ATP Receptor-Mediated Increase of Ca Ionophore-Stimulated Arachidonic Acid Release from PC12 Pheochromocytoma Cells

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ABSTRACT—Phospholipase A_2 has recently been proposed as the effector enzyme involved in the receptor-mediated release of arachidonic acid (AA). Released AA and its metabolites have been demonstrated to play an important role in the regulation of cell functions. [^{3}H]AA release from prelabeled PC12 cells was stimulated by a Ca ionophore such as ionomycin or A23187. Although ATP and its effective analogs, adenosine 5′-*O*-(3-thiotrisphosphate) (ATPγS), 2-methylthio ATP and 3′-*O*-(4-benzoyl)benzoyl ATP, did not stimulate [^{3}H]AA release on their own, they did enhance Ca ionophore-stimulated [^{3}H]AA release. The effect of ATP analogs was dose-dependent. ADP, UTP, GTP, ITP, $\alpha\beta$-methylene ATP, $\beta\gamma$-methylene ATP and 8-bromo ATP showed no effect or very limited effect. The effect of ATPγS was antagonized by suramin, a putative P_{2Y} receptor antagonist. The effective ATP analogs also increased $[Ca^{2+}]_i$ (cytosolic free Ca^{2+} concentration) via Ca^{2+} influx. However, the addition of 50 mM KCl or 10 μM bradykinin, which are well-known to increase $[Ca^{2+}]_i$ by different pathways, did not stimulate [^{3}H]AA release, either with or without the Ca ionophore. The addition of phorbol 12-myristate 13-acetate, an activator of protein kinase C, showed no effect on [^{3}H]AA release, either with or without the Ca ionophore. These data suggest that 1) ATP increased Ca ionophore-stimulated AA release via a P_{2Y}-like ATP receptor, and that 2) the elevation of $[Ca^{2+}]_i$ by ATP does not quantitatively explain the ATP-stimulated AA release in PC12 cells.

Keywords: ATP, Arachidonic acid, Intracellular calcium, PC12 cell

Purine nucleotides and nucleosides have widespread and potent extracellular actions on a variety of excitable and non-excitable cells. Burnstock (1) proposed two types of receptors: the P_1 receptor for adenosine, and the P_2 receptor for ATP. Different subtypes of the P_2 receptor have been defined classically as follows (1-3): P_{2X} and P_{2Y} receptor subclasses have been differentiated on the basis of relative potencies of the ATP analogs and selective antagonism. Thus, for the P_{2X} receptor: $\alpha\beta$-methylene ATP = $\beta\gamma$-methylene ATP > ATP; P_{2Y} receptor: 2-methylthio ATP $\geq$ ATP > $\alpha\beta$-methylene ATP = $\beta\gamma$-methylene ATP. P_{2X} receptors are preferentially located in vascular smooth muscle cells and mediate vasoconstriction. P_{2Y} receptors are located on a variety of cell types and regulate cell functions via the activation of phospholipase C. Suramin and reactive blue 2 have been claimed to be specific antagonists to P_{2Y} receptors, at least over a limited concentration range (4, 5). Since the receptors for ATP on platelets, mast cells, lymphocytes and fibroblasts do not seem to fit this subclassification, they have been termed P_{2T} and P_{2Z} receptors, respectively (1-3). The P_{2T} receptors found in platelets are unique in being activated by ADP rather than ATP. The actual agonist form for P_{2Z} receptors is thought to be the tetrabasic acid ATP^{4-} (6), because Mg^{2+} addition increased the ED_{50} (dose required to achieve 50% of maximal effect) value for ATP. The rank order of agonist potency for P_{2Z} receptors in macrophages (7, 8) and fibroblasts (9) was Bz-ATP (3′-*O*-(4-benzoyl)benzoyl ATP) > ATP > 2-methylthio ATP $\geq$ ATPγS (adenosine 5′-*O*-(3-thiotrisphosphate). Recently, P_{2X} (10, 11), P_{2U} (12) and P_{2Y} (13) receptor cDNSs were cloned, and the existence of distinct P_{2X} subtypes is proposed (10, 11). Further progress in the identification and classification of P_2 receptor subtypes awaits the development of specific ligands and the cloning of the gene encoding these receptors. Additionally, the characterizations of the cell signaling process by extracellular ATP have to be determined for each cell type and response.

The receptor-mediated release of arachidonic acid (AA) has been demonstrated in a variety of neuronal cells (14, 15). In PC12 cells, nerve growth factor caused an

increased release and metabolism of AA with neurite extension (16, 17), and the addition of exogenous phospholipase A_2 induced neurite outgrowth (18). Ray et al. (19) reported that depolarization of nerve growth factor-treated, differentiated PC12 cells by high K^+ resulted in the release of both acetylcholine and AA. They suggested a regulatory role of AA in stimulus-secretion coupling leading to acetylcholine release at the cholinergic nerve terminal. Several reports showed the existence of specific ATP receptors (20–22) and the increase of Ca^{2+} influx by the stimulation of ATP receptors (23–25) in PC12 cells. The addition of ATP is also reported to stimulate catecholamine release in a Ca-dependent manner from PC12 cells (20, 21, 26–28). However, there is no report showing ATP-stimulated AA release and the relationship between AA release and noradrenaline (NA) release in PC12 cells.

In the present study, the effect of ATP on AA release in PC12 cells was examined. We demonstrate that the stimulation of the ATP receptor, which is not effective by itself, enhanced Ca ionophore-stimulated AA release, via P_{2Y}-like receptors. Neither depolarization by KCl nor the addition of bradykinin, which are known to increase $[Ca^{2+}]_i$ (cytosolic free Ca^{2+} concentration), stimulated AA release.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (80–140 Ci/mmol) and *l*-[7,8-^{3}H]NA (30–40 Ci/mmol) were purchased from Amersham Corp. (Buckinghamshire, England). Fura-2AM (fura-2 acetoxymethyl ester) was obtained from Wako Pure Chemical Corp. (Osaka). ATP, ionomycin, A23187, $\alpha\beta$-methylene ATP, $\beta\gamma$-methylene ATP, ADP, GTP, 8-bromo ATP, HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonate) and PMA (phorbol 12-myristate 13-acetate) were purchased from Sigma Chemical Corp. (St. Louis, MO, USA). UTP and ITP were gifts from Yamasa Shoyu Corp. (Chiba). ATPγS, ADPβS (adenosine-5′-*O*-(2-thiodiphosphate) and Bz-ATP were obtained from Boehringer Mannheim (Mannheim, Germany). 2-Methylthio ATP was purchased from Research Biochemicals Inc. (Natick, MA, USA). Suramin was a gift from Bayer Japan (Tokyo).

Cell culture

PC12 (D type) cells were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% horse serum, as previously reported (29). For the experiments, subconfluent PC12 cells were used at 48 hr after the final change of the medium.

Measurement of $[Ca^{2+}]_i$

PC12 cells on dishes were incubated in 0.7 μM fura-2AM for 20 min at 37℃ in modified Tyrode HEPES buffer (137 mM NaCl, 1 mM Na_2HPO_4, 12 mM $NaHCO_3$, 3 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% fatty acid free bovine serum albumin, 20 mM HEPES (pH 7.4)). The PC12 cells were washed and detached from the dish under a gentle stream of the buffer. Detached cells were washed twice by centrifugation ($200 \times g$, 2 min) at 4℃ and resuspended with the buffer. In some experiments, $CaCl_2$ was omitted. An aliquot of $3-5 \times 10^6$ cells was immediately used for autofluorescence measurements at 37℃. Fluorescence readings were taken with a Hitachi F-2000 spectrophotometer, as described previously (29).

Measurement of [^{3}H]AA release

PC12 cells on dishes were incubated with serum-free Dulbecco's modified Eagle's medium and 1 μCi/ml of [^{3}H]AA for 24 hr. The labeled PC12 cells were washed and detached from the dish by incubation with Ca- and Mg-free phosphate-buffered saline (pH 7.4). Detached cells were washed twice by centrifugation ($200 \times g$, 2 min) at 4℃ and resuspended in the modified Tyrode HEPES buffer. Cell suspensions (50–60 μg protein) were incubated with the indicated additions for 20 min at 37℃. The total incubation volume was 200 μl. The final buffer composition was the same as that of the modified Tyrode HEPES buffer. The reaction was terminated by adding 500 μl of ice-cold, Ca-, Mg- and albumin-free Tyrode HEPES buffer containing 5 mM EDTA and 5 mM EGTA followed by centrifugation ($8000 \times g$, 30 sec) at 4℃. The ^{3}H content of the supernatant was estimated by liquid scintillation spectrometry. We estimated the radioactivity in the supernatant as [^{3}H]AA release without separation, because the rate-limiting step in the synthesis of metabolites appears to be the release of AA from the phospholipids. Analysis of the supernatant by means of thin-layer chromatography revealed that AA and its metabolites such as prostaglandins were liberated from cells during the 20-min incubation with ATP and Ca ionophore (data not shown).

Measurement of [^{3}H]NA secretion

PC12 cells on dishes were incubated for 2 hr with 1 μCi/ml of [^{3}H]NA in modified Tyrode HEPES buffer (pH 7.0). The pH of the buffer was lowered to avoid degradation of NA. In some experiments, modified Tyrode HEPES buffer (pH 7.4) containing 0.1 mM ascorbic acid was used for [^{3}H]NA labeling. Data from both procedures were similar. The cell suspensions, prepared as described above, were incubated with additions for 8 min at 37℃. The reaction was terminated by the addition

of ice-cold Tyrode HEPES buffer containing 5 mM EDTA and 5 mM EGTA followed by centrifugation as described above. The ^3H content in the supernatant was estimated.

Statistics

Data were analyzed by Student's *t*-test. P values <0.01 were considered to be significant.

RESULTS

Increase of $[Ca^{2+}]_i$ by ATP stimulation

PC12 cells, labeled with the Ca^{2+} indicator fura-2AM, were challenged with 100 μM ATP (Fig. 1A). In the presence of 1 mM $CaCl_2$, ATP evoked a rapid $[Ca^{2+}]_i$ rise with a peak at 5–10 sec, and the $[Ca^{2+}]_i$ was maintained at a high level after 1 min. The $[Ca^{2+}]_i$ rise evoked by ATP was almost entirely due to Ca^{2+} influx, as the peak $[Ca^{2+}]_i$ rise was reduced by removal of the extracellular $CaCl_2$ (Fig. 1A). The increase of $[Ca^{2+}]_i$ by 300 μM ATP

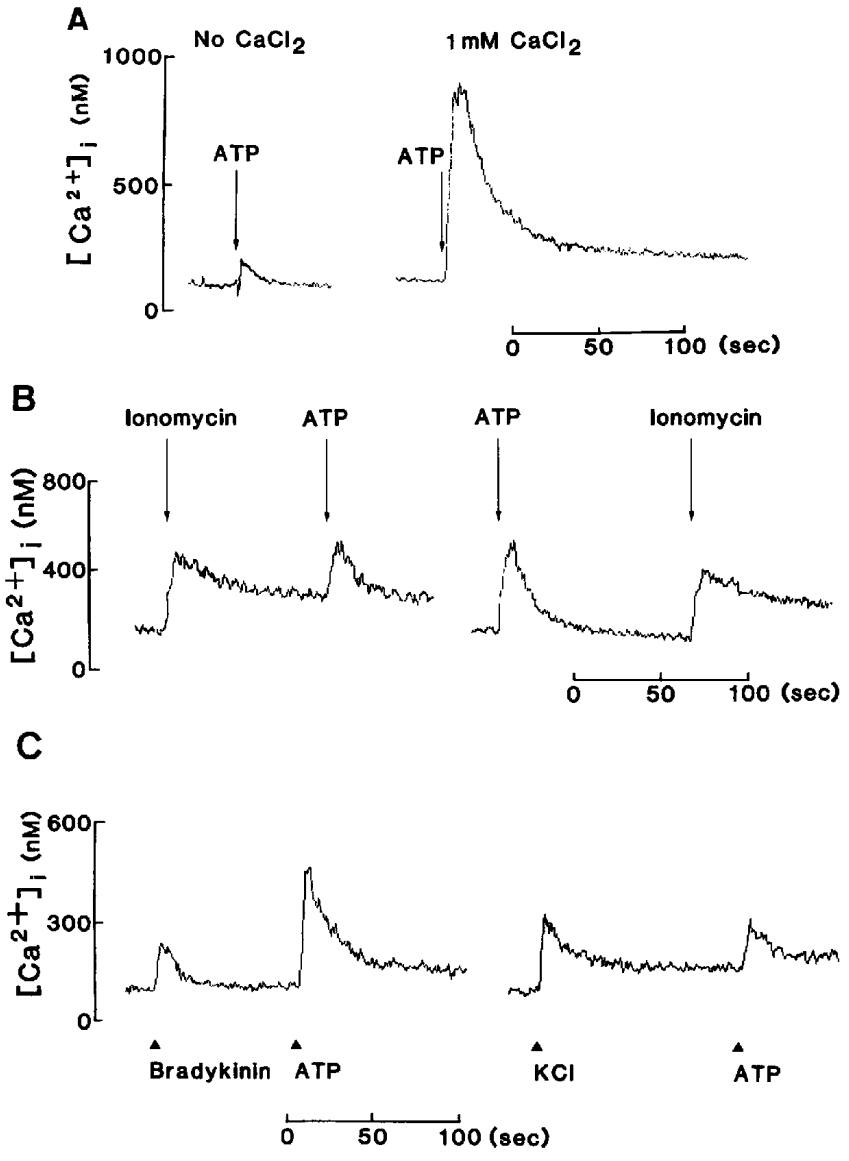

Fig. 1. Fura-2AM fluorescence responses by ATP, Ca ionophore, KCl and bradykinin. Fura-2AM loaded PC12 cells were stimulated with 100 μM ATP in the presence or absence of 1 mM $CaCl_2$ (A). The PC12 cells were stimulated with 5 μM ionomycin and 50 μM ATP in the presence of 1 mM $CaCl_2$ (B). The PC12 cells were stimulated with 1 μM bradykinin, 50 mM KCl and 50 μM ATP in the presence of 1 mM $CaCl_2$ (C). Data show a typical experiment and are representative of 3 independent experiments.

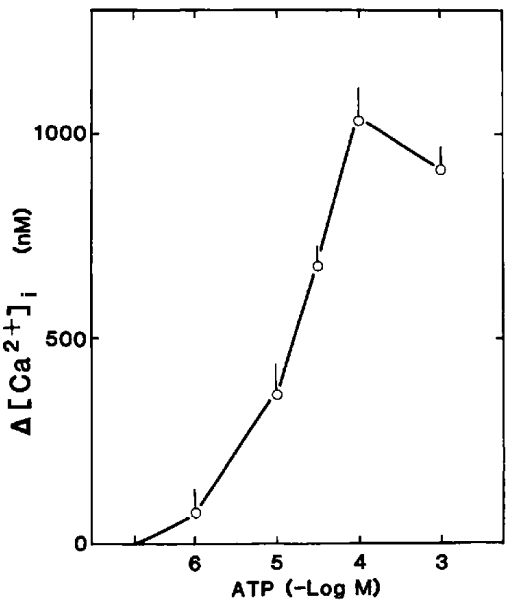

Fig. 2. ATP increases $[Ca^{2+}]_i$ in a dose-dependent manner. Fura-2AM loaded PC12 cells were stimulated with increasing concentrations of ATP in the presence of 1 mM $CaCl_2$. Data are the means±S.E.M. of 3 independent experiments.

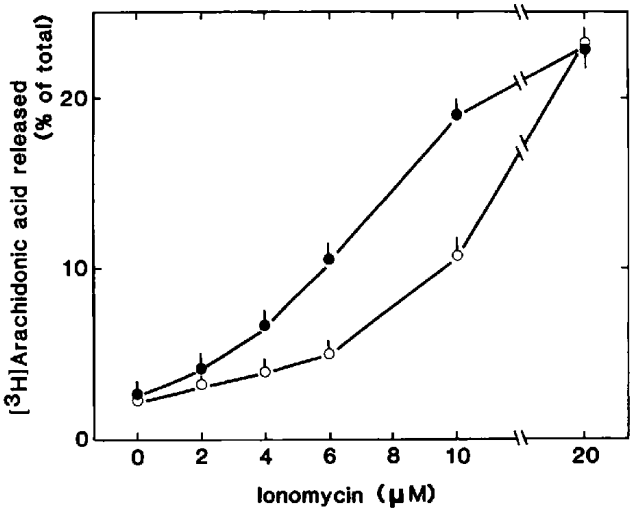

Fig. 3. ATP enhances ionomycin-stimulated [^{3}H]AA release. The PC12 cells prelabeled with [^{3}H]AA were stimulated with increasing concentrations of ionomycin in the presence (●) or absence (○) of 300 μM ATP. The concentration of $CaCl_2$ in the assay mixture was 1 mM. Data are the means±S.D. of triplicate determinations in a typical experiment and are representative of 2 independent experiments.

was 986±71 nM and 89±11 nM (n=3–4), in the presence and absence of extracellular $CaCl_2$, respectively. The effect of ATP on the increase of $[Ca^{2+}]_i$ was dose-dependent, and the ED_{50} value was 14±7 μM (n=4) (Fig. 2). ATPγS stimulated $[Ca^{2+}]_i$ rise as effectively as ATP, with the same order of potency. 2-Methylthio ATP and Bz-ATP were also active. The rank order of potency to increase $[Ca^{2+}]_i$ was: ATPγS ≥ ATP > 2-methylthio ATP > Bz-ATP. In contrast, ADP, ADPβS, GTP, UTP, ITP, $\alpha\beta$-methylene ATP, $\beta\gamma$-methylene ATP and 8-bromo ATP had no effect or only a limited one (data not shown). Additionally, they did not inhibit the effect of ATP on Ca mobilization (data not shown). A submaximal dose (50 μM) of ATP stimulated $[Ca^{2+}]_i$ rise transiently, and the $[Ca^{2+}]_i$ level returned to the basal level after 50 sec (Fig. 1B, right). Ionomycin, a Ca ionophore, evoked a sustained $[Ca^{2+}]_i$ rise (Fig. 1B, left). A long-lasting, steady state elevation of $[Ca^{2+}]_i$ was observed in cells depolarized with 50 mM KCl (Fig. 1C, right), as reported previously (29, 30). Addition of 50 μM ATP stimulated the $[Ca^{2+}]_i$ rise after the addition of KCl, although the effect of ATP was reduced (Fig. 1C, right). Co-addition of ATP and bradykinin induced a long-lasting rise of $[Ca^{2+}]_i$ (Fig. 1C, left), although the addition of either 1 μM bradykinin (Fig. 1C, left) or 50 μM ATP (Fig. 1B, right) only stimulated a transient $[Ca^{2+}]_i$ rise.

Enhancement of Ca ionophore-stimulated [^{3}H]AA release by ATP

It has been reported that $CaCl_2$ is necessary for phospholipase A_2 activation or AA release in many cell types (31–33). Ionomycin, in the presence of extracellular 1 mM $CaCl_2$, stimulated [^{3}H]AA release in a dose-dependent manner from PC12 cells prelabeled with [^{3}H]AA (Fig. 3). The addition of 300 μM ATP had no effect on [^{3}H]AA release, although this concentration of ATP stimulated the $[Ca^{2+}]_i$ increase to the maximal level. ATP, however, enhanced ionomycin-stimulated [^{3}H]AA release when the doses of ionomycin were less than 10 μM. ATP also enhanced A23187-, another Ca ionophore, stimulated [^{3}H]AA release. Neomycin, a phospholipase C inhibitor of phosphoinositide hydrolysis, and RHC 80267, a diacylglycerol lipase inhibitor, did not inhibit ionomycin-stimulated and ATP-enhanced AA releases (data not shown). Two different inhibitors of phospholipase A_2, 10 μM mepacrine and 10 μM 4-bromophenacylbromide, significantly reduced AA releases (data not shown). Thus, AA release from PC12 cells seems to be mediated by phospholipase A_2.

ATP, ATPγS, Bz-ATP and 2-methylthio ATP enhanced the Ca ionophore-stimulated [^{3}H]AA release (Table 1). Figure 4 shows the dose-dependency of effective ATP analogs. The order of ED_{50} was ATPγS ≥ 2-methylthio ATP ≥ ATP > Bz-ATP. The ED_{50} values for ATPγS and ATP were 30.0±8.0 and 62.0±25.1 μM (n=3), respectively. Other ATP analogs that we em-

Table 1. The effect of various ATP analogs on ionophore-stimulated [^{3}H]AA release

Additions	[^{3}H]Arachidonic acid released (% of total)
None	1.87±0.35
20 μM A23187	4.63±0.59
+ATP	15.23±0.41*
+ADP	4.55±0.41
+GTP	5.04±0.71
+UTP	4.53±0.12
+ATPγS	15.02±0.18*
+Bz-ATP	13.99±0.18*
+2-Methylthio ATP	13.64±2.55*
+αβ-Methylene ATP	5.46±0.06
+βγ-Methylene ATP	5.42±0.22
+8-Bromo ATP	5.21±0.04

The PC12 cells prelabeled with [^{3}H]AA were stimulated with 20 μM A23187 and 300 μM ATP analogs. Data are the means±S.D. of triplicate determinations of a typical experiment and are representative of 2 or 3 independent experiments; *$P<0.01$ versus effect of 20 μM A23187 alone.

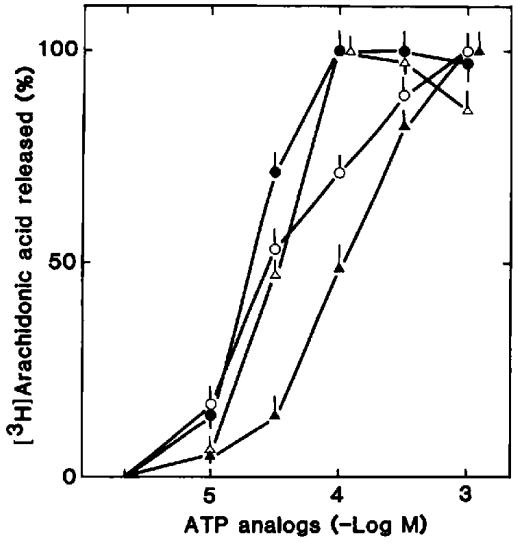

Fig. 4. ATP analogs enhance Ca ionophore-stimulated [^{3}H]AA release in a dose-dependent manner. The PC12 cells prelabeled with [^{3}H]AA were stimulated with increasing concentrations of ATP analogs, ATP (○), ATPγS (●), 2-methylthio ATP (△) and Bz-ATP (▲) in the presence of 20 μM A23187. The concentration of $CaCl_2$ in the assay mixture was 1 mM. The response of each ATP analog is shown as a % of the maximum response of that ATP analog. Data are the means±S.E.M. of 3 or 4 independent experiments.

ployed showed little or no effect. ADP, a P_{2T} receptor agonist, had no effect. Suramin is known to inhibit P_{2Y} receptor-mediated effects (4, 5). The enhancement by ATPγS of A23187-stimulated [^{3}H]AA release was inhibited by the addition of suramin (Fig. 5). The antagonistic effect of suramin was dose-dependent. Reactive blue 2 also inhibited the effect of ATPγS (data not shown). These data suggest that ATP stimulates AA release via P_{2Y} type-like receptors in PC12 cells.

Effects of KCl, bradykinin and PMA on [^{3}H]AA release

A possible explanation for AA release caused by ATP

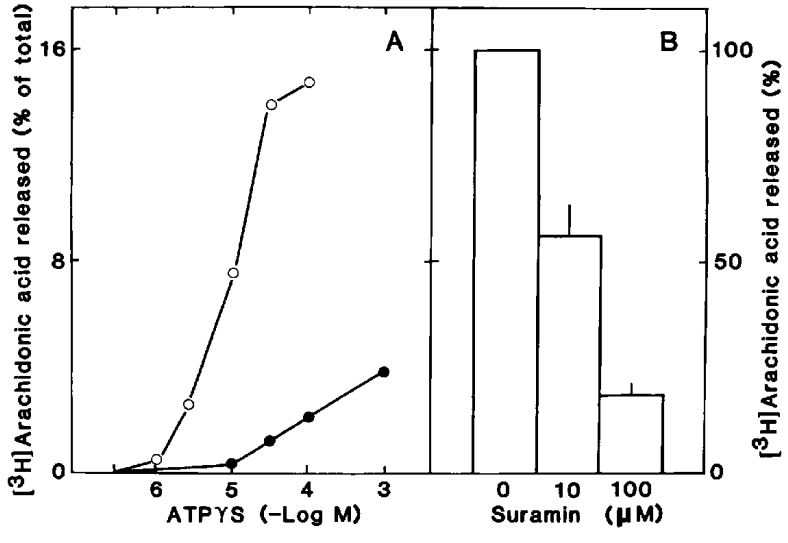

Fig. 5. The effect of ATPγS on [^{3}H]AA release was inhibited by suramin in a dose-dependent manner. The PC12 cells prelabeled with [^{3}H]AA were stimulated with increasing concentrations of ATPγS and 20 μM A23187 in the presence (●) or absence (○) of 100 μM suramin (A). The PC12 cells were incubated with 100 μM ATPγS, 20 μM A23187 and the indicated concentrations of suramin (B). The concentration of $CaCl_2$ in the assay mixture was 1 mM. Data are the means±S.D. of triplicate determinations in a typical experiment and are representative of 2 or 3 independent experiments.

Table 2. The effect of high K^+ or bradykinin on [^{3}H]AA release

Additions	[^{3}H]Arachidonic acid released (% of total)			
	None	ATP	KCl	Bradykinin
None	1.48±0.16	1.71±0.08	2.03±0.27	1.33±0.03
A23187	3.53±0.37	9.30±0.51*	4.49±0.36	2.82±0.05
KCl	2.03±0.27	1.87±0.03	—	n.d.
Bradykinin	1.33±0.03	1.46±0.23	n.d.	—

The PC12 cells prelabeled with [^{3}H]AA were incubated with 20 μM A23187, 50 mM KCl, 1 μM bradykinin, 300 μM ATP or their combination for 20 min. Data are the means±S.D. of triplicate determinations of a typical experiment and are representative of 3 independent experiments; *$P<0.01$ versus effect of 20 μM A23187 alone. n.d., not determined.

Table 3. The effect of phorbol 12-myristate 13-acetate on [^{3}H]AA release

Addition	[^{3}H]Arachidonic acid release (% of total)	
	None	100 nM PMA
None	0.78±0.38	1.15±0.14
+300 μM ATP	0.50±0.06	0.92±0.37
+300 μM ATPγS	1.21±0.42	0.93±0.18
20 μM A23187	2.97±0.15	3.13±0.15
+300 μM ATP	8.86±1.20*	8.68±0.60*
+300 μM ATPγS	10.84±1.51*	8.65±0.77*

The PC12 cells prelabeled with [^{3}H]AA were incubated with additions for 20 min. Data are the means±S.D. of triplicate determinations of a typical experiment and are representative of 2 independent experiments; *$P<0.01$ versus effect of 20 μM A23187 alone.

in the presence of the Ca ionophore is that ATP increased $[Ca^{2+}]_i$ and maintained it at a high level, because $[Ca^{2-}]_i$ is a critical factor in the activity of phospholipase A_2 (31–33). Next, we measured the effects of KCl and bradykinin on [^{3}H]AA release. The addition of KCl induced depolarization and stimulated Ca^{2+} influx from voltage-dependent Ca channels in PC12 cells (Fig. 1C and refs. 29, 30). The addition of 50 mM KCl, however, did not stimulate [^{3}H]AA release from PC12 cells (Table 2). Co-addition of ATP and KCl had no effect on [^{3}H]AA release, although co-addition of 50 μM ATP and 50 mM KCl kept the $[Ca^{2+}]_i$ level high (Fig. 1C). Bradykinin is also known to stimulate the $[Ca^{2+}]_i$ increase in PC12 cells via both inositol 1,4,5-trisphosphate-regulated channels and bradykinin receptor-operated channels (34). In our experiments, addition of 1 μM bradykinin stimulated the $[Ca^{2+}]_i$ rise and enhanced the effect of 50 μM ATP on $[Ca^{2+}]_i$ (Fig. 1C, left). The increase of $[Ca^{2+}]_i$ by 10 μM bradykinin was 372±30 nM and 179±51 nM (n=3–4), in the presence and absence of extracellular $CaCl_2$, respectively. Bradykinin, however, had no stimulatory effect on [^{3}H]AA release, even in the presence of the Ca ionophore (Table 2).

Table 4. The effect of $MgCl_2$ on [^{3}H]AA release and [^{3}H]NA release from PC12 cells

Addition	1 mM $MgCl_2$	Mg-free, 0.2 mM EDTA
Experiment 1	[^{3}H]NA released (% of total)	
None	6.0±0.4	6.5±1.1
100 μM ATP	23.6±1.5*	24.9±0.6*
50 mM KCl	21.7±1.4*	21.7±0.7*
Experiment 2	[^{3}H]AA released (% of total)	
None	2.08±0.48	2.11±0.18
20 μM A23187	4.03±0.15	5.23±0.30
+100 μM ATP	12.85±2.00*	19.83±0.89*,**

The PC12 cells prelabeled with [^{3}H]NA (Experiment 1) or [^{3}H]AA (Experiment 2) were incubated with additions in the presence or absence of 1 mM $MgCl_2$. Data are the means±S.D. of 3 determinations of a typical experiment and are representative of 3 independent experiments; *$P<0.01$ versus effect of none (in Exp. 1) or 20 μM A23187 alone (in Exp. 2); **$P<0.01$ versus effect of ATP in 1 mM $MgCl_2$.

Protein kinase C is known to have a stimulatory effect on phospholipase A_2 activity and AA release in some types of cells (32, 33, 35). Types α and β, not γ, of protein kinase C are present in PC12 cells (36). Therefore, we examined the effect of PMA on AA release. The addition of PMA did not stimulate [^{3}H]AA release in the absence and presence of ATP and/or the Ca ionophore (Table 3). Bradykinin, which is known to accumulate diacylglycerol, an activator of protein kinase C, had no stimulatory effect on AA release (Table 2). These data suggest that the additional $[Ca^{2+}]_i$ rise and activation of protein kinase C are independent of the mechanism by which the ATP receptor can enhance AA release from PC12 cells. In platelets, AA releases by receptor stimulants were reported to be mediated by GTP-binding proteins, which are sensitive to pertussis toxin (37, 38). ATP-stimulated AA release from PC12 cells, however, was not modified by treatment with 1 μg/ml of pertussis toxin, which abolished the inhibition of adenylate cyclase by carbachol (data not shown).

Modification of ATP effects by $MgCl_2$

ATP stimulated [^{3}H]NA release in the presence of extracellular $CaCl_2$ (Table 4), as previously reported (20, 21, 26–28). The ATP receptors coupled to secretions of catecholamines were shown to be P_{2Y} type according to their selectivity to agonists and antagonists. This effect of ATP was observed by itself, although [^{3}H]AA release by ATP was observed only in the presence of the Ca ionophore.

High K^+ also stimulated [^{3}H]NA release without AA release. [^{3}H]NA release by ATP occurred in the absence and presence of extracellular 1 mM $MgCl_2$ to the same degree. On the contrary, the removal of $MgCl_2$ potentiated [^{3}H]AA release by 100 μM (maximal dose) ATP in the presence of the Ca ionophore. The ED_{50} values for ATP in the absence and presence of 1 mM $MgCl_2$ were 10.8 ± 1.6, 62.0 ± 25.1 μM (n=3), respectively. The ATP-stimulated $[Ca^{2+}]_i$ rise was also potentiated 20–30% by the removal of $MgCl_2$ (data not shown). These data demonstrate that [^{3}H]NA release by ATP and KCl from PC12 cells were independent of AA release and suggest the possibility of two types, Mg^{2+}-sensitive and insensitive, of P_{2Y} receptors in PC12 cells.

DISCUSSION

The data presented in this study indicate that ATP stimulated the effect of Ca^{2+} ionophore on [^{3}H]AA release via P_{2Y}-like receptors for the following reasons: 1) $\alpha\beta$-Methylene ATP and $\beta\gamma$-methylene ATP had no effect (Table 1); 2) 2-Methylthio ATP, ATPγS, Bz-ATP showed similar effects to ATP, and the order of potency in enhancing [^{3}H]AA release was ATPγS > 2-methylthio ATP = ATP > Bz-ATP (Fig. 4); 3) Suramin, a P_{2Y} receptor antagonist, inhibited the effect of ATP analogs (Fig. 5); and 4) The involvement of P_{2T} type receptors was excluded because ADP had no effect on [^{3}H]AA release (Table 1). We believe that this is the first report showing the stimulatory effect of ATP on AA release from PC12 cells. In differentiated HL60 cells, Xing et al. (39) found that UTP and ATPγS had stimulatory effects in low doses and inhibitory effects in high doses on AA release. They postulated the existence of "P_{2U}" receptors that recognize ATP and UTP with similar affinities in some cell types including HL60 cells and neutrophils. In PC12 cells, however, UTP did not show any effect on AA release (Table 1).

Several lines of evidence indicate the existence of pore-forming ATP receptors, the so-called P_{2Z} receptors, in a variety of cell types including neuronal cells (1, 2, 6–9). P_{2Z} receptors in these types of cells, which modulate cytotoxity and DNA synthesis, require high doses (over 100 μM) of ATP for activation. The rank order of potency for P_{2Z} receptors was Bz-ATP $\gg$ ATP > ATPγS (1, 2, 6–9). The true agonist for P_{2Z} receptors is believed to be ATP^{4-} because the effects of ATP mediated by P_{2Z} receptors are reduced by $MgCl_2$ addition. In PC12 cells, our present data indicated that the rank order of potency was ATPγS $\geq$ ATP > Bz-ATP for AA release (Fig. 4), the same as that for [^{3}H]NA release (20, 21). The effect of ATP on Ca ionophore-stimulated AA release was observed in the presence of 1 mM $MgCl_2$ and the ED_{50} value for ATP was 50 μM, which is a relatively low concentration (Fig. 4). These data suggest that ATP receptors coupled to AA release in PC12 cells are not P_{2Z} receptors.

Sela et al. (28) reported that agonists for P_{2Y} receptors stimulated the release of [^{3}H]dopamine from PC12 cells in an Mg^{2+}-insensitive manner. In our experiments, 100 μM ATP-stimulated [^{3}H]NA release was not modified by extracellular $MgCl_2$ (Table 4). In contrast, some investigators showed that Mg^{2+} inhibited ATP-stimulated Ca^{2+} influx and NA release in PC12 cells (21, 27), and they concluded that ATP^{4-} was the active ligand for ATP receptor/channels. Kim and Rabin (22) reported a new type of purinergic P_2 receptor on PC12 cells that did not fit the previous classification, although the rank order of potency to increase $[Ca^{2+}]_i$ was similar to that of the P_{2Y} type. Inclusion of Mg^{2+} significantly decreased the specific binding of a ligand to ATP receptors in PC12 cells. In our experiments, the ATP-stimulated [^{3}H]AA release, not NA release, was potentiated by removal of extracellular $MgCl_2$. This result suggests that ATP^{4-} is an active ligand to ATP receptors which activate phospholipase A_2 to release AA. Thus, it is possible to speculate that there are two types of P_{2Y} receptors in our PC12 cells; one is Mg^{2+}-insensitive and coupled to the secretion process, and another is Mg^{2+}-sensitive and coupled to AA release.

$[Ca^{2+}]_i$ is one of the most important and critical factors in the regulation of phospholipase A_2 activity and AA release (19, 31–33, 35). In PC12 cells, [^{3}H]AA release was stimulated by a Ca ionophore and dependent on extracellular $CaCl_2$ (Fig. 3). Actually, ATP and its effective analogs stimulate $[Ca^{2+}]_i$ increase (Fig. 2). PC12 cells are known to be stimulated by ATP to release [^{3}H]NA by activating voltage-insensitive, receptor-operated Ca channels (4, 5). One possible explanation is that the additional $[Ca^{2+}]_i$ rise by ATP enhanced the Ca ionophore-stimulated AA release. This scenario, however, seems unlikely for the following reasons: 1) ATP, by itself, had no effect on AA release, although 0.1 mM ATP stimulated a $[Ca^{2+}]_i$ rise quickly and kept it at a high level for over 3 min (Fig. 1); 2) KCl did not enhance AA release (Table 2), although the $[Ca^{2+}]_i$ increase by high K^+ was sustained for a longer (over 3 min) period than that by ATP; and 3) Bradykinin, which stimulates $[Ca^{2+}]_i$ increase from the inositol 1,4,5-trisphosphate-sensitive pool, did not show any stimulatory effect on AA release (Table 2). Thus, the stimulatory effect of ATP on AA release from PC12 cells seems to be independent of $[Ca^{2+}]_i$ rise.

Kanterman et al. (32) reported a similar receptor-mediated potentiation of A23187-stimulated AA release. They transfected and stably expressed D_2 dopamine receptors to Chinese hamster ovary cells. The A23187-stimulated AA release was potentiated by dopamine,

although dopamine alone had no effect. They showed that the inhibitors of protein kinase C diminish the dopamine-mediated potentiation. Protein kinase C is known to have a stimulatory effect on some types of cells (33, 35) and an inhibitory effect on rabbit platelets (37, 38) with regards to phospholipase A_2 activity or AA release. However, in PC12 cells, bradykinin, which is shown to stimulate phospholipase C, leading to the accumulation of diacylglycerol (34), had no effect on AA release. ATP seems to have a small, very limited effect on phospholipase C activation (27, 28), and the ATP-induced elevation of $[Ca^{2+}]_i$ is almost completely dependent on extracellular $CaCl_2$ (Fig. 1A). The non-stimulated release and the Ca ionophore and/or ATP-stimulated AA releases from PC12 cells were not modified by the addition of PMA (Table 3). Therefore, it is probable that ATP receptor activation stimulates another signal-transducing effector(s), in addition to phosphatidylinositol turnover (diacylglycerol accumulation and Ca mobilization), for the stimulation of phospholipase A_2 in PC12 cells.

In some types of cells, pertussis toxin-sensitive GTP binding proteins are involved in receptor-mediated AA releases or the activation of phospholipase A_2 (31, 33, 37, 38). Recently, the cDNAs for a P_{2U}-type (12) and a P_{2Y}-type (13) receptor were cloned. These sequences exhibit structural features typical of the family of GTP binding protein-coupled receptors. Pretreatment with 1 μg/ml of pertussis toxin, however, showed no effect on basal (non-stimulated) and Ca ionophore-stimulated [^{3}H]AA release in the presence or absence of ATP from PC12 cells. Ligand-gated ion channels defined by P_{2X} receptor were cloned from rat vas deferens (10) and from PC12 cells (11). There is sequence similarity in the pore-forming motif, although the deduced amino acid sequences, the pharmacological profiles, and the sizes and tissue distribution of the receptor mRNA of the two cloned receptors were different. The pharmacological profile including selectivity to antagonists such as suramin in our results resembles that of the cloned P_{2X} receptors from PC12 cells. However, the stimulatory effect of ATP on Ca ionophore-stimulated AA release seems to be separate from Ca^{2+} influx, as described above. The characterization of ATP receptors in PC12 cells and the precise mechanism of ATP action on AA release remains to be determined.

Ray et al. (19) reported that high K^+ stimulated the release of [^{3}H]AA from the nerve growth factor-treated, differentiated PC12 cells. They suggested that AA release is associated with acetylcholine release. In our experiments using non-differentiated PC12 cells, however, high K^+ did not stimulate [^{3}H]AA release, although high K^+ stimulated NA release. Additionally, high K^+ had no effect on [^{3}H]AA release from differentiated PC12 cells (data not shown). The reasons for this discrepancy are unclear at this point.

In conclusion, 1) P_{2Y}-like ATP receptors stimulated AA release from PC12 cells in the presence of Ca ionophore; 2) Addition of Mg^{2+} inhibited the ATP-stimulated AA, not NA release; and 3) The stimulatory effect of ATP on AA release seems to be independent of the elevation of $[Ca^{2+}]_i$ and the activation of protein kinase C.

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