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Two Different Signaling Mechanisms Involved in the Excitation of Rat Sympathetic Neurons by Uridine Nucleotides

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ABSTRACT

UTP stimulates transmitter release and inhibits M-type K^+ channels in rat superior cervical ganglion neurons via G protein-coupled P2Y receptors. To investigate the underlying signaling mechanisms, we treated the neurons with either pertussis or cholera toxin; neither treatment altered the inhibition of M-type K^+ channels by 10 μM UTP. However, pertussis toxin reduced UTP-evoked [^{3}H]noradrenaline release by 66%. UTP, UDP, ATP, and ADP caused accumulation of inositol trisphosphate in a pertussis toxin-insensitive manner. Pharmacological inhibition of inositol trisphosphate-induced Ca^{2+} release (by inhibition of phospholipase C, of inositol trisphosphate receptors, and of the endoplasmic Ca^{2+}-ATPase) prevented the UTP-dependent inhibition of M currents but failed to alter UTP-evoked [^{3}H]noradrenaline release. Chelation of intracellular Ca^{2+} by 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid also reduced the inhibition of M currents by UTP. In addition, all these manipulations attenuated the inhibition of M currents by bradykinin, but hardly affected the inhibitory action of oxotremorine M. These results demonstrate that UTP inhibits M-type K^+ channels via an inositol trisphosphate-dependent signaling cascade that is also used by bradykinin but not by muscarinic acetylcholine receptors. In contrast, the secretagogue action of UTP is largely independent of this signaling cascade but involves pertussis toxin-sensitive G proteins. Thus, UTP-sensitive P2Y receptors excite sympathetic neurons via at least two different signal transduction mechanisms.

Rat superior cervical ganglion (SCG) neurons possess at least two different types of nucleotide receptors that are both excitatory and thus trigger noradrenaline release (Boehm, 1994; Boehm et al., 1995). One of these receptors is a ligand-gated ion channel that is activated by adenine nucleotides (i.e., a P2X purinoceptor; Boehm, 1999). The other receptor is activated by UTP and UDP and is metabotropic rather than ionotropic (Boehm et al., 1995; Boehm and Huck, 1997a). Similar results have been obtained in neurons from thoracolumbal paravertebral sympathetic ganglia of the rat, but the signaling mechanisms underlying the secretagogue action of uridine nucleotides remained elusive (von Kügelgen et al., 1999). Recently, uridine nucleotide-sensitive P2Y receptors of rat SCG neurons were found to inhibit selectively M-type K^+ (K_M) channels (Boehm, 1998). The P2 receptors mediating the induction of transmitter release, on the one hand, and the inhibition of K_M channels, on the other hand, both displayed pharmacological characteristics suggestive of a role of P2Y6-like receptors: 1) UDP and UTP were equipotent agonists, even when interconversion was prevented by hexokinase; 2) the receptors were insensitive to the P2 antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) at 10 μM but blocked by reactive blue 2 at this concentration; and 3) the uridine-nucleotide sensitive receptors showed homologous desensitization but no cross-desensitization with ATP (Boehm et al., 1995; Boehm, 1998). We therefore assumed that the inhibition of K_M channels mediated the secretagogue action of uridine nucleotides. Two independent types of results also suggest that an inhibition of K_M channels may stimulate transmitter release from SCG neurons: 1) activation of B_2 bradykinin receptors inhibits K_M channels of SCG neurons via GTP binding proteins (Jones et al., 1995) and triggers tetrodotoxin (TTX)-sensitive noradrenaline release (Boehm and Huck, 1997b), as do UDP and UTP (Boehm et al., 1995); and 2) direct blockade of K_M channels by either Ba^{2+} or linopirdine also elicits TTX-sensitive transmitter release in these neurons (Kristufek et al., 1999).

Apart from uridine nucleotide-sensitive P2Y receptors (Boehm, 1998) and B_2 bradykinin receptors (Jones et al., 1995), M_1 muscarinic receptors cause, on activation, an inhibition of K_M channels (Marrion et al., 1989; Bernheim et al., 1992). However, most recently, B_2 and M_1 receptors were found to use different signaling pathways that finally lead to the closure of K_M channels: The B_2 receptor activates phospholipase C to cause liberation of Ca^{2+} from inositol trisphosphate (IP_3)-sensitive Ca^{2+} stores (Cruzblanca et al., 1998), and cytosolic Ca^{2+}

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ABBREVIATIONS: SCG, superior cervical ganglion; K_M, M-type K^+ channel; I_M, currents through K_M channels; IP_3, inositol trisphosphate; TTX, tetrodotoxin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester.

at low micromolar concentrations is known to block K_M channels (Selyanko and Brown, 1996). The M_1 receptor, in contrast, was reported to inhibit K_M channels independent of phospholipase C and IP_3-sensitive Ca^{2+} stores (Cruzblanca et al., 1998; del Rio et al., 1999). The mechanisms by which UTP-sensitive P2Y receptors of SCG neurons cause inhibition of K_M channels are unknown (Boehm, 1998). In heterologous expression systems, all known subtypes of P2Y receptors do couple to phospholipase C and thus cause formation of IP_3 and a resulting increase in intracellular Ca^{2+} (Harden et al., 1995; North and Barnard, 1997; King et al., 1998). Here, we investigate the cellular mechanisms that link the uridine nucleotide-sensitive P2Y receptors to K_M channels, on one hand, and to transmitter release, on the other hand. The experiments focus on the role of the phospholipase C-dependent signaling cascade in the two types of UTP-dependent effects in SCG neurons and suggest that the induction of transmitter release and the inhibition of K_M channels are largely independent phenomena.

Experimental Procedures

Cell Culture. Primary cultures of neurons dissociated from SCG of neonatal rats were prepared as previously described in more detail (Boehm, 1994). Briefly, ganglia were dissected from 2- to 6-day-old Sprague-Dawley rat pups, cut into three or four pieces, and incubated in collagenase (no. 9891, 1.5 mg/ml; Sigma Chemical Co., St. Louis, MO) and dispase (no. 165859, 3.0 mg/ml; Boehringer-Mannheim, Indianapolis, IN) for 20 min at 36°C. Subsequently, the ganglia were trypsinized (no. 3703, 0.25% trypsin; Worthington Biochemicals, Freehold, NJ) for 15 min at 36°C, dissociated by trituration, and plated onto 5-mm disks (about 40,000 cells/disk) coated with rat tail collagen (Biomedical Technologies. Cambridge, MA) for superfusion experiments and onto 35-mm culture dishes (no. 153066; Nunc, Naperville, CT) coated with poly(D-lysine) (25 mg/l; Sigma Chemical Co.) for electrophysiological experiments. For the determination of cellular inositol phosphates, cells were plated onto 24 multiwell plates (200,000 cells/well; Nunc) coated with poly(D-lysine) as above. About 50% of the cells in this culture system are neurons; the remainder are provided by non-neural cells, including primarily fibroblasts and glial cells.

Electrophysiology. Electrophysiological experiments were carried out at room temperature (20–24°C) on the somata of isolated neurons using a List EPC-7 amplifier (List Medical, Darmstadt, Germany) and pClamp 6.0 hardware and software (Axon Instruments, Foster City, CA). Unless stated otherwise, currents through K_M channels (I_M) were recorded in the amphotericin perforated-patch configuration (Rae et al., 1991), which prevents rundown of I_M (see Boehm, 1998). Patch pipettes were pulled (Flaming-Brown puller; Sutter Instruments, Novato, CA) from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany) and front-filled with a solution consisting of 75 mM K_2SO_4, 55 mM KCl, 8 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.3 with KOH. Then, electrodes were back-filled with the same solution containing 200 μg/ml amphotericin B (in 0.8% DMSO), which yielded tip resistance values of 1 to 3 MΩ. To apply the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) to the cytosol of the neurons under investigation, I_M was also recorded in the conventional (open-tip) whole-cell configuration (Hamill et al., 1981) of the patch-clamp technique with an intracellular (pipette) solution containing 120 mM K-aspartate, 30 mM KCl, 3.18 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, and 2 mM Li-GTP, adjusted to pH 6.8 with NaOH. Where indicated, 20 mM KCl was replaced by K_4-BAPTA, which reduces the calculated free Ca^{2+} concentration from 1.4 to 0.02 μM.

The bathing solution contained 140 mM NaCl, 6.0 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH. TTX (0.3–1 μM) was included to suppress voltage-activated Na^+ currents, unless otherwise indicated. All channel blocking agents and neurotransmitter receptor agonists were applied via a DAD-12 drug application device (Adams & List, Westbury, NY) that permits a complete exchange of solutions surrounding the cells under investigation within less than 100 ms (see Boehm and Betz, 1997). I_M relaxations were evoked once every 20 s by 1-s hyperpolarizing voltage steps from −30 to −55 mV; the difference between current amplitudes 20 ms after the onset of hyperpolarizations and 20 ms before redepolarization was taken as a measure for I_M. Amplitudes obtained during the application of test drugs (b) were compared with those measured before (a) and after (c) application of these drugs by calculating $200b/(a + c) = \%$ of control or $100 - (200b/[a + c]) = \%$ inhibition (see Boehm, 1998).

Measurement of [^{3}H]Noradrenaline Release. [^{3}H]Noradrenaline uptake and superfusion were performed as described previously (Boehm, 1994). Cultures were labeled with 0.05 μM [^{3}H]noradrenaline (specific activity, 71.7 Ci/mmol) in culture medium supplemented with 1 mM ascorbic acid at 36°C for 1 h. After labeling, culture disks were transferred to small chambers and superfused with a buffer containing (mM) NaCl (120 mM), KCl (6.0 mM), $CaCl_2$ (2.0 mM), $MgCl_2$ (2.0 mM), glucose (20 mM), HEPES (10 mM), fumaric acid (0.5 mM), Na-pyruvate (5.0 mM), ascorbic acid (0.57 mM), adjusted to pH 7.4 with NaOH. Superfusion was performed at 25°C at a rate of about 1.0 ml min^{-1}. Collection of 4-min superfusate fractions was started after a 60-min washout period. When appropriate, thapsigargin (0.3 μM) was included in the superfusion buffer from minute 50 onwards (i.e., 22 min before the application of UTP). [^{3}H] overflow was first induced by inclusion of UTP (10 μM) in the medium from minutes 72 to 74 of superfusion and then by electrical field stimulation (36 monophasic rectangular pulses, 0.5 ms, 0.3 Hz, 50 mA, 50 V cm^{-1}) from minutes 92 to 94. At the end of experiments, radioactivity remaining in the cultures was extracted by immersion of the disks in 1.2 ml of 2% (v/v) perchloric acid, followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting (Tri-Carb 2100 TR; Packard). Radioactivity released in response to electrical field stimulation from rat sympathetic neurons after labeling with tritiated noradrenaline under conditions similar to those of the present study had previously been shown to consist predominantly of the authentic transmitter and to contain only small amounts (≤15%) of metabolites (Schwartz and Malik, 1993). Hence, the outflow of tritium measured in this study was assumed to reflect the release of noradrenaline and not that of metabolites.

The fractional rate of [^{3}H] outflow was obtained by dividing the radioactivity of a collected fraction by the total radioactivity of cultures at the beginning of the corresponding collection period. To obtain the fractional outflow per minute, the values of 4-min samples were then divided by 4. Stimulation-evoked overflow was calculated as the difference between the total [^{3}H] outflow during and after stimulation and the estimated basal outflow, which was assumed to decline linearly throughout experiments. Therefore, basal outflow during periods of stimulation was assumed to equate the arithmetic mean of the samples before and after stimulation, respectively. The difference between the total and the estimated basal outflow was expressed as a percentage of the total radioactivity in the cultures at the beginning of the respective stimulation.

Measurement of IP_3. Measurement of inositol polyphosphate formation was determined as described previously (Nanoff et al., 1990). Briefly, SCG cultures were prelabeled with 7 μCi/ml of *myo*-[1,2-^{3}H]inositol in serum-free culture medium (see earlier) for 24 h. At 30 min before the stimulation with various receptor agonists, the cells were incubated in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) containing 0.2% BSA and 10 mM LiCl. The cells were stimulated with nucleotides, bradykinin, or oxotremorine M in PBS containing 10 mM LiCl for various periods of time, and the incubations were terminated by replacing the buffer with 0.4 ml of 5% trichloroacetic acid. Extracts were collected, and the trichloroacetic acid was removed by washing twice with 4 vol-

umes of water-saturated diethyl ether. The samples were neutralized with 20 mM Tris base and placed on a Dowex AG 1X8 column. Fractions containing inositol, inositol monophosphate, inositol diphosphates, and inositol trisphosphates, respectively, were sequentially eluted (see Nanoff et al., 1991) and probed for their radioactive contents by liquid scintillation counting. The radioactivity in the IP_3 fraction was expressed as a percentage of the radioactivity in the inositol fraction.

Statistical Analysis. All data are given as arithmetic mean ± S.E. (n = number of cell culture disks in release experiments, of multiwell cultures in inositol trisphosphate experiments, and of single cells in electrophysiological recordings). Differences between single data points were evaluated by the Mann-Whitney test.

Materials. (−)-[*ring*-2,5,6-^{3}H]Noradrenaline and *myo*-[1,2-^{3}H]inositol were obtained from NEN (Dreieich, Germany). Bradykinin, Na-UTP, amphotericin B, and TTX were purchased from Sigma (Vienna, Austria). Oxotremorine M, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrollidinedione (U73343), and thapsigargin were obtained from Research Biochemicals Inc. (Natick, MA). BAPTA acetoxymethyl ester (BAPTA-AM) was purchased from Molecular Probes (Eugene, OR). Xestospongin C was obtained from Calbiochem (Bad Soden, Germany). U73122, U73343, xestospongin C, and thapsigargin were first dissolved in DMSO and then diluted into buffer to yield DMSO concentrations of 0.1%. At this concentration, DMSO does not affect any of the parameters investigated.

Results

Effects of Cholera Toxin and PTX on UTP-Induced [^{3}H]Noradrenaline Release and Inhibition of I_M. To obtain insight into the types of GTP binding proteins involved in the actions of UTP in SCG neurons, cultures were treated with either PTX or cholera toxin (both 100 ng/ml for 24 h). Although PTX prevents the signaling of G_i- and/or G_o-coupled receptors in SCG neurons (e.g., Freissmuth et al., 1996), cholera toxin has been shown to down-regulate $G_{s\alpha}$ in sympathetic neurons (Boehm et al., 1996). After toxin treatment, cultures were loaded with [^{3}H]noradrenaline to determine the outflow of radioactivity as a measure of transmitter release or I_M was recorded (Fig. 1). UTP-evoked tritium overflow was reduced by 65.8 ± 5.5% (n = 12) after PTX treatment compared with nontreated cultures (Fig. 1, A and B). This effect was specific for the secretagogue action of UTP, because electrically evoked tritium overflow was not altered. The cholera toxin treatment, in contrast, did not affect UTP-evoked overflow but reduced electrically induced overflow, as described previously (Boehm et al., 1996).

In contrast to the findings with UTP-induced tritium overflow, PTX treatment did not alter the UTP-induced inhibition of I_M (Fig. 1, C and D). Likewise, in cholera toxin-treated neurons, UTP reduced I_M relaxations to the same extent as in nontreated neurons (Fig. 1, C and D). The inhibitory actions of bradykinin (1 μM) and the muscarinic agonist oxotremorine M (10 μM) on I_M relaxations (see below) were also not altered when neurons had been treated with either of the two toxins (data not shown).

Time Course of UTP-Induced Accumulation of IP_3 and Inhibition of I_M. To obtain further insight into the mechanisms underlying the UTP-induced inhibition of I_M, we first investigated the time course of this effect. I_M relax-

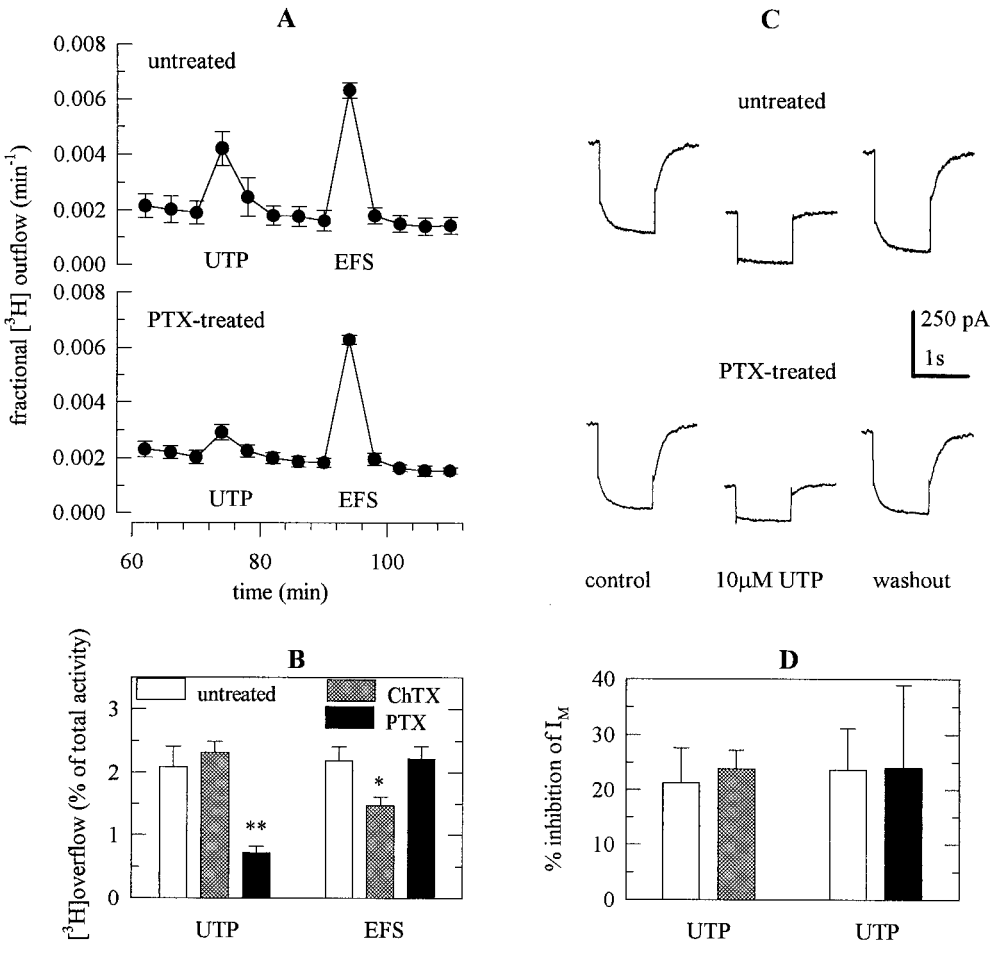

Fig. 1. Effects of cholera toxin and PTX on UTP-induced [^{3}H]noradrenaline release and inhibition of I_M. A and B, neurons, either untreated or treated with PTX or cholera toxin (ChTX) (each 100 ng/ml for 24 h), were labeled with [^{3}H]noradrenaline, superfused, and exposed to 10 μM UTP or to electrical field stimulation (EFS; each for 2 min). A, time course of [^{3}H] outflow (n = 3). B, results obtained in ≥12 cultures by showing UTP and electrically evoked overflow as percent of cellular radioactivity. *P < .05 and **P < .01 versus results obtained in untreated cultures. C, I_M was measured by the amphotericin B-perforated patch technique in an untreated neuron (top traces) and in a neuron treated with PTX (as above; bottom traces). The current traces shown were obtained by clamping the cells at −30 mV and by applying 1-s hyperpolarizing voltage steps to −55 mV; the recordings were performed before (control), during (UTP), and after (washout) the application of 10 μM UTP. D, inhibitory action of 10 μM UTP on I_M determined in five or six neurons, either untreated (open columns) or treated with PTX (filled columns) or cholera toxin (hatched columns) (as above), respectively.

ation amplitudes were slowly reduced when UTP (10 μM) was present for 10 to 60 s, and the effect reached a maximum after about 30 s (Fig. 2A). The B_2 and M_1 receptor-dependent inhibition of I_M in SCG neurons involves $G_{\alpha q}$ and/or $G_{\alpha 11}$ subunits (Jones et al., 1995; Haley et al., 1998), proteins that are commonly linked to phospholipase C (Exton, 1996). Therefore, we investigated whether UTP might cause an accumulation of IP_3 in cultures of SCG neurons and compared the time course of this effect with the time course of I_M inhibition. As shown in Fig. 2B, the UTP-induced IP_3 production showed a delayed onset and reached a maximum after 30 s. Thus, the accumulation of IP_3 and the inhibition of I_M by UTP appeared to occur in parallel.

Accumulation of IP_3 in Presence of Nucleotides: Comparison with Bradykinin and Oxotremorine M. The P2Y receptors of SCG neurons that mediate the inhibition of I_M are activated by UTP, UDP, and ADP but not by ATP (Boehm, 1998). We therefore tested these nucleotides for their capacity to cause an accumulation of IP_3. At concentrations that cause maximal inhibition of I_M (Boehm, 1998), 10 and 100 μM, all of these nucleotides were able to raise the levels of cellular IP_3. However, in comparison with bradykinin (1 μM) and oxotremorine M (10 μM), the nucleotides caused only modest increases in IP_3. As expected, the accumulation of IP_3 in the presence of UTP or oxotremorine M was not altered when cultures had been treated with PTX. However, the UTP- and bradykinin-evoked increases in IP_3 were abolished or largely reduced in the presence of the phospholipase C inhibitor U73122 (1 μM; Jin et al., 1994).

Cultures of dissociated SCG contain not only neurons but also non-neural cells. To exclude that the nucleotides elicited changes in IP_3 only in the fraction of non-neuronal elements, UDP, UTP and ADP, each at 100 μM, were also applied to cultures containing cells dissociated from the connective tissue adherent to SCGs. In these preparations, none of the nucleotides tested caused significant alterations in the levels of cellular IP_3 (not shown).

Effects of a Phospholipase C Inhibitor on Reduction of I_M by UTP, Bradykinin, and Oxotremorine M. The results shown earlier suggested that the inhibition of I_M by UTP may involve a phospholipase C-mediated generation of IP_3. To corroborate this assumption, neurons were treated with U73122, and the effect of UTP on I_M was tested. Before the application of this phospholipase C inhibitor, the neurons under investigation showed a clear-cut inhibition of I_M by UTP (10 μM; 39.9 $\pm$ 7.5% inhibition; $n = 6$), bradykinin (1 μM; 55.3 $\pm$ 12.0% inhibition; $n = 5$), and oxotremorine M (10 μM; 79.7 $\pm$ 7.5% inhibition; $n = 6$). However, when these neurons had been treated with U73122 (1 μM) for 15 min, the subsequent application of UTP (-3.5 ± 3.9% inhibition) and bradykinin (5.5 $\pm$ 5.5% inhibition) failed to cause significant alterations in I_M relaxations, whereas oxotremorine M (51.6 $\pm$ 7.9% inhibition) still caused a significant reduction (Fig. 3, A and C). Furthermore, the effect of U73122 on the receptor-dependent modulation of I_M was irreversible for up to 60 min (Fig. 3B). To exclude the possibility that U73122 had abolished the effects of UTP and bradykinin on I_M by some unspecific action, an isomer that lacks the inhibitory effect on phospholipase C, U73343, was tested (Jin et al., 1994). This agent failed to alter the inhibition of I_M by any of the agonists used (Fig. 3C).

Effects of an IP_3 Antagonist on Reduction of I_M by UTP, Bradykinin, and Oxotremorine M. IP_3 elicits cellular effects by activating IP_3 receptors located at the endoplasmic reticulum (Wilcox et al., 1998). Xestospongins are cell-permeable antagonists at these receptors, with xestospongin C being the most potent isomer (Gafni et al., 1997). When a neuron that displayed a 54% reduction of I_M relaxations in the presence of UTP (10 μM) was superfused with 10 μM xestospongin C for 10 min, the inhibition of I_M by UTP was reduced to 35%. In contrast, the inhibition of I_M by oxotremorine M amounted to 69% before and to 78% after the application of xestospongin C (Fig. 5A). Xestospongin C has been reported to block IP_3-mediated responses in intact PC12 cells more efficiently after prolonged periods of application (Gafni et al., 1997). Therefore, SCG neurons were first incubated in vehicle (0.1% DMSO) for 30 min, and the effects of UTP (10 μM), bradykinin (1 μM), and oxotremorine M (10 μM) on I_M were investigated. Thereafter, neurons in the very same culture dish were exposed to 10 μM xestospongin C again for 30 min, and the three receptor agonists were applied. When the

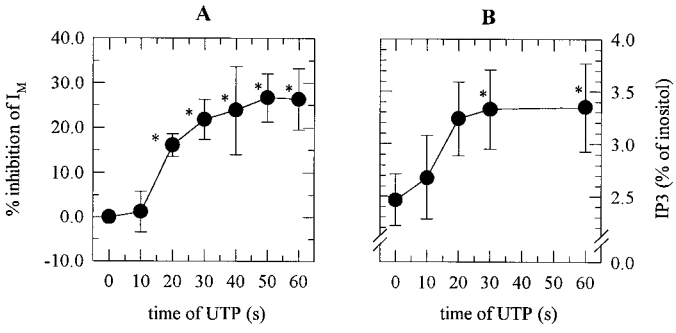

Fig. 2. Time course of the UTP-induced inhibition of I_M and accumulation of IP_3. A, time dependence of the inhibition of I_M relaxation amplitudes (measured as shown in Fig. 1C) by 10 μM UTP applied for the indicated periods of time ($n = 4$; *$P < .05$ versus values obtained in the absence of UTP). B, time dependence of the generation of IP_3 by 10 μM UTP applied for the indicated periods of time ($n = 4$); radioactivity in the IP_3-fraction is depicted as percentage of the radioactivity in the fraction of inositol (*$P < .05$ versus values obtained in the absence of UTP).

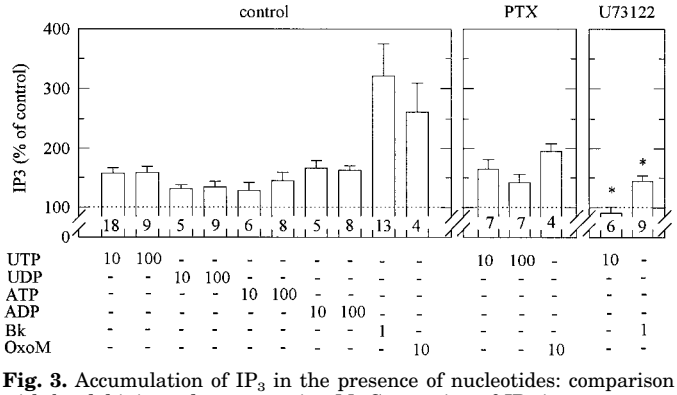

Fig. 3. Accumulation of IP_3 in the presence of nucleotides: comparison with bradykinin and oxotremorine M. Generation of IP_3 in response to UDP, UTP, ADP, ATP, bradykinin (Bk), and oxotremorine M (OxoM), each applied for 60 s, in untreated cultures (control), in cultures treated with PTX (100 ng/ml for 24 h), and in the presence of 1 μM concentration of the phospholipase C inhibitor U73122. The numbers of cultures investigated are given in the columns. Radioactivity in the IP_3 fraction was calculated as a percentage of the radioactivity in the fraction of inositol; values obtained in the presence of agonists are expressed as a percentage of the values obtained in their absence within the same preparation. In untreated and in PTX-treated cultures, all values obtained in the presence of agonists were significantly different from those obtained in their absence (*$P < .05$ versus the effects obtained with the same agonist in the absence of U73122).

results obtained in neurons treated with xestospongin C were compared with those obtained in neurons treated with vehicle, the inhibitory actions of UTP and bradykinin were reduced from 44.2 ± 7.4 to 8.9 ± 2.7% and from 58.3 ± 12.9 to 9.7 ± 5.8% inhibition, respectively. In contrast, the effect of oxotremorine M was not altered (Fig. 5, B and C). Hence, the IP_3 antagonist selectively counteracted the inhibition of I_M by UTP and bradykinin.

Effects of a Ca^{2+} ATPase Inhibitor on UTP-Induced [^{3}H]Noradrenaline Release and Inhibition of I_M. Activation of IP_3 receptors results in liberation of Ca^{2+} from the endoplasmic reticulum into the cytosol (Wilcox et al., 1998). To find out whether intracellular Ca^{2+} stores are required for the inhibition of I_M by UTP, neurons were treated with the Ca^{2+}-ATPase inhibitor thapsigargin, which had been shown previously to entirely deplete Ca^{2+} stores in sympathetic neurons at a concentration of 0.1 μM (Foucart et al., 1995). Before the application of thapsigargin, I_M was reduced by UTP (10 μM), bradykinin (1 μM), and oxotremorine M (10 μM) by 36.1 ± 6.5% (n = 5), 67.7 ± 7.7% (n = 6), and 82.9 ± 6.4% (n = 5), respectively. When neurons had been treated with thapsigargin (1 μM for 15 min) and the receptor agonists were applied again in the continuing presence of the Ca^{2+}-ATPase inhibitor, the inhibitory actions of UTP (9.8 ± 3.0% inhibition) and bradykinin (6.7 ± 2.8% inhibition) were reduced, whereas the inhibition by oxotremorine M (61.1 ± 8.1%) was not significantly altered (Fig. 6, A and B).

Although the modulation of I_M by bradykinin was reduced in the presence of the Ca^{2+}-ATPase inhibitor by 90% (see earlier and Cruzblanca et al., 1998), bradykinin-evoked noradrenaline release from SCG neurons has previously been reported not to be altered by 0.3 μM thapsigargin (Boehm and Huck, 1997b). We therefore investigated the effect of thapsigargin (0.3 μM) on UTP- and electrically evoked [^{3}H]noradrenaline release. Tritium overflow triggered by UTP (10 μM) amounted to 1.78 ± 0.58% of total radioactivity in the absence of thapsigargin (n = 6) and to 1.74 ± 1.25% in its presence (n = 6; $P > .6$). Likewise, electrically evoked tritium overflow was not altered by the Ca^{2+}-ATPase inhibitor (not shown, but see Boehm and Huck, 1997b). Nevertheless, 0.3 μM thapsigargin was sufficient to reduce the inhibition of I_M by UTP (10 μM) from 30.1 ± 7.1 to 1.6 ± 7.7% (n = 6; $P < .01$) and that by bradykinin from 39.3 ± 17.3 to 8.3 ± 4.6% (n = 5; $P < .05$). Hence, intact intracellular Ca^{2+} stores are required for the modulation of I_M but not for the induction of transmitter release by UTP or bradykinin.

Effects of Ca^{2+} Chelators on Reduction of I_M by UTP and Oxotremorine M. To reveal whether increases in cytosolic Ca^{2+} concentrations are required for the inhibition of I_M by UTP, neurons were incubated in 3 μM concentration of the cell-permeable Ca^{2+} chelator BAPTA-AM for 30 min, followed by an incubation in regular bathing solution. In these neurons, UTP (10 μM) did not affect I_M relaxations (2.2 ± 4.0% inhibition, n = 6), whereas in sister cultures not treated with BAPTA-AM the inhibition by UTP amounted to 31.5 ± 5.4% (n = 6; $P < .05$). In contrast, oxotremorine M (10 μM) reduced I_M in neurons treated with BAPTA-AM (85.4 ± 3.0% inhibition; n = 4) to the same extent as in nontreated neurons (88.7 ± 2.7% inhibition; n = 5; Fig. 6C). To corroborate a role of increases in intracellular Ca^{2+} in the UTP-dependent inhibition of I_M, currents were determined in the open-tip whole-cell configuration of the patch-clamp technique with either 20 mM KCl or 20 mM K-BAPTA added to the pipette solution. With BAPTA in the recording electrode, UTP (10 μM) reduced I_M relaxations by 6.4 ± 9.3% (n = 4), with KCl instead of BAPTA, the inhibition amounted to 44.8 ± 11.4% (n = 4; $P < .05$; Fig. 6D).

To learn whether the inhibition of I_M by UTP might depend on the presence of extracellular Ca^{2+}, I_M relaxations were measured again in the perforated-patch configuration, and UTP was applied in the absence and presence of 2 mM Ca^{2+}. The inhibitory action of UTP (10 μM) was the same in both cases (Fig. 6D). Thus, the inhibition of I_M by UTP requires release of intracellular Ca^{2+} but is independent of extracellular Ca^{2+}.

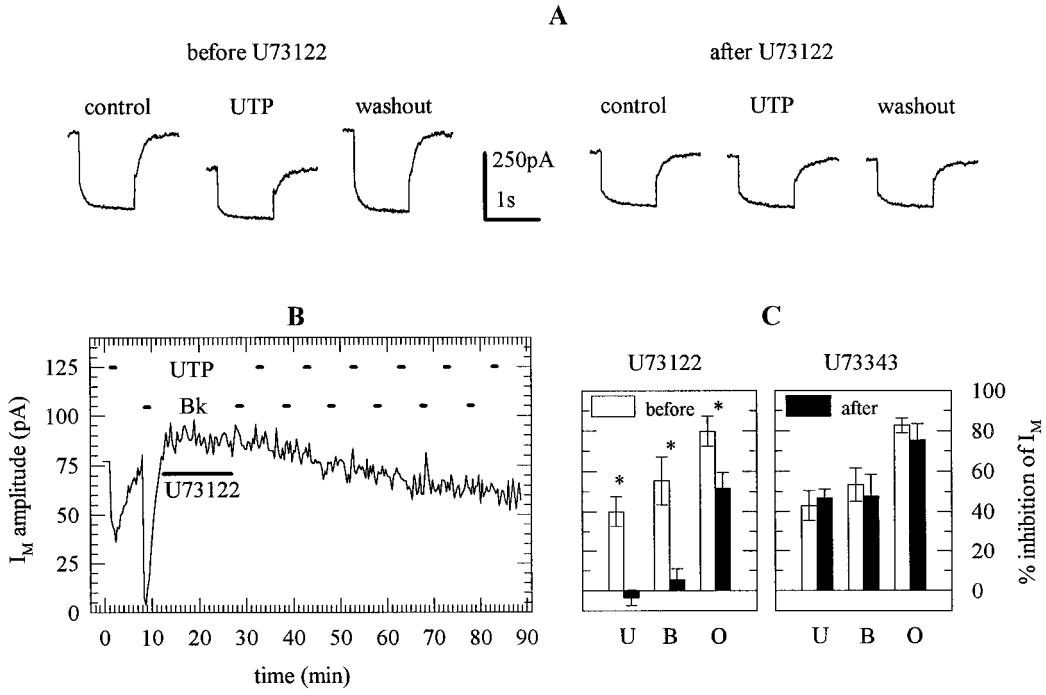

Fig. 4. Effects of a phospholipase C inhibitor on the reduction of I_M by UTP, bradykinin, and oxotremorine M. A, effect of 10 μM UTP on I_M in a SCG neuron was investigated before and after a 15-min application of 1 μM concentration of the phospholipase C inhibitor U73122. The current traces shown were elicited by 1-s hyperpolarizations from −30 to −55 mV and were obtained before (control), during (UTP), and after (washout) the application of 10 μM UTP. B, time course of I_M relaxation amplitudes in an additional SCG neuron. Note that 1 μM U73122 irreversibly abolished the inhibitory actions of UTP (10 μM) and bradykinin (Bk, 1 μM). C, summary of the effects of the active phospholipase C inhibitor U73122 and of the less active isomer U73343 (both at 1 μM) on the inhibitory actions of UTP (U, 10 μM), bradykinin (B, 1 μM), and oxotremorine M (O, 10 μM) on I_M; experiments were performed as shown in B (n = 5 to 6; *$P < .05$ between the effects before and after application of 1 μM U73122 for 15 min).

Discussion

In heterologous expression systems, all types of P2Y receptors couple to phospholipase C to generate inositol polyphosphates, which then liberate Ca^{2+} from intracellular stores. However, the signaling cascades of native P2Y receptors, in particular of those expressed in neurons, are less well characterized (Harden et al., 1995; North and Barnard, 1997; King et al., 1998). In rat SCG neurons, a P2Y6-like G protein-coupled nucleotide receptor mediates UTP-evoked transmitter release, on one hand, and UTP-dependent inhibition of K_M channels, on the other hand (Boehm et al., 1995; Boehm, 1998). The present results demonstrate that these two effects involve different signaling cascades, and only the latter action, the inhibition of K_M channels, involves an accumulation of IP_3 with resulting increases in cytosolic Ca^{2+}.

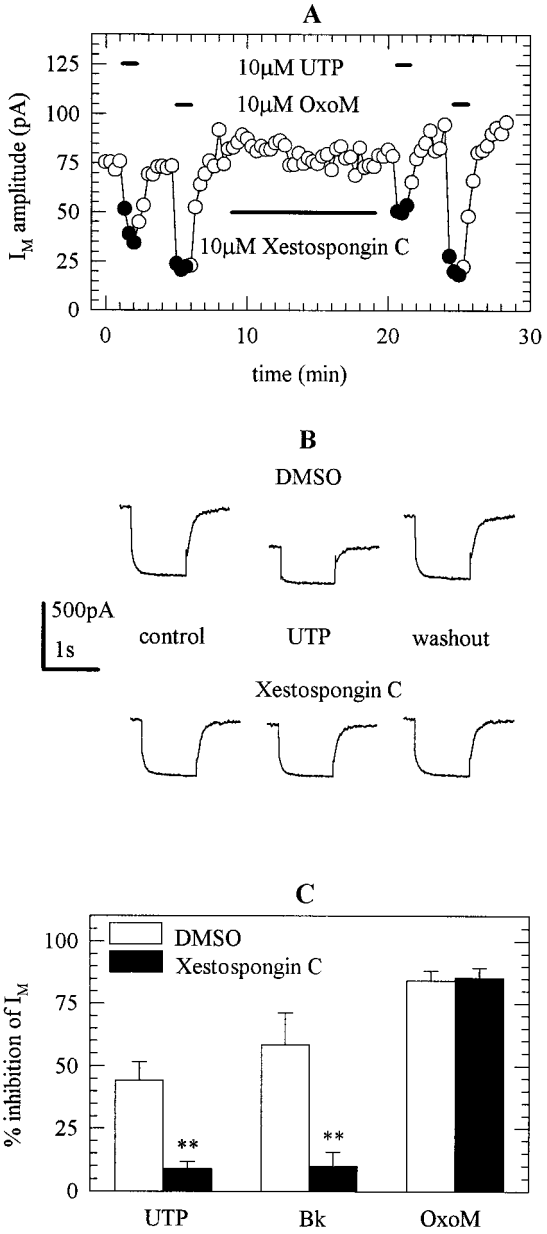

Fig. 5. Effects of an IP_3 antagonist on the reduction of I_M by UTP, bradykinin, and oxotremorine M. A, effects of 10 μM UTP and 10 μM oxotremorine M (OxoM) on I_M in a SCG neuron were investigated before and after a 10-min application of 10 μM concentration of the IP_3 antagonist xestospongin C; the graph shows the time course of I_M relaxation amplitudes. B, effect of 10 μM UTP on I_M was investigated in a SCG neuron incubated for 30 min in 0.1% DMSO (top) and then in a neuron in the same culture dish incubated for 30 min in 10 μM xestospongin C (bottom). The current traces shown were elicited by 1-s hyperpolarizations from −30 to −55 mV and were obtained before (control), during (UTP), and after (washout) the application of 10 μM UTP. C, summary of the effects of incubation of neurons in either 10 μM xestospongin C or 0.1% DMSO, both for 30 min, on the inhibitory actions of UTP (10 μM), bradykinin (Bk, 1 μM), and oxotremorine M (OxoM, 10 μM) on I_M; experiments were performed as shown in B ($n = 4$ or 5; ** $P < .01$ versus the inhibition of I_M in neurons treated with DMSO).

Mechanisms of UTP-Dependent Inhibition of K_M Channels. Our results demonstrate that UTP inhibits K_M channels of SCG neurons via the signal transduction cascade: P2Y receptors → $G_{q/11}$ → phospholipase C → IP_3 → IP_3 receptor → Ca^{2+} release → K_M channel blockade. This conclusion is based on the following results: 1) the UTP-dependent inhibition of I_M was not altered by either PTX or cholera toxin. Hence, members of the toxin-insensitive family of G proteins (Fields and Casey, 1997) must have mediated the inhibition of I_M by UTP. Previously, αq and/or α11 G protein subunits have been shown to be involved in receptor-dependent inhibition of I_M (Caulfield et al., 1994; Jones et al., 1995; Haley et al., 1998). 2) UTP as well as the other nucleotides tested induced the formation of IP_3, and this effect was not altered by PTX but abolished by the phospholipase C inhibitor U73122. The UTP-dependent generation of IP_3 and inhibition of I_M occurred in parallel. Furthermore, U73122 abolished the inhibition of I_M by UTP. This effect appeared specific for phospholipase C, because an isomer (U73343) that fails to block this enzyme (Jin et al., 1994) did not mimic the action of U73122. 3) Xestospongin C, a noncompetitive antagonist of IP_3 receptors (Gafni et al., 1997), largely reduced the inhibition of I_M by UTP, whereas the inhibition by oxotremorine M remained unaltered. This observation verifies that xestospongin C did not interfere with the receptor-mediated modulation of I_M by some unspecific effect. 4) Thapsigargin, which inhibits the endoplasmic Ca^{2+}-ATPase (Thastrup et al., 1990) and thereby depletes intracellular Ca^{2+} stores in SCG neurons (Foucart et al., 1995), significantly attenuated the inhibitory actions of UTP on I_M. 5) Finally, intracellular application of the Ca^{2+} chelator BAPTA prevented the inhibition of I_M by UTP, which, however, was not altered by the removal of extracellular Ca^{2+}. Hence, release of Ca^{2+} from intracellular stores into the cytosol, but not transmembrane Ca^{2+} entry, was involved in the UTP-induced inhibition of I_M. Cytosolic Ca^{2+} concentrations in the submicromolar to low micromolar range have been shown before to directly block K_M channels (Selyanko and Brown, 1996).

Bradykinin obviously used the same signaling pathway to block K_M channels as UTP: the peptide induced the formation of IP_3, and its inhibitory action on I_M was attenuated by U73122, xestospongin C, and thapsigargin by >80%. The inhibition of I_M by the muscarinic agonist oxotremorine M, which also raised IP_3, was not altered by xestospongin C or thapsigargin but somewhat reduced by the phospholipase C inhibitor U73122. Hence, increases in IP_3 are not an absolute prerequisite for the receptor-dependent inhibition of I_M. ATP, at concentrations that do not affect I_M (Boehm, 1998), also caused a rise in IP_3 that was comparable with those elicited by UTP or the other nucleotides tested. Thus, neither an increase in IP_3 (present results; del Rio et al., 1999) nor a

rise in intracellular Ca^{2+} (Pfaffinger et al., 1988; del Rio et al., 1999) does necessarily lead to the inhibition of I_M. It has been speculated that either locally restricted increases in IP_3 and intracellular Ca^{2+} or certain kinetics in the changes of these second messengers are required to lead to the inhibition of I_M (Cruzblanca et al., 1998; Pfaffinger et al., 1988; del Rio et al., 1999). Indeed, the inhibition of I_M by either UTP (Boehm, 1998) or bradykinin (Jones et al., 1995) displays a delayed onset compared with the inhibition by muscarinic agonists. In addition, several Ca^{2+}-dependent enzymes, such as phospholipase A_2 and calcineurin, have been implicated in the receptor-dependent modulation of I_M (for a review, see Marrion, 1997). Therefore, it is not surprising that rises in IP_3 and intracellular Ca^{2+} per se may not be sufficient to reduce I_M; this has also been suggested by experiments in isolated membrane patches of SCG neurons where the direct application of Ca^{2+} blocked K_M channels in only two thirds of the patches investigated (Selyanko and Brown, 1996).

One additional fact should be kept in mind when comparing data of IP_3 assays with those of electrophysiological experiments: biochemical results reflect cellular events in neurons and non-neural cells, which are both present in the culture system used in this study. Patch-clamp recordings, in contrast, are performed with neurons only. Therefore, the apparent discrepancy that receptor agonists such as ATP do raise IP_3, but do not inhibit I_M, may be due to neurotransmitter receptors being expressed in non-neural cells, which contribute to the overall production of IP_3 in the cell cultures. However, our negative results obtained with fibroblasts argue against a major role of non-neuronal elements in the nucleotide-dependent accumulation of IP_3.

Mechanisms of UTP-Evoked Transmitter Release. The PTX treatment, which did not alter the UTP-induced inhibition of I_M or the accumulation of IP_3, clearly reduced the secretagogue action of UTP. Conversely, thapsigargin attenuated the UTP-induced inhibition of I_M but not UTP-evoked noradrenaline release. These results permit several conclusions: 1) UTP-evoked transmitter release involves G proteins other than those involved in the UTP-dependent inhibition of I_M, namely G_i and/or G_o; 2) intact intracellular Ca^{2+} stores are required for the inhibition of I_M, but not for the stimulation of noradrenaline release; and 3) as a consequence, the inhibition of I_M cannot be the major mechanism by which UTP depolarizes SCG neurons to finally evoke transmitter release. The reduction of UTP-evoked noradrenaline release by PTX was not complete but amounted to only 66%. Thus, PTX-insensitive G proteins are also involved in the secretagogue action of the uridine nucleotide. These G proteins cannot include G_s, because down-regu-

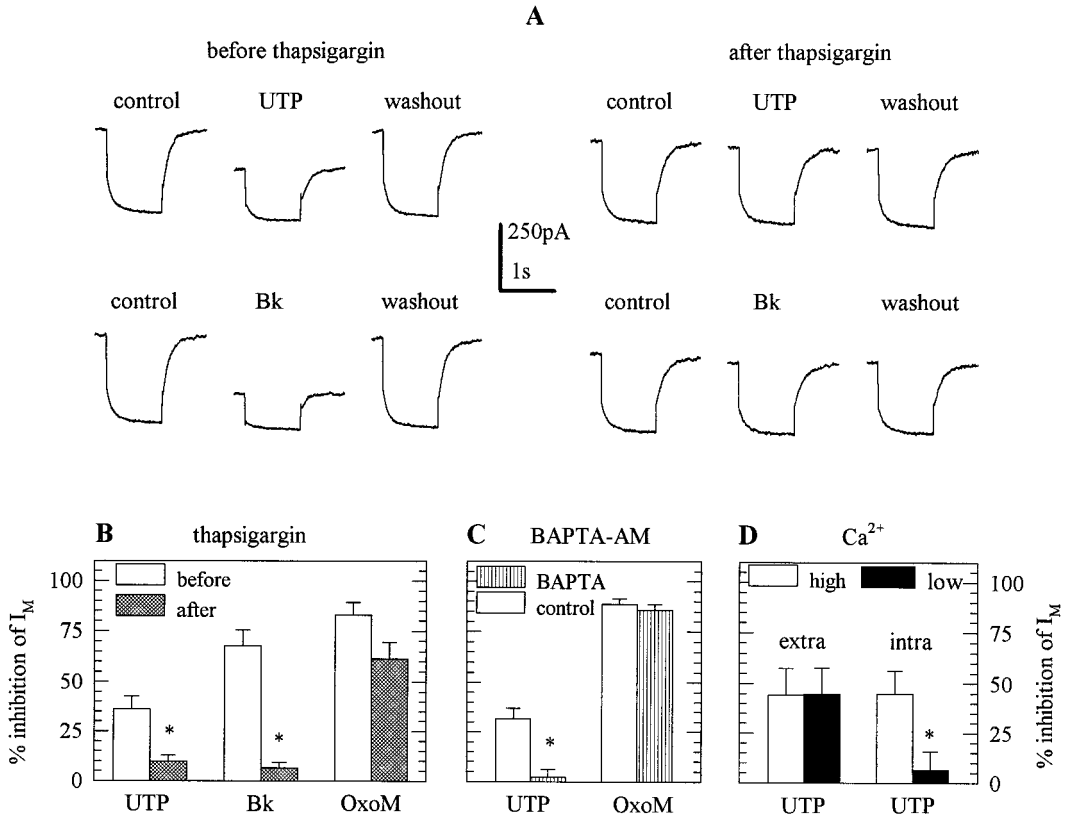

Fig. 6. Effects of Ca^{2+} chelators and Ca^{2+}-ATPase inhibition on the reduction of I_M by UTP. A, effects of 10 μM UTP and 1 μM bradykinin (Bk) on I_M in a SCG neuron were investigated before and after a 15-min application of 1 μM concentration of the Ca^{2+}-ATPase inhibitor thapsigargin. The current traces shown were elicited by 1-s hyperpolarizations from -30 to -55 mV and were obtained before (control), during (UTP, Bk), and after (washout) the application of 10 μM UTP and 1 μM bradykinin, respectively. B, effects of thapsigargin (1 μM) on the inhibitory actions of UTP (10 μM), bradykinin (Bk, 1 μM), and oxotremorine M (OxoM, 10 μM) on I_M; the effects of receptor agonists were tested before and after a 15-min application of 1 μM thapsigargin ($n = 5$ or 6; $^*P < .05$ versus the inhibition of I_M before the application of thapsigargin). C, effects of UTP and oxotremorine M (OxoM; both at 10 μM) were investigated in neurons incubated in 3 μM BAPTA-AM for ≥30 min, followed by a ≥30-min incubation in regular buffer, and were compared with those obtained in control neurons ($n = 4$ to 6; $^*P < .05$ versus the inhibition of I_M in neurons not treated with BAPTA-AM). D, effects of UTP (10 μM) on I_M were investigated in the perforated-patch configuration in the absence and presence extracellular Ca^{2+} (extra) and in the conventional whole-cell configuration with 20 mM KCl or 20 mM BAPTA added to the pipette solution (intra; $n = 4$; $^*P < .05$ versus the inhibition of I_M in neurons dialyzed with pipette solution containing KCl instead of BAPTA).

lation of $G_{s\alpha}$ by cholera toxin (Boehm et al., 1996) failed to affect the induction of transmitter release by UTP. Therefore, it appears likely that the toxin-insensitive G proteins that mediated the formation of IP_3 and the inhibition of I_M (i.e., G_q and/or G_{11}) also contributed to the secretagogue action of UTP. Direct K_M channel blockade by either Ba^{2+} or linopirdine has been found to trigger noradrenaline release from SCG neurons, although the secretagogue actions of these agents are much weaker than those of UTP (Kristufek et al., 1999). However, the lack of effect of thapsigargin on UTP-evoked noradrenaline release argues against an unequivocal role of K_M channel inhibition in the secretagogue action of the nucleotide. Therefore, additional G_q/G_{11}-mediated effects must be involved in the transmitter release stimulated by the activation of P2Y receptors. In accordance with this hypothesis, we found that the protein kinase C inhibitors bisindolylmaleimide I and staurosporine reduced the secretagogue action of UTP by 71 and 91%, respectively (Moskvina et al., 1999).

Previously, G protein-coupled neurotransmitter receptors that either stimulate or facilitate noradrenaline release from sympathetic neurons have all been reported to be linked to PTX-insensitive G proteins (for a review, see Boehm and Huck, 1997a). For example, the PTX pretreatment as performed in this study enhances rather than reduces bradykinin-evoked noradrenaline release from SCG neurons (Boehm and Huck, 1997b). Hence, the P2Y6-like receptors activated by UTP are the first example of G_I- and/or G_o-coupled receptors that trigger transmitter release from SCG neurons. The precise mechanisms underlying the secretagogue action of UTP are currently not known, but $G_{i/o}$-coupled receptors in non-neural cells, such as smooth muscle cells, do cause excitation, and this effect involves protein kinase C but not phospholipase C (Wang et al., 1999).

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