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$P2Y_2$ Nucleotide Receptors Expressed Heterologously in Sympathetic Neurons Inhibit Both N-Type Ca^{2+} and M-Type K^+ Currents

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The $P2Y_2$ receptor is a uridine/adenosine triphosphate (UTP/ATP)-sensitive G-protein-linked nucleotide receptor that previously has been reported to stimulate the phosphoinositide signaling pathway. Messenger RNA for this receptor has been detected in brain tissue. We have investigated the coupling of the molecularly defined rat $P2Y_2$ receptor to neuronal N-type Ca^{2+} channels and to M-type K^+ channels by heterologous expression in rat superior cervical sympathetic (SCG) neurons. After the injection of $P2Y_2$ cRNA, UTP inhibited the currents carried by both types of ion channel. As previously reported [Filippov AK, Webb TE, Barnard EA, Brown DA (1997) Inhibition by heterologously expressed $P2Y_2$ nucleotide receptors of N-type calcium currents in rat sympathetic neurones. Br J Pharmacol 121:849–851], UTP inhibited the Ca^{2+} current ($I_{Ca(N)}$) by up to 64%, with an IC_{50} of ~0.5 μM. We now find that UTP also inhibited the K^+_M current ($I_{K(M)}$) by up to 61%, with an IC_{50} of ~1.5 μM. UTP had no effect on either current in neurons not injected with $P2Y_2$ cRNA. Structure–activity relations for the inhibition of $I_{Ca(N)}$ and $I_{K(M)}$ in $P2Y_2$ cRNA-injected neurons were similar, with UTP $\geq$ ATP $>$ ITP $\gg$ GTP,UDP. However, coupling to these two channels involved different G-proteins: pretreatment with *Pertussis* toxin (PTX) did not affect UTP-induced inhibition of $I_{K(M)}$ but reduced inhibition of $I_{Ca(N)}$ by ~60% and abolished the voltage-dependent component of this inhibition. In unclamped neurons, UTP greatly facilitated depolarization-induced action potential discharges. Thus, the single $P2Y_2$ receptor can couple to at least two G-proteins to inhibit both Ca^{2+}_N and K^+_M channels with near-equal facility. This implies that the $P2Y_2$ receptor may induce a broad range of effector responses in the nervous system.

Key words: nucleotide receptors; uridine triphosphate; adenosine triphosphate; sympathetic neurons; calcium currents; potassium currents; M currents

Nucleotides such as ATP play a significant neurotransmitter role in the mammalian nervous system (Burnstock, 1972, 1990; Edwards and Gibb, 1993; Zimmermann, 1994). There are two families of target nucleotide receptors known at the molecular level—ligand-gated P2X receptors and G-protein-coupled P2Y receptors (North and Barnard, 1997).

The $P2Y_2$ receptor is a member of the family of P2Y G-protein-coupled receptors and is sensitive to both ATP and UTP; it was cloned originally from mouse NG108-15 neuroblastoma X glioma hybrid cells (Lustig et al., 1993). In common with other P2Y receptors (Boarder et al., 1995), $P2Y_2$ receptors from different species all couple to the enzyme phospholipase C (PLC), thereby increasing inositol phosphate production and elevating intracellular [Ca^{2+}] (Erb et al., 1993; Lustig et al., 1993; Parr et al., 1994; Rice et al., 1995; Chen et al., 1996; Nicholas et al., 1996).

Messenger RNA for the $P2Y_2$ receptor, which is found in a range of tissues, is also present in the brain (Lustig et al., 1993), so the question arises as to what effect the activation of this receptor might have on neural function. In native NG108-15 cells, UTP inhibits two membrane ionic currents—an M-like K^+ current ("M-current") and the voltage-gated Ca^{2+} current (Filippov et al., 1994; Filippov and Brown, 1996). The former was to be expected because other receptors that activate PLC inhibit M-currents (Brown, 1988), but Ca^{2+} current inhibition was unexpected, especially because it was mediated (in part, at least) by a different G-protein from that responsible for M-current inhibition (Filippov and Brown, 1996). This raised the question whether both effects actually were produced by the same receptor as that previously cloned from these cells or whether two different receptors were responsible. If the former, was this a peculiarity of this particular cell line, or would it also hold for primary neurons?

To address these questions, we have expressed the recombinant rat $P2Y_2$ receptor in primary cultured rat superior cervical sympathetic (SCG) neurons by microinjecting cRNA, in the manner used by Ikeda et al. (1995) to express heterologous "metabotropic" glutamate receptors, and then recording the effects of activating these exogenous receptors with UTP on the N-type Ca^{2+} currents ($I_{Ca(N)}$; Hirning et al., 1988; Plummer et al., 1989; Regan et al., 1991) and M-type K^+ currents ($I_{K(M)}$; Constanti and Brown, 1981) that are present in these neurons. In preliminary experiments (Filippov et al., 1997) we found that the activation of these expressed receptors did indeed inhibit $I_{Ca(N)}$. In the present experiments we have analyzed this action in more detail and have gone on to test whether the same single receptor also can inhibit the M-type K^+ current. We find that it can; the two currents are inhibited by UTP with near-equal potencies and efficacy, although mediated mainly by different G-proteins, and in joint response increase the excitability of these neurons. Thus, the $P2Y_2$ receptor appears to be unusually promiscuous in terms of its

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coupling to mammalian neuronal G-proteins and ion channels. This has interesting implications for its potential function in nerve cells.

MATERIALS AND METHODS

cRNA preparation. The rat $P2Y_2$ receptor cDNA was obtained from Dr. Zeng-Ping Chen (Department of Neuroscience and Cell Biology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ). A 2 kb *Eco*RI–*Xho*I fragment was cloned into the reciprocal sites of pBK/CMV. The wild-type green fluorescent protein (GFP) cDNA, in pBluescript, also was used. Supercoiled plasmid DNA was prepared from both constructs and linearized with *Xho*I for $P2Y_2$ and with *Eco*RI for GFP before use as a template for cRNA synthesis. Capped cRNA was transcribed with T3 polymerase (Message Machine, Ambion, Austin, TX), and an aliquot of each was analyzed on a denaturing agarose gel to check its integrity before polyadenylation, using poly(A^+) polymerase (Sigma, St. Louis, MO) according to the manufacturer's recommendations. After extractions in turn with phenol, phenol/chloroform, and chloroform and isopropanol precipitation, the cRNA was stored as an ethanol precipitate at −70°C before use.

Neuron preparation and cRNA injection. Single SCG neurons were dissociated from rats aged 15–19 d, and plated on poly-D-lysine-coated glass coverslips bordered by 2 cm plastic rings as previously described (Marrion et al., 1987). At 4 hr after plating, the neurons were microinjected with an equal mixture of cRNA for the $P2Y_2$ receptor (final pipette concentration of 0.5 or 1.25 μg/μl dissolved in water) and cRNA for GFP (used as a marker for foreign cRNA expression; Marshall et al., 1995) or, for controls, with GFP cRNA alone. cRNA solution (1.2 μl) was loaded into prepulled high-resistance (~30 MΩ) Pyrex glass pipettes and injected manually into single neurons by the application of gentle pressure to the back of the pipette with a syringe as described previously for injection of antisera (Caulfield et al., 1994). Successful injection usually resulted in a ~10% increase in cell volume (Ikeda et al., 1995). After injections, cells were incubated for 14–24 hr in a humidified incubator (5% CO_2/95% O_2) at 37°C. Injected neurons that successfully expressed cRNA were identified as bright fluorescent cells, using an inverted microscope (Diaphot 200, Nikon, Tokyo, Japan) equipped with an epifluorescent N B2E block (Nikon). When required, *Pertussis* toxin (PTX) at a final concentration of 500 ng/ml was added to the culture media 1–2 hr after neuron injection. Electrophysiological recordings were made 14–24 hr after injection at room temperature (20°C). Some experiments on neuron excitability were made at 34°C.

Ca^{2+} channel current recording. Currents through voltage-gated Ca^{2+} channels were recorded by the conventional whole-cell patch-clamp method as described previously (Caulfield et al., 1994). Cells were superfused (20–25 ml/min) with a solution consisting of (in mM) 120 tetraethylammonium chloride, 3 KCl, 1.5 $MgCl_2$, 5 $BaCl_2$ (or 5 $CaCl_2$), 10 HEPES, and 11.1 glucose plus 0.5 μM tetrodotoxin. The pH was adjusted to 7.35 with NaOH. Patch electrodes (2–3 MΩ) were filled with a solution containing (in mM) 110 CsCl, 3 Mg Cl_2, 40 HEPES, 3 EGTA, 2 Na_2ATP, and 0.5 Na_2GTP (pH-adjusted to 7.4 with CsOH). Neurons were voltage-clamped with a discontinuous ("switching") amplifier (Axoclamp 2B) with a sampling voltage at 6–8 kHz (50% duty cycle). Commands were generated via a Digidata 1200 interface, using pClamp 6 computer software (Axon Instruments, Foster City, CA). Ca^{2+} channel currents were evoked routinely every 20 sec with a 100 msec depolarizing rectangular test pulse to 0 mV from a holding potential of −90 mV. To obtain current–voltage (*I–V*) relations, we evoked currents by test pulses in 10 mV increments to +40 mV, starting from the holding potential of −90 mV. Where required, *I–V* relations were obtained by using 750 msec ramp depolarizations from −90 to + 40 mV (see Docherty et al., 1991). Currents were digitized and stored on a computer for later analysis by pClamp 6 software (Axon Instruments). Ca^{2+} channel current amplitudes were measured isochronally 10 msec from the onset of the rectangular test pulse (Ikeda et al., 1995), i.e., near to the peak of the control current. To eliminate leak currents, we substituted Co^{2+} for Ca^{2+} and Ba^{2+} in the external solution at the end of each experiment to block all Ca^{2+} channel currents, and we digitally subtracted the residual current from the corresponding currents in Ca^{2+} or Ba^{2+} solution.

M-type K^+ current recordings. Whole-cell M-currents ($I_{K(M)}$) were recorded by the perforated patch-clamp method (Horn and Marty, 1988), as described for the application to SCG neurons by Caulfield et al. (1994). Briefly, patch pipettes (2–4 MΩ) were filled by dipping the tip into a filtered solution containing (in mM) 90 potassium acetate, 20 KCl, 3 $MgCl_2$, 40 HEPES, and 0.1 BAPTA (pH-adjusted to 7.4 by KOH) for 20–60 sec. Then the pipette was back-filled with the same solution containing 0.125 mg/ml amphotericin B as the permeabilizing agent (Rae et al., 1991). Access resistance after permeabilization was 8–15 MΩ. Neurons were superfused (20–25 ml/min) with external modified Krebs' solution containing (in mM) 120 NaCl, 3 KCl, 1.5 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, and 11.1 glucose (pH-adjusted to 7.3 with NaOH). Neurons were voltage-clamped at −20 or −30 mV with a switching amplifier, and M-currents were deactivated with 1 sec hyperpolarizing steps at 5 sec intervals. *I–V* relationships were obtained by using incremental voltage steps of 10 mV between −10 and −100 mV; currents were measured at the end of each hyperpolarizing step. For dose–response curves, currents were measured at −30 mV from steady-state *I–V* relations obtained by using a ramp voltage command of 20 sec from −20 to −90 mV. The leak component of current was estimated in both cases by extrapolating a linear fit to the *I–V* relationship from the negative potential region, where only ohmic currents were observed. All commands, current recordings, and analyses were made with Digidata 1200 interface and pClamp 6 software (Axon Instruments).

Statistical analysis. Data are presented as mean ± SEM as appropriate. Student's *t* test (unpaired) was applied to determine statistical significance. The difference was considered significant if $p \leq 0.05$. Dose–response curves were determined by using concentrations that were added cumulatively, with 1 min exposure times. Curves were fit (using Origin 4.1 software) to pooled data points to the Hill equation: $y = y_{max} \cdot x^{nH}/(x^{nH} + K^{nH})$, where y = the observed percentage of inhibition, y_{max} = extrapolated maximal percentage of inhibition, x = nucleotide concentration (μM), $K = IC_{50}$ (μM), and n_H = the Hill coefficient.

Chemicals. UTP rather than ATP was used as the main agonist throughout to preclude the activation of ATP-sensitive endogenous P2X ligand-gated channels (Cloues et al., 1993). Drugs were applied to the external solution by bath perfusion (bath exchange rate ≤5 sec). Tetrodotoxin was obtained from Calbiochem (La Jolla, CA); uridine 5′-triphosphate (UTP) was from Pharmacia Biotech (Uppsala, Sweden) and from Sigma; ATP, inosine 5′-triphosphate (ITP), guanosine 5′-triphosphate (GTP), acetylcholine chloride, (−)-norepinephrine bitartrate, nifedipine, BAPTA, and amphotericin B were all from Sigma; adenosine 5′-diphosphate (ADP) and uridine 5′-diphosphate (UDP) were from Sigma and Boehringer Mannheim GmbH (Mannheim, Germany); hexokinase was from Boehringer Mannheim GmbH; oxotremorine-M (OxoM) was from Research Biochemicals (Natick, MA); *Pertussis* toxin (PTX) was from Porton Products (Dorset, UK); $CdCl_2$ (AnalaR grade) was from BDH Chemicals (Poole, UK); $BaCl_2$ and CsCl were from Aldrich (Milwaukee, WI). Nifedipine was prepared as a stock solution (10 mM) in ethanol and protected from light during storage and use.

RESULTS

Ca^{2+} channel current inhibition

We have reported previously that, in SCG neurons preinjected with 1.25 μg/μl $P2Y_2$ cRNA (together with GFP cRNA), the application of UTP produced a reversible inhibition of the Ca^{2+} channel current by up to 64.0 ± 0.8%, with an IC_{50} of 0.50 ± 0.03 μM and that, at 0.5 μg/μl $P2Y_2$ cRNA, UTP inhibited the current by up to 50.2 ± 0.6%, with an IC_{50} 0.90 ± 0.05 μM (Filippov et al., 1997). Because no significant inhibition was detected on applying 100 μM UTP to neurons injected with GFP cRNA alone, this effect could be attributed entirely to the activation of newly expressed $P2Y_2$ receptors and not to the activation of any endogenous UTP-sensitive receptors that might have been present (see below).

Voltage dependence

Ca^{2+} current inhibition in SCG neurons produced by activation of some endogenous receptors (see Hille, 1994) or by heterologously expressed mGluR2 receptors (Ikeda et al., 1995) is voltage-dependent—that is, it is reduced at depolarized commands (or by predepolarization; Grassi and Lux, 1989) and is accompanied by "kinetic slowing" resulting from time-dependent

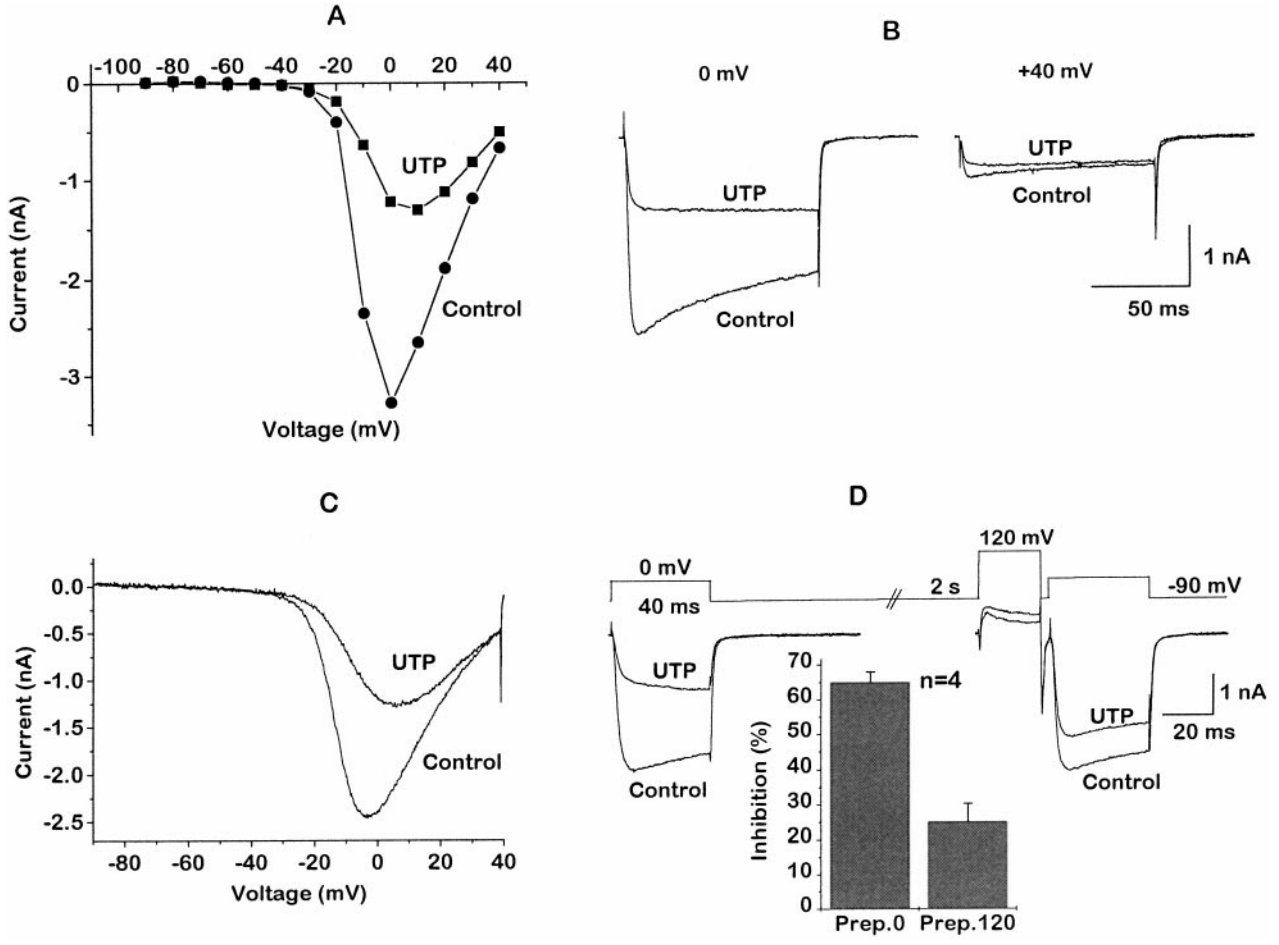

Figure 1. Voltage-dependent inhibition of Ca^{2+} channel currents by UTP in rat SCG neurons expressing heterologous $P2Y_2$ receptors. Neurons were preinjected with 1.25 μg/μl $P2Y_2$ cRNA. Records show leak-subtracted Ca^{2+} channel currents recorded at room temperature (20°C), using the whole-cell (ruptured patch) variant of the patch-clamp technique with 5 mM Ba^{2+} as the charge carrier (I_{Ba}; see Materials and Methods). *A*, I_{Ba} amplitude plotted against membrane voltage (*I–V* relationship) in the absence (*filled circles*) and presence (*filled squares*) of 100 μM UTP. Currents were evoked by 10 mV incrementing test pulses, starting from a holding potential of −90 to +40 mV; amplitudes were measured 10 msec from the onset of the test pulse. *B*, Superimposed currents from *A* at 0 and +40 mV test potentials in the absence and presence of UTP. Note that the inhibition is less at +40 than at 0 mV. *C*, Superimposed currents recorded with 750 msec depolarizing voltage ramps from −90 to + 40 mV (see Materials and Methods) in the absence and presence of 100 μM UTP. Note that the inhibition is less at more positive voltages. *D*, Superimposed currents (*lower traces*) recorded with a double-pulse voltage protocol (*upper traces*) in the absence and presence of 100 μM UTP. The current was recorded first with a 40 msec test pulse to 0 mV; then, after a 2 sec interval, a 25 msec conditioning prepulse to +120 mV was applied, followed 4 msec later by a second 40 msec test pulse to 0 mV. The bar chart at the *bottom* shows the mean percentage of current inhibition (measured after 10 msec at 0 mV command potential) by 100 μM UTP before (*Prep.0*) and after (*Prep.120*) the +120 mV prepulse. Error bars show SEM; n = number of cells. Note that the inhibition is much less after the prepulse. Note also that the prepulse abolished the slowing of the current onset at 0 mV produced by UTP.

relief of block during the depolarizing command (Bean, 1989). Figure 1 shows that the block produced by activating heterologously expressed $P2Y_2$ receptors shares this property. Thus, current–voltage curves constructed by using either stepped (Fig. 1*A,B*) or ramped (Fig. 1*C*) commands showed a greater inhibition by UTP at negative potentials than at positive potentials (resulting in a positive shift of the current peak). For example, in Figure 1*A* peak current inhibition was reduced from ~63% at 0 mV to ~26% at +40 mV. Also, as shown in Figure 1, *B* and *D*, current activation was slowed in the presence of UTP, such that inhibition was less at the end of the 40 msec command than at the beginning. Finally, inhibition was reduced (from 65.0 ± 3.1 to 24.9 ± 5.4%) and the slowing of current activation was abolished when the test command was preceded by a 20 msec depolarizing prepulse to +120 mV (Fig. 1*D*). Such effects have been interpreted to indicate that the activated G-protein (probably the $\beta\gamma$-subunit; Herlitze et al., 1996; Ikeda, 1996; Delmas et al., 1998a,b) interacts directly with the Ca^{2+} channel protein to induce a gating shift (Dolphin, 1995; Jones and Elmslie, 1997).

Pertussis *toxin distinguishes two pathways for $P2Y_2$-mediated Ca^{2+} channel current inhibition*

Voltage-dependent Ca^{2+} current inhibition of the type illustrated in Figure 1 is usually (although not invariably; Ehrlich and Elmslie, 1995) associated with activation by the receptor of a G-protein of the *Pertussis* toxin-sensitive G_i/G_o family (Hille, 1994). We tested whether this applied to expressed $P2Y_2$ receptors by overnight incubation of injected neurons with 0.5 μg/ml *Pertussis* toxin (PTX). In cells preinjected with 1.25 μg/μl $P2Y_2$ cRNA, PTX substantially (~61%) but incompletely reduced the inhibition produced by 10 μM UTP (Fig. 2*A*). In contrast, in the same neurons the inhibition produced by 10 μM norepinephrine (which is mediated primarily by G_o; Caulfield et al., 1994; Del-

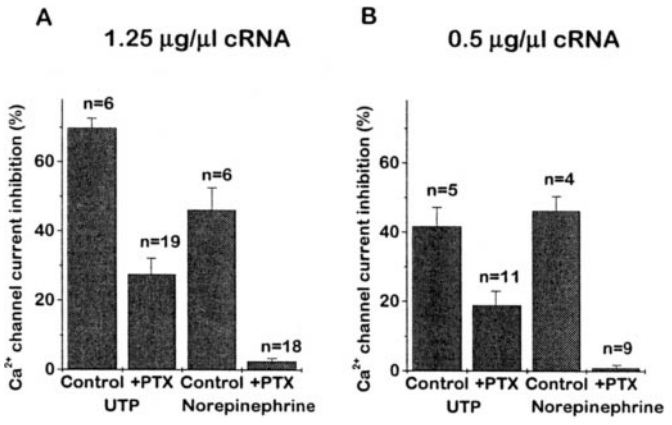

Figure 2. *Pertussis* toxin (*PTX*) distinguishes two pathways for $P2Y_2$-mediated Ca^{2+} channel current inhibition. The bar charts show the mean inhibition of I_{Ba} amplitude by 10 μM UTP and by 10 μM norepinephrine in neurons pretreated with PTX (0.5 μg/ml, overnight; *+PTX*) and in PTX-untreated neurons (*Control*). Error bars show SEM; n = number of cells. Neurons were injected with 1.25 μg/μl $P2Y_2$ cRNA (*A*) or 0.5 μg/μl $P2Y_2$ cRNA (*B*). Currents were recorded by stepping for 100 msec from −90 to 0 mV and measured 10 msec from the onset of the test pulse. Note that PTX pretreatment completely prevented the effect of norepinephrine, but not that of UTP.

mas et al., 1998a) was reduced by >90%, as previously reported (Beech et al., 1992; Chen and Schofield, 1993; Caulfield et al., 1994; Delmas et al., 1998a), thus indicating the effectiveness of the PTX treatment. Nevertheless, because the initial inhibition produced by UTP exceeded that produced by norepinephrine, we were concerned that the substantial component of PTX-resistant inhibition might have resulted from overexpression (and aberrant coupling) of the $P2Y_2$ receptors. This appeared not to be the case, because PTX produced a comparable degree of attenuation (−54%) of the response to UTP in cells preinjected with 0.5 μg/μl $P2Y_2$ cRNA, although the initial inhibition produced by UTP (41.6 ± 5.5%) was now less than that produced by norepinephrine (46.1 ± 4.2%; Fig. 2*D*).

In contrast to the partial antagonism of overall inhibition, PTX pretreatment virtually eliminated the voltage dependence of $P2Y_2$-mediated inhibition; the characteristic slowing of current activation by UTP (compare with Fig. 1) was no longer apparent after PTX treatment (Fig. 3*A*), and the +120 mV depolarizing prepulse did not reverse the inhibition significantly (Fig. 3*B,C*). Thus, the PTX-insensitive component of block also appeared to be voltage-insensitive.

One possible explanation for the PTX-resistant voltage-insensitive component of inhibition is that it reflects the inhibition of an L-type current, rather than the N-type current (Hille, 1994). This possibility is enhanced by previous observations that UTP inhibits both L-type and N-type currents in NG108-15 cells (Filippov and Brown, 1996). To check this, we tested the effect of 2–10 μM nifedipine on the response to UTP of PTX-pretreated SCG neurons preinjected with 1.25 μg/μl $P2Y_2$ cRNA. In agreement with previous observations on non-PTX-treated cells (Filippov et al., 1997), nifedipine itself did not produce any significant inhibition of the current (−2.5 ± 1.7%; n = 13), nor did it affect the inhibitory action of UTP (−nifedipine, −29.4 ± 3.5%; +nifedipine, −27.3 ± 4.25%; n = 6). Thus, the residual PTX-insensitive block is not attributable to the inhibition of L-type channels.

Coupling of $P2Y_2$ receptors to M-type K^+ currents

M-currents are sustained voltage-gated K^+ currents that are activated when SCG neurons are depolarized above −70 mV (Constanti and Brown, 1981) (for review, see Brown, 1988). They can be inhibited by activating endogenous M_1-muscarinic acetylcholine receptors (Marrion et al., 1989; Bernheim et al., 1992), angiotensin receptors (Shapiro et al., 1994), or bradykinin receptors (Jones et al., 1995)—in all cases via PTX-insensitive G-proteins (probably G_q; see Caulfield et al., 1994; Jones et al., 1995).

There is some evidence for the presence of endogenous P2Y receptors in intact SCGs [Connolly et al. (1993); Boehm et al. (1995) and personal communication; Connolly and Harrison (1995); Von Kugelgen et al. (1997)]. However, we found that UTP did not affect the M-current significantly in the uninjected dissociated neurons that we have used, although some other nucleotides did (see below and Fig. 7*A*). We therefore have tested whether the activation of heterologously expressed $P2Y_2$ receptors inhibits the M-current in these neurons, and, if so, how this compares with inhibition of Ca^{2+} current.

Figure 4 illustrates the effect of UTP on M-currents in cells preinjected with 1.25 μg/μl $P2Y_2$ cRNA. The cell was predepolarized to −20 mV to preactivate the M-current and then hyperpolarized in steps of −10 mV for 1 sec each to deactivate the current; deactivation is signaled by the inward tail currents, which reverse at E_K (approximately −90 mV). UTP (10 μM) clearly inhibited the M-current; this is indicated by (1) the inward shift in holding current at −20 mV (reflecting the reduction in outward K^+ current), (2) reduced current responses to depolarizing steps (reduced conductance) and the loss of M-current deactivation tails, and (3) reduced outward rectification in the current–voltage curve positive to −70 mV, with no change in slope negative to −70 mV (indicating no change in "leak" current).

M-current inhibition was quantitated by using voltage ramps to obtain current–voltage curves (see Fig. 6) and then subtracting from the total outward current at −30 mV the extrapolated linear leak currents from potentials negative to −70 mV. UTP (10 μM) inhibited the M-current by 60.8 ± 9.6% (n = 8) in neurons preinjected with 1.25 μg/μl $P2Y_2$ cRNA. As noted above, UTP had no significant effect at 100 μM (0.6 ± 0.4% inhibition) in five noninjected cells (Fig. 5*A*). In four of these same cells the activation of the endogenous muscarinic acetylcholine receptors with 10 μM oxotremorine-M (OxoM) inhibited the current by 55.4 ± 11.4%, in accordance with previous observations (see Caulfield et al., 1994), indicating that the endogenous transduction machinery was intact.

With the use of increasing concentrations of UTP, mean IC_{50} values and extrapolated maximum inhibitions were 1.49 ± 0.18 μM and 48.0 ± 1.2%, respectively, after 1.25 μg/μl $P2Y_2$ cRNA was injected, and 2.41 ± 0.43 μM and 43.2 ± 1.9% after 0.5 μg/μl $P2Y_2$ cRNA was injected (Fig. 6). These IC_{50} values are approximately three times higher than those determined for N-type Ca^{2+} current inhibition (Filippov et al., 1997) (superimposed curves in Fig. 6). The lower apparent maximum inhibition with increasing UTP concentrations than those in Figure 5 may reflect some degree of desensitization (apparent as a slow partial recovery of M-current during prolonged application of UTP); the extent to which this affected IC_{50} estimates is unclear.

In contrast to its effect on Ca^{2+} current inhibition, PTX produced no significant attenuation of UTP-induced M-current inhibition (Fig. 5*B*). Also, inhibition of M-current showed no

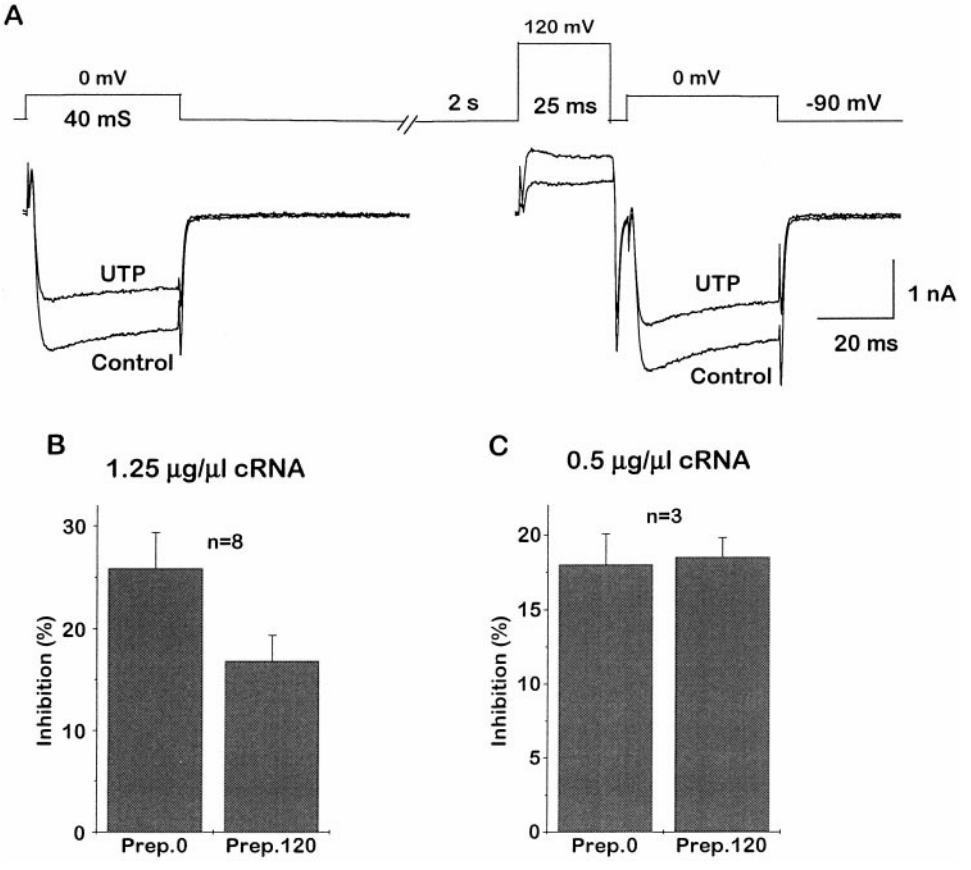

Figure 3. PTX pretreatment eliminates the voltage dependence of $P2Y_2$-mediated Ca^{2+} channel current inhibition. Cells were pretreated with 500 ng/ml PTX. Records in *A* show superimposed I_{Ba} traces generated with the same double-pulse voltage protocol as in Figure 1*D* in the absence and presence of 10 μM UTP. The bar charts show the mean inhibition of I_{Ba} by 10 μM UTP before (*Prep.0*) and after (*Prep.120*) a +120 mV prepulse in neurons injected with 1.25 $\mu g/\mu l$ $P2Y_2$ cRNA (*B*) and 0.5 $\mu g/\mu l$ $P2Y_2$ cRNA (*C*). Note that, after PTX treatment, UTP no longer slowed the recorded currents and that the prepulse no longer significantly reduced inhibition (compare with Fig. 1).

clear voltage dependence in that neither the current–voltage curve nor the kinetics of the deactivation relaxations was altered appreciably in the presence of UTP.

We further tested the effects of several other nucleotides, using a standard concentration of 10 μM (Fig. 7*A*). The approximate order of activity was UTP $\geq$ ATP $>$ ITP $\gg$ GTP,UDP. This accords with their relative activities in inhibiting the Ca^{2+} current in $P2Y_2$-expressing SCG neurons (Fig. 7*B*) (see Filippov et al., 1997) and also with stimulation of inositol phosphate production or intracellular Ca^{2+} elevation in other cells expressing cloned $P2Y_2$ receptors (Lustig et al., 1993; Chen et al., 1996; Nicholas et al., 1996) and in NG108-15 cells expressing the endogenous receptor (Lin et al., 1993); the strong activity of ITP and relatively weak activity of UDP (see below) are particularly noteworthy in this respect. The inhibitory activity of ADP was less than that of UTP and appeared to be less than that of ATP, but it was difficult to quantitate because ADP produced some inhibition of both M- and Ca^{2+} currents in control (GFP cRNA-injected) cells (Fig. 7, *shaded columns*)—possibly via a low-abundance endogenous $P2Y_1$ receptor. ATP also partly inhibited $I_{K(M)}$ in control cells; the other nucleotides tested (UTP, UDP, ITP, GTP) did not produce any significant effect on either Ca^{2+}- or M-current in the cells preinjected with GFP cRNA alone. [It should be noted that, because the test concentration of UTP was near-maximal, the relative heights of the *bars* in Fig. 7 do not provide an accurate index of numerical potency ratios. For example, ADP was ~50 times less potent than UTP in inhibiting I_{Ca} when measured from full dose–response curves (Filippov et al., 1997). Thus the dinucleotides ADP and UDP are likely to be at least one or two orders of magnitude less potent than UTP in inhibiting the M-current. Unfortunately, because of desensitiza-

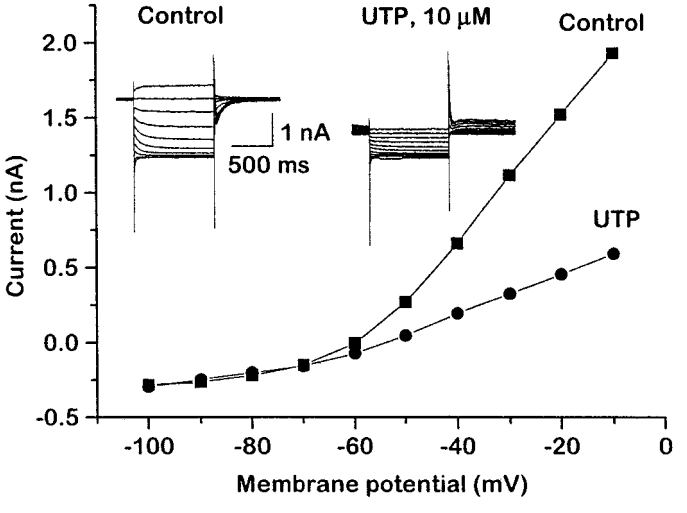

Figure 4. Activation of heterologously expressed $P2Y_2$ receptors inhibits the M-type K^+ current (M-current; $I_{K(M)}$) in rat SCG neurons. The neuron was injected 18 hr beforehand with 1.25 $\mu g/\mu l$ $P2Y_2$ cRNA. M-current was recorded with a perforated patch electrode by predepolarizing the neuron to −20 mV and then deactivating the current with 1 sec hyperpolarizing steps in increments of 10 mV at 5 sec intervals, as shown in the current records. The graph shows the current amplitude at the end of each 1 sec step measured as change from zero current. Currents were recorded before (*filled squares*; *Control*) and after (*filled circles*) the addition of 10 μM UTP. Note that UTP produced an inward current at the holding potential of −20 mV, reduced the amplitude of the M-current deactivation tail currents during the hyperpolarizing steps, and reduced the outward rectification of the current–voltage curve positive to −70 mV. (The slight outward drift of the holding current in the presence of UTP reflects slow receptor desensitization.)

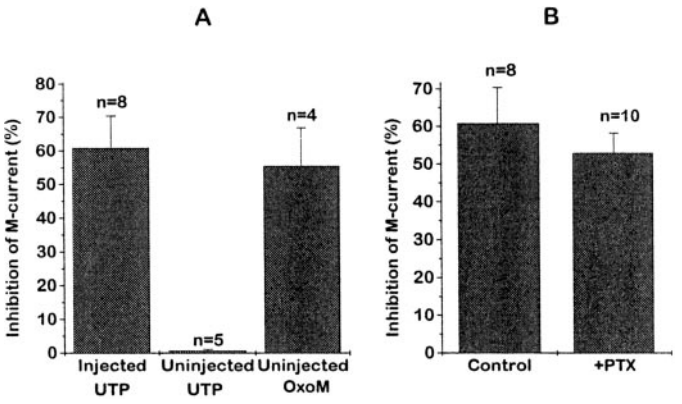

Figure 5. M-current inhibition by UTP requires heterologous $P2Y_2$ receptor expression (*A*) and is not prevented by PTX (*B*). The bar chart in *A* shows the mean percentage of inhibition of the M-current at −30 mV (see Materials and Methods) by 10 μM UTP in neurons injected with 1.25 μg/μl $P2Y_2$ cRNA (*injected*) and in uninjected neurons. Current inhibition by 10 μM oxotremorine-M (*OxoM*) in uninjected neurons is shown for comparison. Error bars show SEM; n = number of cells tested. The bar chart in *B* shows the mean percentage of inhibition of the M-current by 10 μM UTP at −30 mV in neurons preinjected with $P2Y_2$ cRNA without (*Control*) or with (*+PTX*) overnight pretreatment with 0.5 μg/ml PTX.

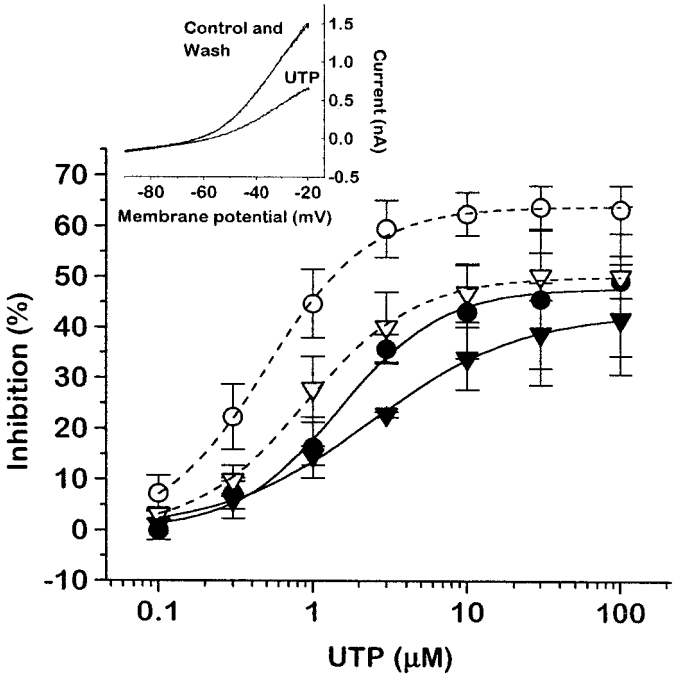

Figure 6. Concentration dependence for UTP inhibition of M-current (*solid lines*) and N-type Ca^{2+} channel current (*dashed lines*) in cells preinjected with 1.25 μg/μl $P2Y_2$ cRNA (*circles*) or 0.5 μg/μl $P2Y_2$ cRNA (*triangles*). M-current was recorded by using a voltage ramp protocol (*inset*) and was measured at −30 mV (see Materials and Methods). The *points* show the mean ± SEM of measurements in three to four cells; concentrations were added cumulatively, with 1 min exposure times. Curves were fit to pooled data points, using Origin 4.1 software to the Hill equation: $y = y_{max} \cdot x^{nH}/(x^{nH} + K^{nH})$, where y = the observed percentage of inhibition, y_{max} = extrapolated maximal percentage of inhibition, x = nucleotide concentration (μM), K = IC_{50} (μM), and n_H = the Hill coefficient. Values of constants (mean ± SEM) for M-current inhibition were y_{max} = 48.0 ± 1.20%, K = 1.49 ± 0.14 μM, n_H = 1.29 ± 0.14 for neurons injected with 1.25 μg/μl $P2Y_2$ cRNA; and y_{max} = 43.2 ± 1.95%, K = 2.41 ± 0.43 μM, n_H = 0.88 ± 0.11 for neurons injected with 0.5 μg/μl $P2Y_2$ cRNA. Data for Ca^{2+} channel current inhibition (*dashed lines*) are taken from Filippov et al. (1997) and are superimposed for comparison. (Values for constants were y_{max} = 64.0 ± 0.75%, K = 0.50 ± 0.03 μM, n_H = 1.29 ± 0.0714 for neurons injected with 1.25 μg/μl $P2Y_2$ cRNA; and y_{max} = 50.2 ± 0.61%, K = 0.90 ± 0.05 μM, n_H = 1.21 ± 0.06 for neurons injected with 0.5 μg/μl $P2Y_2$ cRNA).

tion and slow recovery, it was not possible to construct full dose–response curves for the inhibitory action of the weaker analogs on the M-current.]

To check whether the effects of UDP and ADP in cRNA $P2Y_2$ preinjected cells arise from possible contamination of these nucleotides by UTP and ATP (see Nicholas et al., 1996), we performed such tests by using purified UDP and ADP from Boehringer Mannheim (99% pure) immediately after their dissolution in the medium. In addition, we used samples of UDP and ADP pretreated for 1 hr with hexokinase (1 mM UDP or ADP incubated with 10 U/ml hexokinase plus 22 mM glucose at 37°C); this should convert any UTP and ATP that was present to their diphosphates (Nicholas et al., 1996). The hexokinase pretreatment did not significantly reduce the inhibitory effects of 10 μM UDP or ADP, and the pure UDP and ADP produced inhibitions within the range of those observed with the same nucleotides from Sigma.

Effects on excitability

The inhibition of M-type K^+ currents and N-type Ca^{2+} currents through endogenous G-protein-coupled receptors increases the excitability of SCG neurons. This occurs because the M-current itself acts as a "braking" current on action potential discharges (see Brown, 1988), and the entry of Ca^{2+} through N-type Ca^{2+} channels opens Ca^{2+}-activated K^+ channels and thereby induces a long-lasting afterhyperpolarization (AHP), which further limits subsequent spike activity [see Davies et al. (1996) and references therein]. Thus, the activation of expressed $P2Y_2$ receptors also might be expected to enhance spike activity.

Figure 8 shows that this was the case. In Figure 8*A*, the cells were challenged with long depolarizing or hyperpolarizing current injections from a preset potential of −60 mV at room temperature, at 20°C (Fig. 8*Aa*), and at 34°C (Fig. 8*Ab*). The long depolarizing current induced a brief burst of two to three spikes, followed by silence. The application of UTP had no effect on this spike discharge in control cells preinjected with GFP cRNA but greatly prolonged the discharge in cells preinjected with $P2Y_2$ cRNA. This effect of UTP was increased dramatically at 34°C (Fig. 8*Ab*); this occurs probably because M-current kinetics are faster at 34°C (Brown, 1988), thereby exerting a faster and more effective "brake" on firing (see Cuevas et al., 1997). UTP also increased the voltage response to hyperpolarizing current pulses (Fig. 8*Ab*), as expected after M-current inhibition (see Adams et al., 1982). These effects closely resemble the effect of activating endogenous muscarinic receptors in SCG neurons (see Brown and Constanti, 1980).

Figure 8*B* shows the effect of stimulating expressed $P2Y_2$ receptors on the Ca^{2+}-activated afterhyperpolarization that follows an action potential: UTP abbreviated the afterhyperpolarization in a manner resembling the effect of stimulating endogenous α2-adrenergic receptors with norepinephrine (Horn and McAfee, 1980), which results from the inhibition of the N-type Ca^{2+} current (Galvan and Adams, 1982; Schofield, 1990).

DISCUSSION

The principal points emerging from these and the preceding experiments (Filippov et al., 1997) are that the recombinant rat

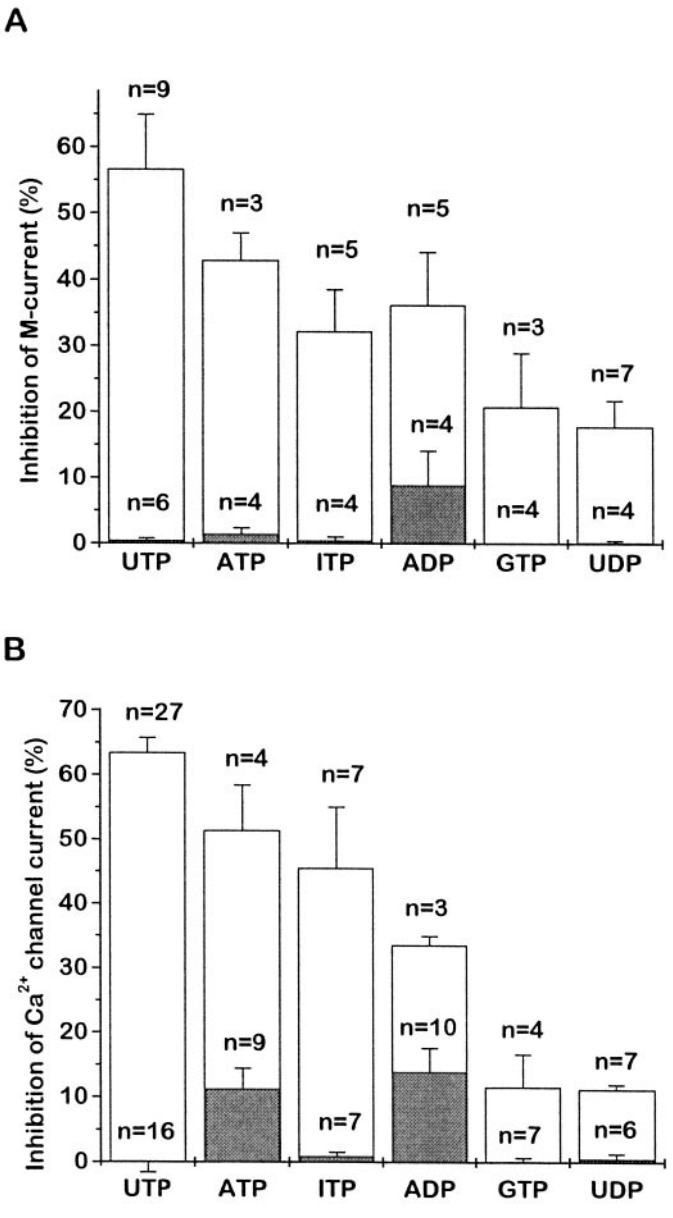

Figure 7. Mean effects of different nucleotides on M-current (*A*) and Ca^{2+} channel current (*B*). Nucleotides were applied at 10 μM. *Open bars* show the mean percentage of inhibition in neurons preinjected with 1.25 $\mu g/\mu l$ $P2Y_2$ cRNA; the *shaded bars* show the effects in control cells (preinjected with GFP cRNA, but without $P2Y_2$ cRNA). Error bars show SEM; n = number of cells. Data for $P2Y_2$ cRNA-injected cells in *B* were recalculated from Filippov et al. (1997). Inhibition was measured as described in Figures 2 and 6.

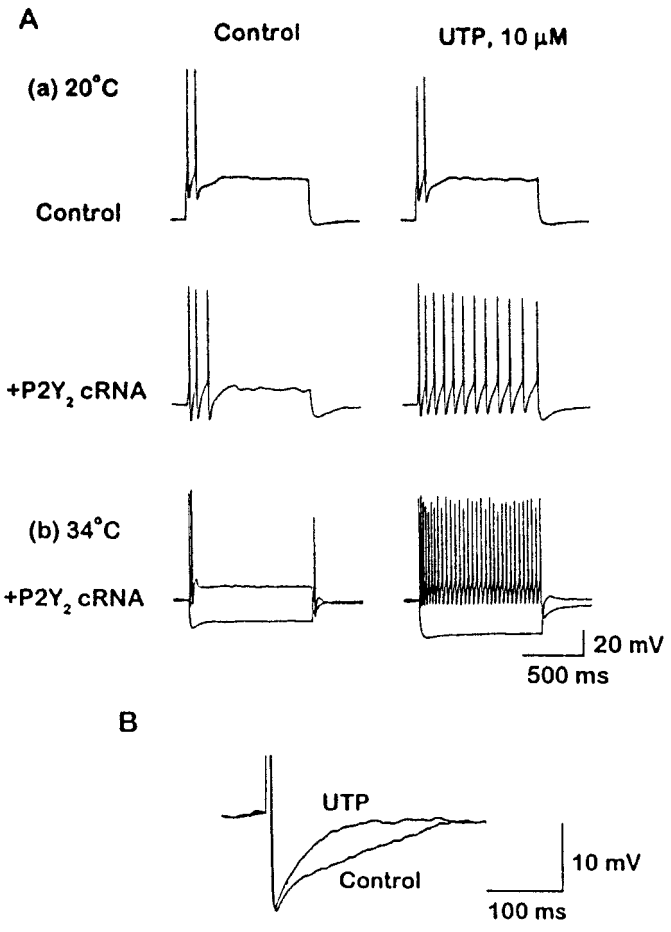

Figure 8. Activation of heterologously expressed $P2Y_2$ receptors enhances repetitive firing (*A*) and reduces the Ca^{2+}-activated spike afterhyperpolarization (*B*) in SCG neurons. *Aa*, Voltage responses to depolarizing current pulses (1 sec) recorded from a control (uninjected) SCG neuron (*top panel*, 0.4 nA pulse) and from a $P2Y_2$ cRNA (1.25 $\mu g/\mu l$) preinjected neuron (*middle panel*, 0.1 nA pulse) before and during application of UTP at 20°C. *Ab*, Voltage responses to depolarizing (0.3 nA) and hyperpolarizing (−0.2 nA) current pulses from $P2Y_2$ cRNA (1.25 $\mu g/\mu l$) preinjected neuron before and during the application of UTP at 34°C. *B*, Afterhyperpolarization (AHP) that followed an action potential evoked by a brief depolarizing current pulse (1 nA, 1 msec) recorded from a $P2Y_2$ cRNA (1.25 $\mu g/\mu l$) preinjected neuron before and during the application of UTP at 34°C.

$P2Y_2$ receptor can couple with near-equal facility to two quite different neural ion channels—the N-type voltage-gated Ca^{2+} channel and the M-type K^+ channel—when expressed in rat SCG neurons and that this involves the intermediation of at least two different G-proteins. Dual coupling to these particular channels by one species of G-protein-linked receptor is unusual (see below) and suggests that the $P2Y_2$ receptor can have a potentially broader range of effects on neurons than many other neurotransmitter receptors.

This apparent cross-talk is unlikely to be an artifact of receptor overexpression, for two reasons. First, it was preserved after injections of two different amounts of the receptor cRNA, such that, at the lower level (0.5 $\mu g/\mu l$), the maximum response to UTP was less than that obtained on stimulating endogenous adrenergic and muscarinic receptors. Thus, although (in the absence of appropriate antibodies) we have not been able to measure the number of receptors expressed, they are unlikely to be "excessive" in comparison to other endogenous serpentine receptors in these cells. Second, the IC_{50} values for UTP observed in the present experiments (0.5–0.9 μM for Ca^{2+} current inhibition and 1.5–2.4 μM for M-current inhibition) are not dissimilar to those observed for UTP to elevate intracellular $[Ca^{2+}]$ [1.1 μM, Lustig et al. (1993); ~1.1 μM, Parr et al. (1994); 0.2 μM, Chen et al. (1996)] or to stimulate inositol phosphate production (~0.1 μM; Nicholas et al., 1996) when applied to recombinant $P2Y_2$ receptors expressed in other cell lines. (IC_{50} values likely will vary from one expression system to another, depending on the level of receptor expression, as indicated by the dose–response curves in Fig. 6.) More pertinently, perhaps, the present IC_{50} values also

accord with those that follow the stimulation of the endogenous $P2Y_2$ receptor in NG108-15 cells [~3 μM for inositol phosphate production (Lin, 1994); 0.8 μM for M-like current inhibition (Filippov et al., 1994); 2.8 μM for N-type Ca^{2+} current inhibition (Filippov and Brown, 1996)]. The agreement between the results obtained on stimulating the endogenous receptors in this neural cell line and the exogenously expressed receptors in SCG neurons strongly suggests that the effects we see are likely to be a general phenomenon, applicable in other cells in which the same receptor might be expressed.

These divergent responses start at the level of the G-protein. M-current inhibition was insensitive to PTX; although we have not positively identified the species of PTX-resistant G-protein(s) responsible, previous experiments that used site-directed antibodies on the analogous effects of muscarinic agonists (Caulfield et al., 1994) and bradykinin (Jones et al., 1995) suggest that it is most likely G_q and/or G_{11} (principally G_q; Haley et al., 1997). In contrast, Ca^{2+} current inhibition is mediated by at least two G-proteins: a PTX-sensitive G-protein, responsible for the gating shift and for ~60% of the peak inhibition measured at 0 mV, and a PTX-insensitive G-protein responsible for the residual voltage-insensitive component of inhibition. By analogy with the voltage-dependent effects of norepinephrine, somatostatin, and M_4 muscarinic stimulation, the former (PTX-sensitive) G-protein is probably G_{oA} (Caulfield et al., 1994; Delmas et al., 1998a,b), and the gating shift probably results from the interaction of the dissociated free $\beta\gamma$-subunits with the Ca^{2+} channel protein (see Herlitze et al., 1996; Ikeda, 1996; Delmas et al., 1998a,b). The PTX-insensitive G-protein responsible for the voltage-insensitive component of Ca^{2+} current inhibition could well be the same as that (G_q?) postulated to cause M-current inhibition, because M_1 muscarinic acetylcholine receptor stimulation produces the same dual-effector response *via* G_q (Delmas et al., 1998b).

In effect, therefore, stimulating the $P2Y_2$ receptor imitates the combined effects of stimulating two separate endogenous muscarinic acetylcholine receptors, the M_1 and the M_4 receptors (see Hille, 1994). This is summarized in Figure 9; at a minimum, it requires parallel coupling of the $P2Y_2$ receptor to two G-proteins: G_q (leading to inhibition of the M-current and voltage-independent inhibition of the Ca^{2+} current) and G_o (producing a gating shift of the Ca^{2+} channels).

There are other instances of "promiscuity" in receptor/G-protein coupling (see Milligan, 1997). This can result in effects on more than one ion channel—for example, the inhibition of Ca^{2+} currents and the activation of inward rectifier (K_{ir}) K^+ currents (Surprenant et al., 1992). However, dual coupling to $Ca^{2+}{}_N$ and $K^+{}_M$ channels through two G-proteins by a single, defined receptor is more unusual. Thus, although many transmitters can, collectively, modulate Ca^{2+} currents or M-currents in SCG neurons in a manner similar to that produced by $P2Y_2$ receptors (see Hille, 1994), this usually involves the activation of separate receptors, each coupled to a different G-protein. As indicated already, muscarinic acetylcholine agonists can produce the same dual response as UTP, but to do so they have to activate two *different* molecular species of muscarinic receptor; activation of G_o (and the Ca^{2+} channel gating shift) requires M_4 receptors, whereas activation of G_q (and consequential voltage-independent inhibition of M-current and Ca^{2+} current) requires M_1 receptors (see Beech et al., 1992; Delmas et al., 1998b). This also applies to other heterologous receptors expressed by this method. Thus, there is negligible cross-talk between heterologous mGluR1a and mGluR2 receptors expressed from cRNA injections; the former inhibit M-currents via a PTX-insensitive G-protein with insignificant effect on the Ca^{2+} current, whereas mGluR2 receptors inhibit Ca^{2+} currents entirely through a PTX-sensitive G-protein without any effect on M-currents (Ikeda et al., 1995). Likewise, heterologously expressed cannabinoid receptors selectively inhibit Ca^{2+} currents through a PTX-sensitive G-protein (Pan et al., 1996). The closest analogy is provided by the action of angiotensin, which also couples through two G-proteins (PTX-sensitive and insensitive) to inhibit Ca^{2+} and M-currents (Shapiro et al., 1994); however, it has not yet been established whether both coupling routes are activated by the same or different molecular species of angiotensin receptor.

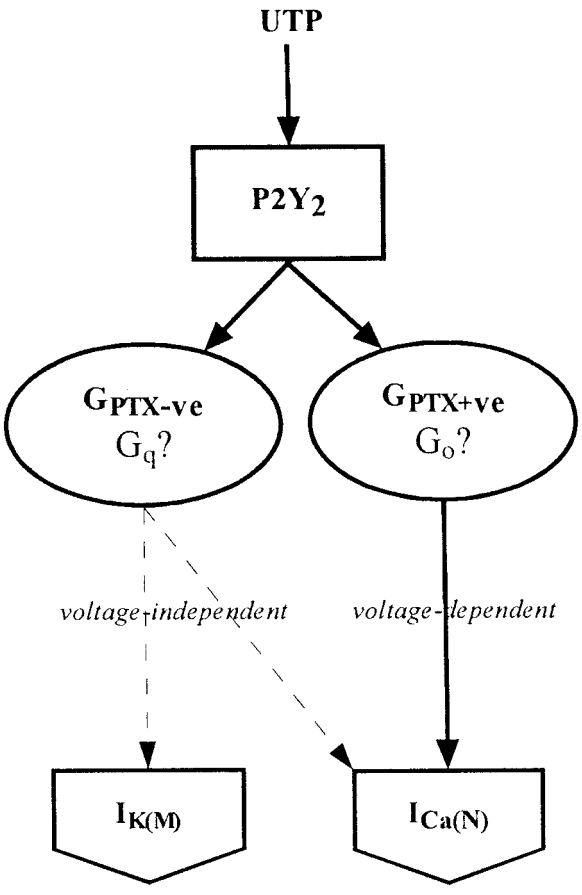

Figure 9. Schematic diagram representing dual coupling of $P2Y_2$ receptor to $Ca^{2+}{}_N$ and $K^+{}_M$ channels via two different G-proteins. *Solid lines*, Direct connections; *dashed lines*, indirect connections.

Physiological significance

From a functional viewpoint, the net effect of stimulating expressed $P2Y_2$ receptors in SCG neurons is to increase their excitability (see Fig. 8*A*). This is the expected result of inhibiting the M-current and the N-type Ca^{2+} current, because reducing the latter will decrease the Ca^{2+}-dependent K^+ current and abbreviate the spike afterhyperpolarization (as shown in Fig. 8*B*), and K_M and K_{Ca} currents act as synergistic braking currents on spike discharge (see Jones and Adams, 1987).

This is essentially a postsynaptic response; it mimics the natural postsynaptic effect of activating the endogenous muscarinic receptors by synaptically released acetylcholine in SCG neurons (see Brown, 1988), so it would provide a mechanism for slow postsynaptic excitation by a nucleotide transmitter. Although no

such nucleotide-mediated *synaptic* responses have been reported yet, comparable excitatory effects of *exogenous* UTP and ATP have been described in, for example, frog sympathetic (Siggins et al., 1977; Adams et al., 1982; Akasu et al., 1983; Lopez and Adams, 1989), frog sensory (Tokimasa and Akasu, 1990), and rat intracardiac (Cuevas et al., 1997) ganglion cells. However, the species of P2Y receptor responsible for these effects has not been determined, nor is there yet any direct evidence for equivalent effects on central neurons.

On the other hand, if endogenous $P2Y_2$ receptors were located *presynaptically*, then the most likely effect of their stimulation would be to reduce transmitter release via the inhibition of the N-type Ca^{2+} current, in the same manner as stimulating endogenous presynaptic adrenergic or muscarinic receptors in SCG neurons (Boehm and Huck, 1996; Koh and Hille, 1997). This would provide a mechanism for autoinhibition in nucleotide-releasing nerve terminals. There is some evidence for P2Y-mediated autoinhibition in peripheral sympathetic nerves (Fuder and Muth, 1993) and chromaffin cells (Currie and Fox, 1996); also, P2Y-mediated inhibition of norepinephrine release from isolated brain tissue by exogenous nucleotides has been reported (Von Kugelgen et al., 1994), but the identity of these receptors has not yet been established.

As pointed out in the introductory remarks, there is now direct evidence for the synaptic release of ATP in the brain and the consequent activation of postsynaptic P2X receptors (Edwards and Gibb, 1993). Although there is, as yet, no evidence for the synaptic release of UTP, the latter nucleotide can be released from cells by other mechanisms (Lazarowski et al., 1997). Because mRNA for the $P2Y_2$ receptor is expressed in the brain (Lustig et al., 1993), were this receptor to be activated by either of these endogenously released nucleotides, its dual coupling would provide scope for some unusually divergent effects on neural signaling.

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