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Presence of dinucleotide and ATP receptors in human cerebrocortical synaptic terminals

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Abstract

Human cerebrocortical synaptic terminals elicited concentration-dependent Ca^{2+} transients after Ap_5A (diadenosine pentaphosphate) and ATP stimulation, with EC_{50} values of 23.44 ± 3.70 μM and 11.48 ± 2.12 μM, respectively. The lack of cross-desensitisation and the selective antagonism by Ip_5I (diinosine pentaphosphate), suggests the activation of a dinucleotide receptor by Ap_5A, and a P2X receptor by ATP. Ap_5A Ca^{2+} transients were partially abolished by ω-conotoxin GVI-A (53%), suggesting the participation of a N-type Ca^{2+} channel in the dinucleotide response. ATP effect on Ca^{2+} entry was abolished by nicardipine (44%) and by ω-conotoxin GVI-A (52%), suggesting the participation of L- and N-type Ca^{2+} channels. These data suggest that Ap_5A and ATP activate dinucleotide and P2X receptors, respectively, in human brain synaptic terminals.

Keywords: ATP; Ap_5A (diadenosine pentaphosphate); Diadenosine polyphosphate; Ip_5I (diinosine pentaphosphate); Ca^{2+} channels, voltage-dependent

1. Introduction

There is increasing interest in the study of the components of synaptic vesicles which are co-stored together with the classical neurotransmitters. Among these substances, nucleotides have appeared as new neurotransmitters since they fulfil the basic criteria for a chemical transmitter: they are co-stored with acetylcholine and catecholamines, they are released after synaptic terminal stimulation, interact with specific receptors and they are inactivated by means of the ecto-nucleotidases (Burnstock, 1996; Zimmermann, 1996).

The best representative of the nucleotides as a neurotransmitter is adenosine-5′-triphosphate (ATP), but it is not the only one stored with other neurotransmitters (Pintor et al., 1997c). A family of dinucleotides, termed diadenosine polyphosphates (or adenine dinucleotides), are also stored with ATP (Lüthje and Ogilvie, 1983; Rodriguez del Castillo et al., 1988; Pintor et al., 1992b). Diadenosine polyphosphates are formed by two adenosine moieties linked by a phosphate bridge which varies from 2 to 6 (Ap_nA, $n = 2$–6) (for a review, see the work of Baxi and Vishwanatha (1995)). Ap_4A (diadenosine tetraphosphate), Ap_5A (diadenosine pentaphosphate) and Ap_6A (diadenosine hexaphosphate) are present in rat brain synaptic terminals and are released after terminal depolarisation in a Ca^{2+}-dependent way (Pintor et al., 1992a). Ap_nA can stimulate different subtypes of purinergic receptors, mainly P2-receptors and dinucleotide receptors (Pintor and Miras-Portugal, 1995; Pivorun and Nordone, 1996). Dinucleotide receptors are receptor-operated Ca^{2+} channels, sensitive to the new antagonist Ip_5I (diinosine pentaphosphate) and are strongly modulated by protein kinases and phosphatases (Pintor et al., 1997a,b).

ATP and its actions in the CNS (central nervous system) have been studied since the late 1970s (White, 1977). ATP has been shown to activate ionic currents in central neurones (Harms et al., 1992; Ueno et al., 1992; Shen and North, 1993). Effects of ATP in the CNS reflect an increase in the firing rate of cortical neurones (Stone and Perkins, 1981), locus coeruleus neurones (Fröhlich et al.,

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1996) and dorsal root ganglia neurones (Krishtal et al., 1988). These effects were mimicked by diadenosine polyphosphates, but the nature of the receptors responsible for the adenine dinucleotide response is not yet clear.

Diadenosine polyphosphate receptors in rat midbrain synaptosomes could be involved in processes related to the potentiation of the synaptic transmission, since their activation by Ap_5A (or Ap_4A) leads to a Ca^{2+} entry to the pre-synaptic terminal which is enlarged by the further opening of the voltage-dependent Na^+ and Ca^{2+} channels (Klishin et al., 1994; Pintor and Miras-Portugal, 1995).

All experiments related to Ap_nA in the CNS so far have been carried out in rat midbrain, guinea pig and deermouse brain (for a review, see the work of Pintor et al. (1997c)). Nothing has been reported regarding these receptors in human brain tissue and their possible implications in pathological states.

Several studies have demonstrated that some neural pathologies are associated with an increase or decrease in the levels of the neurotransmitters at the synaptic cleft (Marsden, 1982). Since diadenosine polyphosphates and ATP are active substances at the pre-synaptic terminals, there is a chance for these substances to become the source of new therapeutic drugs.

In the present experimental work, the nature of the purinergic receptors for Ap_5A and ATP in the synaptic terminals of human brain are investigated. Their connection with the voltage-dependent Ca^{2+} channels and their ability to change the synaptosomal membrane potential are also studied.

2. Experimental procedures

2.1. Synaptosomal preparation

Two samples of healthy cerebral cortex (250–500 mg) were obtained post-surgically from patients undergoing resection of a parietal or temporal lobe tumour. Although the samples were surgically redundant, ethical permission was given by the Hospital Ethical Committee. Synaptosomes were prepared from human brain following the method described by Pintor et al. (1992a). Synaptosomal pellets containing 1 mg of protein were resuspended in 1 ml incubation medium (composition (in 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).

2.2. Ca^{2+} measurements

The cytosolic free Ca^{2+} concentration was determined using FURA-2 as described by Grynkiewicz et al. (1985). A total of 5 min after synaptosomal resuspension, $CaCl_2$ (1.33 mM) and FURA-2-acetoxymethyl ester (5 μM) were added. Following incubation for 35 min, the synaptosomes were pelleted at 800 rpm for 1 min, washed twice and resuspended into 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.

2.3. Membrane potential measurements

Synaptosomal plasma membrane potential was monitored with a potential-sensitive cationic cyanide dye, 3′,3-diethylthiadicarbocyanide iodine ($DiSC_2(5)$) as described by Simms et al. (1974). Synaptosomes were pre-incubated at 37°C for 1 h, then pelleted and re-suspended in incubation medium containing $DiSC_2(5)$ (5 μM) and $CaCl_2$ (1.33 mM). After 5 min equilibration, ATP and Ap_5A were added at a concentration of 100 μM. Fluorescence was determined at 643 and 680 nm. Data were collected at 2-s intervals.

2.4. Pharmacological studies

Ap_5A and ATP were assayed at concentrations ranging from 10^{-7} to 10^{-3} M to obtain the dose–response curves. In some cases, the specific *dinucleotide receptor* antagonist Ip_5I was assayed under conditions described elsewhere (Pintor et al., 1997a). Briefly, the Ip_5I was pre-incubated at a concentration of 100 nM for 2 min together with the synaptosomes before the application of the corresponding agonist. Each agonist was applied at a final concentration of 100 μM and the Ca^{2+} increase in the presence of Ip_5I was compared with its effect in the absence of the antagonist. The ATP receptor antagonist PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid) was also assayed following the same protocol as that used for Ip_5I. Cross-desensitisation studies were performed applying the nucleotide or dinucleotide (100 μM) and, after 1 min, the second nucleotide was assayed at the same concentration, the response of which was then compared with its corresponding control.

2.5. Ca^{2+} channel blocker assay

ω-Agatoxin IV-A (ω-Aga, 5 μM), ω-conotoxin GVI-A (ω-Cono, 5 μM) and nicardipine (200 nM) were incubated with synaptosomes 30 min before the application of the agonists (Pintor and Miras-Portugal, 1995).

2.6. Materials

ATP and Ap_5A were from Boehringer Mannheim (Germany). PPADS was purchased from RBI (USA). Fura-2 and $DiSC_2(5)$ were from Molecular Probes (USA). ω-Aga, ω-Cono and nicardipine were obtained from Calbiochem (USA). Other reagents were analytical grade purchased from Merck (Germany).

2.7. Statistical analysis

Data are presented as mean ± S.E.M. of two determinations in triplicate. Significant differences were determined by Student's two-tailed *t*-test. When appropriate, single experimental traces are represented in the figures.

3. Results

3.1. Effect of Ap_5A and ATP on human cerebrocortical synaptosomes

Ap_5A and ATP evoked measurable Ca^{2+} transients in the human cortical synaptic terminals which were concentration-dependent (basal synaptosomal Ca^{2+} concentration 117 ± 5.2 nM). At a variable range of concentrations tested, from 10^{-7} to 10^{-3} M, both nucleotides showed dose–response curves very similar to each other in their respective maximal effects and slopes, and with a small difference in terms of EC_{50} values (Fig. 1A). ATP was more potent than Ap_5A, the EC_{50} values being 11.48 ± 2.12 μM ($n = 6$) for ATP and 23.44 ± 3.70 μM for Ap_5A ($n = 6$).

3.2. Membrane potential studies

Ap_5A and ATP (both at 100 μM) evoked depolarisation as shown in Fig. 1B. The change in the fluorescence observed as a consequence of membrane depolarisation was 8.0 ± 0.7 Fau (fluorescent arbitrary units) for ATP and 7.2 ± 0.8 Fau for Ap_5A ($n = 6$, in both cases). The change in the membrane potential was significantly less pronounced than that elicited by a 60-mM K^+ concentration (31.1 ± 1.2 Fau, $n = 12$). This response was abolished in the absence of extrasynaptosomal Ca^{2+} indicating that depolarisation depends on the cation entry mediated by receptor activation.

3.3. Effect of extracellular Ca^{2+} removal

In the absence of extrasynaptosomal Ca^{2+}, both ATP and Ap_5A were not able to induce any increase in the cytosolic Ca^{2+} signal (Fig. 2A). This is suggesting that the Ca^{2+} transients elicited by ATP or Ap_5A depends on the extracellular Ca^{2+} and not on its cytosolic reservoires.

3.4. Cross-desensitisation studies and antagonism by Ip_5I and PPADS

In order to see whether Ap_5A evoked the Ca^{2+} transients by acting on the same receptor as ATP, or by an independent receptor, cross-desensitisation studies were carried out. A study was also made of the specific *dinucleotide receptor* antagonist Ip_5I and of the P2 receptor antagonist PPADS.

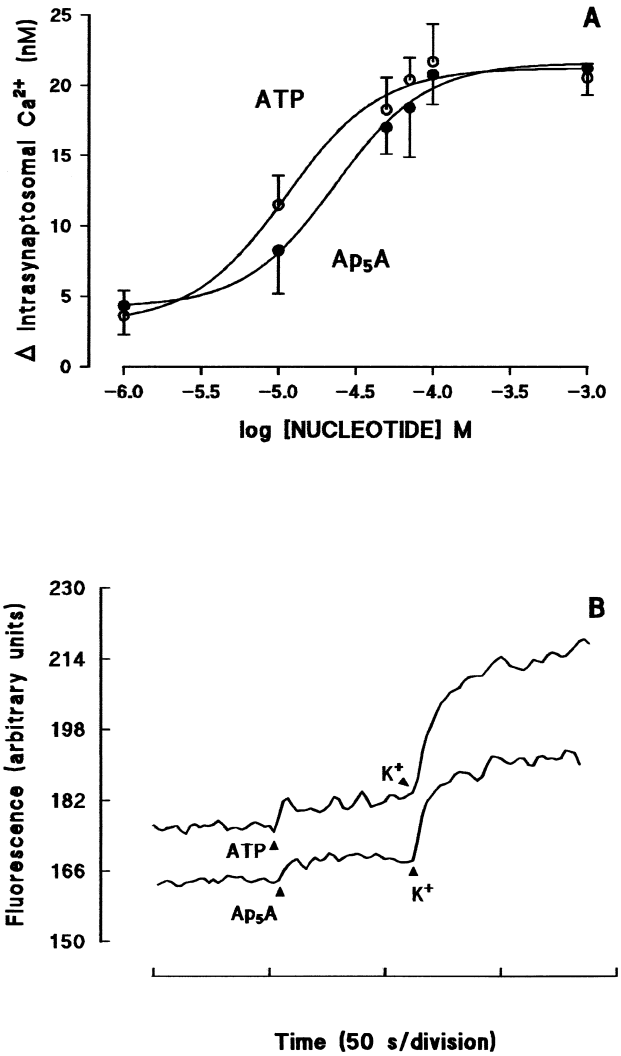

Fig. 1. Effect of Ap_5A and ATP on human cerebrocortical synaptic terminals. (A) Concentration–response relationship for Ap_5A (●) and ATP (○) on intracellular Ca^{2+} increase in human synaptic terminals. All the experiments represent mean ± S.E.M. of two determinations in triplicate. (B) Ap_5A (lower trace) and ATP (upper trace) elicited changes in the membrane potential after their application at a final concentration of 100 μM. K^+ (60 mM) was used as a control for the synaptic terminal functionality.

Pre-incubation for 2 min with 100 μM ATP did not significantly modify the response to a subsequent application of 100 μM Ap_5A. In the converse experiment, pre-incubation of the synaptosomes with Ap_5A (100 μM) did not significantly modify the response to the subsequent application of ATP (100 μM). Nevertheless, there was always desensitisation when the same agonist was applied twice, reducing the Ca^{2+} transient in the second pulse to almost zero (Fig. 2B).

Ip_5I, a selective *dinucleotide receptor* antagonist (Pintor et al., 1997a), was incubated 2 min before the application of Ap_5A or ATP. The Ca^{2+} transient elicited by Ap_5A was inhibited to 67% when compared to the control. ATP (100 μM), on the other hand, was not significantly

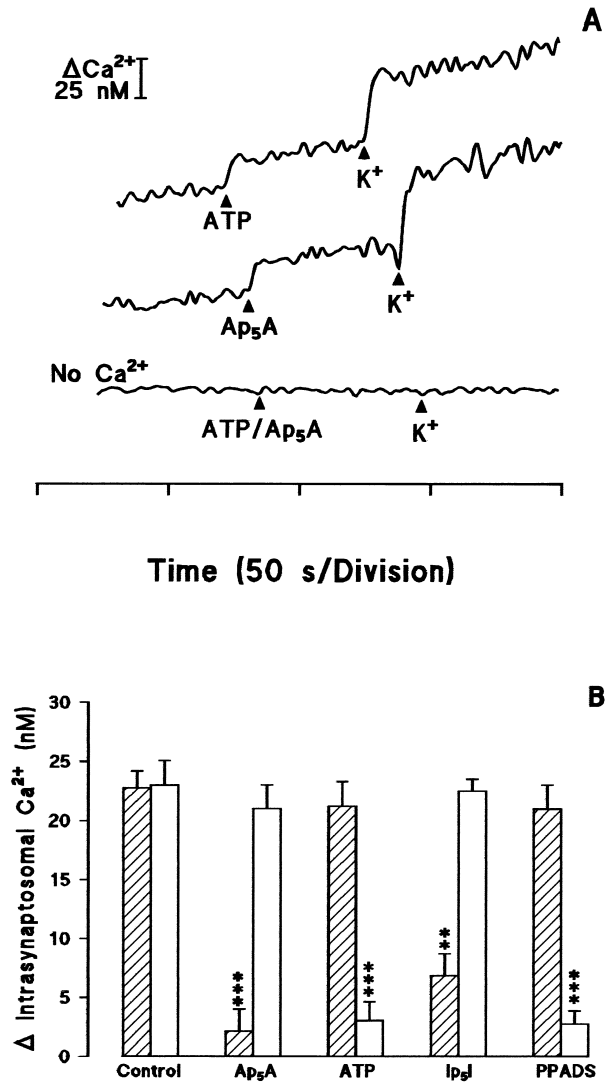

Fig. 2. Extracellular Ca^{2+} dependence, cross-desensitisation studies and purinergic antagonist pattern of human synaptic terminals after stimulation with Ap_5A and ATP. (A) Representative experiments of both ATP and Ap_5A (100 μM applications) in the presence (upper traces) and in the absence (lower trace) of extrasynaptosomal Ca^{2+}. (B) Ap_5A (dashed bars) or ATP (open bars), both 100 μM, were applied after 2-min incubation with the corresponding agonist or antagonist described in each case. The concentrations for each compound were as follows, Ap_5A 100 μM, ATP 100 μM, Ip_5I 100 nM and PPADS 100 μM. Results represent mean ± S.E.M. of two determinations in triplicate. $**$ $P < 0.01$ vs. control; $***$ $P < 0.001$ vs. control.

affected by Ip_5I. The pre-incubation of the synaptic terminals with the ATP receptor antagonist PPADS blocked significantly the response elicited by ATP and did not alter the Ca^{2+} transient induced by 100 μM Ap_5A (Fig. 2B).

3.5. Effect of Ca^{2+} channel blockers

Depolarisation carried out by Ap_5A and ATP, as a consequence of the receptor stimulation (dinucleotide and P2, respectively), might permit the opening of voltage-dependent Ca^{2+} channels present in the human synaptosomal plasma membranes. To test this possibility, different compounds were assayed: ω-Aga (a P-type Ca^{2+} channel blocker), ω-Cono (a N-type Ca^{2+} channel blocker) and nicardipine (a L-type Ca^{2+} channel blocker).

Ap_5A Ca^{2+} response was not significantly modified by the action either of the P-type Ca^{2+} channel blocker ω-Aga or of the L-type nicardipine (Fig. 3A). The N-type Ca^{2+} channel blocker ω-Cono diminished the response elicited by 100 μM Ap_5A to 53% (Fig. 3B) which indicates the involvement of N-type Ca^{2+} channels in the dinucleotide response.

The effects of the channel blockers on ATP Ca^{2+} transients were different from those observed with Ap_5A (Fig. 4A). ATP Ca^{2+} responses were inhibited by ω-Aga

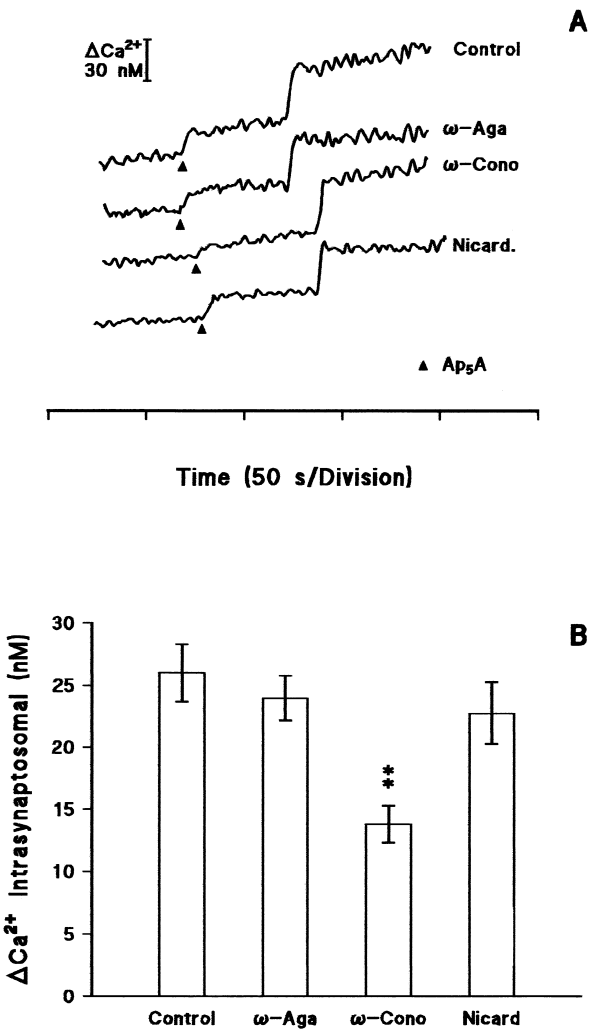

Fig. 3. Effects of voltage-dependent channel blockers on the Ca^{2+} transients elicited by Ap_5A. (A) Representative experiment of the effect of 100 μM Ap_5A (arrows) in the absence (control) and in the presence of ω-Aga, ω-Cono and nicardipine (Nicard), pre-incubated as described in Section 2. The second Ca^{2+} transient present in all records, represent a K^+ pulse to check the synaptosomal functionality. (B) Effects of different Ca^{2+} channel blockers on Ap_5A (100 μM) responses. Results are mean ± S.E.M. of two determinations in triplicate. $**$ $P < 0.01$ vs. control.

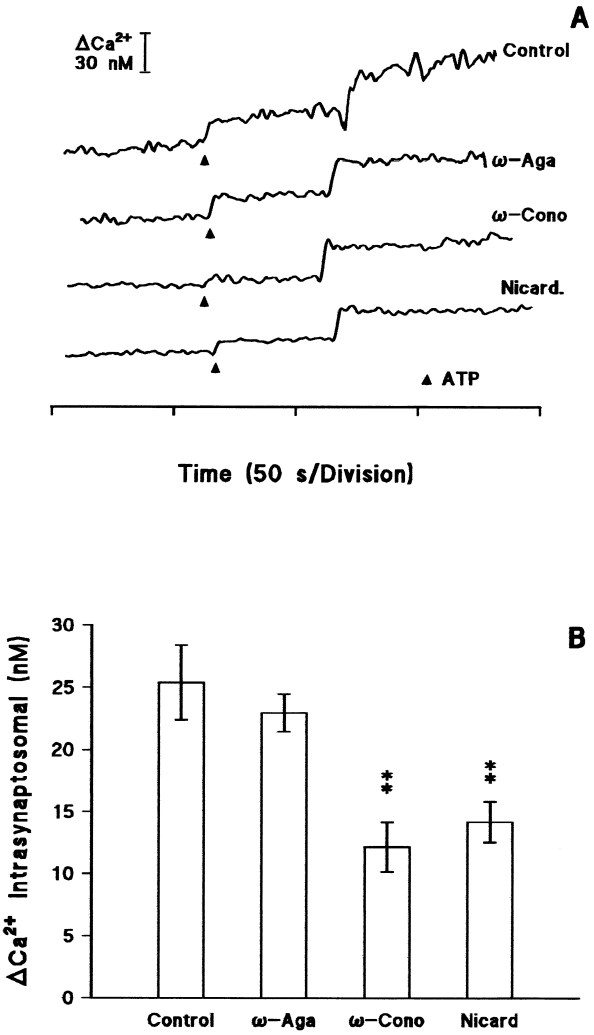

Fig. 4. Effects of voltage-dependent channel blockers on the Ca^{2+} transients elicited by ATP. (A) Representative experiment of the effect of 100 μM ATP, indicated by the arrows, in the absence (control) and in the presence of ω-Aga, ω-Cono and nicardipine (Nicard), under the conditions described in Section 2. The second Ca^{2+} transient present in all traces, represent a K^+ pulse to check the synaptosomal functionality. (B) Effects of different Ca^{2+} channel blockers on 100 μM ATP responses. Results are mean ± S.E.M. of two determinations in triplicate. $**P <$ 0.01 vs. control.

and also by nicardipine, reducing the transients to 52% and 44%, respectively. The P-type Ca^{2+} channel blocker did not significantly modify the response elicited by ATP as occurred by Ap_5A (Fig. 4B).

To test the possible involvement of the T-channels, 40 μM Ni^{2+} was tested with either Ap_5A (100 μM) or ATP (100 μM). No changes were observed in the Ca^{2+} responses elicited by Ap_5A or ATP (results not shown).

4. Discussion

Human brain synaptic terminals present receptors for both diadenosine polyphosphates and ATP, eliciting Ca^{2+} transients the amplitude of which depends on the extracellular concentration of the agonist. In terms of EC_{50}, both display a similar value in the micromolar range, as well as the same maximal effect. The cross-desensitisation studies suggested the agonists tested, ATP and Ap_5A, do not share the same receptors. After a maximal dose of one of the agonists, a second application of the other induced a typical response with no change in the amplitude when compared to control. These results were further confirmed by using two receptor antagonists, PPADS (Ziganshin et al., 1993) and Ip_5I (Pintor et al., 1997a). PPADS, which blocks the effects of ATP and diadenosine polyphosphates mediated by P2-receptors, effectively diminished the Ca^{2+} entry induced by ATP but was unable to reduce or modify the Ca^{2+} transient elicited by Ap_5A. This clearly indicates that Ap_5A does not induce the Ca^{2+} transient through ATP receptors. To further confirm this, the newly synthesised specific *dinucleotide receptor* antagonist, Ip_5I, was assayed. After pre-incubation with this compound ATP was still an active agonist, while Ap_5A response was reduced to 67% of the control value. In the model of rat midbrain synaptosomes this antagonist blocked completely the response to Ap_5A, while in the human brain it was able to reduce the response only to half the control. This could indicate that this receptor is not so sensitive to this antagonist as is that of the rat. Another possibility could be the existence of a subpopulation of dinucleotide receptors sensitive to Ap_5A which are not blocked by Ip_5I that are located in mammalian cortical areas.

The activation of the dinucleotide receptor by Ap_5A produces an increase in the ω-Cono sensitive component of the Ca^{2+} channels in human cortical synaptic terminals. The specificity of the mentioned toxin (Miller and Fox, 1990; Tsien and Tsien, 1990), and the lack of effect by the other blockers, indicate that Ap_5A facilitates only the activity of N-type Ca^{2+} channels, the other channels being uninvolved in the dinucleotide action. This result is in agreement with those described in rat brain synaptosomes (Pintor and Miras-Portugal, 1995) and CA3 hippocampal neurones (Panchenko et al., 1996). In both cases, Ap_5A acting through dinucleotide receptors facilitate the Ca^{2+} transients through N-type Ca^{2+} channels.

Studies performed on rat hippocampal slices demonstrated a complex action of diadenosine polyphosphates on synaptic transmission. One of the effects was the appearance of an overshoot of the post-synaptic transmission which might be connected with a Ca^{2+} entry in the pre-synaptic terminals induced by the Ap_5A released to the synaptic cleft (Klishin et al., 1994). Considering the Ca^{2+} results obtained for human synaptosomes, a similar situation could be supposed at the post-synaptic level.

The non-association of the Ap_5A stimulation with the activation of a L-type Ca^{2+} channel, has been pointed out in rat brain synaptosomes, hippocampal neurones and also deermouse synaptosomes (Pintor and Miras-Portugal, 1995; Panchenko et al., 1996). The L-type channel blockers, such

as verapamil or nifedipine did not significantly modify the Ca^{2+} responses induced by the dinucleotide in those neural models (Pivorun and Nordone, 1996).

ATP behaved in a different way since it activated both the N- and L-type Ca^{2+} channels (Carbone and Swandulla, 1989). These results are consistent with those found in the rat midbrain where both channels were also activated (Pintor and Miras-Portugal, 1995), and contrary to those described in the heart nodal cells where ATP blocks the activity of the L-type Ca^{2+} channel (Qi and Kwan, 1996).

It has been reported that ATP receptors stimulate the release of dopamine in rat brain by a pre-synaptic mechanism suggesting a role of this nucleotide in parkinsonism (Zhang et al., 1995). In a similar way, if the presence of dinucleotide receptors could be demonstrated in dopaminergic terminals, the release of this neurotransmitter could be modulated by blocking the *dinucleotide receptor* with Ip_5I, thus some perspectives related to the use of diadenosine polyphosphates and the antagonist Ip_5I as therapeutic purposes arise. The presence of these receptors in the terminals where the release of a particular neurotransmitter is pathologically altered, provide a new target for drug action and development.

In summary, human cortical synaptic terminals present dinucleotide and P2 receptors eliciting Ca^{2+} transients which can be partially blocked by specific purinergic antagonists and voltage-dependent Ca^{2+} channel inhibitors. The present experiments, considering that they have been carried out in human tissue, have yielded findings which should provide new perspectives in the search of purinergic drugs for human therapeutically purposes.

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