly show that P2Y receptors can exert two independent effects on GIRK currents in sympathetic neurons, through activation of different G proteins: stimulation (via a PTX-sensitive G protein) and inhibition (via a PTX-insensitive G protein). They further show that different P2Y receptors vary in their propensity to exert one or other of these two actions. Thus, $P2Y_{12}$ receptors show only stimulation (Simon et al., 2002); $P2Y_4$ and $P2Y_6$ receptors are predominantly inhibitory, whereas $P2Y_1$ receptors exert both effects. Previous experiments on oocytes suggest that $P2Y_2$ receptors might also act

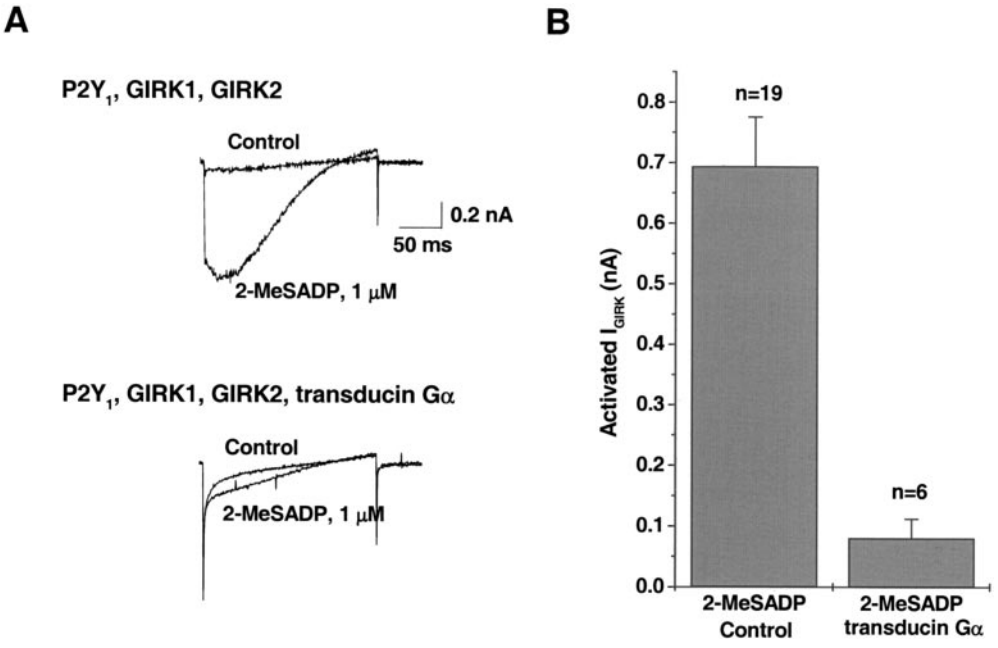

Fig. 5. $P2Y_1$-mediated I_{GIRK} activation is prevented by coexpression of a βγ-scavenger, transducin-Gα. Currents were evoked as shown in Fig. 1. Traces are control currents and maximal currents activated by $P2Y_1$ agonist 2-MeSADP (1 μM), with and without transducin-Gα coexpression. Columns show I_{GIRK} activated by 2-MeSADP (1 μM) with or without transducin Gα coexpressed. Activated current was measured as maximal current after agonist application minus current before agonist application.

like $P2Y_1$ receptors, because, in the recordings of Mark et al. (2000), the activation of the expressed I_{GIRK} by stimulating expressed $P2Y_2$ receptors can be seen to decline in the continued presence of nucleotide agonist in a manner resembling that illustrated in Fig. 1.

The relative expression of these two properties, GIRK

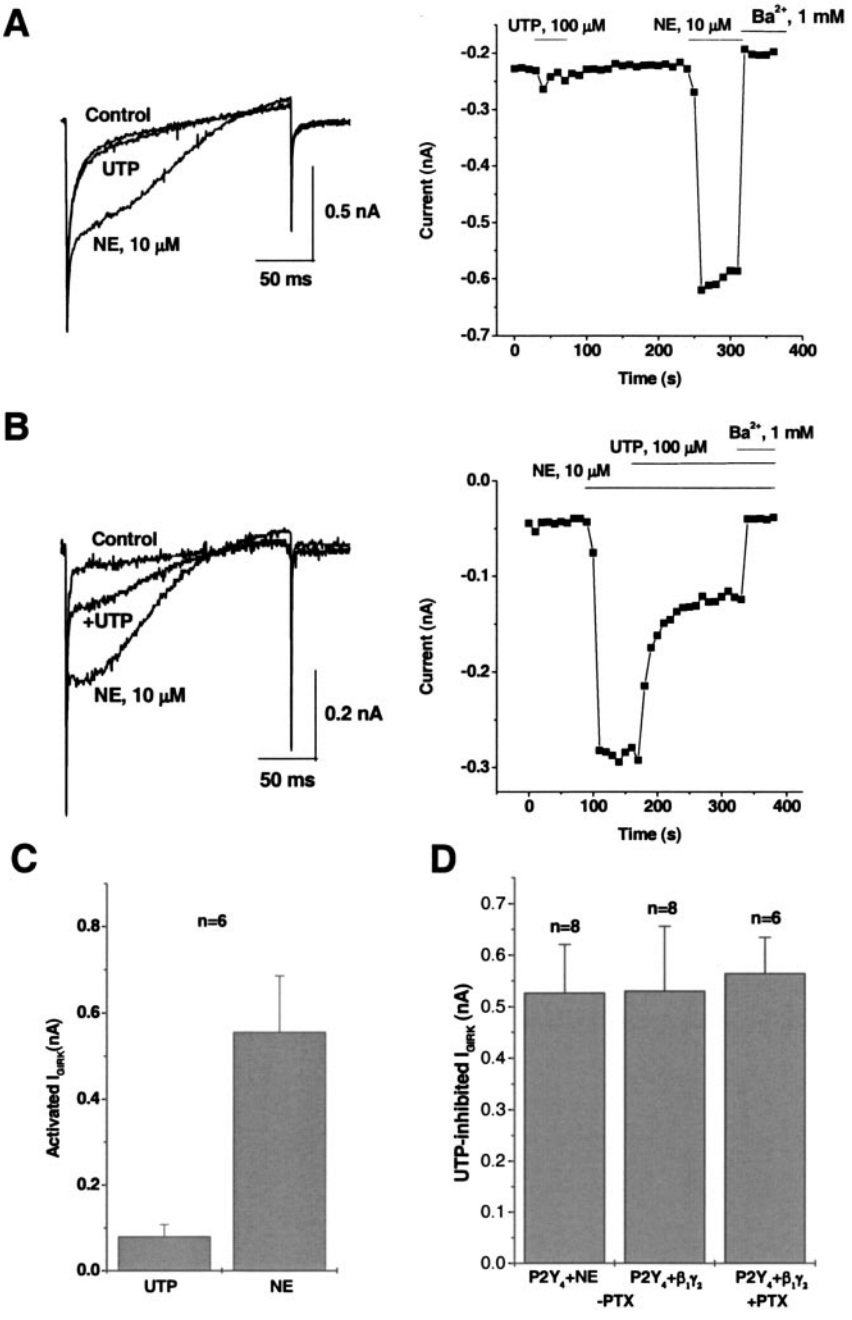

Fig. 6. Inhibition of GIRK1+GIRK2 channel current via the $P2Y_4$ receptor. Same experimental protocol as in Fig. 1 except that $P2Y_4$ cDNA was injected instead of $P2Y_1$ cDNA and UTP (100 μM) was used as agonist. A, currents recorded before and after application of UTP, followed by application of norepinephrine (NE; 10 μM). The time-plot shows absolute current values measured every 10 s (at 20 ms after ramp start). Note that UTP does not activate I_{GIRK} but norepinephrine does. B, currents recorded before and after application of norepinephrine followed by application of UTP. The time-plot shows absolute currents measured every 10 s. Note that UTP does not activate but only inhibits I_{GIRK} preactivated by norepinephrine. C, I_{GIRK} activated by UTP (100 μM) or norepinephrine (10 μM). Activated current was measured as maximal current after agonist application minus current before agonist application. D, amount of current preactivated by norepinephrine or by prior expression of G$\beta\gamma$ that is inhibited by UTP (100 μM) with or without pretreatment of the neurons with PTX. (Whole-cell mode recording.) Inhibited current was measured as maximal activated current minus current at the end of the UTP application (before Ba^{2+} was added to the solution). Note that PTX pretreatment did not change UTP-induced inhibition of I_{GIRK}. Also note that UTP produced a similar inhibition of I_{GIRK} preactivated either by norepinephrine or by coexpression of $G\beta_1\gamma_2$ subunits.

TABLE 1

Activation and inhibition of the GIRK current by agonists of P2Y nucleotide receptors co-expressed in rat sympathetic neurons with G protein-gated potassium GIRK1 and GIRK2 channel subunits

Activated current was measured as maximal current after agonist application minus current before agonist application. Inhibited current was measured as maximal preactivated current minus current at the end of 100-s agonist application.

Receptor	Agonist	GIRK Activated		GIRK Inhibited[a]	
		−PTX	+PTX	−PTX	+PTX
		nA		*pA/pF*	
$P2Y_1$	1 μM 2-MeSADP	0.65 ± 0.09 (15)	0.16 ± 0.05 (7)	13.4 ± 2.5 (10)	10.7 ± 1.4 (8)
$P2Y_4$	100 μM UTP	0.09 ± 0.03 (5)		8.3 ± 1.6 (8)	9.3 ± 1.4 (6)
$P2Y_6$	10 μM UDP	0.10 ± 0.07 (7)		9.1 ± 2.0 (6)	11.0 ± 3.0 (6)

[a] $G\beta_1$ and $G\gamma_2$ subunits were co-expressed in neurons to preactivate GIRK channels.

stimulation and inhibition, among the different receptors correlates with their effects on voltage-gated N-type Ca^{2+} and M-type K^+ channels in these neurons (Fig. 10). Thus, those receptors that solely activate I_{GIRK} ($P2Y_{12}$) also inhibit the Ca^{2+} currents without affecting M currents (Simon et al., 2002). On the contrary, $P2Y_4$ receptors, which predominantly inhibit I_{GIRK}, preferentially inhibit M currents rather than Ca^{2+} currents (Filippov et al., 2003), whereas those that both activate and inhibit GIRK currents ($P2Y_1$ and $P2Y_2$) inhibit both Ca^{2+} and M currents with approximately equal potency (Brown et al., 2000). Finally, although $P2Y_6$ receptors can inhibit the Ca^{2+} current, this is mediated by a (PTX-insensitive) G protein different from that used by $P2Y_1$, $P2Y_2$, or $P2Y_{12}$ for this connection. This emphasizes another feature of P2Y receptor signaling: that the pathway followed (Ca^{2+} channel inhibition/GIRK channel activation, M channel inhibition/GIRK channel inhibition, or both) is determined, in the first place, by the selectivity with which the individual receptors couple to the two classes of PTX-sensitive and -insensitive G proteins.

Mechanism of GIRK Activation. $P2Y_1$ -mediated activation of I_{GIRK} was PTX-sensitive and strongly inhibited by over-expressing the $G\beta\gamma$-scavenging protein, $G\alpha$-transducin. This implies that—as for GIRK-activation by α_2 adrenoceptors or M_2 muscarinic receptors in these neurons (Ruiz-Velasco and Ikeda, 1998; Fernandez-Fernandez et al., 2001)—P2Y-induced activation is mediated by $\beta\gamma$-subunits liberated from stimulated G_i protein heterotrimers. This accords with the mechanism of GIRK activation by G protein-coupled receptors deduced from many other studies (see Wickman and Clapham, 1995; Stanfield et al., 2002).

Mechanism of GIRK Inhibition. In contrast, I_{GIRK} inhibition by P2Y receptors is probably mediated by the α-subunit of $G_{q/11}$ because 1) it was attenuated by RGS2, which specifically interacts with $G\alpha_{q/11}$ (Heximer et al., 1997), and 2) was unaffected by RGS11, which interacts with $G\beta_q$ (see *Results*) (Lei et al., 2000, 2001). This corresponds with the most likely G protein-coupling required for receptor-mediated inhibition of M currents in these neurons (Haley et al., 1998; Kammermeier and Ikeda, 1999) and hence would explain the apparent relationship between these two effects among the different P2Y receptors.

$G_{q/11}$-linked receptor-mediated inhibition of GIRK channels has been reported in a number of reconstituted systems, in atrial myocytes, and in some central neurons (for review, see Stanfield et al., 2002; Wellner-Kienitz et al., 2003). Several mechanisms consequential upon $G_{q/11}$-mediated activation of phospholipase C and resultant hydrolysis of PIP_2 have been advanced: disruption of channel gating by PIP_2 as a result of the reduction in membrane PIP_2 concentrations (Kobrinsky et al., 2000; Cho et al., 2001a; Lei et al., 2001; Meyer et al., 2001; Wellner-Kienitz et al., 2003); activation of PKC by the PIP_2 hydrolysis product diacylglycerol (Takano et al., 1995; Sharon et al., 1997; Stevens et al., 1999; Hill and Peralta, 2001; Leaney et al., 2001); and release of intracellular Ca^{2+}, perhaps contributing to PKC activation (Hill and Peralta, 2001) or activating phospholipase A_2 (Rogalski et al., 1999). Release of intracellular Ca^{2+} also seems to contribute to P2Y-mediated M current inhibition in sympathetic neurons (Bofill-Cardona et al., 2000). These mechanisms are not mutually exclusive—for example, PKC activation might modify the gating of GIRK channels by PIP_2 (Cho et al., 2001a), whereas phosphorylation of GIRK channels can also modify gating by $G\beta\gamma$ (Medina et al., 2000).

Notwithstanding this, in the present experiments, we have been unable to obtain direct evidence to establish any of these mechanisms as responsible individually for P2Y-mediated GIRK inhibition. Thus, unlike the responses to TRH of GIRK channels expressed in HEK293 cells (Lei et al., 2001), P2Y-mediated inhibition of GIRK currents in sympathetic neurons was not diminished by expressing a mini-gene coding for the PH-domain of PLCδ, which sequesters PIP_2, and was not affected by the specific PKC inhibitor GF 109203X (Leaney et al., 2001) or by buffering intracellular Ca^{2+} with

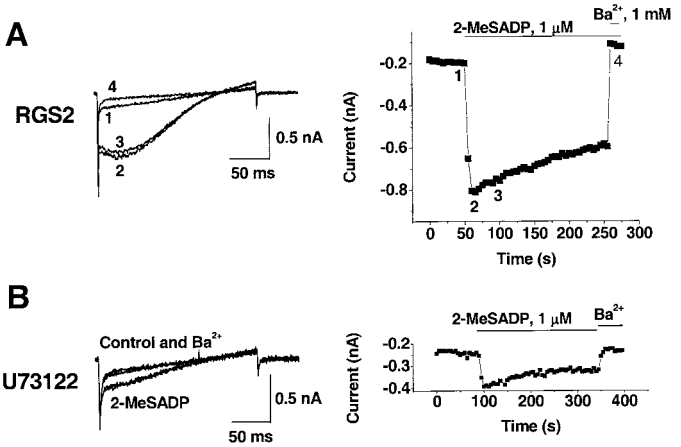

Fig. 7. Inhibition of I_{GIRK} is reduced by RGS2 protein or by an inhibitor of PLC. Records and time-plots show I_{GIRK} evoked and measured as shown on Fig. 1A after coexpression of RGS2 protein (A) and after 20 min pretreatment with the PLC inhibitor, U73122 (1 μM) (B). Traces shown are: 1, control; 2, maximal I_{GIRK} activated; 3, current recorded 30 s after maximal activation; and 4, current recorded after adding 1 mM Ba^{2+}. Note that in both cases, little inhibition can be seen 30 s after maximal activation of I_{GIRK} (compare with Fig. 1A). Note also that U73122 reduced GIRK activation.

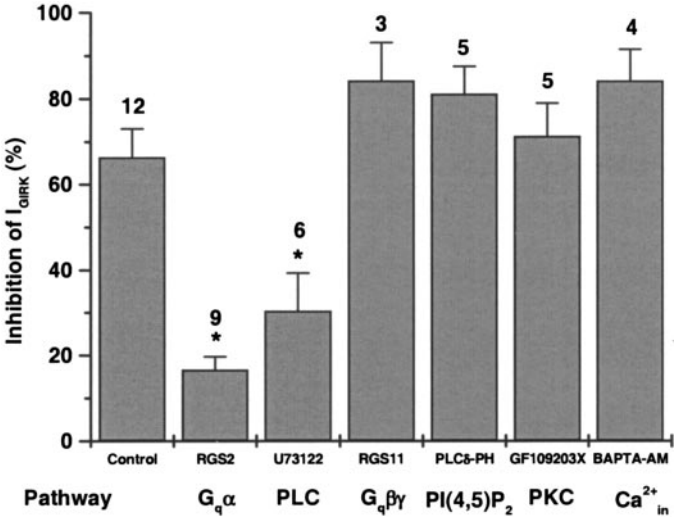

Fig. 8. $G_q\alpha$ pathway mediates inhibition of I_{GIRK} by the $P2Y_1$ receptor. Columns show percentage inhibition of I_{GIRK} (mean ± S.E.M.,), measured as percentage decline from the peak current recorded 30 s after maximal activation by 2-MeSADP (1 μM) (e.g., see point 3 in Fig. 7A). Note that coexpression of the RGS2 protein, which inhibits $G_q\alpha$, significantly reduced $P2Y_1$ mediated inhibition of I_{GIRK}. A similar effect is observed after pretreatment of the neurons with the PLC inhibitor U73122. *, significantly different from control, $P < 0.05$.

BAPTA-AM. The latter two effects were likewise found lacking in the system of Lei et al. (2001) noted above. Furthermore, although the PLC inhibitor U73122 did suppress inhibition by $P2Y_1$ receptor activation [as reported previously for nucleotide-mediated M current inhibition in these neurons (Bofill-Cardona et al., 2000)], interpretation of this is complicated by the facts that U73122 also reduced GIRK activation [through an effect unrelated to PLC (Cho et al., 2001b)] and that the inactive isomer U73343 totally suppressed I_{GIRK} and hence could not be tested against P2Y-mediated inhibition.

Nevertheless, the fact that expression of PLCδ-PH strongly reduced the amount of current activated by stimulating $P2Y_1$ receptors does suggest that in these cells, as in others, PIP_2 is required for effective GIRK channel opening. Furthermore, the membrane-to-cytosol translocation of the GFP-tagged PLCδ-PH that accompanies $P2Y_1$-mediated GIRK-inhibition also suggests an intimate connection between PIP_2 hydrolysis and current inhibition. The lack of effect of PIP_2 sequestration by the PLCδ-PH construct on P2Y-mediated inhibition might not necessarily negate membrane PIP_2 hydrolysis/depletion as the cause of GIRK inhibition. Thus, if the individual channels required the binding of one (or a very few) PIP_2 molecules to attain the open state, then channels from which PIP_2 had dissociated (and were therefore closed) after PIP_2 sequestration could not be further closed by P2Y-induced PIP_2 hydrolysis. On the other hand, those that retained bound PIP_2 in the presence of PLCδ-PH would also retain their normal sensitivity to P2Y receptor stimulation.

Channel closure as a consequence of PIP_2 hydrolysis might contribute to the higher agonist potency against GIRK channels (Fig. 4) than when measured by GTPase hydrolysis (Waldo and Harden, 2004), because each G_q-activated PLC molecule will hydrolyze many PIP_2 molecules, thereby amplifying the G protein signal.

Functional Significance. Some P2Y receptors—most notably, $P2Y_1$—are strongly expressed in the brain and in neurons therein (see Introduction). Furthermore, $P2Y_1$ receptors are exquisitely sensitive to adenine nucleotides, with EC_{50} values in the low nanomolar range (Fig. 4) (Filippov et al., 2000), so they should be readily activated after the release of ATP from neurons or glial cells and its subsequent hydrolysis. Activation of GIRK channels would lead to neuronal hyperpolarization and inhibition, in the manner produced by a variety of neurotransmitters (North, 1989), whereas inhibition of GIRK channels could depolarize neurons and increase their excitability, in the manner produced by, for

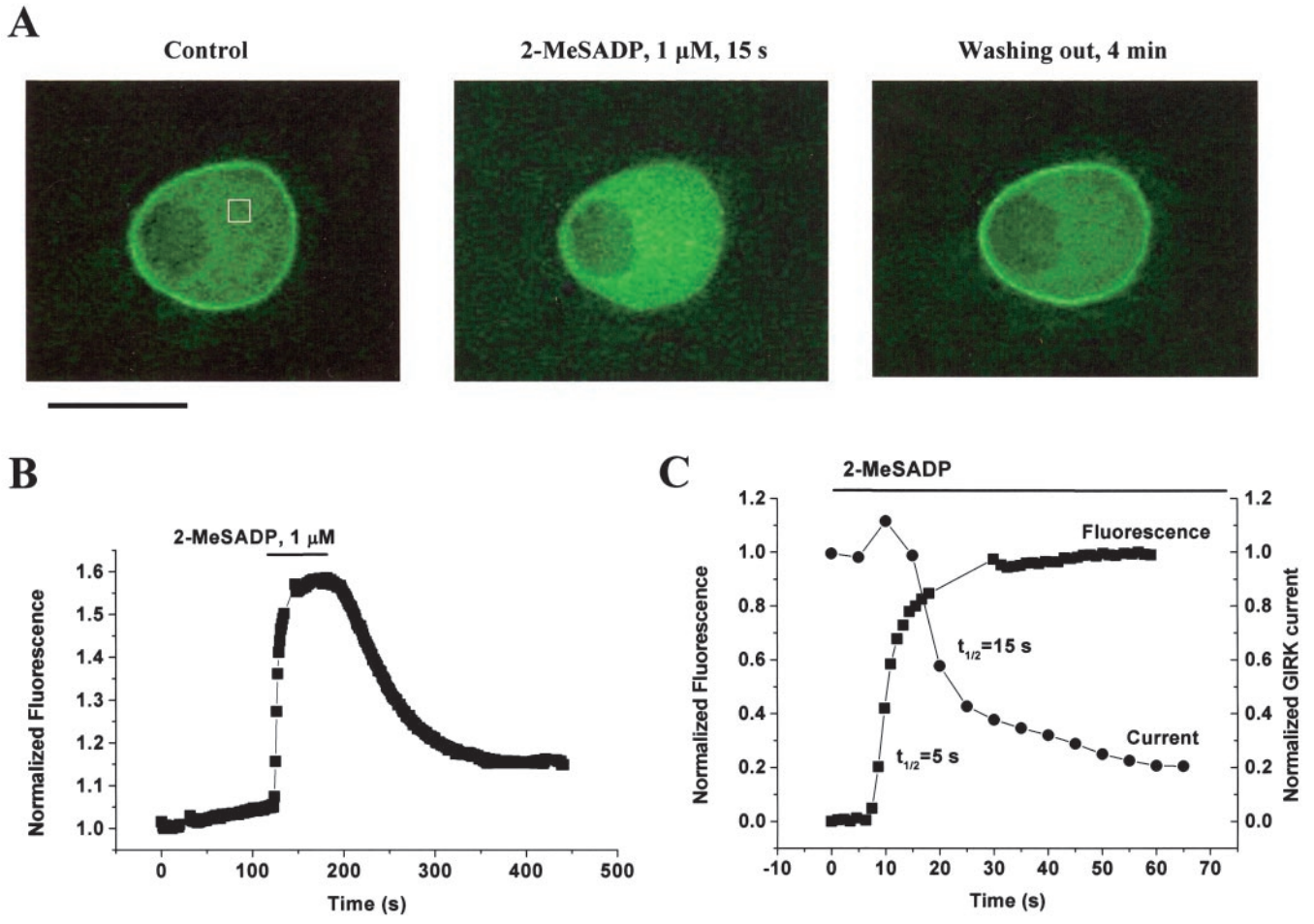

Fig. 9. Concurrent stimulation of PIP_2 hydrolysis and inhibition of I_{GIRK} mediated by stimulating the $P2Y_1$ receptor. $P2Y_1$ receptor activation by 1 μM 2-MeSADP induced a translocation of the coexpressed PIP_2 indicator GFP-PLCδ-PH from the plasma membrane to the cytosol. The images shown in A were recorded before (control), 15 s after addition of 2-MeSADP, and 4 min after washing out the agonist. Scale bar, 10 μM. B, relative fluorescence intensity in the cytosol (measured in the white box marked in the left image in A) plotted against time. C, representative examples of the time course of current inhibition (●) and increased cytosolic GFP-PLCδ-PH fluorescence (■) after application of 1 μM 2-MeSADP to cells in which the I_{GIRK} had been preactivated with 10 μM norepinephrine. Note that the time course of translocation matched that of I_{GIRK} inhibition.

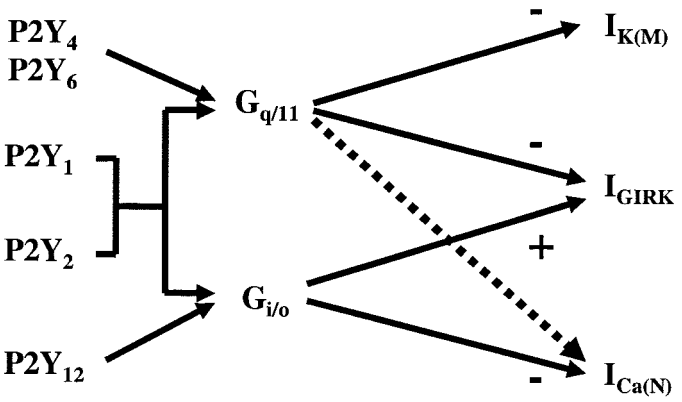

Fig. 10. Simplified schematic of the principal pathways for P2Y receptor coupling to neural ion channels in sympathetic neurons. −, inhibition; +, activation; interrupted line, pathway present for $P2Y_6$ in perforated-patch recording, not in whole-cell recording.

example, substance P (Stanfield et al., 2002), or might powerfully oppose the activation of these channels by other transmitters (Velimirovic et al., 1995). Adenine nucleotides have been reported to activate K^+ channels in a variety of central neurons (Nishizaki and Mori, 1998) and to inhibit K^+ currents in *X. laevis* spinal neurons (Brown and Dale, 2002) via P2Y receptors. However, these currents and channels were outwardly rectifying and the species of P2Y receptor involved is unclear. The present results suggest that further investigations into the effects of P2Y receptor stimulation on neural GIRK channels should be informative.

Acknowledgments

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