Coexpression of Ligand-gated P<sub>2X</sub> and G Protein-coupled P<sub>2Y</sub> Receptors in Smooth Muscle

P<sub>2</sub> receptor subtypes and their signaling mechanisms were characterized in dispersed smooth muscle cells. UTP and ATP stimulated inositol 1,4,5-triphosphate formation, Ca<sup>2+</sup> release, and contraction that were abolished by U-73122 and guanosine 5′-O-(3-thio)diphosphate, and partly inhibited (50–60%) by pertussis toxin (PTX). ATP analogs (adenosine 5′-(α,β-methylene)-triphosphate, adenosine 5′-(β,γ-methylene)triphosphate, and 2-methylthio-ATP) stimulated Ca<sup>2+</sup> influx and contraction that were abolished by nifedipine and in Ca<sup>2+</sup>-free medium. Micromolar concentrations of ATP stimulated both Ca<sup>2+</sup>-influx and Ca<sup>2+</sup> release.

ATP and UTP activated G<sub>aq/11</sub> and G<sub>bg</sub> in gastric and aortic smooth muscle and heart membranes, G<sub>aq/11</sub> and G<sub>bg</sub> in liver membranes, and G<sub>bg</sub> in brain membranes. Phosphoinositide hydrolysis stimulated by ATP and UTP was mediated concurrently by PLC<sub>bq</sub> and PLC<sub>bq</sub> and PLC<sub>bq</sub> and PLC<sub>bq</sub>

The pattern of responses implied that P<sub>2Y</sub> receptors in visceral and vascular smooth muscle are coupled to PLC<sub>bq</sub> via G<sub>aq/11</sub> and to PLC<sub>bq</sub> via Gβ<sub>bg</sub>. These receptors co-exist with ligand-gated P<sub>2X</sub> receptors activated by ATP analogs and high levels of ATP.

P<sub>2</sub> receptors have been classified recently into two classes comprising ligand-gated cationic channels or P<sub>2X</sub> receptors and G protein-coupled P<sub>2Y</sub> receptors (1, 2); P<sub>2X</sub> and P<sub>2Y</sub> receptors have been subsumed into the P<sub>2Y</sub> class of receptors. The term P<sub>2</sub> recognizes the fact that purine and pyrimidine nucleotides can act as preferential ligands of various receptor subtypes (2). Up to seven P<sub>2X</sub> receptor subtypes (3–9) and eight P<sub>2Y</sub> receptor subtypes (10–16) have been cloned from mammalian and avian species. Fuller understanding of the functions subserved by discrete receptor subtypes is hampered by the organization of native P<sub>2X</sub> receptors into homopolymers or heteropolymers (5) and by the co-existence of P<sub>2X</sub> and P<sub>2Y</sub> receptors on the same cell (17). Earlier classifications based on agonist potency profiles had been confounded by the paucity of selective antagonists and radioligands (2), and by the rapid degradation of some nucleotides, mainly ATP and 2-methylthio-ATP, by ecto-nucleotidases (18), and the interconversion of adenine and uridine nucleotides by ecto-nucleoside diphosphokinases (19, 20).

P<sub>2X1</sub> is the main P<sub>2X</sub> receptor subtype expressed in visceral and vascular smooth muscle (21), whereas P<sub>2X2</sub> and P<sub>2X3</sub> are the main receptor subtypes expressed in peripheral sensory ganglia (8, 21–23). Both P<sub>2X1</sub> and P<sub>2X3</sub> receptors have high affinity for ATP and AMP-PCP<sup>1</sup> and are rapidly desensitized (23, 24). P<sub>2X2</sub>, P<sub>2X4</sub>, and P<sub>2X6</sub> receptors are the predominant receptor subtypes expressed in the adult brain where they are present in various heteromeric combinations; these receptor subtypes exhibit lower affinity for ATP, are insensitive to AMP-PCP, and are not readily desensitized (7, 8, 22, 23). Their insensitivity to AMP-PCP restricts the usefulness of this analog as a radioligand for all but the P<sub>2X1</sub> and P<sub>2X3</sub> receptor subtypes (24).

P<sub>2Y</sub> receptors exhibit variable affinity for purine and pyrimidine nucleotides. P<sub>2Y1</sub> are purinoceptors and are adenine nucleotide-specific (10, 13), whereas P<sub>2Y2</sub> receptors (P<sub>2U</sub> in earlier classifications) have equal affinity for adenine and uridine nucleotide triphosphates (UTP ≥ ATP) (11, 19); P<sub>2Y3</sub>, P<sub>2Y4</sub>, and P<sub>2Y6</sub> are pyrimidinoceptors; P<sub>2Y4</sub> is UTP selective whereas P<sub>2Y3</sub>, P<sub>2Y4</sub>, and P<sub>2Y6</sub> are UDP selective (14, 19). The functional status of P<sub>2Y5</sub> which has low homology to other P<sub>2Y</sub> receptors has not been resolved (16, 25), while the P<sub>2Y7</sub> receptor has now been identified as the leukotriene B<sub>4</sub> receptor (26). P<sub>2Y</sub> receptors are variously coupled to pertussis toxin-sensitive and -insensitive G proteins which activate or inhibit various effector enzymes including phospholipase C-β (PLC-β) (15, 16, 27–30), phospholipase D (31, 32), phospholipase A<sub>2</sub> (38, 39), and adenylyl cyclase (28, 30, 34).

ATP, UTP, and AMP-PCP can mobilize Ca<sup>2+</sup> and elicit contractile responses in vascular and visceral smooth muscle suggesting that both P<sub>2X</sub> and P<sub>2Y</sub> receptors are present (15, 17, 35–37). Their co-existence raises the question as to which receptor subtype mediates preferentially the action of the endogenous ligand, ATP. In the present study, we have used a series of purine and pyrimidine agonists to characterize P<sub>2</sub> receptors in dispersed gastric smooth muscle cells and identify the sig-

<sup>1</sup>The abbreviations used are: AMP-PCP, adenosine 5′-{(α,β-methylene)triphosphate; AMP-CPP, adenosine 5′-(β,γ-methylene)triphosphate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; GDPβS, guanosine 5′-O-3-thio-diphosphate; DPCPX, cyclopentyl-1,3-dipropylxanthine; CGS-15943, 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine; PTX, pertussis toxin; GTPγS, guanosine 5′-O-3-thiotriphosphate; PLC, phospholipase C; [Ca<sup>2+</sup>], intracellular Ca<sup>2+</sup>; PI, phosphoinositol.

From the Departments of Medicine and Physiology, Medical College of Virginia, Richmond, Virginia 23298-0711

Karnam S. Murthy and Gabriel M. Makhlouf†

(Received for publication, August 16, 1997, and in revised form, November 10, 1997)
naling pathways to which they are coupled. Comparative studies characterized the coupling of \( \beta_{2} \) to receptors to G proteins in vascular smooth muscle, heart, liver, and brain. \( \beta_{2X} \) and \( \beta_{2Y} \) receptors were shown to co-exist on gastric smooth muscle cells and to mediate \( \mathrm{Ca}^{2+} \) mobilization and muscle contraction via three distinct pathways. UTP and nanomolar concentrations of ATP activated exclusively \( \beta_{2X} \) Receptors, whereas micromolar concentrations of ATP activated additionally \( \beta_{2Y} \) receptors. The pattern suggests that contraction induced by purine and pyrimidine nucleotides may be preferentially mediated by G protein-coupled receptors.

**EXPERIMENTAL PROCEDURES**

**Dispersion of Gastric Smooth Muscle Cells**—Smooth muscle cells were isolated from the circular muscle layer of rabbit stomach by sequential enzymatic digestion, filtration, and centrifugation as described previously (38–40). The cells were resuspended in enzyme-free medium consisting of 120 mM NaCl, 4 mM KCl, 2.6 mM KH2PO4, 2 mM CaCl\(_2\), 0.6 mM MgCl\(_2\), 25 mM HEPES, 14 mM glucose, and 2.1% Eagle’s essential amino acid mixture. The muscle cells were harvested by filtration through 500- \( \mu \)m Nitex mesh and centrifuged twice at 350 \( \times \) g for 10 min.

In these experiments, the muscle cells were reversibly permeabilized using the TransPort reagent (Life Technologies, Inc.) as described previously (40). The cells were washed in \( \mathrm{Ca}^{2+} \)- and Mg\(^{2+}\)-free HEPES medium and re-suspended in a medium containing 10 mM NaCl, 140 mM KCl, 2.4 mM MgCl\(_2\), and 10 mM HEPES. TransPort reagent (15 \( \mu \)l/ml) was added with or without GDP\(\beta\)S (10 \( \mu \)M) and the mixture incubated at 31°C for 20 min. Permeabilization was terminated by addition of Stop solution (30 \( \mu \)l/ml) and the cell suspension centrifuged for 15 min at 350 \( \times \) g. The cells were resuspended in control HEPES medium containing 0.1% bovine serum albumin and incubated at 31°C for 1 h. The reselled cells were shown to exclude trypan blue and respond to contractile agonists and depolarizing concentrations of KCl.

**Identification of G Protein Subtypes and PLC-\( \beta \) Isozymes in Gastric Smooth Muscle by Western Blot**—The expression of G proteins and PLC-\( \beta \) isozymes was determined by Western blot analysis as described previously (41–43). Homogenates prepared from dispersed muscle cells were solubilized on ice for 1 h in 20 \( \mu \)l Tris (pH 8.0), 1 mM dithiothreitol, 100 mM NaCl, and 0.5% sodium cholate. The suspension was centrifuged at 13,000 \( \times \) g for 5 min. Solubilized proteins were resolved by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. The blots were incubated for 12 h at 4°C with subtype-specific G protein or PLC-\( \beta \) antibodies, and then for 1 h with secondary antibody conjugated with horseradish peroxidase. The bands were identified by enhanced chemiluminescence.

**Selective Protection of \( \beta_{2} \) Receptors**—A technique of selective receptor protection previously used to determine the co-existence and function of various G protein-coupled receptors (44–48) was used to determine the presence and function of \( \beta_{2} \) receptor subtypes. The technique involves protection of one receptor subtype with selective agonists or antagonists followed by inactivation of all unprotected receptors with a low concentration of N-ethylmaleimide (5 \( \mu \)M). Freshly dispersed muscle cells were incubated with one agonist (AMP-PCP, AMP-CPP, UTP, or ATP) for 10 min at 31°C. The cells were centrifuged twice at 150 \( \times \) g for 10 min and resuspended in control HEPES medium for 60 min to ensure complete re-sensitization. The contractile response of cells treated in this fashion was compared with the response of untreated cells. As previously shown (44–48), muscle cells incubated with N-ethylmaleimide without protective agent did not contract in response to receptor-linked agonists, but they responded fully upon addition of agents that bypass receptors (e.g. ionomycin, KCl, and forskolin), implying that post-receptor mechanisms were intact.

**Measurement of Contraction in Dispersed Muscle Cells**—Contraction of dispersed muscle cells was measured by scanning micrometry as described previously (38–40). The length of 50 muscle cells treated with one concentration of a contractile agent was measured by scanning micrometry and compared with the length of 50 untreated muscle cells. All measurements were done in the presence of adenosine A\(_1\) and A\(_2\) antagonists (1 \( \mu \)M DPCPX and 0.1 \( \mu \)M CGS-15943, respectively) (47).

Time course measurements were done at intervals ranging from 5 s to 5 min. As with other agonists, peak contraction was measured at 30 s and the response used to construct concentration-response curves. Contraction was expressed as the mean decrease in cell length from control in micrometers or as the percent decrease in cell length (range of control cell length in various experiments 96 ± 4 to 103 ± 5 \( \mu \)m).

**Measurement of Cytosolic Free \( \mathrm{Ca}^{2+} \) in Dispersed Muscle Cells**—Cytosolic free \( \mathrm{Ca}^{2+} \) was measured by fluorescence in suspensions of muscle cells loaded with the fluorescent \( \mathrm{Ca}^{2+} \) dye, fura 2, as described previously (40, 45). Autofluorescence of unloaded cells was determined in each suspension and subtracted from the fluorescence of fura 2-loaded cells. Measurements were done in the presence of adenine A\(_1\) and A\(_2\) antagonists. Ca\(^{2+}\) levels were calculated under basal conditions and upon addition of agonist from the ratios of observed, minimal and maximal fluorescence (49).

**Inositol 1,4,5-Trisphosphate (IP\(_3\)) Radioreceptor Assay**—IP\(_3\) was measured in dispersed muscle cells by a radioreceptor assay which utilizes \( ^{3} \)H-labeled d-myoinositol and bovine brain microsomes as described previously (41, 42). Agonists were added for 30 s in the presence of adenosine A\(_1\) and A\(_2\) antagonists to 1 ml of muscle cell suspension (10\(^6\) cells/ml) and the reaction terminated with an equal volume of ice-cold 10% perchloric acid. The supernatant was extracted and IP\(_3\) content in the aqueous phase was measured. The results were expressed as picomoles of IP\(_3\)/10\(^6\) cells.

**Assay of PLC-\( \beta \) Activity in Plasma Membranes**—PLC-\( \beta \) activity was determined in plasma membranes by a modification of the method of UHING et al. (50) as described previously (43, 51). The membranes were isolated from dispersed muscle cells labeled with \( ^{3} \)H-Inositol. PLC-\( \beta \) assay was initiated by addition of 0.4 mg of membrane protein to 25 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 10 mM MgCl\(_2\), 300 mM free \( \mathrm{Ca}^{2+}\), 1 mM GTP\_S, 5 mM phosphoethanolamine, and 50 units/ml creatine phosphokinase in a total volume of 0.4 ml. After incubation at 31°C for 60 s, the reaction was terminated with 0.6 ml of 25% trichloroacetic acid (w/v). The supernatant was extracted four times with 2 ml of diethyl ether and the amount of labeled inositol phosphates in the aqueous phase was counted. All measurements were done in the presence of adenine A\(_1\) and A\(_2\) antagonists. PLC-\( \beta \) activity was expressed as counts/min/mg protein/min.

**Identification of Receptor-activated G Proteins**—G proteins selectively activated by \( \beta_{2} \) receptor agonists in muscle cell membranes were identified by the method of OKOMOTO et al. (52) as described previously (41, 42, 48). Muscle cell homogenates were centrifuged at 27,000 \( \times \) g for 15 min, and the crude membranes solubilized for 60 min at 4°C in 20 mM HEPES medium (pH 7.4) containing 2 mM EDTA, 240 mM NaCl, and 1% CHAPS. The membranes were diluted 10-fold and incubated at 37°C with 60 mM [\( ^{32} \)GTP\_]S in a medium containing 10 mM HEPES (pH 7.4), 100 mM EDTA, and 10 mM MgCl\(_2\). The reaction was stopped with 10 volumes of 100 mM Tris-HCl medium (pH 8.0) containing 10 mM MgCl\(_2\), 100 mM NaCl, and 20 mM GTP, and the mixture incubated for 2 h on ice in a precoated nitrocellulose (4-G) protein antibodies. The wells were washed three times with phosphate buffer solution containing 0.05% Tween 20 and the radioactivity from each well was counted. Coating with G protein antibodies (1:1000) was done after the wells were first coated with anti-rabbit IgG (1:1000) for 2 h on ice. The coated nitrocellulose plates were washed with 4-G protein antibodies of various G proteins or PLC-\( \beta \) antibodies, and then for 1 h with secondary antibody conjugated with horseradish peroxidase. The bands were identified by enhanced chemiluminescence.

**Materials**—\( \beta_{2} \) receptor agonists were obtained from American Peptide Company, Sunnyvale, CA and Sigma Chemicals Co., St. Louis, MO, and all other chemicals were of analytical grade.
RESULTS

Contraction and Ca\(^{2+}\) Mobilization in Dispersed Smooth Muscle Cells by Purine and Pyrimidine Nucleotides—Exposure of muscle cells to 1 \(\mu\)M UTP or ATP caused immediate contraction that was virtually linear during the first 20 s and attained a peak at 30 s. Muscle cell contraction was measured by scanning micrometry and expressed as percent decrease in cell length from control. B, muscle cells were exposed for various intervals to either 1 \(\mu\)M UTP or 1 \(\mu\)M AMP-PCP (\(\alpha\)-MeATP). The cells were washed and peak response to the same agonist was measured at 30 s by scanning micrometry. The results are expressed as percent of the control response before desensitization.

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Time course and rate of desensitization of contractile response to purine and pyrimidine nucleotides. A, time course of contraction in response to 1 \(\mu\)M ATP and UTP. The initial response detected within 5 s was virtually linear during the first 20 s and attained a peak at 30 s. Muscle cell contraction was measured by scanning micrometry and expressed as percent decrease in cell length from control. B, muscle cells were exposed for various intervals to either 1 \(\mu\)M UTP or 1 \(\mu\)M AMP-PCP (\(\alpha\)-MeATP). The cells were washed and peak response to the same agonist was measured at 30 s by scanning micrometry. The results are expressed as percent of the control response before desensitization.

Contraction and Ca\(^{2+}\) mobilization in dispersed smooth muscle cells by purine and pyrimidine nucleotides—Exposure of muscle cells to 1 \(\mu\)M UTP or ATP caused immediate contraction that was virtually linear during the first 10 s and attained a peak in 30 s followed by a decline to lower levels (Fig. 1A). The biphasic time course was identical to that observed with other contractile agonists (38, 53). The peak response at 30 s was used to construct concentration-response curves. Prolonged exposure of muscle cells to purine or pyrimidine agonists resulted in time-dependent desensitization that was more rapid with P\(_{2X}\) receptor agonists (e.g., AMP-PCP) than with P\(_{2Y}\) receptor agonists (e.g., UTP) (Fig. 1B). With either type of agonist, however, there was minimal desensitization (<2% of control response) during the initial 30-s period when peak response was measured.

UTP, ATP, and ATP analogs caused concentration-dependent contraction of dispersed smooth muscle cells yielding curves with EC\(_{50}\) values of 33 ± 6 nM for ATP and UTP, respectively, 78 ± 17 and 85 ± 20 nM for AMP-PCP and AMP-CPP, respectively, and 178 ± 23 nM for 2-methylthio-ATP (Fig. 2). Except for the response to 2-methylthio-ATP, maximal contraction induced by all agonist (29 ± 3 to 30 ± 2% decrease in cell length) was similar to that elicited by other contractile agonists, such as cholecystokinin octapeptide (30 ± 3%) or acetylcholine (31 ± 4%).

ATP, UTP, AMP-PCP, and AMP-CPP increased cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) by 1-fold at 10 nM and by 3-fold at 10 \(\mu\)M (Table II). The increase induced by 2-methylthio-ATP was also concentration-dependent but significantly lower (Table II).

Contraction and the increase in [Ca\(^{2+}\)]\(_i\) induced by ATP, UTP, AMP-PCP, and AMP-CPP increased cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in dispersed smooth muscle cells by 1-fold at 10 nM and by 3-fold at 10 \(\mu\)M (Table II). The increase induced by 2-methylthio-ATP was also concentration-dependent but significantly lower (Table II).

In contrast, contraction and the increase in [Ca\(^{2+}\)]\(_i\) induced by 10 nM or 10 \(\mu\)M UTP, and by 10 nM ATP were not affected by nifedipine or Ca\(^{2+}\)-free medium but were abolished by GDP\(_{\beta}\)S or U-73122 (Tables I and II). Pertussis toxin partly inhibited
Contraction induced by purine and pyrimidine nucleotides (10 nM and 10 μM) in dispersed smooth muscle cells

Table I

| Control | 0 Ca\(^{2+}\)/EGTA | Nifedipine | U-73122 | U-73122 and nifedipine | GDP/βS | PTX |
|---------|----------------|------------|---------|------------------------|-------|-----|
| AMP-PCP (10 nM) | 98 ± 23 | 10 ± 10\(^a\) | 26 ± 18\(^b\) | 75 ± 13 | 25 ± 15\(^a\) | 74 ± 14 | 99 ± 34 |
| AMP-PCP (10 μM) | 305 ± 42 | 22 ± 20\(^a\) | 37 ± 19\(^b\) | 315 ± 24 | 17 ± 4\(^a\) | 304 ± 40 | 321 ± 34 |
| AMP-CPP (10 nM) | 106 ± 24 | 25 ± 16\(^a\) | 19 ± 9\(^a\) | 91 ± 24 | 9 ± 5\(^a\) | 91 ± 28 | 96 ± 25 |
| AMP-CPP (10 μM) | 351 ± 35 | 28 ± 14\(^a\) | 50 ± 15\(^a\) | 308 ± 23 | 23 ± 12\(^a\) | 327 ± 34 | 295 ± 35 |
| 2-Methylthio-ATP (10 nM) | 52 ± 10 | 10 ± 7\(^a\) | 22 ± 13\(^a\) | 74 ± 22 | 11 ± 6\(^a\) | 64 ± 18 | 69 ± 20 |
| 2-Methylthio-ATP (10 μM) | 191 ± 28 | 31 ± 21\(^a\) | 21 ± 20\(^a\) | 167 ± 16 | 9 ± 9\(^b\) | 199 ± 26 | 168 ± 16 |
| ATP (10 nM) | 94 ± 22 | 96 ± 12 | 88 ± 18 | 20 ± 10\(^a\) | 15 ± 14\(^b\) | 52 ± 11\(^b\) |
| ATP (10 μM) | 346 ± 38 | 302 ± 18 | 263 ± 21 | 241 ± 25\(^b\) | 17 ± 12\(^a\) | 254 ± 29\(^b\) | 278 ± 52 |
| UTP (10 nM) | 121 ± 27 | 103 ± 16 | 83 ± 22 | 19 ± 16\(^b\) | 14 ± 16\(^b\) | 11 ± 6\(^b\) | 53 ± 23\(^b\) |
| UTP (10 μM) | 334 ± 36 | 292 ± 27 | 302 ± 35 | 25 ± 14\(^a\) | 16 ± 17\(^a\) | 26 ± 7\(^a\) | 108 ± 12\(^a\) |

\(^a\) p < 0.01. \(^b\) p < 0.05.

Increase in [Ca\(^{2+}\)]\(_i\), induced by purine and pyrimidine nucleotides in dispersed smooth muscle cells

Table II

| Control | 0 Ca\(^{2+}\)/EGTA | Nifedipine | U-73122 | U-73122 and nifedipine | GDP/βS | PTX |
|---------|----------------|------------|---------|------------------------|-------|-----|
| AMP-PCP (10 nM) | 98 ± 23 | 10 ± 10\(^a\) | 26 ± 18\(^b\) | 75 ± 13 | 25 ± 15\(^a\) | 74 ± 14 | 99 ± 34 |
| AMP-PCP (10 μM) | 305 ± 42 | 22 ± 20\(^a\) | 37 ± 19\(^b\) | 315 ± 24 | 17 ± 4\(^a\) | 304 ± 40 | 321 ± 34 |
| AMP-CPP (10 nM) | 106 ± 24 | 25 ± 16\(^a\) | 19 ± 9\(^a\) | 91 ± 24 | 9 ± 5\(^a\) | 91 ± 28 | 96 ± 25 |
| AMP-CPP (10 μM) | 351 ± 35 | 28 ± 14\(^a\) | 50 ± 15\(^a\) | 308 ± 23 | 23 ± 12\(^a\) | 327 ± 34 | 295 ± 35 |
| 2-Methylthio-ATP (10 nM) | 52 ± 10 | 10 ± 7\(^a\) | 22 ± 13\(^a\) | 74 ± 22 | 11 ± 6\(^a\) | 64 ± 18 | 69 ± 20 |
| 2-Methylthio-ATP (10 μM) | 191 ± 28 | 31 ± 21\(^a\) | 21 ± 20\(^a\) | 167 ± 16 | 9 ± 9\(^b\) | 199 ± 26 | 168 ± 16 |
| ATP (10 nM) | 94 ± 22 | 96 ± 12 | 88 ± 18 | 20 ± 10\(^a\) | 15 ± 14\(^b\) | 52 ± 11\(^b\) |
| ATP (10 μM) | 346 ± 38 | 302 ± 18 | 263 ± 21 | 241 ± 25\(^b\) | 17 ± 12\(^a\) | 254 ± 29\(^b\) | 278 ± 52 |
| UTP (10 nM) | 121 ± 27 | 103 ± 16 | 83 ± 22 | 19 ± 16\(^b\) | 14 ± 16\(^b\) | 11 ± 6\(^b\) | 53 ± 23\(^b\) |
| UTP (10 μM) | 334 ± 36 | 292 ± 27 | 302 ± 35 | 25 ± 14\(^a\) | 16 ± 17\(^a\) | 26 ± 7\(^a\) | 108 ± 12\(^a\) |

\(^a\) p < 0.01. \(^b\) p < 0.05.

Contraction induced by purine and pyrimidine nucleotides (10 nM and 10 μM) in dispersed smooth muscle cells

The effect of ATP-induced contraction mediated by P\(_{2X}\) receptors was evaluated at different concentrations of ATP in the presence of 1 μM U-73122. The concentration-response curve was shifted to the right by U-73122 (Fig. 2), and the EC\(_{50}\) for ATP acting via P\(_{2X}\) receptors was 0.61 ± 0.07 μM (compared with 3.3 ± 0.9 μM in the absence of U-73122). In Ca\(^{2+}\)-free medium, the contractile response to all concentrations of ATP was abolished by U-73122 (Fig. 2).

Stimulation of IP\(_3\) Formation in Dispersed Smooth Muscle Cells by ATP and UTP—Both ATP and UTP caused a prompt increase in IP\(_3\) formation in dispersed smooth muscle cells (5.1 ± 0.4 and 5.2 ± 0.4 pmol/10\(^6\) cells above basal level with 10 μM ATP and UTP, respectively; basal levels: 3.3 ± 0.5 pmol/10\(^6\) cells) (Fig. 3). IP\(_3\) formation induced by ATP and UTP was partly inhibited by PTX (60 ± 7 and 63 ± 4%), and more potently by GDP/βS (82 ± 5 and 80 ± 3%) (Fig. 3). AMP-PCP, AMP-CPP, and 2-methylthio-ATP did not induce IP\(_3\) formation in muscle cells (0.1 ± 0.2 to 0.7 ± 0.4 pmol/10\(^6\) cells).

Identification of PLC-β Isozymes and G Proteins Activated by ATP and UTP in Smooth Muscle—Western blot analysis dis-
closed the presence of PLC-\(\beta_1\), PLC-\(\beta_3\), and PLC-\(\beta_4\) with minimal expression of PLC-\(\beta_2\) in gastric muscle cell homogenates (Fig. 4). The analysis disclosed the presence also of \(G_{q11}\), \(G_s\), \(G_i\), \(G_o\), \(G_\|\) and \(G_\|/11\) (Fig. 4). The pattern of G protein and PLC-\(\beta\) expression was similar to that previously reported in intestinal muscle cell homogenates (41–43).

The isoforms of PLC-\(\beta\) and G proteins activated by ATP and UTP in smooth muscle were identified by functional blockade with specific antibodies. PLC-\(\beta\) antibodies and G protein antibodies were used at a concentration of 10 \(\mu\)g/ml shown previously to be maximally effective (41–43, 48, 51, 54). PLC-\(\beta\) activity (\[^{3}H\]inositol phosphate formation) in membranes derived from dispersed smooth muscle cells increased by 218 ± 20 and 236 ± 54% with 10 \(\mu\)M ATP and UTP, respectively; AMP-PCP, AMP-CPP, and 2-methylthio-ATP had no effect (3 ± 4 to 9 ± 9%). Pretreatment of plasma membranes for 1 h with 10 \(\mu\)g/ml PLC-\(\beta_1\) antibody or PLC-\(\beta_3\) antibody inhibited ATP-stimulated PLC-\(\beta\) activity by 47 ± 5 and 59 ± 8%, respectively (Fig. 5). The effect of a combination of both antibodies was additive eliciting complete inhibition (91 ± 6%) (Fig. 5). Pretreatment with PLC-\(\beta_2\) or PLC-\(\beta_4\) antibody had no effect on ATP-stimulated PLC-\(\beta\) activity (6 ± 5 and 8 ± 4%). Identical results were obtained for UTP-stimulated PLC-\(\beta\) activity which was inhibited 41 ± 7% by PLC-\(\beta_1\) antibody, 49 ± 8% by PLC-\(\beta_3\) antibody, and 93 ± 5% by a combination of PLC-\(\beta_1\) and PLC-\(\beta_3\) antibodies (Fig. 5). The results implied that PI hydrolysis induced by ATP and UTP was mediated additively by PLC-\(\beta_1\) and PLC-\(\beta_3\).

PLC-\(\beta\) activity stimulated by ATP or UTP was inhibited by \(G_{q11}\) antibody or a common antibody to \(G_\|\); the antibodies were used at a concentration of 10 \(\mu\)g/ml previously shown to be maximally effective (41–43, 48, 51, 54). PLC-\(\beta\) activity stimulated by ATP was inhibited 47 ± 8% by \(G_{q11}\) antibody, 56 ± 6% by \(G_\|\) antibody, and 92 ± 5% by a combination of both antibodies (Fig. 5). \(G_{\|}\), \(G_{\|2}\), and \(G_\|/11\) antibodies had no effect on ATP-stimulated PLC-\(\beta\) activity (4 ± 5 to 5 ± 10%). Identical results were obtained for UTP-stimulated PLC-\(\beta\) activity which was inhibited 40 ± 7% by \(G_{q11}\) antibody, 57 ± 8% by \(G_\|\) antibody, and 92 ± 4% by a combination of both antibodies (Fig. 5).

The effect of a combination of PLC-\(\beta_1\) antibody and \(G_\|\) antibody was additive, eliciting complete inhibition of PLC-\(\beta\) ac-

![Image](http://www.jbc.org/)

**Figure 4.** Expression of G proteins and PLC-\(\beta\) isoforms in gastric smooth muscle. Homogenates were prepared from dispersed gastric circular muscle cells and solubilized with sodium cholate in Tris buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes, and probed with specific antibodies to the PLC-\(\beta\) isoforms and \(\alpha\)-subunits of various G proteins and then with anti-rabbit IgG conjugated to horseradish peroxidase. The proteins were identified by enhanced chemiluminescence.

**Figure 5.** Inhibition of ATP- and UTP-stimulated PLC-\(\beta\) activity in smooth muscle membranes by PLC-\(\beta\) and G protein antibodies. PLC-\(\beta\) activity induced by 10 \(\mu\)M ATP and UTP in the presence of 1 \(\mu\)M GTP-S was measured in plasma membranes isolated from dispersed smooth muscle cells. The measurements were repeated in membranes treated for 1 h with 10 \(\mu\)g/ml of each antibody separately. Antibodies to PLC-\(\beta_2\), PLC-\(\beta_4\), \(G_{\|}\), \(G_{\|2}\), \(G_{\|/11}\), and \(G_\|/11\) had no effect on ATP- or UTP-stimulated PLC activity and are not depicted. Results are expressed as counts/min/mg of protein/min of inositol phosphates (\[^{3}H\]IPs) above basal level (1105 ± 110 cpm/mg/min). Values are mean ± S.E. of four experiments. ***, significant inhibition, \(p < 0.01\) to \(p < 0.001\).
stimulated PLC- either antibody alone). Identical results were obtained for UTP-

activity (91 ± 4%), whereas the effect of a combination of PLC-β3 antibody and Gβ antibody was not additive (62 ± 6% inhibition by the combination versus 59 ± 8 and 56 ± 6% inhibition for either antibody alone). Identical results were obtained for UTP-stimulated PLC-β activity which was inhibited 90 ± 6% by the combination of PLC-β1 and Gβ antibodies and 53 ± 7% by a combination of PLC-β3 and Gβ antibodies (Fig. 5).

Pretreatment of the cells for 1 h with 400 ng/ml PTX before membrane isolation inhibited ATP- and UTP-stimulated PLC-β activity by 59 ± 6 and 60 ± 7%, respectively (Fig. 6). ATP- and UTP-stimulated PLC-β activities were abolished by a combination of PTX with either PLC-β1 or Goq11 antibody (96 ± 3 to 98 ± 5% inhibition). In contrast, inhibition by a combination of PTX with either PLC-β2 or Gβ antibody was not significantly different from inhibition by PTX, PLC-β3 antibody, or Gαi antibody alone (range of inhibition 58 ± 5 to 61 ± 5%) (Fig. 6).

The results implied that phosphoinositide (PI) hydrolysis induced by ATP and UTP was mediated by Goq11-dependent activation of PLC-β1, and by PTX-sensitive, Gβ-dependent activation of PLC-β3. The pattern is consistent with PTX-sensitive and -insensitive stimulation of IP₃ formation in dispersed muscle cells by ATP and UTP (Fig. 3).

Identification of G Proteins Coupled to P₂Y Receptors—The identification of G proteins coupled to P₂Y receptors in smooth muscle cells by using an antibody against either P₂X or P₂Y receptor was performed by the method of Lamerton and Hokin (1970). Membranes were isolated from dispersed smooth muscle cells before and after treatment of the cells with 400 ng/ml PTX for 1 h. Membranes isolated from cell pretreated with PTX were incubated for 1 h with 10 μg/ml of each antibody separately. Results are expressed as counts/min/mg protein/min of inositol phosphates ([3H]IPs) above basal level (4700 ± 150 cpm/mg/min). Values are mean ± S.E. of four experiments.

**,** significant inhibition, p < 0.01 to p < 0.001.

PTX-sensitive and -insensitive G protein(s) activated by ATP and UTP in gastric smooth muscle were identified by a technique that did not involve functional blockade with antibodies. Solubilized muscle cell membranes were incubated with [35S]GTPγS (60 nM) with or without ATP or UTP and added to wells precoated with different Gα antibodies; an increase in the binding of [35S]GTPγS to Gα complexes to a specific Gα antibody reflected activation of the corresponding G protein. Addition of ATP (10 μM) caused a time-dependent increase in the binding of [35S]GTPγS to Gαq11 and Goq3 antibodies (Fig. 7), but not to Goa1–2, Goa3, or Goa5 antibody (Table III). An identical pattern was observed with UTP which stimulated the binding of [35S]GTPγS to Goa1–2 and Goa3 antibodies but not to Goa1–2, Goa3, or Goa5 antibodies (Table III). Pretreatment of muscle cells with 400 ng/ml PTX for 1 h before membrane isolation abolished the ATP- and UTP-stimulated increase in steady-state binding of [35S]GTPγS to Goα3 antibody, but not to Goa1–2 antibody (Table III).

Peptide I (KNNKECGLY), comprising the G protein sequence against which the Goa5 antibody was raised, inhibited selectively ATP- and UTP-stimulated activation of Goa5 (Table...
Values are mean ± S.E. of four experiments.

**Significant increase in binding above binding to GTP-β-S alone, p < 0.01.**

a Significant inhibition of binding, p < 0.001, by peptides I and II (sequences against which Gaq, and Gaq11 antibodies were raised, respectively). Values are mean ± S.E. of four experiments. c NT, not tested.

III). Conversely, peptide II (QLNLKEYNVLV), comprising the G protein sequence against which Gaq11 antibody was raised, inhibited selectively ATP- and UTP-stimulated activation of Goq11 (Table III). Peptides I and II inhibited both activation of G proteins by GTP-β-S as well as the increase in activation induced by ATP or UTP. Peptides I and II were used at a concentration (1 μM) previously shown to abolish activation of Goq3 and Gaq11, respectively (48). AMP-PCP, AMP-CPP, and 2-methylthio-ATP did not cause activation of Gaq11, Goq12–13, Goq3, and Gaq (Table III).

To determine whether P2X receptors were invariably coupled to the same G proteins, similar measurements were done on solubilized membranes from rabbit aortic smooth muscle, heart, liver, and whole brain (Figs. 8 and 9). The results obtained in heart and vascular smooth muscle membranes were identical to those obtained in visceral smooth muscle membranes: UTP and ATP activated Goq11 and Goq3 but not Gβ1, Gβ2, Gαs, or Go (Fig. 8). In liver, ATP and UTP activated Goq11 and Gβ1 and/or Gαs, but did not activate Goq3, Go or Gαs (Fig. 9). In contrast to vascular and visceral smooth muscle, heart, and liver, ATP and UTP activated predominantly Go as well as Goq3 and Gβ1, and/or Gαs in brain membranes, but did not activate Goq11 or Go (Fig. 9). The extent of activation of specific G proteins by ATP and UTP was similar in all tissues except brain where activation of all inhibitory G proteins by ATP was more pronounced, suggesting interaction of ATP with P2Y receptors and ATP-prefering P2X receptors.

**Identification of P2Y and P2X Receptors in Smooth Muscle Cells by Selective Receptor Protection**—The pattern of PI hydrolysis, IP3 formation, Ca2+ mobilization and contraction suggested that UTP and ATP interacted with a common P2Y receptor coupled to PTX-sensitive and -insensitive G proteins, whereas AMP-PCP, AMP-CPP, and 2-methylthio-ATP interacted with a distinct ligand-gated P2X receptor; the latter was also activated by high concentrations of ATP. This notion was corroborated by selective receptor protection to enrich the muscle cells with one receptor subtype. After selective receptor protection, muscle cells were incubated for 60 min in control medium to allow complete resensitization of the cells (see "Experimental Procedures").

Receptor protection with 10 nM AMP-PCP preserved completely the contractile response to 10 nM AMP-PCP (15 ± 3% decrease in cell length) and AMP-CPP (14 ± 2%) (see Fig. 2 and Table I for comparison with responses to untreated muscle cells), but not the responses to ATP or UTP. An identical pattern was obtained by receptor protection with 10 nM AMP-CPP. In contrast, receptor protection with 10 nM UTP preserved completely the responses to 10 nM UTP (15 ± 1%) and ATP (13 ± 2%), but not the responses to AMP-PCP and AMP-CPP. An identical pattern was obtained by receptor protection with 10 nM ATP. Receptor protection with a high concentration of ATP (10 μM) preserved completely the responses to UTP and ATP as well as the responses to AMP-PCP and AMP-CPP.
implying that at this concentration ATP interacted with P2X and P2Y receptors on muscle cells.

After selective protection of P2Y receptors with UTP, the contractile response to a high concentration of ATP (10 μM) could be abolished by U-73122 (control response: 28 ± 1% decrease in cell length; with U-73122: 3 ± 2%) implying that it was exclusively mediated by PI hydrolysis. Following desensitization of ligand-gated P2X receptors by preincubation of muscle cells for 30 min with 10 μM AMP-PCP, the contractile response to 10 μM ATP was virtually abolished by GDPβS and U-73122 (control, 30 ± 2% decrease in cell length; GDPβS, 3 ± 1%; U-73122, 4 ± 2%) and partly inhibited by PTX (control, 30 ± 2%; PTX, 13 ± 2%). Thus, after selective desensitization of P2X receptors or selective protection of P2Y receptors, the response to a high concentration of ATP (10 μM) reflected exclusively activation of G protein-coupled pathways.

**DISCUSSION**

This study demonstrates the co-existence of ligand-gated P2X and G protein-coupled P2Y receptors on freshly dispersed gastric smooth muscle cells and suggests that ATP activates preferentially P2Y receptors to elicit Ca^{2+} mobilization and muscle contraction. The P2Y receptors selectively activated by UTP and ATP were coupled to PLC-β1 via G_α_{q/11} and to PLC-β3 via G_βγ_{13}. Concurrent activation of the two effector enzymes resulted in PTX-sensitive and -insensitive IP_3 formation and IP_3-dependent Ca^{2+} release from sarcoplasmic stores. The high affinity for ATP and UTP suggested that these were P2Y2 receptors: UTP-selective P2Y4 receptors and UDP-selective P2Y6 receptors coupled to PLC-β can be expressed in smooth muscle but they exhibit low or minimal affinity for ATP (11, 19). The P2X receptors selectively activated by AMP-PCP and AMP-CPP-mediated Ca^{2+} influx via dihydropyridine-sensitive, voltage-gated Ca^{2+} channels; the Ca^{2+} channels were activated by depolarization of the plasma membrane that resulted from the opening of ligand-gated cation P2X receptors (22, 23). Their presence on smooth muscle (which predominantly expresses P2X1 receptors (21)), and their activation by AMP-CPP (which selectively interacts with P2X1 receptors on smooth muscle (36)), and by AMP-CPP (which interacts with P2X1 and P2X3 receptors (23, 24, 36)), suggested that the receptors were of the P2X1 subtype. Although the activity profile in visceral smooth muscle (AMP-CPP > AMP-CPP > 2-methylthio-ATP > ATP) resembled that seen in vascular smooth muscle (17), it differed from the activity profile determined in patch-clamp studies of the cloned human and rat P2X receptors where ATP and 2-methylthio-ATP were more potent than AMP-CPP (4, 5, 23). It seems unlikely that the difference reflected degradation of ATP or 2-methylthio-ATP by ecto-nucleotidases, since the measurements of response, particularly those of [Ca^{2+}]_i, in both visceral and vascular smooth muscle were virtually instantaneous (<2 s), and the ratio of medium to cell volume (5000:1) was very high. It is possible that the rabbit P2X1 receptor is different from the human and rat homologs or that its conformation or extent of polymerization is different when it is expressed in smooth muscle.

The evidence for the co-existence and subtype of P2Y and P2X receptors in smooth muscle may be summarized as follows. First, ATP and UTP stimulated IP_3 formation, Ca^{2+} release, and contraction in dispersed smooth muscle cells. The responses to UTP and low nanomolar concentrations of ATP were abolished by GDPβS and the PLC-β inhibitor, U-73122, and partly inhibited by PTX, implying the participation of PTX-sensitive and -insensitive G proteins in IP_3 formation and IP_3-dependent Ca^{2+} release. In contrast, ATP analogs with high affinity for P2Y receptors did not stimulate IP_3 formation; contraction and the increase in [Ca^{2+}]_i were abolished by nifedipine and in Ca^{2+}-free medium implying that they were mediated by Ca^{2+} influx. Higher micromolar concentrations of ATP stimulated both Ca^{2+} influx and IP_3-dependent Ca^{2+} release. A similar pattern was observed by Pacad et al. (17) for the [Ca^{2+}]_i response in single aortic smooth muscle cells.

Second, the interaction of ATP analogs with P2X receptors, and UTP and ATP with P2Y receptors was corroborated in experiments using smooth muscle cells enriched with one receptor subtype. The validity of this approach was previously established for several agonists (44–48). Selective protection of P2Y receptors with either UTP or ATP preserved the contractile response to both UTP and ATP, whereas selective protection of P2X receptors with AMP-PCP or AMP-CPP preserved the response to both analogs and to high concentrations of ATP.

Third, ATP and UTP activated both G_α_{q/11} and G_βγ in muscle membranes; activation of G_βγ was suppressed by pretreatment of muscle cells with PTX. PI hydrolysis stimulated by ATP and UTP in plasma membranes was mediated concurrently by the
α-subunit of G_{α11} and the βγ-subunits of G_i; the α-subunit of G_{α11} activated PLC-β1, whereas the βγ-subunits of G_i activated PLC-β3. The activation of PLC-β1 by G_{αq/11} and PLC-β3 by Gβγ3 were independent and additive. Gαα11 and G_i antibodies elicited partial inhibition of PI hydrolysis separately and complete inhibition in combination; similarly, PLC-β1 and PLC-β3 antibodies elicited partial inhibition separately and complete inhibition in combination. Complete inhibition was also obtained by combining PLC-β1 and G_i antibodies, PLC-β1 antibody and PTX, and G_{αq/11} antibody and PTX. It should be emphasized that PLC-β or G proteins antibody were used at maximally effective concentrations (41–43, 48, 51, 54), so that when additive effects were observed with combinations of antibodies, they reflected the involvement of both G proteins or PLC-β isoforms. The specificities of these interactions were confirmed by studies with antibodies to other PLC-β isomers (PLC-β2 and PLC-β4) and G proteins (Gαα11, G_{α11-2γ}, and Gααi), none of which inhibited PI hydrolysis.

The activation of PLC-β3 by the βγ-subunits of G_i in smooth muscle cells confirmed a pattern previously established for other agonists (41–43). Thus, PI hydrolysis induced by somatostatin (via somatostatin type 3 receptors) was mediated by the βγ-subunits of G_11 and G_i (41), whereas PI hydrolysis induced by opioid agonists (via μ, δ, and κ receptors) was mediated by the βγ-subunits of G_12 and G_2 (42); PI hydrolysis induced by adenosine (via A_1 receptors) was distinctive in that it required activation of PLC-β3 by both α- and βγ-subunits of G_i (43).

Comparative studies in other tissues to determine whether coupling of P_2Y receptors to G_{α11} and G_i was an invariant characteristic showed identical coupling to these G proteins in vascular smooth muscle and heart. Coupling in hepatocytes was to G_i and/or G_α, and coupling in brain was confined to inhibitory G proteins, particularly G_i, but also G_{α11}, G_{α2δ}, and G_{α3δ}. The extent of activation of G proteins in visceral and vascular smooth muscle, heart, and liver was similar for ATP and UTP, suggesting interaction with P_2Y2 receptors. Activation of inhibitory G proteins in brain was greater for ATP than UTP, suggesting interaction with P_2X1 and P_2Y1 receptors.

It is noteworthy that ATP exhibited a 200-fold higher affinity for P_2X1 receptors than for P_2Y1 receptors in smooth muscle cells. Ca^{2+} mobilization and contraction induced by nanomolar concentrations of ATP were mediated exclusively by IP_3-dependent Ca^{2+} release. At higher concentrations (0.1 to 100 μM), ATP antagonized additionally with P_2X1 receptors eliciting both Ca^{2+} influx and IP_3-dependent Ca^{2+} release. Whether P_2X1 receptors participate in the physiological response of smooth muscle to endogenous ATP would depend on the ambient concentration of ATP. ATP released from cells is rapidly metabolized by ecto-nucleotidases and ecto-nucleoside diphosphokinases (18–20). ATP is co-released with soluble nucleotidases at neuromuscular junctions where it is rapidly hydrolyzed to adenosine (56). In the present study, both adenosine A_1 and A_2 antagonists were added to the medium to ensure against adventitious effects resulting from degradation of ATP. As shown previously (43), adenosine acting on A_1 receptors coupled via G_i to PLC-β3 can mimic to some extent the responses mediated by ATP. Recent studies (57) suggest that UTP like ATP can be released by mechanical stimulation raising the possibility that P_2Y1 receptors with high affinity for UTP (P_2Y4 and P_2Y1) and UDP (P_2Y3 and P_2Y8) may have a functional role (14, 19). The protein nature of purine and pyrimidine nucleotides which can be modified by ecto-nucleotidases or interconverted by nucleoside diphosphokinases determines to some extent which specific P_2Y receptor subtypes are activated.
P$_{2X}$ and P$_{2Y}$ Receptors on Smooth Muscle

261, 2140–2146

51. Murthy, K. S., and Makhlouf, G. M. (1995) Am. J. Physiol. 269, C969–C978
52. Okomoto, T., Ikeru, T., Murayama, Y., Ogata, E., and Nishimoto, I. (1992) FEBS Lett. 305, 125–129
53. Bitar, K. N., Bradford, P. G., Putney, J. W., Jr., and Makhlouf, G. M. (1986) J. Biol. Chem. 261, 16591–16596
54. Macrez, N., Morel, J.-L., Kalkbrenner, F., Viard, P., Schultz, G., and Mironneau, J. (1997) J. Biol. Chem. 272, 23180–23185
55. Simon, J., Webb, T. E., and Barnard, E. A. (1995) Pharmacol. Toxicol. 76, 302–307
56. Todorov, L. D., Mihaylova-Todorova, S., Westfall, T. D., Sneddon, P., Kennedy, C., Bjur, R. A., and Westfall, D. P. (1997) Nature 387, 76–79
57. Lazarowski, E. R., Homolya, L., Boucher, R. C., and Harden, T. K. (1997) J. Biol. Chem. 272, 24348–24354
