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Modulation of the dinucleotide receptor present in rat midbrain synaptosomes by adenosine and ATP

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1 Diadenosine polyphosphates activate dinucleotide receptors in rat midbrain synaptic terminals. The agonist with highest affinity at this receptor, diadenosine pentaphosphate (Ap_5A), elicits Ca^{2+} transients at concentrations ranging from 10^{-7} to 10^{-3} M with a single-phase curve and an EC_{50} value of 56.21 ± 1.82 μM.

2 Treatment of synaptosomal preparations with alkaline phosphatase (AP) changes the dose-response control curve into a biphasic one presenting two EC_{50} values of 6.47 ± 1.25 nM and 11.16 ± 0.83 μM respectively.

3 The adenosine A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) reversed the biphasic concentration-response for Ap_5A curve in the presence of AP, to a monophasic one with an EC_{50} value of 76.05 ± 7.51 μM.

4 The application of adenosine deaminase produced the same effect as DPCPX, the EC_{50} value for Ap_5A, in the presence of AP being 18.62 ± 4.03 μM.

5 Activation of the adenosine A_1 receptor by means of cyclohexyladenosine (CHA) shifted the dose response curve for Ap_5A to the left, resulting in a monophasic curve with an EC_{50} of 5.01 ± 0.02 pM.

6 The destruction of extrasynaptosomal nucleotides by AP or the addition of pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), a broad P2 antagonist compound, enhance maximal effect of the Ap_5A up to 55.6% on the dose response curve, thus suggesting a negative modulation by P2 receptors.

7 In a summary, ATP and adenosine present at the extra-synaptosomal space, are relevant natural modulators of the dinucleotide receptor, *via* P2 and adenosine A_1 receptors respectively.
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Keywords: ATP; diadenosine polyphosphates; adenosine; dinucleotide receptor; alkaline phosphatase; adenosine receptor

Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; AP, alkaline phosphatase; Ap_5A, diadenosine pentaphosphate; ATP, adenosine 5′-triphosphate; CHA, cyclohexyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; H.P.L.C., high performance liquid chromatography; β,γ-meATP, β,γ-methylene adenosine 5′-triphosphate; NBTI, Nitrobenzylthioinosine; PKA, protein kinase A; PKC, protein kinase C; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid

Introduction

Diadenosine polyphosphates, also termed adenine dinucleotides, are a family of nucleotidic compounds formed by two adenosines linked by a variable number of phosphates (Baxi & Vishwanatha, 1995; McLennan, 1992). Diadenosine tetraphosphate (Ap_4A), diadenosine pentaphosphate (Ap_5A), and ATP, are present in rat brain synaptosomes, and are stored in secretory vesicles by means of a high affinity nucleotide vesicular transporter (Gualix *et al*., 1997). ATP and adenine dinucleotides are released after synaptic terminal stimulation (Potter & White, 1980; Pintor *et al*., 1992) and activate different purinergic receptors in neural and non-neural cells once in the extracellular medium (Hoyle, 1990; Pintor *et al*., 1997b). Actions of diadenosine polyphosphates mediated by purine receptors in the central nervous system are the modulation of the firing rate in cortical neurones (Stone & Perkins, 1981), inhibition of the synaptic transmission in hippocampal slices (Klishin *et al*., 1994) and the facilitation of action potentials in locus coeruleus neurones (Fröhlich *et al*., 1996). In rat, guinea-pig and deermouse synaptosomes the presence of independent receptors for adenine dinucleotides and ATP have been reported (Pintor & Miras-Portugal, 1995b; Pintor *et al*., 1997b; Pivorun & Nordone, 1996). Considering that all of these are receptor operated Ca^{2+} channels, they might be involved in facilitatory presynaptic mechanisms in central neurones (Pintor & Miras-Portugal, 1995b; Pintor *et al*., 1997b). The Ca^{2+} signal mediated by these presynaptic receptors can be modulated by the action of protein kinases and phosphatases. Effectors inducing the activation of either protein kinase A (PKA) and protein kinase C (PKC) result in a considerable Ca^{2+} signal reduction. (Pintor *et al*., 1997a).

ATP and its breakdown product adenosine can activate P2 and adenosine receptors, respectively, promoting modulatory mechanisms involved, in many cases, in the presynaptic control of transmitter release (Schubert *et al*., 1995; Ribeiro, 1995). Keeping in view the fact that diadenosine polyphosphates, ATP and adenosine can co-exist extracellularly in neural models and in synaptosomal preparations, and given their physiological importance, we sought to describe their effect of the Ca^{2+} responses elicited by Ap_5A through the dinucleotide receptor.

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Methods

Synaptosomal preparation and dye loading

Synaptosomes were prepared from rat midbrain of cervically dislocated and decapitated male Wistar rats (Pintor *et al*., 1992). Synaptosomal pellets containing 1 mg of protein were re-suspended in 1 ml of incubation medium (composition mM: NaCl 122, KCl 3.1, KH_2PO_4 0.4, $NaHCO_3$ 5, $MgSO_4$ 1.2, glucose 10 and TES buffer 20, pH 7.4).

The cytosolic free calcium concentration was determined using FURA-2 as described by Grynkiewicz *et al*. (1985). Five minutes after synaptosomal re-suspension, $CaCl_2$ (1.33 mM) and FURA-2-acetoxymethyl ester (5 μM) were added. Following incubation for 25 min the synaptosomes were pelleted at 13,000 r.p.m. for 1 min, washed twice and re-suspended in fresh medium containing 1.33 mM $CaCl_2$. Fluorescence was measured in a Perkin Elmer Spectrofluorimeter LS-50, and monitored at 340 and 510 nm. Data were collected at 0.5 s intervals.

Calcium measurements

Ca^{2+} measurements were performed by incubating 1 mg of synaptosomes in 1 ml of Elliot's medium containing 1.33 mM Ca^{2+}. After 1 min, the corresponding dose of agonist was applied to the cuvette and the corresponding fluorescence change was recorded. One min after the agonist application a 30 mM K^+ pulse was applied to verify the synaptosomal integrity. Again, after 1 min a mixture EGTA (5 mM)/TRIS (30 mM) was applied to eliminate extracellular Ca^{2+} followed by 20 μl of Triton X-100 (0.2%) to obtain the F_{min}. This was accompanied with 30 μl of (15 mM) Ca^{2+} to obtain the F_{max}. Once this calibration was obtained, F_{min} and F_{max} were calculated and applied to Grynkiewicz equation to transform fluorescence into Ca^{2+} concentrations (Grynkiewicz, 1985).

Experimental procedures

Synaptosomes were pre-incubated with 1 u ml^{-1} of alkaline phosphatase (EC 3.1.3.1) 2 min before diadenosine pentaphosphate was assayed. Doses of Ap_5A ranging from 10^{-12} to 10^{-3} M were assayed in the presence of alkaline phosphatase and pyridoxalphosphate-6-azophenyl-2′,5′-′disulphonic acid (PPADS) 50 μM, cyclohexyladenosine (CHA) 1 μM, β,γ-meATP 50 μM, and the results were presented as dose-response curves. Also, dose-response curves of Ap_5A alone, in the same concentration range as above, were performed as control experiments.

Dose response analysis was also carried out with Ap_5A in the presence of alkaline phosphatase (1 u ml^{-1}) and with the A_1 adenosine receptor antagonist DPCPX (50 nM).

Adenosine deaminase (0.2 u ml^{-1}) was pre-incubated for 2 min together with alkaline phosphatase prior to Ap_5A dose-response curve analysis to transform adenosine into inosine and thus to see the effect of adenosine removal.

Nitrobenzylthioinosine (NBTI) 10 μM, was pre-incubated for 25 min at a concentration of 10 μM before the application of diadenosine pentaphosphate to block the adenosine transporter (Fideu *et al*., 1994). The effect of the A_1 agonist cyclohexyladenosine (CHA) on Ap_5A dose-response curve was studied by pre-incubating CHA for 2 min at a concentration of 1 μM in the presence of alkaline phosphatase (1 u ml^{-1}) and adenosine deaminase (0.2 u ml^{-1}).

Dibutiryl-cyclic AMP (100 μM) was pre-incubated 3 min before the application of the corresponding dose of Ap_5A, to verify the involvement of protein kinase A.

Chemicals

Nitrobenzylthioinosine (NBTI) were purchased from Sigma (U.S.A.). FURA-2 was obtained from Molecular Probes (U.S.A.). Diadenosine pentaphosphate, adenosine deaminase (EC 3.1.1.7) and alkaline phosphatase, (EC 3.1.3.1) molecular biology grade, were from Boehringer Mannheim (Germany). Pyridoxalphosphate - 6 - azophenyl - 2′,5′ - ′disulphonic acid (PPADS), Dibutiryl-cyclic AMP and cyclohexyladenosine (CHA) were purchased from RBI (U.S.A.). Other analytical grade reagents were purchased from Merck (Darmstadt, Germany).

H.P.L.C. procedures

To study the presence of extracellular adenine nucleotides and adenosine in the control synaptosomal preparation together with the transformation after enzymatic treatment to adenosine and inosine (by adenosine deaminase), experiments were carried out using high performance liquid chromatography (H.P.L.C.). The chromatographic equipment consisted of a Waters 600E delivery system, a Waters 717+ autosampler and a Waters 2487 dual wavelength absorbance detector, all managed by Millennium 2010 software running on a NEC 486DX computer. The analysis was performed under ion-pair chromatography conditions by equilibrating the system with 0.1 mM KH_2PO_4, 2 mM tetrabutyl ammonium, 10% acetonitrile, pH 7.5. The column was a Spherisorb ODS-2 (25 cm length and 0.4 cm inner diameter) from Waters. Detection was monitored at 260 nm wavelength. The peak areas were transformed to concentrations by correlation with commercial standards.

Data analysis

Data are presented as mean$\pm$s.e.mean of at least four determinations in duplicate from different synaptosomal preparations. Comparisons between experimental samples and untreated controls were carried out using non-parametric Mann-Whitney *U*-test (two tails). Dose-response curves plotting and fitting was carried out by the computer program FigP (Biosoft, Cambridge).

Results

Effect of Ap_5A on synaptosomal Ca^{2+} transients: changes induced by alkaline phosphatase

Ap_5A has been described as the best agonist of the dinucleotide receptor eliciting Ca^{2+} transients in rat midbrain synaptosomes (Pintor & Miras-Portugal, 1995a). For this reason this dinucleotide was chosen for assays throughout the experimental work described here.

Diadenosine pentaphosphate was assayed in synaptic terminals in the absence of any additional compound to obtain a control dose-response curve (Figure 1A). The curve obtained was monophasic, with an EC_{50} of 56.21$\pm$1.82 μM and a maximal Ca^{2+} increase of 30.52$\pm$1.64 nM which corresponds to 100% of the control maximal effect (Table 1).

As the preparation of synaptic terminals implies tissue disruption and resealing of neural membranes, the presence of

abundant cytosolic compounds, such as nucleotides, at the extrasynaptosomal space cannot be discarded. In this way, a substantial change in the dose-response curve for Ap_5A was observed after the treatment of synaptosomes with alkaline phosphatase, since lower doses of Ap_5A, ineffective in the absence of the enzyme, were effective (Figure 1A). A biphasic dose-response curve was then obtained with a new component in the nanomolar range and a second component in the micromolar range. This micromolar component, compared to control curve showed an increase of 66.8% above the control, changing the Ap_5A maximal Ca^{2+} transients from 30.52 ± 1.64 to 50.84 ± 1.57 nM (Figure 1B).

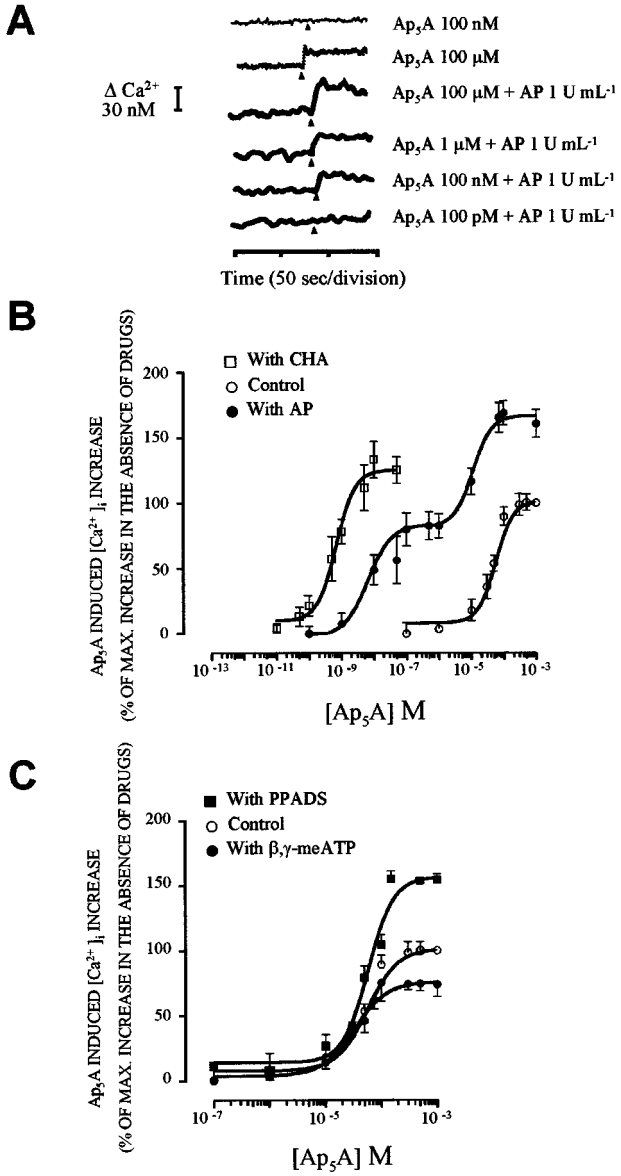

Figure 1 Concentration-response curve for diadenosine pentaphosphate (Ap_5A) in the presence and in the absence of alkaline phoshatase (AP). Effect of PPADS on Ap_5A response. (A) Representative experiment of the effect Ap_5A (arrows) in absence (upper traces) and in the presence (lower traces) of AP. (B) Ap_5A in concentration ranging from 10^{-7} to 10^{-3} M was assayed alone with 1 mg of synaptosomes as described in the Methods section. When the same protocol was carried out in the presence of alkaline phosphatase (1 u ml^{-1}) or CHA 1 μM, concentrations of Ap_5A ranging from 10^{-11} to 10^{-3} M were sufficient to complete the dose response curve. 100% of effect corresponds to the maximal Ca^{2+} transient elicited by Ap_5A in the absence of any substance or enzyme. (C) Dose-response curve for Ap_5A in the presence of the P2 antagonist PPADS (50 μM) or β,γ-meATP (50 μM) preincubated for 2 min as described in the Methods section. 100% of effect corresponds to the maximal Ca^{2+} transient elicited by Ap_5A in the absence of any substance or enzyme (which corresponds to 30.53 ± 1.64 nM). Values are the mean $\pm$ s.e.mean of five experiments performed in duplicate.

To analyse whether the elimination of background ATP present in this preparation was responsible for the change in the dose response curve, experiments with the P2 antagonist PPADS, in the absence of alkaline phosphatase were performed. As is shown in Figure 1C, PPADS was able to change the maximal response induced by Ap_5A but was unable to produce any further change in the dose-response curve. The P2 antagonist produced an enhancement in the Ca^{2+} response elicited by Ap_5A, corresponding to a 55.6% increase above the control value (Figure 1C). The EC_{50} value for Ap_5A in the presence of PPADS was not significantly changed relative to the control curve (Table 1). To verify the involvement of P2 receptors in the change of the maximal effect in the Ap_5A dose-response curve, the P2 receptor agonist, β,γ-meATP, was used at a concentration of 50 μM. With the nucleotide it was possible to measure a change in the maximal effect, this being a reduction in the presence of β,γ-meATP (Figure 1C, Table 1). Statistically significant differences ($P<0.05$, Mann-Whitney U-test) were corroborated between the observed values of EC_{50} (observed values of experiments vs observed values of controls) and maximal values.

Effect of adenosine on Ca^{2+} transients elicited by Ap_5A

Adenosine, which is generated by alkaline phosphatase action from extracellular ATP, might be the substance involved in the appearance of a high affinity component in the Ap_5A dose-response curve. To test this possibility, adenosine deaminase (which transforms adenosine in inosine) was assayed and the possible reversion of the Ap_5A dose-response curve to control values examined. This enzyme shifted the dose-response curve to an EC_{50} value in the micromolar range, with a maximal Ca^{2+} increase of 51.8 % above the maximal effect measured on the control curve. This shifting to micromolar EC_{50} values might suggest that adenosine is inducing the appearance of a high affinity component (Figure 2). To further confirm this hypothesis, the adenosine transporter inhibitor NBTI (nitrobenzylthioinosine) was added together with alkaline phosphatase to the synaptosomal preparation. The result obtained for the Ap_5A concentration-response curve was a significant shift to the left (Figure 2), showing an EC_{50} value about 0.1 nM (Table 1).

Since adenosine A_1 receptors have been described in rat midbrain synaptosomes, the A_1 adenosine receptor antagonist DPCPX (50 nM) was assayed together with alkaline phosphatase to investigate the involvement of this adenosine receptor in the change of Ap_5A dose-response curve. Concentration-response analysis for Ap_5A under this condition showed a curve with an EC_{50} value in the micromolar range (Figure 3), with no significant variation in the maximal effect. The results obtained with this antagonist strongly suggested the involvement of an adenosine A_1 receptor. To further confirm this point the adenosine A_1 agonist cyclohexyladenosine (CHA) was assayed in the presence of alkaline phosphatase and adenosine deaminase. CHA was able to shift Ap_5A dose-response to picomolar values as it is shown in Figure 3 (Table 1). In the absence of the two enzymes, AP and adenosine deaminase, the A1 agonist CHA produced a shift to the left of the dose-response curve, showing a EC_{50} value of 685.2 ± 23.5 pM, as shown in Figure

Table 1. EC_{50} values for the Ap_5A concentration-response curves in the presence of various pharmacological tools and enzymes

Experimental conditions	*EC_{50} first component*	*EC_{50} second component*	*Effect (%) of maximal response*
Control	N.A.	56.21±1.82 μM	100±6.3
Alkaline phosphatase	6.47±1.25 nM	11.16±0.83 μM	166.8±9.2
β,γ-meATP	N.A.	33.1±2.45 μM	76.1±2.3
PPADS	N.A.	58.53±11.84 μM	155.6±4.5
CHA	685.2±23.51 pM	N.A.	125.3±3.6
Alkaline phosphatase and adenosine deaminase	N.A.	18.62±4.03 μM	151.8±8.9
Alkaline phosphatase and NBTI	104.51±17.91 pM	N.A.	125.8±7.5
Alkaline phosphatase, adenosine deaminase and CHA	5.01±0.02 pM	N.A.	117.8±10.8
Alkaline phosphatase and DPCPX	N.A.	76.05±7.51 μM	128.5±10.7
Alkaline phosphatase and dibutiryl cyclic AMP	N.A.	5.79±1.92 μM	89.3±4.1

The substances and enzymes were assayed under the conditions described in the Methods. The EC_{50} values represent the concentration of Ap_5A which is necessary to produce 50% of the maximal effect in each step. The EC_{50} values for the first and second component were all statistically significant compared to control ($P<0.05$ Mann-Whitney *U*-test), except for the PPADs experiment ($P>0.05$). Maximal effect values were all statistically significant when compared to control ($P<0.05$ Mann Whitney *U*-test), which correspond to increase on intrasynaptosomal Ca^{2+} of 30.52±1.64 nM. These results are the means±s.e.mean of four experiments in duplicate. N.A. = not applicable.

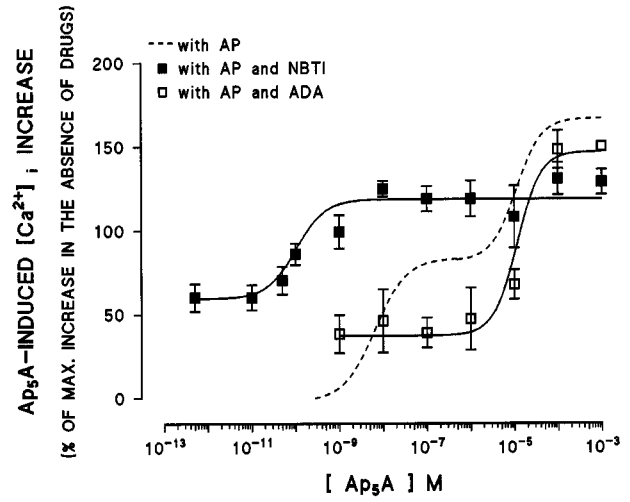

Figure 2 Concentration-response curves for Ap_5A in the presence of NBTI and adenosine deaminase. The effect of the adenosine present due to the action of alkaline phosphatase (AP) was studied in two ways: (1) by blocking the adenosine transporter by means of nitrobenzyl thioinosine (NBTI) 10 μM, and (2) by adenosine destruction with adenosine deaminase 0.2 u ml^{-1}, following the protocol described in the Methods section. 100% of effect corresponds to the maximal Ca^{2+} transient elicited by Ap_5A in the absence of any substance or enzyme (which corresponds with 30.53±1.64 nM). To allow comparison, the dose-response curve for Ap_5A in the presence of alkaline phosphatase, from Figure 1, is represented as a dashed line. Values are the mean±s.e.mean of five experiments performed in duplicate.

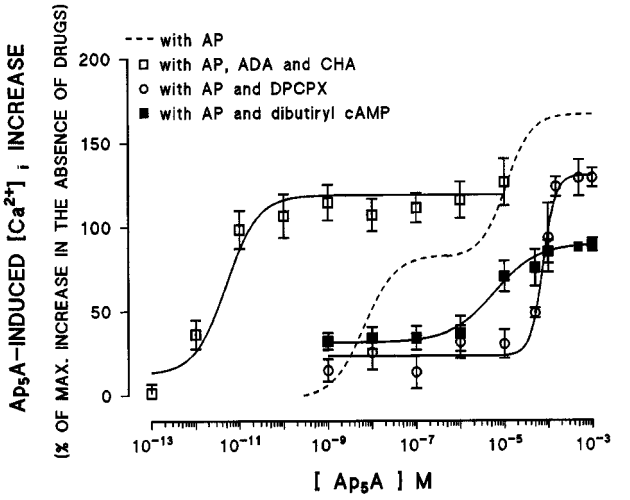

Figure 3 Dose-response curves for diadenosine pentaphosphate in the presence of adenosine A_1 receptor modulators. The effect of the adenosine present due to the action of alkaline phosphatase (AP) was studied by blocking the adenosine A_1 receptors with antagonist DPCPX 50 nM. Also the involvement of the A_1 receptor was studied by incubating the synaptosomes with adenosine deaminase and the adenosine A_1 agonist cyclohexyladenosine (CHA) 1 μM. The effect of the protein kinase A (PKA) activator dibutyril cyclic AMP 100 μM was assayed following the protocol described in the Methods. 100% of effect corresponds to the maximal Ca^{2+} transient elicited by Ap_5A in the absence of any substance or enzyme, which value is 30.52±1.64 nM. To allow comparison, the dose-response curve for Ap_5A in the presence of alkaline phosphatase, from Figure 1, is represented as a dashed line. Values are the mean±s.e. mean of five experiments performed in duplicate.

1B. Statistically significant differences ($P<0.05$, Mann–Whitney *U*-test) were corroborated between the observed values of EC_{50} (observed values of experiments vs observed values of controls) and maximal values.

As the adenosine A_1 receptor is negatively coupled to adenylate cyclase, resulting in a decrease of PKA activity (Fredholm *et al.*, 1994), experiments were done to analyse whether the direct activation of PKA would produce an effect opposite to the one observed for CHA. In this way, synaptosomal incubation with dibutiryl cyclic AMP in the presence of alkaline phosphatase produced a shift in the dose-response curve to micromolar values as observed in Figure 3, together with a significant decrease in the maximal Ca^{2+} response (Table 1).

Extracellular adenosine metabolites

The levels of extracellular adenosine were measured by high performance liquid chromatography (H.P.L.C.). Experiments were carried out in the absence of any added substance to measure adenosine background (Table 2). Once the extra-synaptosomal adenosine levels were calculated (control value), it was possible to establish the effect of different substances. The application of alkaline phosphatase produced a clear enhancement of 7.2 fold in adenosine levels when compared to control (Table 2). NBTI, an adenosine transporter inhibitor also increased the extrasynaptosomal amounts of adenosine to a similar extent, that is 7.4 fold the control value. Adenosine

Table 2 Extrasynaptosomal levels of adenosine, inosine and AMP

Experimental conditions	*Adenosine* (nM)	*AMP* (nM)	*Inosine* (nM)
Control	11.92±2.37	39.54±11.22	N.D.
After incubation	51.51±10.30	50.72±9.58	N.D.
Alkaline phosphatase	86.21±12.84	N.D.	N.D.
NBTI	89.16±14.97	57.15±11.43	N.D.
Adenosine deaminase	N.D.	52.60±10.66	44.29±4.42
NBTI and alkaline phosphatase	117.01±14.38	N.D.	N.D.
Adenosine deaminase and alkaline phosphatase	N.D.	N.D.	78.64±15.33

All the data presented in this table were obtained with the experimental conditions described in the Methods, with 1 mg protein of synaptosomal preparation in 1 ml assay media. The extracellular concentration values of ATP and ADP were 71.2±14.6 and 32.4±6.1 nM respectively at a temperature between 0° and 4°C. When the incubation was performed at 37°C for 2 min period and an additional 1 min centrifugation, no ATP, no ADP were detectable due to the presence of ecto-nucleotidases in the synaptosomal preparation. All the values are mean±s.e.mean of four experiments in duplicate. N.D. = not detectable.

deaminase completely transformed adenosine levels into inosine without any effect on AMP (adenosine 5′-monophosphate) levels (Table 2), and the combination of alkaline phosphatase and NBTI generated a 10 fold increase in the adenosine concentration. During the incubation time ATP and ADP (adenosine 5′-diphosphate) are degraded and a significant increase in the adenosine and AMP levels is observed (Table 2). At the same time, adenosine is being internalized by an NBTI sensitive membrane transporter as it is shown in Table 2.

Discussion

In the present manuscript, the effect of alkaline phosphatase (AP) on the Ca^{2+} transients elicited by Ap_5A in midbrain synaptic terminals is explained on the basis of nucleotide destruction and adenosine formation at the extracellular space. The pre-incubation of synaptosomes with AP produced a dramatic change in the dose-response curve for Ap_5A. This was originally a sigmoid curve with an EC_{50} value in the micromolar range and was turned into a biphasic one with two clear saturation steps, and EC_{50} values in the nanomolar and in the micromolar range respectively. Moreover, the maximal effect on the Ca^{2+} transients was significantly increased with respect to control. The experimental data reported here indicate that nucleotide and adenosine receptors play a leading role modulating the dinucleotide receptor affinity and maximal effect.

The presence of adenine nucleotides in the extracellular space was demonstrated by means of H.P.L.C. analysis. Leakage during synaptosomal preparation or physiological release in the absence of synaptic depolarization are at the possible origin of extrasynaptosomal nucleotides (Hamann & Attwell, 1996). The background levels of ATP and ADP can account for P2 receptors stimulation that are present in this preparation (Pintor & Miras-Portugal, 1995b). Moreover, PPADS, a P2 antagonist, (Ziganshin *et al*., 1993) increased the maximal response elicited by Ap_5A, without the previous enzymatic treatment, in agreement with the postulated modulatory role for a P2 receptor in the reduction of the maximal effect. This hypothesis was confirm by the effect of P2 agonist, β,γ-meATP, which produced a reduction in the maximal effect of Ap_5A dose-response curve. The P2 receptor subtype/s involved in this effect needs further pharmacological characterization. Nevertheless, P2 receptors do not appear to play a role on the high affinity step of Ap_5A doses-response curve originated after AP treatment. Thus, the effect of the last reaction product, adenosine, which levels were significantly increased, as quantified by H.P.L.C., was studied.

The presence of A_1, A_{2A} and A_{2B} adenosine receptors has been demonstrated in the rat brain, being the A_1 subtype the most abundant at the presynaptic level (Fastbom & Fredholm, 1990; Fideu *et al*., 1994; Pintor & Miras-Portugal 1995b).

Adenosine is formed by the action of alkaline phosphatase on extracellular adenine nucleotides, as monitored in the H.P.L.C. analysis. The presence of this nucleoside permits the activation of the adenosine A_1 receptors as further demonstrated by means of its agonists and antagonists. Receptor blockade by DPCPX produced a return to control dose-response values, indicating that when adenosine is present at the extracellular space the changes observed are mediated by A_1 receptors. Their activation by adenosine, or the agonist CHA, allows the dinucleotide receptor to reach a high affinity state, thus being stimulated by lower concentrations of adenine dinucleotides. Adenosine destruction to inosine by adenosine deaminase prevents A_1 receptor activation, thus the dinucleotide receptor exhibiting as low affinity values as the control. The latter effect together with all the other experimental results is strongly suggesting the participation of adenosine A_1 receptors in these effects. To further confirm this, the inhibition of adenosine transport by NBTI, which is very efficient in this preparation as previously demonstrated (Fideu *et al*., 1994), would allow higher adenosine levels at the extrasynaptosomal space, activating A_1 receptors and thus increasing the sensitivity of the dinucleotide receptor to Ap_5A. The former hypothesis was completely established and affinity values in the picomolar were experimentally obtained in the presence of NBTI. The adenosine extrasynaptosomal levels, both in the presence of NBTI and after adenosine deaminase treatment also correlates well with its modulatory role acting on adenosine receptors. Therefore, experimental situations where A1 receptors are occupied by the endogenous adenosine or specific synthetic agonist result in a significantly increase in the dinucleotide receptor affinity.

Ectonucleotidases present in neural models can degrade ATP to adenosine (Zimmermann, 1996; Mateo *et al*., 1996). This effect is the same one obtained when AP is added to the synaptosomal preparation. This is also the situation in our synaptic terminals where after 2 min pre-incubation at 37°C, the ATP and ADP appear mostly as AMP and adenosine.

Nucleotides and adenosine acting at plasma membrane receptors should induce intracellular signalling. Besides, it is known that the dinucleotide receptor is intracellularly modulated by protein kinases and phosphatases (Pintor *et al*., 1997a). Activation of protein kinase A (PKA) leads to an inhibition of this receptor activity. Since adenosine A_1 receptor is negatively coupled to adenylate cyclase (Linden, 1991), it seems reasonable to think that adenosine, by acting through this receptor, decreases the activity of PKA, thus allowing a

lower degree of phosphorylation of the dinucleotide receptor (Pintor *et al*., 1997a). This lack of phosphorylation would permit higher affinity of the receptor for the diadenosine polyphosphates allowing its activation even at nanomolar concentrations elicited by Ap_5A through the dinucleotide receptor (Pintor *et al*., 1997a).

The presence of an ionotropic presynaptic receptor for diadenosine polyphosphates, and its complex regulation by related compounds, deserves an analysis about its possible physiological relevance. It is well known that most of the neurones contain presynaptic receptors, which are involved in many cases in the inhibition of neurotransmitter release. Most of these effects are mediated by G-protein mechanisms and could involve the inhibition of the N-type voltage-dependent Ca^{2+} channel together with stimulation of K^+ channels (Yawo & Chuhma, 1993; Wu & Saggau, 1994). This is the situation in cortical rat brain synaptosomes where adenosine, *via* A_1 receptors, inhibits the Ca^{2+} dependent release of glutamate (Barrie & Nicholls, 1993; Herrero *et al*., 1996). This fact is in sharp contrast with the present case, where adenosine *via* A_1 receptors is facilitating the activity of the dinucleotide receptor, even at very low concentration of the agonist, and this permits the release of neurotransmitters in synaptic terminals. A related question is whether or not the amount of Ca^{2+} entering the synaptic terminals as a consequence of the dinucleotide receptor stimulation is enough to induce transmitter release, or if it is increasing the basal Ca^{2+} levels to facilitate any further exocytotic event.

In summary, extracellular purines have a dual effect on dinucleotide receptor activity. On the one hand, extracellular ATP negatively modulates the maximal effect in the Ca^{2+} transients elicited by Ap_5A by means of P2 receptors. On the other hand, adenosine, generated from the ATP breakdown, positively modulates the affinity of the dinucleotide receptor permitting Ap_5A to be active at lower concentrations than in the absence of the nucleoside. This effect is mediated by adenosine A_1 receptors and may involve reduction of PKA activity.

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