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Cytosolic Calcium Responses to Extracellular Adenosine 5′,5‴-P_1,P_4-Tetraphosphate in PC12 Cells

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NORDONE, A. J. AND E. B. PIVORUN. *Cytosolic calcium responses to extracellular adenosine 5′,5‴-P_1,P_4-tetraphosphate in PC12 cells.* PHARMACOL BIOCHEM BEHAV **52**(1) 85-91, 1995.—Binding of adenosine 5′,5‴-P_1,P_4-tetraphosphate (Ap_4A) to a purinoceptor on nerve growth factor-differentiated (NGF) pheochromacytoma (PC12) cells modulated cytosolic Ca^{2+} levels. Both Ap_4A and ATP elicited an influx of extracellular Ca^{2+}, but both the sensitivity of the response and the flux profile were different. Preincubation of the PC12 cells with the compounds adenosine 5′-0-(2-thio)diphosphate (ADP-β-S) and periodate-oxidized ATP had differential effects upon the Ap_4A and ATP-induced response. These results indicate that Ap_4A and ATP were either interacting with distinct purinoceptor subclasses or with the same purinoceptor with differing affinities. Simultaneous depolarization and application of either Ap_4A or ATP to the PC12 cells induced an additive effect on the calcium flux. Preincubation with verapamil negated the effects of depolarization without significantly modifying the ligand-elicited Ca^{2+} fluxes, suggesting the presence of Ap_4A ligand-gated channels that may function as modulators of PC12 cell function.

Cytosolic calcium Diadenosine tetraphosphate Nerve growth factor PC12 cells

ADENOSINE 5′,5‴-P_1,P_4-tetraphosphate (Ap_4A) was first reported to be formed in biologic systems as a secondary reaction product of aminoacyl-tRNA synthetases (43). Subsequently, the presence of intracellular and extracellular Ap_4A has been shown to modulate a variety of cellular events. Intracellular Ap_4A has been implicated in the initiation of DNA replication (41), via binding to DNA polymerase α (16,34), in the modulation of ADP-ribosylation of nuclear proteins (39), and in intracellular signaling during adaptive responses to cellular stress (4,11). Extracellular Ap_4A and its analogs antagonize ADP-induced platelet aggregation (42), induce calcium release from perfused liver preparations (8), modulate the contractile state of arterial preparations (7), and activate glycogen phosphorylase in isolated liver cells (12).

Neuronal cell function is also influenced by Ap_4A. Excitation of the rat nodose ganglion in the presence of Ap_4A (24) and inhibition of ATP-elicited excitation of this ganglion (23) have been observed. Ap_4A has been shown to increase basal secretion of catecholamines from isolated chromaffin cells (10) and is costored with ATP and the catecholamines within adrenal chromaffin granules (36). Ap_4A may have a neuromodulatory or cotransmitter role in mammalian brain, in a manner analogous to ATP cotransmission (23,24). Amphetamine induces the release of Ap_4A and adenosine 5′,5‴-P_1,P_5-pentaphosphate (Ap_5A) from the caudate putamen of conscious rats (32) and corelease of Ap_4A and ATP from the adrenal chromaffin granules occurs concomitantly with secretagogue-induced release of catecholamines (29). Ap_4A and ATP are costored within the synaptic vesicles of the *Torpedo* electric organ and within mammalian brain synaptosomes (30,31), from which the depolarizing agents, 4-aminopyridine and veratridine, elicit Ca^{2+}-dependent release of Ap_4A.

This laboratory demonstrated that membrane preparations from a variety of mammalian tissues (19), synaptosomes, and pheocromacytoma (PC12) cells (unpublished observations) exhibit specific and saturable receptor sites for Ap_4A. In addition, the Ap_4A receptor has been identified as a 42-kD polypeptide at the surface of individual mouse heart cells (20). The existence of high-affinity binding sites for Ap_4A has been

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confirmed in chromaffin cells (29), which display a putative P_{2y}-purinoceptor pharmacologic profile (6).

There is little information concerning the transduction events associated with diadenosine polyphosphate purinoceptor binding. It has recently been demonstrated that Ap_4A increases the cytosolic Ca^{2+} levels in chromaffin cells by activating release from intracellular stores (9). Activation of P_2 purinoceptors by ATP binding has been shown to elicit a rapid depolarization of sensory neurons via increased cation conductance (23) and to induce an extracellular Ca^{2+} influx in dorsal root ganglion cells (1) and a variety of neuronal cell lines (19). Highly specific ATP-gated Ca^{2+} channels have been demonstrated in PC12 cells that are distinct from the classical voltage-gated Ca^{2+} channels (22,27).

This study was initiated to determine and contrast the effects of Ap_4A, its analogs, and ATP receptor activation on temporal changes in cytosolic Ca^{2+} levels in nerve growth factor (NGF)-differentiated PC12 cells, which resemble sympathetic neurons morphologically and functionally (38). We present evidence that NGF-differentiated PC12 cells respond to extracellular diadenosine polyphosphates and ATP with distinct Ca^{2+} influx profiles and differential sensitivities to these nucleotides. Both Ap_4A and ATP activate ligand-gated verapamil-insensitive Ca^{2+} channels.

METHOD

Cell culture materials were obtained from Gibco BRL (Grand Island, NY). Fura-2 acetoxymethyl ester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR). All nucleotides and verapamil were purchased from Sigma (St. Louis, MO), with all other reagents of analytical grade or better.

Cell Culture

PC12 cells were obtained from American Type Culture Collection (Rockville, MD). The clones were cultured at 37°C in media consisting of RPMI 1640 with L-glutamine, 1000 U/l penicillin, 1000 mg/l streptomycin, 10% heat-inactivated horse serum, and 5% fetal bovine serum in a humidified atmosphere with 5% CO_2. Cells were plated onto collagen-coated six-well plates (35 mm in diameter) or canted-neck flasks (25 cm^2) at a density of 0.25 and 0.45 $\times$ 10^6 cells/cm^2, respectively, and allowed to grow for 2 days. At the end of this period, the neuronal phenotype was induced by the addition of 50 ng/ml NGF to the culture medium for 5–7 days.

Measurement of Cytosolic Ca^{2+} Concentrations

Cytosolic-free calcium was measured using the calcium-sensitive fluorescent dye fura-2/AM. Cells were detached from the flasks, centrifuged at 300 $\times$ *g* for 3 min, and washed once with incubation medium containing (mM/l): NaCl 125, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.0, glucose 6.0, Hepes 25 (pH 7.4). PC12 cell suspensions (5 $\times$ 10^6 cells/ml) were loaded with dye by incubation in 1.0 μM fura-2 AM in dimethyl sulfoxide (0.05% final concentration) for 15 min at 37°C. The cells were then washed twice with fresh medium. Cell suspensions (0.5–1.0 $\times$ 10^6 cells/ml) supplemented with 250 μM sulfinpyrazone (to prevent dye leakage) were transferred to a 3-ml thermostatted (37°C) cuvette maintained under continuous stirring. The fura-2 signal was analyzed on an SLM 8000C spectrofluorimeter (SLM AMINCO, Urbana, IL) as described (17) using a K_d for fura-2 of 224 nM. F_{max} was determined using 35 μM of the Ca^{2+} ionophore 4-bromo A-23187 and a saturating concentration of Ca^{2+}. F_{min} was determined in the presence of 10 μM alkaline EGTA. Autofluorescence (normally $<3.0\%$) was recorded for each batch of cells and subtracted before calculating Ca^{2+} concentration. Data were processed and stored on an IBM microcomputer interfaced to the spectrofluorimeter. Excitation and emission wavelengths were set to 340/380 and 510 nm, respectively. All figures show results from representitive cultures.

Data Analysis

Pairwise comparisons were made using Student's *t*-test (one-tailed, paired, based a priori hypothesis regarding the direction of the expected difference between means) and multiple comparisons using analysis of variance (ANOVA). $P \leq 0.05$ was considered statistically significant.

RESULTS

Binding of Ap_4A and ATP elicited a rapid rise in PC12 cell cytosolic Ca^{2+} levels (Figs. 1A and B). Dose-response curves for the two ligands (Fig. 2) indicated that changes in Ca^{2+} levels were more sensitive to ATP than Ap_4A, with estimated

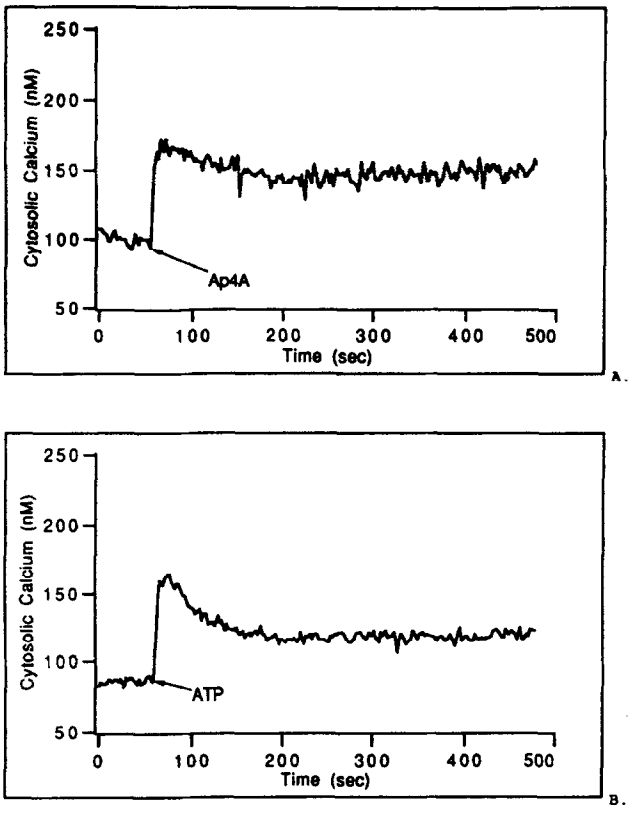

FIG. 1. Alterations in PC12 cell cytosolic Ca^{2+} elicited by the extracellular application of nucleotides. Cell suspensions (0.5–1.0 $\times$ 10^6 cells/ml) were loaded with fura2/AM. Ca^{2+} levels were monitored for 5 min before and 8 min subsequent to the application of nucleotide. Nucleotide added at the time indicated by the arrow. (A) Effects elicited by the addition of 500 μM Ap_4A. (B) Effects elicited by the addition of 5.0 μM ATP.

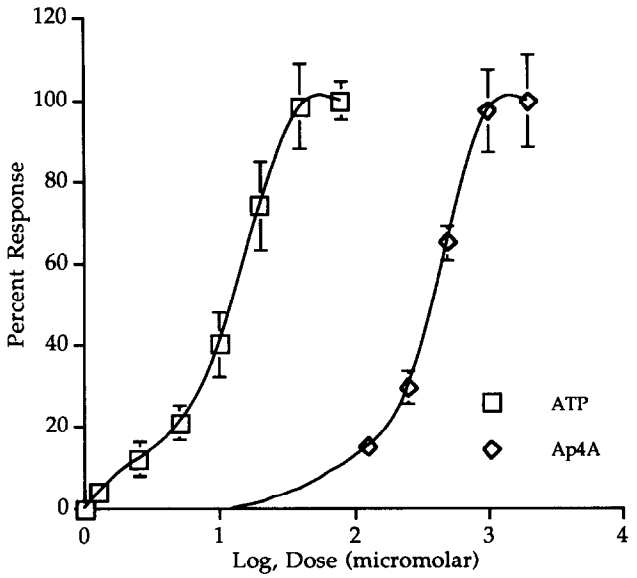

FIG. 2. Dose-response curves for the effect of extracellular Ap_4A and ATP on the cytosolic Ca^{2+} concentration of PC12 cells. Results are expressed as mean ± SE of seven experiments.

EC_{50} values of 11.75 and 371.54 μM, respectively. Dosages of 500 μM Ap_4A and 5.0 μM ATP were chosen for further analyses, because these doses produce similar changes in peak cytosolic Ca^{2+} levels and good signal-to-noise ratios. The maximal elevation in Ca^{2+} was 78 ± 3 nM (n = 18) after 500 μM Ap_4A application and 88 ± 4 nM (n = 17) after ATP application.

The temporal dynamics of the response, both before and after peak Ca^{2+} levels were attained, differed for the two ligands. Duration to peak Ca^{2+} levels was greater ($P < 0.05$) for Ap_4A (24 ± 2 s) than for ATP (16 ± 1 s). A residual elevation in Ca^{2+} levels was observed after the application of each ligand; it was greater ($P < 0.05$) for Ap_4A than ATP (54 ± 2 nM vs. 41 ± 3 nM), representing a decline from peak Ca^{2+} levels of 30 ± 2% and 53 ± 3%, respectively. Decline to residual Ca^{2+} levels was also slower ($P < 0.05$) for Ap_4A (99 ± 5 s) than for ATP (71.25 ± 4.73 s). The decline to residual Ca^{2+} levels was calculated as the time between peak and the beginning of the residual Ca^{2+} levels. With each ligand, the residual Ca^{2+} levels remained relatively constant for the remainder of the 8-min recording period.

Our results indicate that Ap_3A, Ap_4A, and Ap_5A elicited a rise in PC12 cytosolic Ca^{2+} levels as a consequence of ligand binding to extracellular purinoceptors, but only Ap_4A and Ap_5A caused physiologically significant increases. Exposure to 500 μM Ap_3A and Ap_5A resulted in Ca^{2+} flux profiles different from that elicited by Ap_4A. The maximal Ca^{2+} rise was only 7 ± 1 nM (n = 5) with Ap_3A, and no decline from this level was observed (data not shown). The maximal rise for Ap_5A was 30 ± 2 nM (n = 5) with time to peak level (36 ± 3 s) longer ($P < 0.05$) than for Ap_4A. There was no decline from peak Ca^{2+} levels (data not shown). PC12 cells exposed to 500 μM adenosine Ap_2A and Ap_6A exhibited no changes in cytosolic Ca^{2+} levels (data not shown).

To determine the relative contributions of intra- and extracellular Ca^{2+} pools to the calcium response, PC12 cells were exposed to 6 mM Na^+ Hepes-buffered EGTA. The Ap_4A elicited response was completely negated in the presence of EGTA, whereas that of ATP was significantly ($P < 0.001$) altered (Fig. 3A and B). Following ATP application, cytosolic Ca^{2+} rose only 43 ± 3 nM (n = 5) in 12 ± 1 s. A return to baseline level was achieved in 61 ± 2 s.

Activation of voltage-dependent Ca^{2+} channels with depolarizing levels of KCl (60 mM final concentration) elicited a change in cytosolic Ca^{2+} levels that had similar temporal dynamics to the response following ATP application but with a lower (63 ± 4 nM, n = 5) maximal increase (data not shown). The simultaneous application of Ap_4A or ATP with KCl modified the typical profiles elicited by the two ligands (Fig. 4A and B). Peak increases in cytosolic Ca^{2+} levels rose 41% to 109 ± 12 nM (n = 5) for Ap_4A and 45% to 127 ± 9 nM (n = 5) for ATP. Residual Ca^{2+} levels of 54 ± 6 and 45 ± 7 nM following Ap_4A and ATP application, respectively, were essentially unaltered, but represented greater percentage declines from peak levels (49 ± 4 and 64 ± 5%, respectively). The additional increase in Ca^{2+} influx was the result of activation of voltage-operated Ca^{2+} channels.

Ca^{2+} flux elicited by KCl was inhibited by the dihydropyridine Ca^{2+} channel blocker verapamil (data not shown), but exposure of the PC12 cells to 30 μM verapamil for 15 min had very little effect on the temporal dynamics of the Ca^{2+} profile following Ap_4A or ATP application; however, it did reduce

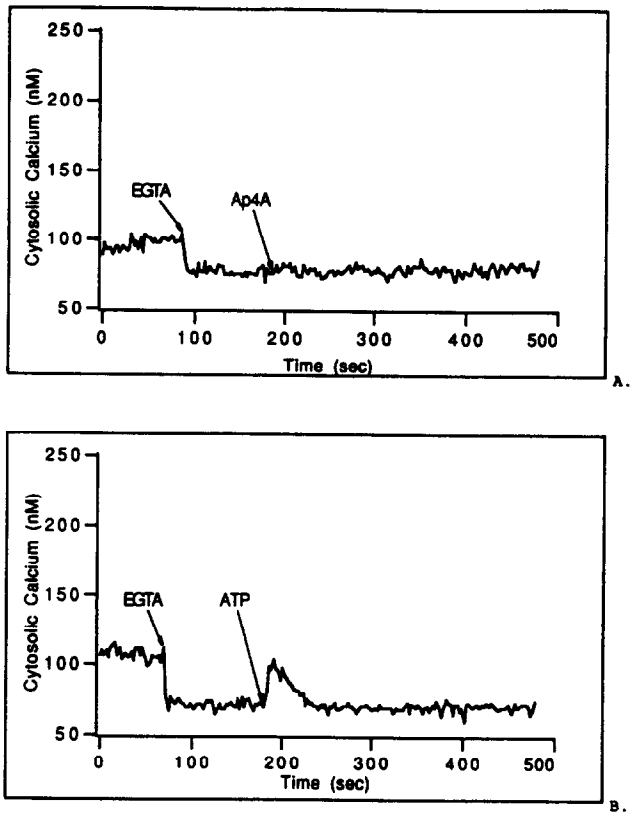

FIG. 3. Effects of 6.0 mM EGTA on the Ap_4A and ATP-induced Ca^{2+} flux. Nucleotide added at the time indicated by the arrow. (A) Effects elicited by the addition of 500 μM Ap_4A. (B) Effects elicited by the addition of 5.0 μM ATP.

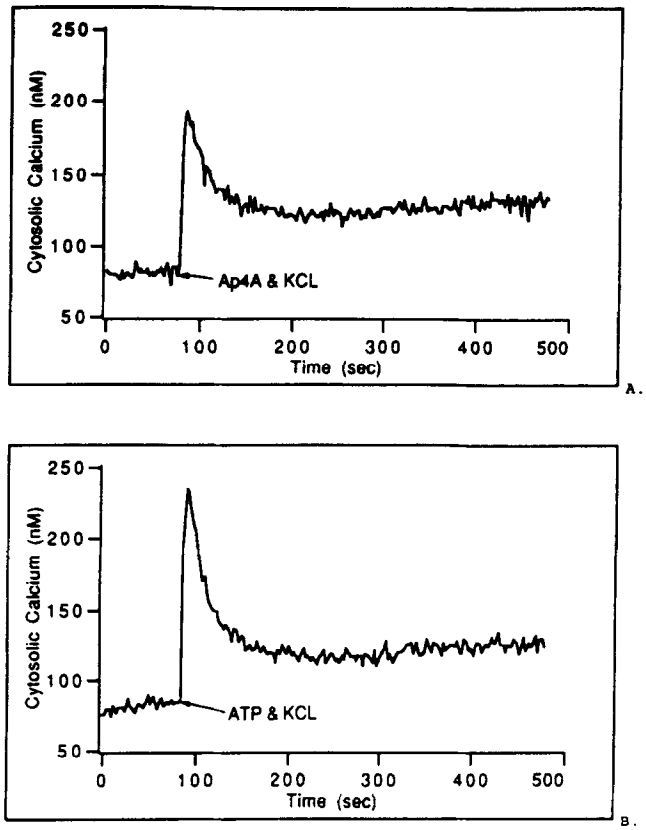

FIG. 4. Effects of the simultaneous application of 60 mM KCl (final concentration) and nucleotide on the PC12 cell cytosolic Ca^{2+} profile. Nucleotide added at the time indicated by the arrow. (A) Effects elicited by the addition of 500 μM Ap_4A and KCl. (B) Effects elicited by the addition of 5.0 μM ATP and KCl.

peak Ca^{2+} levels by approximately 10 nM in both cases (Fig. 5A and B). Preincubation of the cells with verapamil negated the additive effect demonstrated by simultaneous exposure to KCl and Ap_4A or ATP (Fig. 6A and B). These results are consistant with the activation of ligand-gated Ca^{2+} channels by both Ap_4A and ATP and the inhibition of depolarization-induced Ca^{2+} influx by verapamil.

In an attempt to distinguish pharmacologically between the Ap_4A and ATP effects, we investigated the ability of various nucleotides to antagonize Ap_4A and ATP binding. Application of 100 μM of the putative Ap_4A antagonist adenosine 5′-0-(2-thio)diphosphate (ADP-β-S) produced a Ca^{2+} flux profile similar to ATP, with a peak increase of 80 ± 3 nM (n = 6). ADP-β-S also had a differential effect on subsequent ATP and Ap_4A-induced Ca^{2+} flux compared to controls (Fig. 7A and B). Although the peak levels associated with Ap_4A application were significantly ($P < 0.05$) reduced, 53% to 36 ± 2 nM (n = 4), there was no effect on the ATP-induced Ca^{2+} flux (peak levels of 86 ± 3 nM). Ca^{2+} flux associated with ATP was also not affected by prior application of 500 μM of the putative ATP antagonist periodate oxidized ATP, whereas the peak increase induced with Ap_4A application was significantly ($P < 0.001$) reduced, 86% to 10 ± 2 nM (n = 4) compared to control (Fig. 8A and B). Prior exposure of the PC12 cells to 200 μM of a number of putative ATP antagonists including β,γ-methyladenosine 5′-triphophate (AMP-PCP), adenosine 5′-0-(3-thio)triphosphate (ATP-γ-S), or α,β-methyladenosine 5′-triphosphate (AMP-CPP) had no effect on ATP or Ap_4A-induced Ca^{2+} flux (data not shown). Both ATP-γ-S and AMP-CPP application induced a Ca^{2+} flux profile similar to ATP, but with smaller maximal increases of 85 ± 3 nM (n = 4) and 39 ± 3 nM (n = 4), respectively (data not shown).

DISCUSSION

Low micromolar doses of ATP have been shown to elicit increases in cytosolic Ca^{2+} levels in both differentiated and nondifferentiated PC12 cells (15,37); however, this report presents the first evidence of an Ap_4A-elicited Ca^{2+} flux in this cell type. The considerable differences in the sensitivity and temporal aspects of PC12 cell responses to Ap_4A and ATP suggest the possible presence of two purinoceptor subclasses or that Ap_4A may act as a partial agonist at the ATP site. This latter explanation may account for the fact that a concentration of Ap_4A greater than the EC_{50} initiates a Ca^{2+} flux similar to that initiated by a concentration of ATP below the EC_{50}. Initiation by Ap_4A and ATP of their characteristic calcium profiles could induce distinct Ca^{2+}-dependent intracellular responses. These results provide further support for a neuromodulatory role for Ap_4A in the CNS.

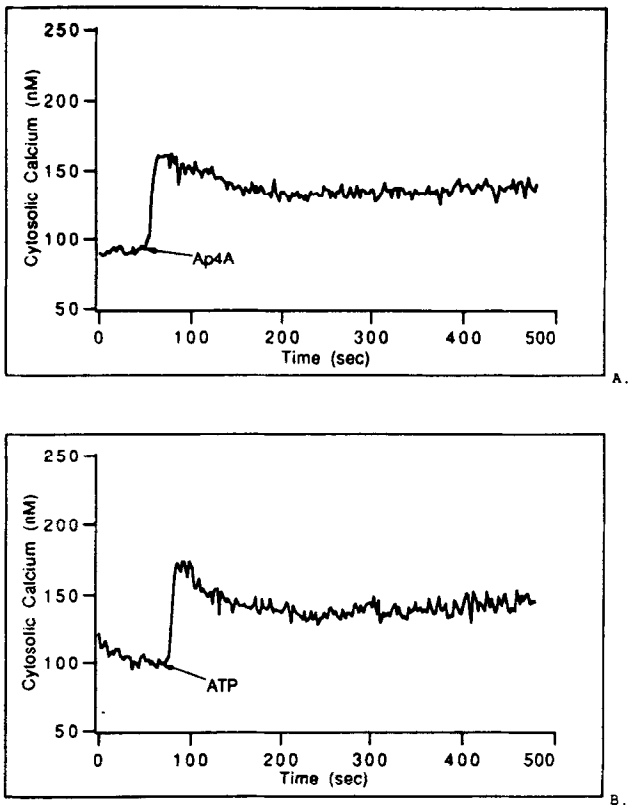

FIG. 5. Effects of 15 min verapamil preincubation (30 μM) on the PC12 cell cytosolic Ca^{2+} profile elicited by nucleotide application. (A) Nucleotide added at the time indicated by the arrow. Effects elicited by the addition of 500 μM Ap_4A. (B) Effects elicited by the addition of 5.0 μM ATP.

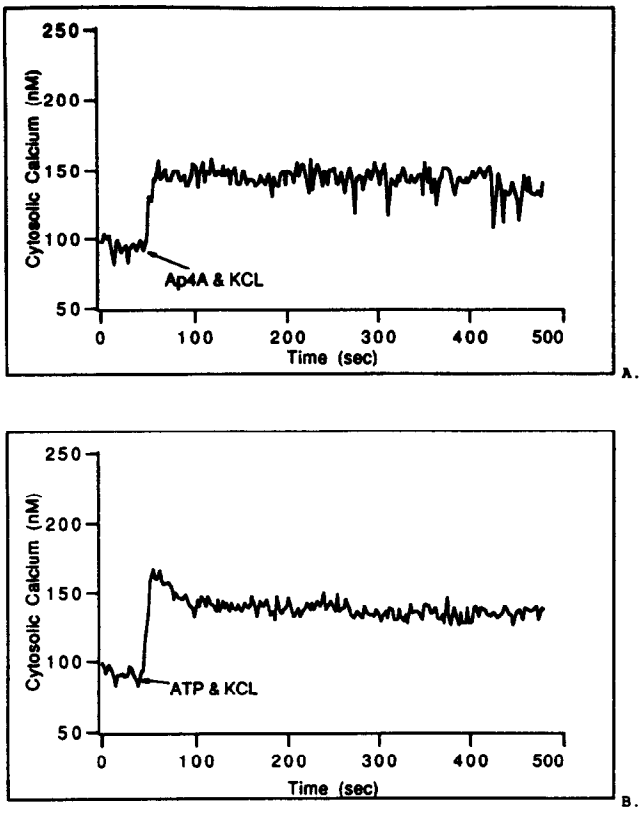

FIG. 6. Effects of 15 min verapamil preincubation (30 μM) on the PC12 cell cytosolic Ca^{2+} profile elicited by simultaneous nucleotide and 30 mM KCl application. Nucleotide added at the time indicated by the arrow. (A) Effects elicited by the addition of 500 μM Ap_4A and KCl. (B) Effects elicited by the addition of 5.0 μM ATP and KCl.

In contrast to the PC12 cells, lower concentrations of Ap_4A are required to elicit a concentration-dependent increase in cytosolic Ca^{2+} levels in resting chromaffin cells (9). These cells display an EC_{50} value of 28 ± 7 μM. However, a concentration of 100 μM Ap_4A is needed to modulate the evoked release of catecholamines from the same cell type (10).

There are no widely accepted competitive antagonists of Ap_4A binding; however, ADP-β-S has been shown to inhibit Ap_4A-induced cytosolic Ca^{2+} increases in chromaffin cells (9) and to inhibit binding of [^{3}H]Ap_4A to midbrain synaptosomes (29). The application of 100 μM ADP-β-S to PC12 cells produces a peak Ca^{2+} level approximately half of that demonstrated in a previous report (9) on chromaffin cells. It is not explained in this report whether ADP-β-S exerts its own agonistic effect via a putative Ap_4A receptor, but its inhibitory effect is assumed to be an inhibition of Ap_4A binding. Following the application of 100 μM ADP-β-S, the expected elevation in Ca^{2+} elicited by Ap_4A is significantly reduced, but there is no effect on the ATP-induced response. We did not attempt to determine whether the ADP-β-S-induced flux was due to binding at the same site as Ap_4A but, because of the ligand-gated, extracellular Ca^{2+} influx associated with Ap_4A, we believe that ADP-β-S inhibits the binding of Ap_4A to the extracellular membrane. These results suggest that ADP-β-S acts as a partial agonist in this preparation and differ from the observations of Castro et al. (9), who suggested that ADP-β-S may selectively block the Ap_4A receptor, which exhibits a putative P_2y pharmacologic profile.

Periodate-oxidized ATP has been demonstrated to antagonize ATP-induced responses in guinea pig vas deferens (14) and the mouse macrophage cell line J774 (26). In contrast, we found that application of 500 μM periodate-oxidized ATP to the PC12 cells primarily inhibits Ap_4A-induced Ca^{2+} influx without affecting the ATP response. Periodate-oxidized ATP would not appear to be an antagonist of the ATP receptor in PC12 cells. Previous studies used higher doses of, or longer incubation periods with, periodate-oxidized ATP to antagonize ATP-induced responses (14,26). Higher doses or longer incubation periods may have also altered the ATP-induced response in PC12 cells. However, the differential response obtained with ADP-β-S and periodate-oxidized ATP suggests the possibility of two purinoceptor subtypes on NGF-differentiated PC12 cells, although this evidence alone is inconclusive.

The diadenosine polyphosphates agonistic activity to induce Ca^{2+} flux is $Ap_4A > Ap_5A > Ap_3A$ $Ap_2A = Ap_6A$. Ap_3A and Ap_5A are also found to be less effective than Ap_4A in suppressing nicotine-evoked catecholamine release from dispersed chromaffin cells (10), despite binding studies indicating that Ap_4A and Ap_5A had similar binding affinities (9). Ap_2A and Ap_3A do not elicit Ca^{2+} release from muscle sarco-

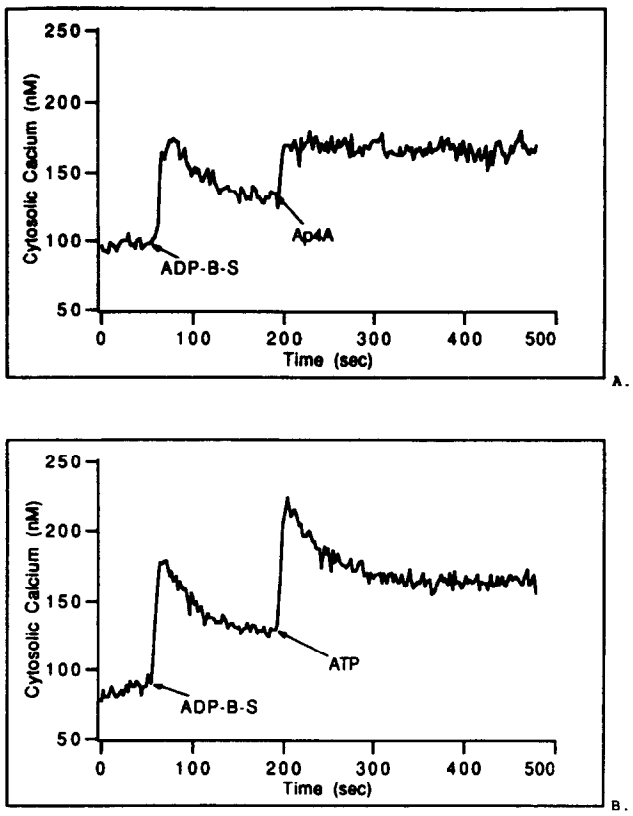

FIG. 7. Effects of the application of 100 μM ADP-β-S on the PC12 cell cytosolic Ca^{2+} profile elicited by nucleotide application. Nucleotide added at the time indicated by the arrow. (A) Effects elicited by the addition of 500 μM Ap_4A. (B) Effects elicited by the addition of 5.0 μM ATP.

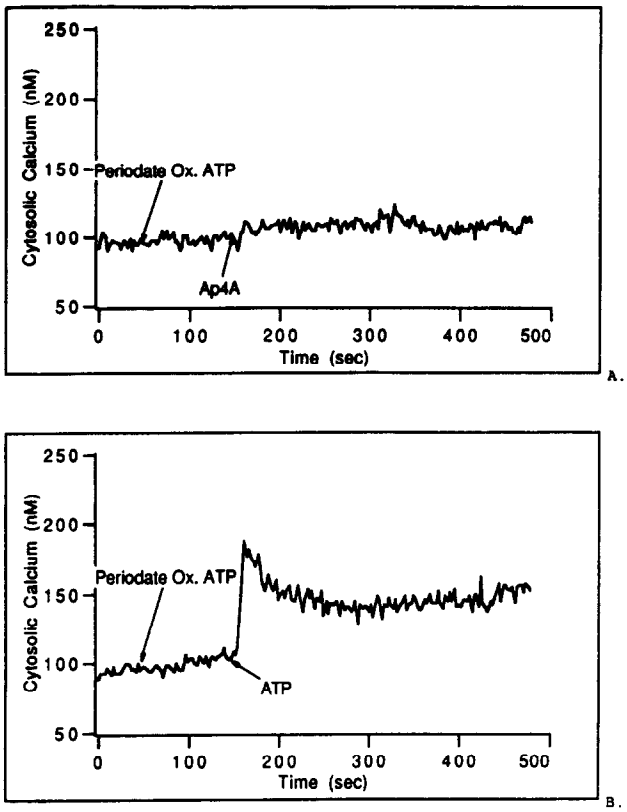

FIG. 8. Effects of the application of 500 μM periodate oxidised ATP on the PC12 cell Ca^{2+} calcium profile elicited by nucleotide application. Nucleotide added at the time indicated by the arrow. (A) Effects elicited by the addition of 500 μM Ap_4A. (B) Effects elicited by the addition of 5.0 μM ATP.

plasmic reticulum, whereas Ap_4A and Ap_5A induce a significant increase in Ca^{2+} release (25). In contrast to the results presented here, Ap_6A is the most potent stimulator of Ca^{2+} flux in the sarcoplasmic reticulum model, inducing greater inceases than similar concentrations of Ap_4A by fivefold. Despite some conflicting results, the similarities of the effects of the diadenosine polyphosphates on induction of Ca^{2+} flux in these different models suggest that Ap_4A is physiologically significant, inducing similar changes in intracellular Ca^{2+} to those induced by ATP. Ap_4A may induce Ca^{2+} channel activation by a pathway that is common to both internal and external cellular membranes.

Ca^{2+} influx across the extracellular membrane appears to be the only source of the Ap_4A-induced increase in Ca^{2+} levels observed in this PC12 cell clone. However, the increased levels associated with ATP application appear to be a combination of transmembrane flux and redistribution from internal stores. A number of researchers (3,13,35) have indicated that ATP-mediated Ca^{2+} flux in PC12 cells is predominantly of extracellular origin, although it has been demonstrated that in a significant proportion of a PC12 cell population, the response can be due partly to internal mobilization (37). This heterogeneity is attributed to the coexistance of multiple cell clones within a PC12 cell population. In other neuronal cell lines (NG 108-15, GOTO, and C_6), the response is also attributed largely to Ca^{2+} influx with some release from internal stores (21). In contrast to our observations with PC12 cells, other researchers (9) conclued that the Ap_4A-stimulated increase in chromaffin cell Ca^{2+} levels are due entirely to release from internal stores.

An increase in transmembrane Ca^{2+} flux depends on activation of voltage-gated Ca^{2+} (18) or ligand-gated channels (2). Preincubation with verapamil causes a nonsignificant reduction in the Ap_4A and ATP-induced peak Ca^{2+} level, but has no effect on the residual levels. This indicates that the Ca^{2+} fluxes elicited by Ap_4A and ATP in PC12 cells appear largely to depend on activation of ligand-gated channels, but that at least part of the flux may be due to the opening of voltage-gated channels. Activation of voltage-gated channels may depend on the influx of other ions through the Ap_4A-activated channels. In many preparations including PC12 cells, smooth muscle, and sensory neurons, ATP cation channel activation is not specific and allows for the passage of both Na^+ and Ca^{2+} (2,22,23). It has also been demonstrated (13) that ATP activates ligand-gated Ca^{2+} channels in PC12 cells and that part of the Ca^{2+} flux is sensitive to verapamil and other inhibitors of voltage-regulated channels. Using patch clamp methods, other researchers have shown that ATP activates ligand-gated channels in PC12 cells (27) and smooth muscle preparations (2,3). These channels allow the passage of Ca^{2+} and monovalent cations with activation of voltage-regulated calcium channels occurring in some studies (2).

We have demonstrated that Ap_4A binding to NGF-differentiated PC12 cells elicits a physiologically significant transmembrane Ca^{2+} flux profile that is predominantly due to the activation of ligand-gated Ca^{2+} channels. This profile is distinct from that elicited by ATP purinoceptor activation. We demonstrated that Ap_4A can modulate depolarization-induced Ca^{2+} flux in a manner consistant with a neuromodulatory role.

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