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Characterization of diadenosine tetraphosphate (Ap_4A) binding sites in cultured chromaffin cells: evidence for a P_{2y} site

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1 Diadenosine tetraphosphate (Ap_4A) a dinucleotide, which is stored in secretory granules, presents two types of high affinity binding sites in chromaffin cells. A K_d value of $8 \pm 0.65 \times 10^{-11}$ M and B_{max} value of 5420 ± 450 sites per cell were obtained for the high affinity binding site. A K_d value of $5.6 \pm 0.53 \times 10^{-9}$ M and a B_{max} value close to 70,000 sites per cell were obtained for the second binding site with high affinity.

2 The diadenosine polyphosphates, Ap_3A, Ap_4A, Ap_5A and Ap_6A, displaced [^{3}H]-Ap_4A from the two binding sites, the K_i values being 1.0 nM, 0.013 nM, 0.013 nM and 0.013 nM for the very high affinity binding site and 0.5 μM, 0.13 μM, 0.062 μM and 0.75 μM for the second binding site.

3 The ATP analogues displaced [^{3}H]-Ap_4A with the potency order of the P_{2y} receptors, adenosine 5'-O-(2 thiodiphosphate) (ADP-β-S) > 5'-adenylyl imidodiphosphate (AMP-PNP) > α,β-methylene ATP (α,β-MeATP), in both binding sites. The K_i values were respectively 0.075 nM, 0.2 nM and 0.75 nM for the very high affinity binding site and 0.125 μM, 0.5 μM and 0.9 μM for the second binding site.

Keywords: Chromaffin cells; diadenosine polyphosphates; diadenosine tetraphosphate binding sites; ectophosphodiesterases, P_2-purinoceptors

Introduction

The presence of diadenosine polyphosphates, namely adenosine-(5')-triphospho-(5')-adenosine (Ap_3A), adenosine-(5')-tetraphospho-(5')-adenosine (Ap_4A), and adenosine-(5')-pentaphospho-(5')adenosine (Ap_5A) has been described in the secretory granules of neural and non-neural cells (Luthje & Ogilvie, 1983; Rodriguez del Castillo *et al.*, 1988). Considerable amounts of Ap_3A and Ap_4A are co-stored with 5-hydroxytryptamine and adenine nucleotides in the dense granules of human platelets (Flodgaard & Klenow, 1982; Luthje & Ogilvie, 1983). These compounds are released to the extracellular media during thrombin-induced aggregation. Ap_3A causes a gradual aggregation of platelets; in contrast, Ap_4A competitively inhibits ADP-induced platelet aggregation (Luthje & Ogilvie, 1984; Louie *et al.*, 1988). Both compounds also have vasoactive properties on peripheral arteries with intact or damaged endothelium (Busse *et al.*, 1988).

Chromaffin cells, which can be considered as the homologues of adrenergic neurones, store significant amounts of Ap_4A and Ap_5A. The diadenosine polyphosphates are co-stored in secretory granules with catecholamines and ATP (Rodriguez del Castillo *et al.*, 1988) and exocytotically released by the action of secretagogues (Pintor *et al.*, 1991). Recently reported are the effects of Ap_3A, Ap_4A and Ap_5A on catecholamine release, all of which increase the basal secretion but have an inhibitory action in nicotine-evoked release from isolated chromaffin cells (Castro *et al.*, 1990).

The extracellular receptors for adenine nucleotides (P_2) have been characterized by the pioneering work of Burnstock, who proposed the existence of two subtypes, P_{2x} and P_{2y} (Burnstock & Kennedy, 1985). This family of receptors is now increasing in number, due to the availability of specific ligands (Gordon, 1986; Burnstock, 1989; Cusack & Hourani, 1989). P_2-purinoceptors can be linked to several effector systems. The P_{2x} receptors appear to be coupled to calcium channels (Benham & Tsien, 1987). P_{2y} receptors seem to stimulate the inositol phospholipid metabolism (Forsberg *et al.*, 1987; Cooper *et al.*, 1989; Boyer *et al.*, 1990).

Concerning the diadenosine polyphosphate receptors, attempts have been made to characterize them. In sensory neurones, Ap_4A and Ap_5A are able to activate the same receptors as adenosine 5'-triphosphate (ATP), but to a lesser extent (Krishtal *et al.*, 1988). No single type of receptor seems to exist, and analogies with P_{2x}, P_{2y}, or even some different type of purinoceptor have been suggested (Busse *et al.*, 1988; Hoyle 1990). The presence of ectophosphodiesterases able to hydrolyze the Ap_xA with high affinity needs to be considered when studying the extracellular actions and binding sites of diadenosine polyphosphates (Goldman *et al.*, 1986; Miras-Portugal *et al.*, 1990; Rotllán *et al.*, 1991).

Since Ap_4A mediates an inhibitory action on induced catecholamine release in chromaffin cells, the aim of the present study was to determine its possible binding sites, as well to characterize the subtype of purinoceptor involved.

Methods

Isolation and culture of chromaffin cells

Chromaffin cells were isolated from bovine adrenal glands according to the method of Miras-Portugal *et al.* (1985). The cells were isolated by collagenase (EC 3.4.24.3) action and purified through a percoll gradient, carefully collected and washed with Ca^{2+}, Mg^{2+}-free Locke solution. Finally, cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal calf serum, 50 μM cytosine arabinofuranoside and 10 μM fluorodeoxyuridine. Cells were plated in 6-well Costar cluster dishes and incubated at 37°C in 5% CO_2 and 95% air at a density of 3×10^6 cells per well. Cells were employed for binding studies during the first five days of culture. Cellular viability was studied by trypan blue exclusion.

Ap_4A binding experiments and displacement studies

[^{3}H]-Ap_4A (6.3 Ci mmol^{-1}) binding assays were performed with 3×10^6 cultured chromaffin cells. The culture medium

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was aspirated and cells were washed twice with 1 ml Locke solution before the experiments were carried out. The binding assays were carried out in a volume of 1 ml Locke solution containing graded concentrations of [^{3}H]-Ap_4A ranging from 0.025 nM to 2.5 nM. The incubation period was 6 h at a temperature of 4°C to avoid the ectophosphodiesterase activity present in these cells (Miras-Portugal *et al.*, 1990). No hydrolysis of Ap_4A occurred in these conditions. Controls were studied by means of high performance liquid chromatography (h.p.l.c.), at the end of incubation period. Incubation was terminated by aspirating the fluid and washing twice with 1 ml cold Locke solution containing 10 μM of non-labelled Ap_4A, after which 1 ml of 10% trichloroacetic acid was added. The precipitated material was scraped out of the plastic dish, placed in a vial with 10 ml of scintillation liquid for aqueous samples, and counted in a Beckman LS 3.801. Non-specific binding was obtained by incubation of 10 μM non-labelled Ap_4A and subtracting the result from assays. This represented 5%–10% of the total.

The displacement studies with adenosine receptor ligands (R-phenylisopropyl adenosine (R-PIA) and N-ethyl-carboxamidoadenosine (NECA)), nucleotide receptor ligands (5'-adenylyl-imidodiphosphate (AMP-PNP), α,β-methylene ATP, (α,β-MeATP), adenosine 5'-O-(2-thiodiphosphate) (ADP-β-S)), Ap_4A analogues (Ap_3A, Ap_5A, and Ap_6A) were carried out as described above for cultured cells. The [H^3]-Ap_4A concentration employed in these assays was 2.5×10^{-10} M, because this value was three times the K_d value obtained (K_d = 0.08 nM). An exponential graded concentration ranging from 10^{-12} to 10^{-3} M was generally employed for every compound under study. Binding and displacement studies were analyzed by the computer programme LIGAND (Mundson & Robard, 1980). At the end of the incubation period the cellular integrity was measured by trypan blue exclusion and the lactate dehydrogenase was measured in the incubation media (Bergmeyer, 1974); one aliquot medium was removed for h.p.l.c. analysis.

The h.p.l.c. was carried out with a Waters 600 E pump with automated gradient controller, a variable volume injector U6K, and a 481-LC Spectrophotometer λ max (Waters). The detector responses were recorded, as were the integrated areas and retention times, with a 745 data module integrator (Waters).

Chromatography was performed with a C_{18} reverse phase column (C_{18} id. 3.9 mm, 22 cm long from Kontron) according to Rodriguez del Castillo *et al.* (1988) and adapted by Torres *et al.* (1990) for the ectonucleotidase activities present in chromaffin cells. The mobile phase was 10 mM potassium phosphate, 2 mM tetrabutylammonium (PIC A), a final pH of 7.5 and 15% (v/v) of acetonitrile. The retention times were 3 min for AMP, 4 min for ADP, 5.2 min for ATP and 9.4 min for Ap_4A, at a flow rate of 2 ml min^{-1}.

Materials

Nucleoside analogues and nucleotides, Ap_3A, Ap_4A and Ap_5A were from Boehringer (Mannheim, F.R.G.). Ap_6A was purchased from Sigma (St. Louis, U.S.A.). Phosphodiesterase from *Crotalus durissus* (EC 3.1.4.1), alkaline phosphatase (EC 3.1.3.1) and collagenase (EC 3.4.24.3) were from Pharmacia (Upsala, Sweden). Culture media and products were from Gibco (Glasgow, U.K.). [^{3}H]-Ap_4A was obtained from Amersham (United Kingdom).

Results

Measurements of [^3H]-diadenosine tetraphosphate binding to cultured chromaffin cells

The presence of an asymmetrical ectophosphodiesterase in chromaffin cells is a very important factor to consider in the study of Ap_4A binding sites (Miras-Portugal *et al.*, 1990; Rotllán *et al.*, 1991). To prevent the Ap_4A destruction, the binding studies were done at 4°C. As shown in Figure 1, no Ap_4A hydrolysis was observed after 8 h of incubation.

The time course of [^{3}H]-Ap_4A association with chromaffin cells is shown in Figure 2. The equilibrium was reached after 6 h incubation. The value obtained for the rate constant for the association, k_{+1} was 1.65 h^{-1} nM^{-1} (4°C). Controls for cellular integrity showed that, at the end of the experimental time, all the cells excluded the trypan blue and no release of lactate dehydrogenase activity was observed.

Once the assay conditions were established, the binding experiments were carried out, and the data obtained represented as a Scatchard plot (Figure 3). The results were analyzed by the computer programme LIGAND (Mundson & Robard, 1980) showing the presence of two binding sites. The first one presented a very high affinity as indicated by its K_d value of 0.080 ± 0.006 nM. The number of high affinity binding sites, estimated by extrapolation to the abscissa scale, gave a value of 27 fmol/3 × 10^6 cells which corresponds to 5420 ± 450 receptors per cell. The second binding site showed lower affinity; the K_d value obtained was 5.6 ± 0.53 nM, roughly two orders of magnitude higher. The bound maximum obtained for this second component was 345 ± 30 fmol/3 × 10^6 cells which corresponds to a value close to 70,000 binding sites per cell.

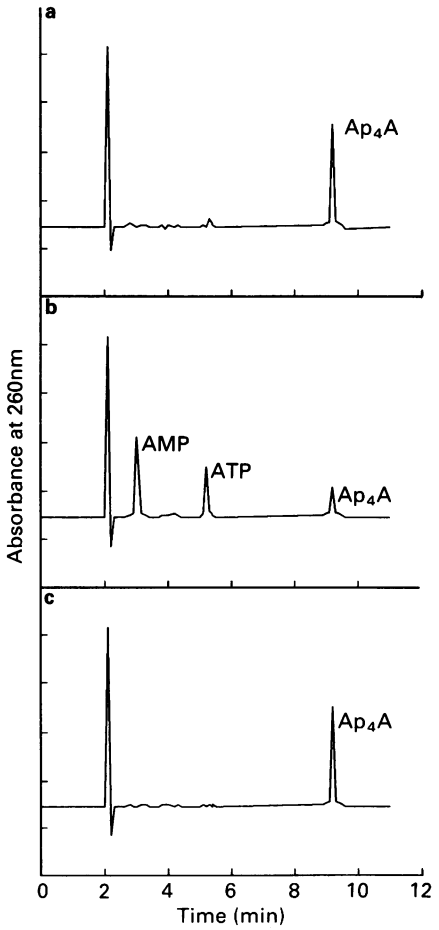

Figure 1 Ectophosphodiesterase activity in cultured chromaffin cells: (a) High performance liquid chromatography (h.p.l.c.) elution profile of 250 pmol diadenosine tetraphosphate (Ap_4A) standard in the elution condition as described in methods; (b) h.p.l.c. elution profile obtained with 3 × 10^6 cells incubated in the presence of 10 μM Ap_4A after 10 min incubation at 37°C. (c) The same as (b) but at 4°C and after 8 h incubation. Samples of 25 μl were injected. The first peak of the chromatogram corresponds with the solvent front and does not represent any adenosine compound.

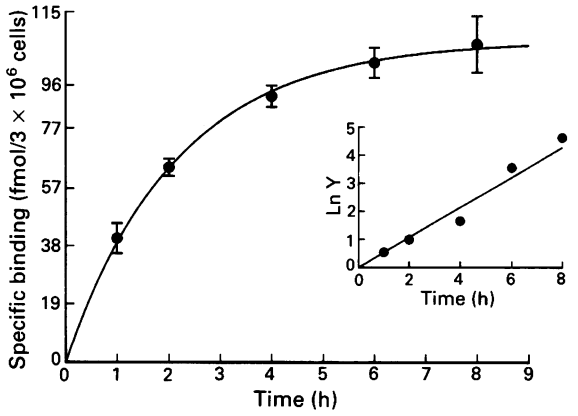

Figure 2 Determination of association rate for diadenosine tetraphosphate (Ap_4A) with cultured chromaffin cells at 4°C. The Ap_4A concentration was 0.250 nM. Controls of Ap_4A integrity were done for the maximal experimental time period (see Figure 1). In the insert, ln Y corresponds with $\text{Ln}\,Y = [B_e]/([B_e] - [B])$, where B_e is the bound of the equilibrium at this concentration and B is the bound at every experimental time. k_{+1} is deduced from the slope considering $k_{+1} = \text{slope}/([L]B_{max}/[B_e])$.

Displacement studies with diadenosine polyphosphates

Indirect binding assays of diadenosine polyphosphates, Ap_3A Ap_4A, Ap_5A and Ap_6A, were made by displacement studies of labelled [^{3}H]-Ap_4A. This compound was used at 0.25 nM, which is three times the high affinity K_d value (0.08 nM) in our experimental conditions.

In Figure 4 the displacement curves obtained for the diadenosine polyphosphates are shown. The percentage displacement with high affinity was about 10% of the total for each one.

The characteristic of Ap_4A, Ap_5A and Ap_6A displacement binding was very similar at the high affinity step (Table 1). Nevertheless, Ap_6A had a lower affinity for the second binding site. Ap_3A showed lower displacement capacity for both the high and the very high affinity binding sites. In Table 1 the K_i values for the two stepped shape of the curve are summarized.

Hill numbers (nH) confirmed the presence of more than one binding site. The nH values were very similar for all diadenosine polyphosphates studied, the mean value being 0.46 ± 0.18 (Table 1).

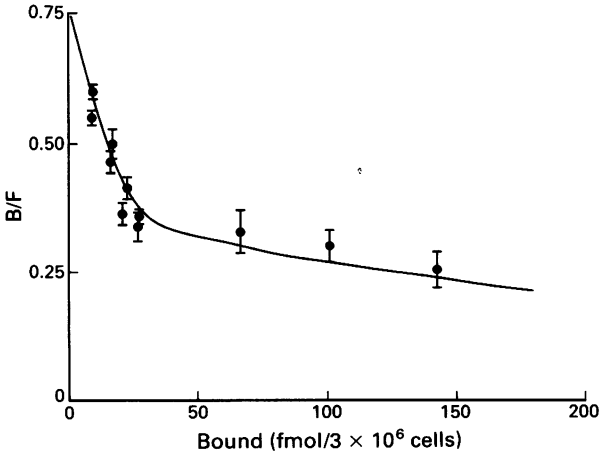

Figure 3 Scatchard analysis of equilibrium of [^{3}H]-diadenosine tetraphosphate ([^{3}H]Ap_4A) binding to chromaffin cells in culture. Binding was studied with 3 × 10^6 cells as described in methods. Results are means for six determinations in duplicate; s.d. shown by vertical bars. K_d and B_{max} values were obtained by the computer programme LIGAND (Mundson & Robard, 1980).

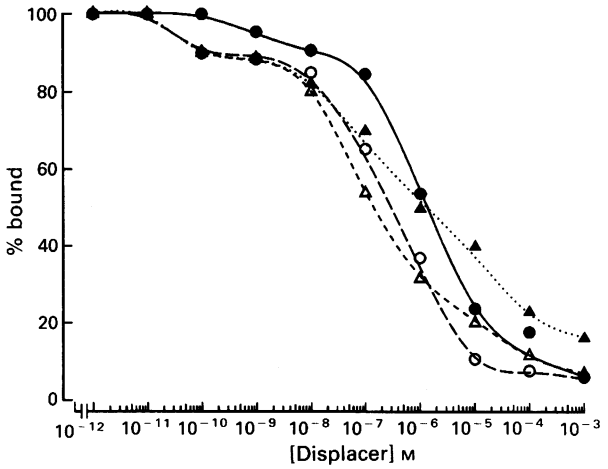

Figure 4 Inhibition of site-specific binding of [^{3}H]-diadenosine tetraphosphate ([^{3}H]-Ap_4A) to cultured chromaffin cells by Ap_xA compounds. Cells (3 × 10^6) were incubated with [^{3}H]-Ap_4A as described in methods in the presence of each, Ap_3A (●), Ap_4A (○), Ap_5A (△) or Ap_6A (▲). Results are plotted as a percentage of site-specific binding of [^{3}H]-Ap_4A in the absence of inhibitor. Results are means of three experiments in triplicate.

Binding displacement studies with P_1 and P_2 purinoceptor ligands

Compounds which have activity at P_2-purinoceptors were employed in displacement studies. The compounds used were ADP-β-S, AMP-PNP and α,β-MeATP, which are representative ligands of P_2-purinoceptors. The order is in the rank of potency for P_{2y} (Cooper *et al.*, 1989; Berrie *et al.*, 1989; Burnstock 1990; Cusack & Hourani, 1990). The displacement curves for these ligands are represented in Figure 5. As with the displacement of diadenosine polyphosphates, a two-stepped curve was obtained for each compound. The first step represented 10–12% of the total binding sites. ADP-β-S was the most potent displacer; however, it reached only one order of magnitude lower than Ap_4A (Table 1). AMP-PNP and α,β-Me-ATP, in this order of potency, were good displacers of

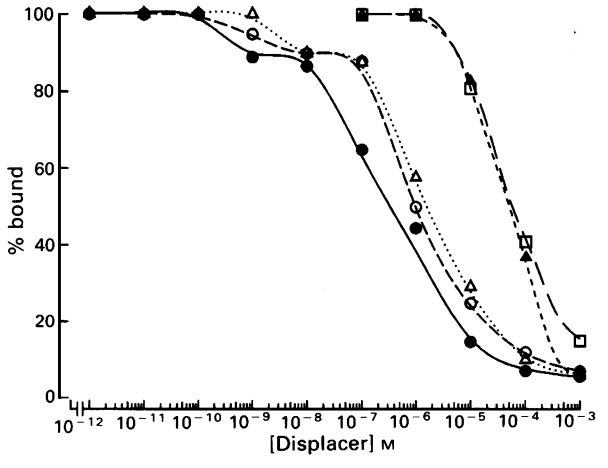

Figure 5 Displacement studies of diadenosine tetraphosphate (Ap_4A) binding by P_1 and P_2 receptor agonists. Cultured chromaffin cells (3 × 10^6) were incubated in the presence of P_1 receptor agonists phenylisopropyl adenosine (▲) and N-ethyl carboxamido adenosine (□) as described in methods. The P_2 agonists employed for the displacements were adenosine 5'-O-(2-thiodiphosphate) (●), 5'-adenylyl imidodiphosphate (○) and α,β methylene ATP (△). All the experiments represent the mean of three experiments in duplicate. The K_i values are summarized in Table 1.

Table 1 K_i values for the [^{3}H]-diadenosine tetraphosphate ([^{3}H]-Ap_4A) binding displacement by diadenosine polyphosphates, P_2 receptor agonists and P_1 agonists

Compound	(n)	*Very high affinity binding site* K_i (nM)	*High affinity binding site* K_i (μM)	*Hill number* nH
Ap_3A	(3)	1.000 ± 0.100	0.500 ± 0.050	0.49
Ap_4A	(3)	0.013 ± 0.002	0.130 ± 0.010	0.58
Ap_5A	(3)	0.013 ± 0.001	0.062 ± 0.006	0.38
Ap_6A	(3)	0.013 ± 0.002	0.750 ± 0.070	0.41
ADP-β-S	(3)	0.075 ± 0.006	0.130 ± 0.010	0.42
AMP-PNP	(3)	0.200 ± 0.015	0.500 ± 0.040	0.50
α,β-Me-ATP	(3)	0.750 ± 0.060	0.870 ± 0.070	0.60
NECA	(3)	—	15.000 ± 2.000	1.03
R-PIA	(3)	—	12.500 ± 1.500	1.02

The K_i values for the diadenosine polyphosphate analogues were obtained from the IC_{50} values of Figure 4. The values for P_1 and P_2 purinoceptor agonists were obtained from Figure 5. The programme ligand (Mundson & Robard, 1980) was employed. Values are the means ± s.d. of (*n*), number of individual experiments. The K_d value for the very high affinity binding site was 0.08 ± 0.0065 nM (6). The K_d value for the high affinity binding site was 5.6 ± 0.53 nM (6).
ADP-β-S: adenosine 5'-O-(2-thiodiphosphate); AMP-PNP: 5'-adenylyl imidodiphosphate; α,β Me-ATP: α,β methylene adenosine 5'-triphosphate; NECA: N-ethyl carboxamido adenosine; PIA: phenylisopropyl adenosine.

Ap_4A but with lower affinity as observed for the IC_{50} and K_i values. The displacer capacity at the low affinity binding site also presented the range order of P_{2y} receptors. The existence of two different binding sites for Ap_4A is confirmed by these results and the nH value which is close to 0.5 for these compounds.

The agonist ligands for P_1 receptors were **R**-PIA which is more specific for the A_1 receptor and NECA which is more selective for the A_2 receptor. The binding displacement curves for these compounds did not present the two-stepped shape (Figure 5). The displacement values IC_{50} and K_i were six orders and two orders of magnitude lower when compared with the high affinity and low affinity binding displacement for Ap_4A and Ap_5A (Table 1). There were no differences between NECA and **R**-PIA as displacers for Ap_4A. The Hill number obtained for both compounds was 1.

Discussion

The presence of two high affinity binding sites for Ap_4A and related compounds is described in this work, on cultured bovine chromaffin cells. Thus, the presence and release of these compounds from secretory granules reaches a physiological significance; which provides information about their inhibitory control of exocytosis in the same cellular model (Rodriguez del Castillo *et al.*, 1988; Castro *et al.*, 1990; Pintor *et al.*, 1991).

The first binding site for Ap_4A, had a very high affinity K_d value of 0.08 nM (8×10^{-11} M), the highest affinity reported for a nucleotide binding site, although Ap_4A is a naturally occurring compound. The second high affinity binding site had a K_d of 5.6 nM (5×10^{-9} M) which is closer to the values reported in the literature (Williams, 1987; Cooper *et al.*, 1989; Keppens *et al.*, 1989). The coincidence in the same cell of very high and high affinity binding sites has also been shown for peptidic hormones (Lipkin *et al.*, 1986; Shapiro *et al.*, 1986; Betschart *et al.*, 1986; Delicado *et al.*, 1987).

The estimated values of very high and high affinity binding sites for Ap_4A for one chromaffin cell were 5420 ± 450 and 70,000, respectively. Similar estimated values are described for very high and high affinity insulin receptors in mammalian cells. The percentage of the very high affinity binding sites is close to 10% of the total.

The displacement studies indicate that the Ap_4A, Ap_5A and Ap_6A have identical or very similar affinities ($K_i = 1.3 \times 10^{-11}$) for the very high affinity binding sites. However, Ap_3A shows an affinity two orders of magnitude lower ($K_i = 1 \times 10^{-9}$ M). Nevertheless, the second binding site is more specifically displaced by Ap_4A and Ap_5A than by Ap_6A, which presents an effect similar to the Ap_3A (Table 1). In this cellular model, diadenosine polyphosphates have an inhibitory action on nicotine-evoked release (Castro *et al.*, 1990). Their effectiveness corresponds to a potency order of $Ap_4A > Ap_5A \simeq Ap_3A$. Ap_4A is the most active compound, but other factors in addition to binding have to be considered to explain the behaviour of Ap_3A and Ap_5A. There are no data available about Ap_6A effects on catecholamine secretion.

ATP analogues displace the Ap_4A binding with the potency order of the P_{2y} receptors, adenosine 5'-O-(2 thiodiphosphate) (ADP-β-S) > 5'-adenylyl-imidodiphosphate (AMP-PNP) > α,β-Me-ATP, in both binding sites. The K_i values ranged from 7×10^{-11} to 7×10^{-10} M for the high affinity displacement and from 1×10^{-7} to 9×10^{-7} M for the second binding site (Gordon, 1986; Burnstock & Kennedy, 1985; Burnstock, 1989; Cooper *et al.*, 1989; Cusack & Hourani, 1990).

R-PIA and NECA exhibited the same displacement capacity with a very low affinity ($K_i = 1 \times 10^{-5}$ M). Although chromaffin cells and their tumoral homologue, the PC 12, seem to present the A_2 purinoceptor, (Delicado *et al.*, 1990; Noronha-Blob *et al.*, 1986; Williams, 1987; Williams *et al.*, 1987).

The presence of these two high affinity binding sites for Ap_4A deserves special considerations. The first is related to the extracellular levels of Ap_4A and Ap_5A reached after carbachol-induced release. Chromaffin cells can release about 27 pmol/1 $\times 10^6$ cells of both compounds, the volume of 10^6 chromaffin cells being about 1 μl (Pintor *et al.*, 1991). Thus, if the extracellular volume of distribution is similar to (or less than) the intracellular volume, the concentration reached ought to be 27 μM at the surrounding area of stimulated cells under the most unfavourable circumstances. In this situation both sites would be saturated by the ligand. This could also be the case for a synaptic cleft. Additionally the Ap_4A or Ap_5A concentration would maintain their actions through the very high affinity receptor even after 10^5–10^6 times dilution, as long as diadenosine polyphosphates are diffusing. Moreover, target cells endowed with the very high affinity receptor could also respond to these compounds, even if they are located far from the releasing sites. This aspect takes special relevance at the vascular system where the effects of Ap_4A have been found (Busse *et al.*, 1988).

Another important consideration related to the previously noted is whether Ap_4A and Ap_5A are the natural ligands for the P_{2y} receptors or a different species of purinoceptors. Pharmacological studies in the vas deferens and urinary bladder suggest a possible action of diadenosine polyphosphates through P_{2x} receptors, but many of their actions cannot be explained in terms of activation of the already known purinoceptors. Hoyle suggests that classes of receptors for adenine dinucleotides and nucleotides in general, are yet to be discovered (Hoyle, 1990).

In conclusion, our studies demonstrate the presence of very high and high affinity receptors for Ap_4A in a homogeneous neural cell population, the chromaffin cells, with a P_{2y} profile in displacement binding studies and in the absence of ectophosphodiesterase activity. A broad and careful study in other homogeneous and well defined cellular systems is necessary for further understanding of the physiological relevance of these natural compounds.

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