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Effect of diadenosine polyphosphates on catecholamine secretion from isolated chromaffin cells

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1 The action of several diadenosine polyphosphates (AP_3A, AP_4A and AP_5A) on basal, and on nicotine- and high K^+-evoked, catecholamine (CA) release has been investigated. Each of the three diadenosine polyphosphates weakly but significantly increased basal CA secretion. This enhancement represented about 10% of the response evoked by 2 μM nicotine.

2 The evoked secretory response to diadenosine polyphosphates had an absolute requirement for extracellular Ca^{2+}.

3 In contrast, these compounds had an inhibitory action on nicotine-evoked release. This response was concentration-dependent, EC_{50} values being $3.2 \pm 0.4\,\mu$M, $4.0 \pm 1.6\,\mu$M and $19.3 \pm 4.0\,\mu$M for AP_3A, AP_4A, and AP_5A, respectively. The lower the concentration of nicotine used to evoke secretion, the higher the inhibitory power of these compounds.

4 The CA secretion evoked by K^+-rich solutions was further enhanced by AP_3A and AP_5A, whereas AP_4A inhibited it. The possible physiological role of these dual actions is discussed.

Introduction

The use of adrenal medullary chromaffin cells as a model has been largely responsible for the improvement in our understanding of neurosecretory responses, their molecular mechanism and modulation (Winkler & Carmichael, 1982; Burgoyne, 1984; Bader *et al.*, 1986). Many substances, including opioid peptides, substance P, γ-aminobutyric acid and peptides derived from cromogranin A, appear to modulate acetylcholine-mediated catecholamine (CA) release from these cells (Mizobe *et al.*, 1979; Kumakura *et al.*, 1980; Castro *et al.*, 1988; Simon *et al.*, 1988).

Another of these putative neuromodulator substances is adenosine and its analogues. Today, adenosine receptors and their actions are well documented (Williams, 1987), and recently adenosine triphosphate (ATP) itself has been shown to influence many biological processes (Gordon, 1986; Reilly & Burnstock, 1987).

ATP is one of the main components of chromaffin granules and it is released in the exocytotic process. This nucleotide can be degraded extracellularly by the action of ecto-nucleotidases (Richardson *et al.*, 1987; Newby, 1988) to form adenosine. The effect of adenosine and adenosine nucleotides on CA secretion from chromaffin cells has therefore been studied. ATP, adenosine diphosphate (ADP) and adenosine inhibit acetylcholine-evoked CA release, probably by prior conversion to adenosine (Chern *et al.*, 1987). In contrast, adenosine can enhance, in a quite complex manner, forskolin-mediated secretion (Chern *et al.*, 1988). Chromaffin cells present a single class of high affinity adenosine transporters of the neural type (Miras-Portugal *et al.*, 1986; Torres *et al.*, 1986; 1988). These transporters are active enough to control the termination of the effects of adenosine.

ATP is not the only nucleotide component co-stored in secretory granules. In effect, diadenosine polyphosphates (AP_xA) have been demonstrated to exist in platelet (Flodgaard & Klenov, 1982; Lütje & Ogilvie, 1983) and in chromaffin granules (Rodriguez del Castillo *et al.*, 1988). The AP_xA have been demonstrated to be responsible for multiple biological effects inside the cells (Zamecnik, 1983), but their extracellular role, if any, after release, is still not fully known (Lütje & Ogilvie, 1987; 1988). Recently, Louie *et al.* (1988) found an antithrombotic action for AP_4A.

AP_3A, AP_4A and AP_5A are present in chromaffin granules and the purpose of the present experiments was to study the effects of these dinucleotides on CA release from isolated chromaffin cells.

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Methods

Bovine adrenal glands supplied by the local slaughter house were immediately placed in ice-cold physiological saline solution and processed within 1–2 h following the death of the animal.

Isolation of bovine adrenal chromaffin cells

Chromaffin cells were prepared from adrenal medullae according to the method of Miras-Portugal *et al.* (1985). In brief, glands were cannulated and washed by retrograde perfusion with Ca^{2+}-free Locke medium containing 5% bovine serum albumin. Medullary tissue was digested with 0.1% collagenase (Boehringer) perfused continuously for 1 h. Collected cells were washed twice and purified in a percoll gradient (50% isotonic percoll, centrifuged at 15000 *g* for 30 min at 20°C). Collected cells were suspended in Dulbecco's modified Eagle's medium, DMEM (GIBCO), and washed twice. Cell viability was checked by trypan blue exclusion. The purity of the chromaffin cells was assessed by the specific incorporation of neutral red into these cells. Viability and purity were greater than 90%.

Purified cells were dispersed at a density of 10^6 cells ml^{-1} in DMEM containing 10% foetal calf serum (GIBCO), standard antibiotics (100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 40 μg ml^{-1} gentamicin, all from Sigma), 50 μM cytosine arabinoside (Aldrich), 50 μM 5-fluorodeoxyuridine (Aldrich) and 100 μM sodium ascorbate (Sigma). This suspension was kept at 4°C and used during the 2–3 days following cell isolation, as described by Greenberg & Zinder (1982). Under these conditions, third-day cells were able to grow when seeded in plastic Petri dishes (Costar) and maintained at 37°C in 5% CO_2/95% air.

Chromaffin cell secretory response

CA release was measured by direct electrochemical detection of CA eluting from a superfused cell bed, in a monitoring system similar to that described by Green & Perlman (1981) and Kumakura *et al.* (1986). Chromaffin cells (10^6 cells) were introduced into a perfusion chamber, formed by a Millex GS filter (0.22 μm pore size, 25 mm ϕ) and perfused at 2 ml min^{-1} with Locke solution (composition in mM: NaCl 140, KCl 4.4, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 4.0, glucose 5.6 and HEPES 10, pH 7.5). In potassium-rich solution (K^+-rich, 25 mM), KCl concentration was increased at the expense of NaCl.

The cell bed was stimulated by injection of chemical stimuli into the flow stream, in a volume of 50 μl, through a loop injector (Rheodyne 7010, Cotati, California). When taking into account the filter volume and the continuous perfusion, the maximum concentrations of the secretagogue in the cell bed (referred to as final concentrations) were 4.7 times lower than the secretagogue concentration in the injected solution (referred to as initial concentrations). This ratio was measured experimentally, by injecting different adrenaline concentrations under the same experimental conditions and referring peak height to the signal produced by continuous perfusion with those concentrations of adrenaline in the medium. The electrochemical detector was adjusted to +500 mV to avoid K^+ effect on support current. The electrochemical detector provided a continuous signal proportional to the concentration of catecholamines in the perfusate. None of the drugs used in our experiments gave electrochemical signals detected by this system.

Results are presented as the mean ± s.e.mean of at least three experiments, each performed in triplicate. For each experiment, cells from adrenal glands of four animals were pooled. The level of significance was established at $P < 0.05$, obtained by use of Student's t test. EC_{50} values were derived by logit-log regression.

Results

Effects on basal secretion: diadenosine polyphosphate-evoked release

All three diadenosine polyphosphates had a weak secretory action, which was only about 10% of control 2 μM (final concentration) nicotine-evoked release. However, this effect could not be attributed to a reagent artifact since, under the same experimental conditions but without cells present in the perfusion chamber, no signal was observed.

CA release by chromaffin cells was increased when cells were challenged with AP_xA in a concentration-dependent manner. AP_xA-evoked release was low, amounting to 27 ± 5, 19 ± 3 and 23 ± 3% of previous basal release for AP_3A, AP_4A and AP_5A, respectively.

The secretory response had an absolute requirement for extracellular calcium. Challenging cells with each AP_xA, or nicotine as a control, in a Ca^{2+}-free medium failed to elicit a secretory response (Figure 1).

Effect of diadenosine polyphosphates on nicotine-evoked catecholamine release

When CA secretion was stimulated with 10 μM nicotine (initial concentration), the three AP_xA compounds studied exerted an inhibitory effect (Figure 2), in a concentration-dependent fashion. EC_{50} values were 3.2 ± 0.4 μM for AP_3A, 4.0 ± 1.6 μM for AP_4A and 19.3 ± 4.0 μM for AP_5A. The inhibitory effect of

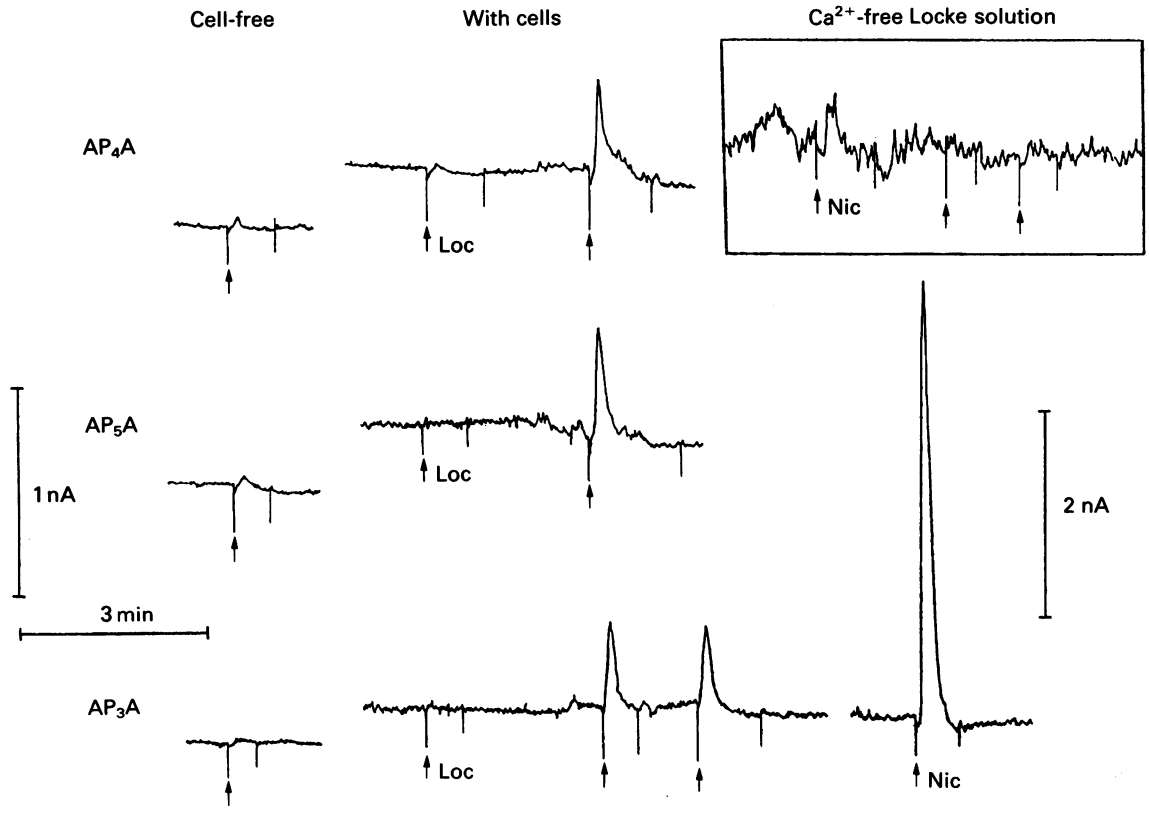

Figure 1 Effect of diadenosine polyphosphates (AP_xA) on basal catecholamine (CA) release. Typical records of electrochemically monitored current-time peaks from a perfusion chamber without cells (cell-free), with cells in normal medium containing Ca^{2+} and in the absence of Ca^{2+}. Drugs (100 μM, initial concentration) were injected at the arrows. Loc, normal Locke solution injected as negative control. The nicotine peak referred to as CA release in normal Ca^{2+}-containing medium evoked by 10 μM (initial concentration) nicotine. Note the different scale.

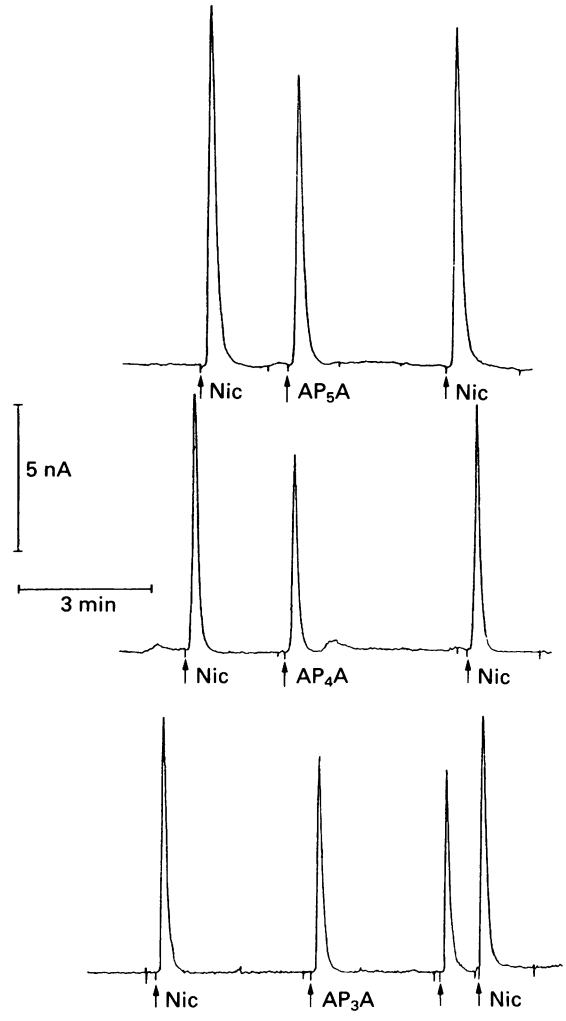

Figure 2 Effect of diadenosine polyphosphates (AP_xA) on catecholamine (CA) release evoked by nicotine. The figure represents typical records of electrochemically monitored current-time peaks from nicotine (Nic) 10 μM (initial concentration) and nicotine (10 μM) plus the respective diadenosine polyphosphate (100 μM, initial concentration).

these polyphosphates was not further increased at concentrations higher than 100 μM (initial concentration). The greatest inhibitory effect was achieved with AP_4A, 40.0 ± 0.5% maximum inhibition, whereas the inhibitions caused by AP_3A and AP_5A, though significant, were only 15 ± 0.5% and 18 ± 3%, respectively (Figure 3).

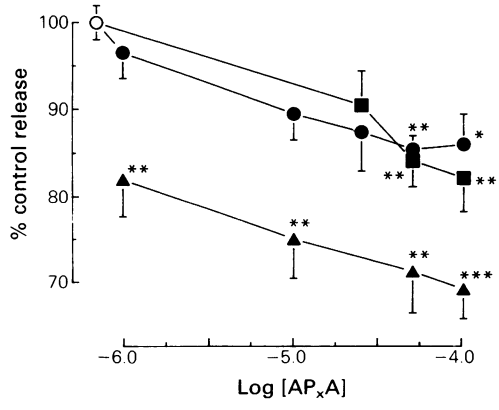

Figure 3 Inhibition of nicotine-evoked catecholamine (CA) release by diadenosine polyphosphates (AP_xA). Control release was evoked by 10 μM (initial concentration) nicotine. Each point was determined as shown in Figure 2 for each concentration of AP_3A (●), AP_4A (▲) and AP_5A (■). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Vertical lines show s.e.mean.

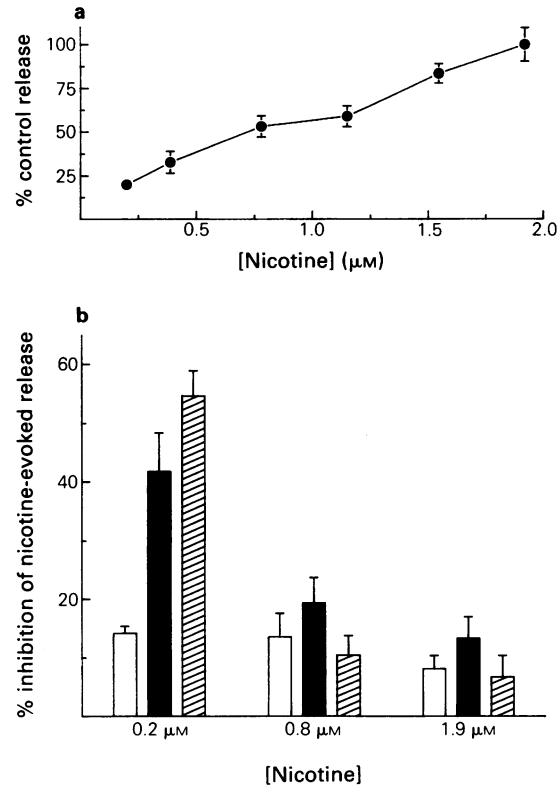

Figure 4 (a) Control values of catecholamine (CA) release evoked by increasing concentrations of nicotine. (b) Effect of 25 μM (initial concentration) diadenosine polyphosphates (AP_xA) on CA release evoked by different nicotine concentrations. Open columns, effect of AP_3A; solid columns, AP_4A; hatched columns, AP_5A.

At a final concentration of 5.3 μM, AP_3A, AP_4A and AP_5A caused a statistically significant reduction in the CA release evoked by nicotine (0.2–1.9 μM, final concentration). This inhibitory effect was greater when cells were stimulated with low concentrations of nicotine, and became reduced with increasing nicotine concentrations (Figure 4).

Effect of diadenosine polyphosphates on catecholamine release evoked by 25 mM K^+

In the presence of K^+-rich solution, CA secretion was elevated. AP_3A and AP_5A further enhanced this evoked release, whereas AP_4A inhibited it (Table 1).

Effect of continuous perfusion with diadenosine polyphosphates

When long-term effects of AP_XA were studied by bathing the cells with the appropriate drug and testing secretory responses to nicotine, the inhibitory action of all three compounds increased with time. The effect was reversed when the drug

Table 1 Effect of diadenosine polyphosphates (19 μM) on catecholamine release evoked by 25 mM K^+ solution

Effector	*% effect*
KCl alone	100 ± 6
+ AP_3A	151 ± 12
+ AP_4A	63 ± 2
+ AP_5A	173 ± 6

Concentrations refer to final concentrations. Figures are mean ± s.e.mean for six cell beds corresponding to three different preparations.

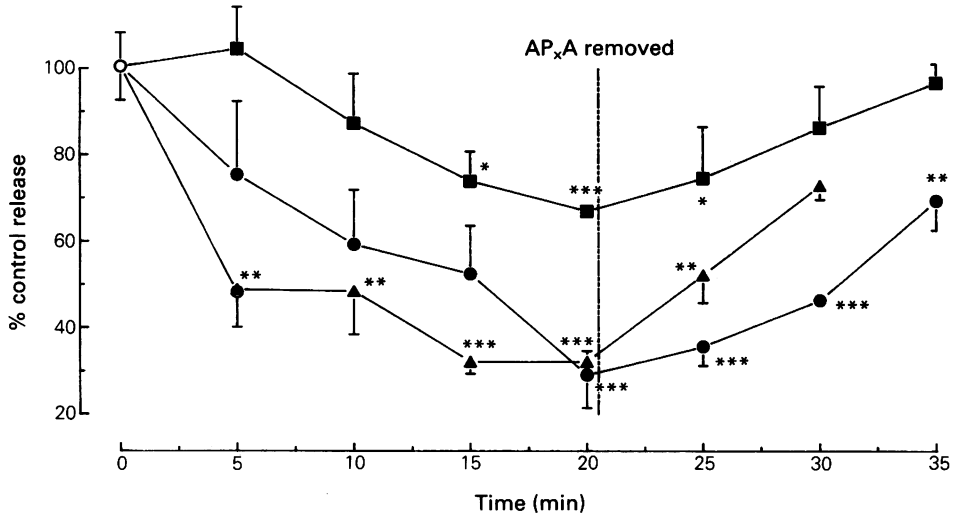

Figure 5 Effect of continuous perfusion of 10 μM diadenosine polyphosphates (AP_xA) on 10 μM (initial concentration) nicotine evoked catecholamine (CA) release with respect to time. Cells were perfused with drug-containing medium. At the vertical line the medium was replaced by a drug-free one. (○) Control release prior to drug perfusion, (●) AP_3A, (▲) AP_4A and (■) AP_5A. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Vertical bars show s.e.mean.

was removed from perfusing medium (Figure 5). The rank order of these inhibitory agents appeared to be $AP_4A \geqslant AP_3A > AP_5A$.

Discussion

The results presented here demonstrate, for the first time, that adenosine polyphosphates can play a role in nicotine-evoked CA release. This novel effect adds to the emerging extracellular actions being shown for these compounds, for instance on macrophage-induced cell growth (Ogilvie & Lüthje, 1987) and platelet aggregation (Louie *et al.*, 1988). It is important to realize that in our model diadenosine polyphosphates are active in the low μM range, whereas in the other studies high μM or μM concentrations were needed to obtain the effects.

Although chromaffin cells have a low hydrolytic activity, the effects seems to be due to diadenosine polyphosphates themselves, rather than to degradation products. Since in our experimental system drugs are in contact with the cell bed only for a few seconds, the possible degradation would be negligible (<1% per hour in cultured cells, M.T. Miras-Portugal, unpublished results). In these conditions ATP, ADP, AMP or adenosine concentrations resulting from diadenosine phosphate degradation must be far below the μM range. Since the K_d values for ATP and adenosine at purinoceptors (Reilly & Burnstock, 1987) and the known effects of adenosine on CA release (Chern *et al.*, 1988) are produced at concentrations in the order of 100 μM, an action through these degradation products is probably precluded.

The action of these effectors, especially that of AP_4A and AP_5A, may be physiologically important in the local control of CA release, since they are stored at an intragranular concentration of about 6 mM in chromaffin cells (Rodriguez del Castillo *et al.*, 1988), and are released together with CAs in the exocytotic process. The finding that the lower the concentration of nicotine used to stimulate the cells, the higher the modulator potential, is concordant with results on other systems, such as phorbol ester modulation of glutamate release from synaptosomes (Diaz-Guerra *et al.*, 1988). This behaviour points to a predominant role of these compounds on the basal/sub-maximal levels of secretion. In fact Malhotra & Wakade (1987) have shown that in the adrenal medulla *in situ* acetylcholine is not the major component of splanchnic nerve stimulation input to chromaffin cells.

The results with AP_3A and AP_5A seem confusing, since they are inhibitors of nicotine-evoked release, but are activators when CA secretion is evoked by high K^+. However, similar results have been obtained with substance P, which modulates nicotine-evoked release but has no action on K^+-evoked release (Livett *et al.*, 1983). Similar opposing effects of different diadenosine polyphosphates on the same response have been found previously (Chao & Zamecnik, 1984; Lüthje *et al.*, 1985). CA secretion from chromaffin cells is triggered by a fast rise in cytosolic calcium concentration, $[Ca^{2+}]_i$ (Kao & Schneider, 1986), but there is growing evidence to support the idea that nicotine and nicotinic agonists activate an alternative second messenger system, in addition to the rise in free cytosolic $[Ca^{2+}]$ (Cobbold *et al.*, 1987; Minenko *et al.*, 1987). It may be possible that the inhibition of nicotine-evoked release produced by these compounds could be mediated through this alternative pathway. Enhancement of K^+-evoked release could be related to their own secretory action on basal output.

We have shown that AP_3A, AP_4A and AP_5A could play a physiological role in the modulation of basal and evoked release of CA from chromaffin cells, though the assay method does not permit any conclusion to be drawn concerning any differential effects these compounds may have on the output of adrenaline and noradrenaline. These compounds could serve as valuable tools for the study of secondary and later steps in exocytosis and offer a new direction for the development of pharmacologically active drugs.

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