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

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ABSTRACT—Phospholipase A2 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. [3H]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) (ATPrS), 2-methylthio ATP and 3′-O-(4-benzoyl)benzoyl ATP, did not stimulate [3H]AA release on their own, they did enhance Ca ionophore-stimulated [3H]AA release. The effect of ATP analogs was dose-dependent. ADP, UTP, GTP, ITp, aβ-methylene ATP, βγ-methylene ATP and 8-bromo ATP showed no effect or very limited effect. The effect of ATPrS was antagonized by suramin, a putative P2Y receptor antagonist. The effective ATP analogs also increased [Ca2+]i (cytosolic free Ca2+ concentration) via Ca2+ influx. However, the addition of 50 mM KCl or 10 μM bradykinin, which are well-known to increase [Ca2+]i by different pathways, did not stimulate [3H]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 [3H]AA release, either with or without the Ca ionophore. These data suggest that 1) ATP increased Ca ionophore-stimulated AA release via a P2Y-like ATP receptor, and that 2) the elevation of [Ca2+]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 P1 receptor for adenosine, and the P2 receptor for ATP. Different subtypes of the P2 receptor have been defined classically as follows (1–3): P2X and P2Y receptor subclasses have been differentiated on the basis of relative potencies of the ATP analogs and selectivite antagonism. Thus, for the P2X receptor: αβ-methylene ATP = βγ-methylene ATP > ATP; P2Y receptor: 2-methylthio ATP ≥ ATP > αβ-methylene ATP = βγ-methylene ATP. P2X receptors are preferentially located in vascular smooth muscle cells and mediate vasoconstriction. P2Y 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 P2Y 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 P2Y receptors, respectively (1–3). The P2 receptors found in platelets are unique in being activated by ADP rather than ATP. The actual agonist form for P22 receptors is thought to be the tetra-basic acid ATP4− (6), because Mg2+ addition increased the ED50 (dose required to achieve 50% of maximal effect) value for ATP. The rank order of agonist potency for P22 receptors in macrophages (7, 8) and fibroblasts (9) was Bz-ATP (3′-O-(4-benzoyl)benzoyl ATP) > ATP > 2-methylthio ATP ≥ ATP/ (adenosine 5′-O-(3-thiotriphosphate). Recently, P2X (10, 11), P2U (12) and P2Y (13) receptor cDNAs were cloned, and the existence of distinct P2X subtypes is proposed (10, 11). Further progress in the identification and classification of P2 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 A2 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 Ca2+ 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 P2Y-like receptors. Neither depolarization by KCl nor the addition of bradykinin, which are known to increase [Ca2+], (cytosolic free Ca2+ concentration), stimulated AA release.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-3H]AA (80–140 Ci/mmol) and [7,8-3H]NA (30–40 Ci/mmol) were purchased from Amersham Corp. (Buckinghamshire, England). Fura-2 AM (fura-2 acetoxymethyl ester) was obtained from Wako Pure Chemical Corp. (Osaka). ATP, ionomycin, A23187, αβ-methylene ATP, βγ-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 Wako Pure Chemical 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 [Ca2+]i

PC12 cells on dishes were incubated in 0.7 μM fura-2 AM for 20 min at 37°C in modified Tyrode HEPES buffer (137 mM NaCl, 1 mM Na2HPO4, 12 mM NaHCO3, 3 mM KCl, 5 mM glucose, 1 mM MgCl2, 1 mM CaCl2, 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 x g, 2 min) at 4°C and resuspended with the modified Tyrode HEPES buffer. An aliquot of 3–5 x 10⁶ cells was immediately used for auto-fluorescence measurements at 37°C. Fluorescence readings were taken with a Hitachi F-2000 spectrophotometer, as described previously (29).

Measurement of [3H]AA release

PC12 cells on dishes were incubated with serum-free Dulbecco's modified Eagle's medium and 1 μCi/ml of [3H]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 x g, 2 min) at 4°C 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°C. 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 x g, 30 sec) at 4°C. The 3H content of the supernatant was estimated by liquid scintillation spectrometry. We estimated the radioactivity in the supernatant as [3H]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 [3H]NA secretion

PC12 cells on dishes were incubated for 2 hr with 1 μCi/ml of [3H]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 [3H]NA labeling. Data from both procedures were similar. The cell suspensions, prepared as described above, were incubated with additions for 8 min at 37°C. 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 $^3$H content in the supernatant was estimated.

**Statistics**

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

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**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...

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**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.
was 986 ± 71 nM and 89 ± 11 nM (n = 3–4), in the presence and absence of extracellular CaCl₂, respectively. The effect of ATP on the increase of [Ca²⁺]i was dose-dependent, and the ED₅₀ value was 14 ± 7 µM (n = 4) (Fig. 2). ATP₇S stimulated [Ca²⁺]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 [Ca²⁺]i was: ATP₇S > ATP > 2-methylthio ATP > Bz-ATP. In contrast, ADP, ADPβS, GTP, UTP, ITP, αβ-methylene ATP, βγ-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 [Ca²⁺]i rise transiently, and the [Ca²⁺]i level returned to the basal level after 50 sec (Fig. 1B, right). Ionomycin, a Ca ionophore, evoked a sustained [Ca²⁺]i rise (Fig. 1B, left). A long-lasting, steady state elevation of [Ca²⁺]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 [Ca²⁺]i rise after the addition of KCl (Fig. 1C, right), although the effect of ATP was reduced (Fig. 1C, right). Co-addition of ATP and bradykinin induced a long-lasting rise of [Ca²⁺]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 [Ca²⁺]i rise.

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

It has been reported that CaCl₂ is necessary for phospholipase A₂ activation or AA release in many cell types (31–33). Ionomycin, in the presence of extracellular 1 mM CaCl₂, stimulated [³H]AA release in a dose-dependent manner from PC12 cells prelabeled with [³H]AA (Fig. 3). The addition of 300 µM ATP had no effect on [³H]AA release, although this concentration of ATP stimulated the [Ca²⁺]i increase to the maximal level. ATP, however, enhanced ionomycin-stimulated [³H]AA release when the doses of ionomycin were less than 10 µM. ATP also enhanced A23187-, another Ca ionophore, stimulated [³H]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 A₂, 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 A₂.

ATP, ATP₇S, Bz-ATP and 2-methylthio ATP enhanced the Ca ionophore-stimulated [³H]AA release (Table 1). Figure 4 shows the dose-dependency of effective ATP analogs. The order of ED₅₀ was ATP₇S ≥ 2-methylthio ATP ≥ ATP > Bz-ATP. The ED₅₀ values for ATP₇S and ATP were 30.0 ± 8.0 and 62.0 ± 25.1 µM (n = 3), respectively. Other ATP analogs that we em-

Fig. 2. ATP increases [Ca²⁺]i in a dose-dependent manner. Fura-2AM loaded PC12 cells were stimulated with increasing concentrations of ATP in the presence of 1 mM CaCl₂. Data are the means ± S.E.M. of 3 independent experiments.

Fig. 3. ATP enhances ionomycin-stimulated [³H]AA release. The PC12 cells prelabeled with [³H]AA were stimulated with increasing concentrations of ionomycin in the presence (○) or absence (□) of 300 µM ATP. The concentration of CaCl₂ 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.
ployed showed little or no effect. ADP, a P_{2\alpha} receptor agonist, had no effect. Suramin is known to inhibit P_{2Y} receptor-mediated effects (4, 5). The enhancement by ATP\_S of A23187-stimulated [3H]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 ATP\_S (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 [3H]AA release**

A possible explanation for AA release caused by ATP...
The PC12 cells prelabeled with \([^{3}H]AA\) were incubated with 20 \(\mu M\) A23187, 50 mM KCl, 1 \(\mu M\) bradykinin, 300 \(\mu M\) ATP or their combination for 20 min. Data are the means \(\pm\) S.D. of triplicate determinations of a typical experiment and are representative of 3 independent experiments; \(^*P<0.01\) versus effect of 20 \(\mu M\) A23187 alone. n.d., not determined.

| 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.    | n.d.      |
| Bradykinin         | 1.33±0.03 | 1.46±0.23 | n.d.    | n.d.      |

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

| Addition                                      | \([^{3}H]Arachidonic acid released (\% of total)\) |
|-----------------------------------------------|-------------------------------------------------|
|                                                | None    | 100 nM PMA                                      |
|                                                | 0.78±0.38 | 1.15±0.14                                       |
| +300 \(\mu M\) ATP                           | 0.50±0.06 | 0.92±0.37                                       |
| +300 \(\mu M\) ATP;S                         | 1.21±0.42 | 0.93±0.18                                       |
| 20 \(\mu M\) A23187                         | 2.97±0.15 | 3.13±0.15                                       |
| +300 \(\mu M\) ATP                           | 8.86±1.20* | 6.68±0.60*                                      |
| +300 \(\mu M\) ATP;S                         | 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 \(\pm\) S.D. of triplicate determinations of a typical experiment and are representative of 2 independent experiments; \(^*P<0.01\) versus effect of 20 \(\mu M\) A23187 alone.

in the presence of the Ca ionophore is that ATP increased \([Ca^{2+}]\); and maintained it at a high level, because \([Ca^{2+}]\); is a critical factor in the activity of phospholipase A2 (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 \(\mu M\) ATP and 50 mM KCl kept the \([Ca^{2+}]\); level high (Fig. 1C). Bradykinin is also known to stimulate the \([Ca^{2+}]\); 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 \(\mu M\) bradykinin stimulated the \([Ca^{2+}]\); rise and enhanced the effect of 50 \(\mu M\) ATP on \([Ca^{2+}]\); (Fig. 1C, left). The increase of \([Ca^{2+}]\); by 10 \(\mu M\) bradykinin was \(372\pm30\) nM and \(179\pm51\) nM (n = 3–4), in the presence and absence of extracellular CaCl2, respectively. Bradykinin, however, had no stimulatory effect on \([^{3}H]AA\) release, even in the presence of the Ca ionophore (Table 2).

Protein kinase C is known to have a stimulatory effect on phospholipase A2 activity and AA release in some types of cells (32, 33, 35). Types \(\alpha\) and \(\beta\), not \(\gamma\), 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).

Modification of ATP effects by MgCl2

ATP stimulated \([^{3}H]NA\) release in the presence of extracellular CaCl2 (Table 4), as previously reported (20, 21, 26–28). The ATP receptors coupled to secretions of catecholamines were shown to be P2Y 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 \(\mu M\) (maximal dose) ATP in the presence of the Ca ionophore. The \(E_{50}\) values for ATP in the absence and presence of 1 mM MgCl\(_2\) were 10.8 ± 1.6, 62.0 ± 25.1 \(\mu M\) (n = 3), respectively. The ATP-stimulated \([\text{Ca}^{2+}]_i\) 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, ATP\(_\gamma\)S, Bz-ATP showed similar effects to ATP, and the order of potency in enhancing \(^{3}H\)AA release was ATP\(_\gamma\)S > 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\(_{2Y}\) 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 ATP\(_\gamma\)S had stimulatory effects in low doses and inhibitory effects in high doses on AA release. They postulated the existence of “P\(_{2X}\)” 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\(_{2X}\) receptors, in a variety of cell types including neuronal cells (1, 2, 6–9). P\(_{2X}\) receptors in these types of cells, which modulate cytotoxicity and DNA synthesis, require high doses (over 100 \(\mu M\)) of ATP for activation. The rank order of potency for P\(_{2X}\) receptors was Bz-ATP \(\gg\) ATP \(\geq\) ATP\(_\gamma\)S (1, 2, 6–9). The true agonist for P\(_{2X}\) receptors is believed to be ATP\(^{4-}\) because the effects of ATP mediated by P\(_{2X}\) receptors are reduced by MgCl\(_2\) addition. In PC12 cells, our present data indicated that the rank order of potency was ATP\(_\gamma\)S \(\geq\) ATP \(\gg\) 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 \(E_{50}\) value for ATP was 50 \(\mu M\), which is a relatively low concentration (Fig. 4). These data suggest that ATP receptors coupled to AA release in PC12 cells are not P\(_{2Y}\) 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 \(\mu M\) 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 \([\text{Ca}^{2+}]_i\) 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.

\([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) increase by high K\(^+\) was sustained for a longer (over 3 min) period than that by ATP; and 3) Bradykinin, which stimulates \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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 A₂ activity or AA release. However, in PC12 cells, bradykinin, which is known 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 [Ca²⁺], is almost completely dependent on extracellular CaCl₂ (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 effectors(s), in addition to phosphatidylinositol turnover (diacylglycerol accumulation and Ca mobilization), for the stimulation of phospholipase A₂ 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 A₂ (31, 33, 37, 38). Recently, the cDNAs for a P₂X₄-type (12) and a P₂Y-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 [³H]AA release in the presence or absence of ATP from PC12 cells. Ligand-gated ion channels defined by P₂X 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 P₂X receptors from PC12 cells. However, the stimulatory effect of ATP on Ca ionophore-stimulated AA release seems to be separate from Ca²⁺ 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 [³H]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 [³H]AA release, although high K⁺ stimulated NA release. Additionally, high K⁺ had no effect on [³H]AA release from differentiated PC12 cells (data not shown). The reasons for this discrepancy are unclear at this point.

In conclusion, 1) P₂X₄-like ATP receptors stimulated AA release from PC12 cells in the presence of Ca ionophore; 2) Addition of Mg²⁺ 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 [Ca²⁺], and the activation of protein kinase C.

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