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Diinosine Polyphosphates, a Group of Dinucleotides with Antagonistic Effects on Diadenosine Polyphosphate Receptor

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SUMMARY

A new family of dinucleotide derivatives, diinosine polyphosphates, has been synthesized through the use of the enzyme 5′ adenylic acid deaminase from *Aspergillus* sp., starting from the corresponding diadenosine polyphosphates. Functional studies were performed on rat brain synaptic terminals in which a dinucleotide receptor has been described that is specific for adenine dinucleotides. The results demonstrated that diinosine polyphosphates did not behave as agonists on the diadenosine polyphosphate receptor (also know as P_4 purinoceptor), but they were very efficient as antagonists in abolishing the Ca^{2+} responses elicited by diadenosine pentaphosphate. The IC_{50} values for diinosine triphosphate, diinosine tetraphosphate, and diinosine pentaphosphate were 4.90 ± 0.10 μM, 8.33 ± 0.22 μM, and 4.23 ± 0.12 nM, respectively. The diinosine polyphosphates also antagonized the ATP receptors present in synaptic terminals, showing IC_{50} values of 100.08 ± 5.72 μM for diinosine triphosphate, 29.51 ± 1.40 μM for diinosine tetraphosphate and 27.75 ± 1.65 μM for diinosine pentaphosphate. The antagonistic ability of these diinosine nucleotides was studied in comparison with other P_1 and P_2 purinoceptor antagonists, such as suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, and 8-cyclopentyl-1,3-dipropylxanthine. These purinergic antagonists did not inhibit the response of the P_4 purinoceptor; only the diinosine polyphosphates were able to act as antagonists on the dinucleotide receptor. Suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid attenuated the responses elicited by ATP, as did the diinosine polyphosphate compounds. The most antagonistic diinosine polyphosphate for the dinucleotide and ATP receptors was diinosine pentaphosphate, which was 6000 times more selective for the P_4 purinoceptor than it was for the ATP receptor.

Ap_nAs (where n ranges from three to six phosphates) are a family of compounds formed by two adenosine moieties bridged by a variable number of phosphates. These compounds are active substances in neural and non-neural tissues (1). Their action is generally mediated by ATP purinergic receptors and in some cases by adenosine-like receptors in specific areas of the central nervous system (2, 3). Recently, a new subtype of presynaptic receptor that is exclusive to Ap_nAs has been described in the central nervous system (4). This receptor, characterized in rat brain synaptic terminals, has tentatively been termed P_4 purinoceptor, or dinucleotide receptor. The activation of the P_4 purinoceptor by Ap_5A and Ap_4A is coupled to the Ca^{2+} entry to the synaptic terminals via a voltage-independent mechanism. The main feature of the dinucleotide receptor is the nonsensitivity to ATP and its synthetic analogs, being activated only by Ap_nAs (5). Very recently, a receptor sensitive to Ap_4A was also described in deermouse brain synaptosomes (6).

Receptors for Ap_nAs and adenine nucleotides have been pharmacologically characterized by means of ATP synthetic analogs due to the scarcity and poor specificity of the available antagonists (7). The use of agonistic profiles has not allowed a clear characterization of the receptors present in different tissues. Several compounds have been tested as P_2 antagonists, among which suramin and PPADS are still the most widely used (8, 9). Although initially both were used to discriminate within different P_2 purinoceptor subtypes, only PPADS seems to be quite specific for the P_{2X} purinoceptors, with suramin suitable for distinguishing between P_1 and P_2 purinoceptors (10).

The existence of nonspecific adenylate deaminases isolated from organisms such as the snail *Helix pomatia* and microorganisms suc as *Aspergillus* sp. allows the enzymatic transformation of the Ap_nAs and adenosine nucleotides to inosine

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ABBREVIATIONS: Ap_nA, diadenosine polyphosphate or adenine dinucleotide; Ip_nI, diinosine polyphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; HPLC, high performance liquid chromatography; Ap_5A, diadenosine pentaphosphate; Ap_4A, diadenosine tetraphosphate; Ip_3I, diinosine triphosphate; Ip_4I, diinosine tetraphosphate; Ip_5I, diinosine pentaphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

derivatives (11). The transformation of Ap_nA by adenylate deaminases yields compounds with the structure inosine(5′)oligophospho(5′)inosine (Ip_nI). The Ip_nIs could enlarge the number of different nucleotidic compounds acting as agonists or antagonists on purinergic receptors, especially on the P_4 purinoceptor, due to their structural similarities to Ap_nAs.

In the current study, we describe the synthesis of Ip_nI from *Aspergillus* sp. by means of a commercial AMP deaminase. The relevant antagonistic properties of these dinucleotides on the P_4 purinoceptor and ATP receptors is reported in rat brain synaptic terminals. Special attention is devoted to Ip_5I because its high antagonistic activity on Ap_nA receptors makes this compound a useful tool with which to discriminate between ATP receptors and P_4 purinoceptors.

Experimental Procedures

Synthesis of Ip_nIs. Ip_nIs were obtained by starting with the corresponding Ap_nAs through the action of 5′adenylic acid deaminase from *Aspergillus* sp. The enzyme (0.12 unit) was incubated with 10 mM Ap_3A, Ap_4A, or Ap_5A in a final volume of 1 ml of 50 mM HEPES, pH 6.5. Incubation was carried out at 37°. Aliquots of 10 μl were taken at different times, placed in a 100° water bath for 5 min (to stop the reaction), and diluted 1/100 with distilled water before injection into the HPLC system to follow the process of deamination. After 1.5 hr, the reaction was finally stopped by boiling the incubation mixture at 100° for 5 min followed by centrifugation at 13,000 × *g* to eliminate the protein debris. Boiling did not affect the stability of the formed Ip_nI as previously described for Ap_nAs (12).

Treatment with phosphodiesterase from *Crotalus durissus* (EC 3.1.15.1) was carried out to verify the nature of the formed compounds. Aliquots of 15 μl of the final reaction mixture were diluted 1:100 with distilled water and incubated with 3 munits of phosphodiesterase at 37°. Samples of 20 μl were taken, at different times, to follow the appearance of the inosine mononucleotides by HPLC.

Chromatographic procedures. The chromatographic equipment consisted of a Waters (Milford, MA) 600E system controller, a Waters 717+ autosampler, and a 481 λmax spectrophotometer, all of which were managed by Millenium 2010 software running on an NEC 486DX computer. Analysis was performed under ion-pair chromatography conditions by equilibrating the chromatographic system with the following mobile phase: 0.1 mM KH_2PO_4, 2 mM tetrabutylammonium hydrogen sulfate, and 17% acetonitrile, pH 7.4. The column was a NovaPak C-18 (15-cm length, 0.4-cm diameter; Waters). Detection was monitored at 260-nm wavelength.

Synaptosomal preparation. Synaptosomes for functional studies were prepared from middle rat brain cerebral cortices of cervically dislocated and decapitated male Wistar rats (12). Synaptosomal pellets containing 1 mg of protein were resuspended in 1 ml of incubation medium (122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose, and 20 mM TES, pH 7.4).

Intrasynaptosomal Ca^{2+} measurements. The cytosolic free calcium concentration was determined using Fura-2 as described by Grynkiewicz *et al*. (13). Five minutes after resuspension, 1.33 mM $CaCl_2$ and 5 μM Fura-2-acetoxymethyl ester were added. After incubation for 35 min, the synaptosomes were pelleted (through centrifugation at 800 rpm for 1 min), washed twice, and resuspended in fresh medium containing 1.33 mM $CaCl_2$. Fluorescence was measured in a Perkin-Elmer Cetus (Norwalk, CT) Spectrofluorimeter LS-50 and monitored at 340 and 510 nm. Data were collected at 0.5-sec intervals.

Pharmacological studies. Ap_5A and ATP were tested at a final concentration of 100 μM. Also, Ip_nIs were assayed at the same concentration before any antagonistic experiment was performed. The capacities of Ip_3I, Ip_4I, and Ip_5I to act as possible antagonists was analyzed by assaying them at different concentrations ranging from 10^{-10} to 10^{-3} M. When tested as antagonists, Ip_nIs were preincubated 2 min before the addition of the agonists. Other P_2 antagonists, such as suramin and PPADS, were assayed at a final concentration of 100 μM 2 min before the application of the agonist. DPCPX, an A_1 adenosine antagonist, was preincubated at a final concentration of 250 nM at 2 min before the agonist was added to the synaptosomal preparation.

Studies were also performed to obtain the equilibrium dissociation constant (K_B) for Ip_5I. A series of Ap_5A dose curves (ranging from 10^{-7} to 10^{-3} M) were obtained in the presence of Ip_5I at the following concentrations: 0, 4, 40, and 400 nM. These curves were analyzed according to the method of Stone and Angus (14) to obtain the K_B value for the antagonist.

Materials. Ap_3A, Ap_4A, Ap_5A, ATP, ITP, IDP, IMP, and 5′ adenylic acid deaminase from *Aspergillus* sp. were obtained from Sigma Chemical (St. Louis, MO). Suramin was kindly provided by Dr. A. IJzerman (Center for Drug Research, Division of Medicinal Chemistry, Leiden, The Netherlands). PPADS and DPCPX were purchased from Research Biochemicals (Natick, MA). Phosphodiesterase from *C. durissus* (EC 3.1.15.1) was purchased from Boehringer-Mannheim Biochemica (Mannheim, Germany). Fura-2 was obtained from Molecular Probes (Eugene, OR). Other analytical-grade reagents were purchased from Merck (Darmstadt, Germany).

Statistical analysis. Data are presented as mean ± standard error (for IC_{50} values) of curves fitted by means of Fig P version 6.0 (Biosoft, Cambridge, UK). Experiments were performed at least four times in duplicate and in different synaptosomal preparations. Significant differences were determined by two-tailed Student's *t* test. When appropriate, single experiment traces are represented in the figures; they are representative of at least four determinations in duplicate with equivalent results.

Results

Chromatographic characterization of Ip_nIs. Ip_nIs were synthesized by starting with Ap_nAs through sequential deamination of both adenosine moieties. The 5′ adenylic acid deaminase from *Aspergillus* sp. was very efficient with the three Ap_nAs that were converted to the corresponding Ip_nI (Fig. 1). The reaction had a yield of 100% after 1.5 hr with no further transformation of the products into other compounds. At the intermediate times, it was possible to note the presence of peaks with retention times between those of the initial Ap_nA and the final Ip_nI. It seems possible that those peaks could correspond to the intermediate reaction products in which one of the adenosines remain unchanged while the other had already been deaminated (Fig. 1).

To verify the nature of the final products, samples of the putative Ip_nIs were collected and incubated with phosphodiesterase from *C. durissus.* This enzyme cleaves dinucleotides giving a nucleotide monophosphate plus another mononucleotide with an $n-1$ phosphate. The presence of IMP and IDP was determined for the putative Ip_3I (Fig. 2A), of IMP and ITP for Ip_4I (Fig. 2B), and of IMP and a compound putatively identified as inosine-5′-tetraphosphate for Ip_5I (Fig. 2C). In all of the Ip_nI compounds treated with the phosphodiesterase, a gradual increase in the inosine mononucleotides and a disappearance of the dinucleotide were observed. Once the identity of the synthesized Ip_nI was confirmed, they were assayed as active molecules in rat brain synaptic terminals.

Effect of Ip_nIs on rat brain synaptic terminals. The application of Ip_nIs to the rat brain synaptic terminals in a concentration of ≤100 μM did not induce any Ca^{2+} movement

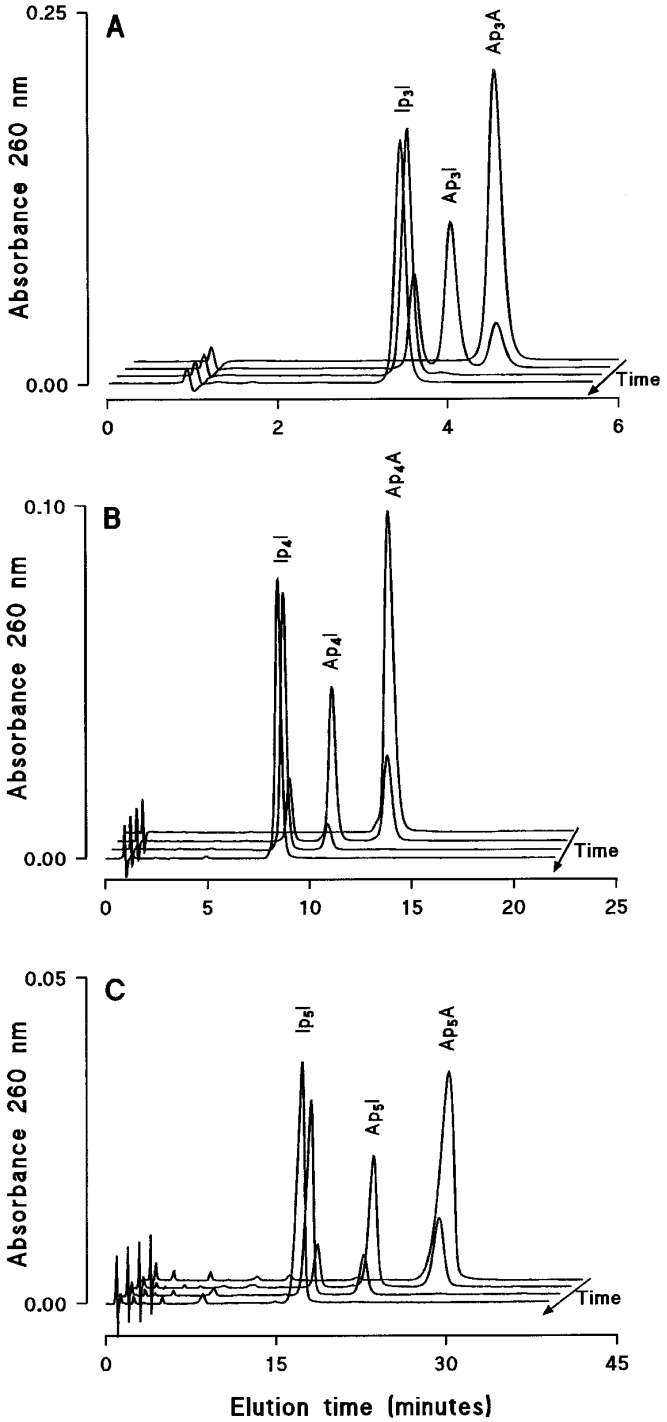


Fig. 1. Enzymatic synthesis of inosine(5′)oligophospho(5′) inosines occurred with reverse-phase chromatography. A, HPLC elution profile for the synthesis of Ip_3I. Ap_3A (10 mM) was incubated with *Aspergillus* sp. adenylate deaminase. Aliquots of the reaction mixture were taken at different incubation times (0, 15, 45, and 90 min). Then, the reaction was stopped, and HPLC was used to follow the reaction development, as described in Experimental Procedures. The HPLC profiles obtained at increasing incubation times are represented as consecutive chromatograms, starting from time zero (*rear trace*). At low times, hybrid compounds are formed as a consequence of the partial deamination of the Ap_nA. Ip_3I was the only compound present after a 90-min incubation (*front trace*). B, Chromatographic profile of the Ip_4I synthesis. Ap_4A (*rear trace*) transformation was followed after 15, 45, and 90 min (*front trace*) of incubation with *Aspergillus* sp. deaminase, as described in A for the Ip_3I formation. C, Analysis of the Ip_5I formation under the same conditions as described in A and B.

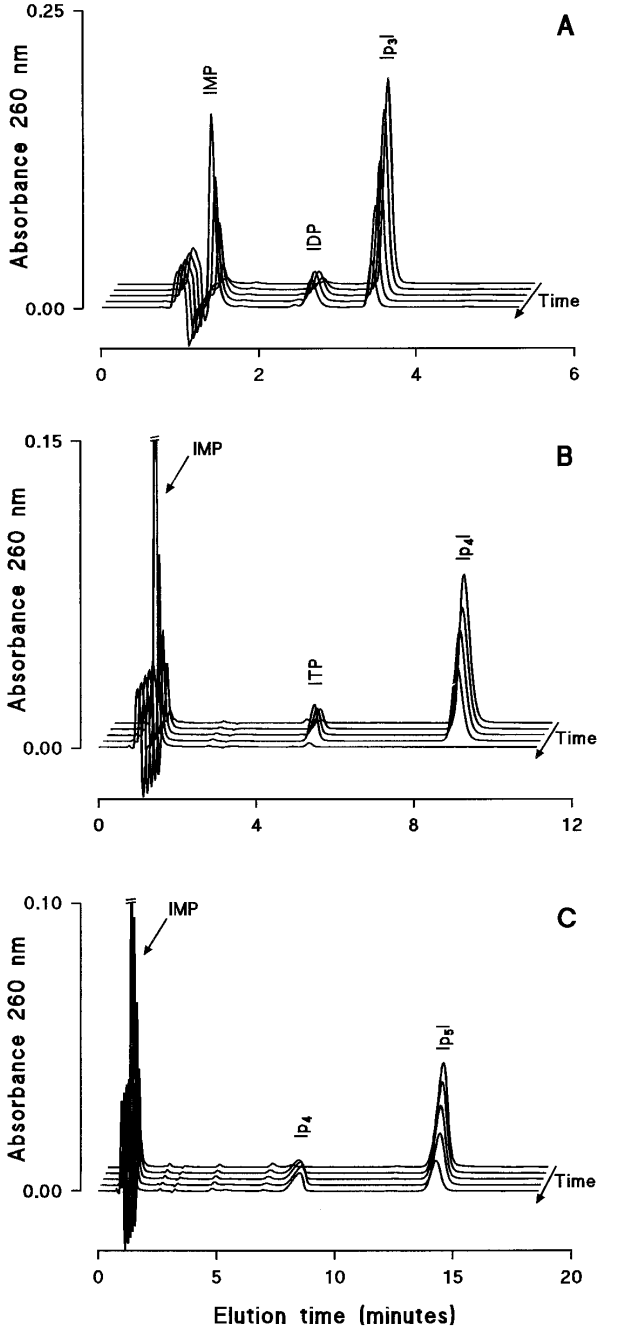


Fig. 2. Characterization of Ip_xI through phosphodiesterase digestion. Putative Ip_nIs were incubated with phosphodiesterase from *C. durissus*, as described in Experimental Procedures. Aliquots taken at 0, 3, 6, 10, and 20 min were injected and analyzed by ion-pair chromatography to follow the Ip_xI disappearance and the appearance of the corresponding degradation products. A, Characterization of Ip_3I through digestion with phosphodiesterase. Putative Ip_3I was treated as previously described. The consecutive chromatograms correspond to the HPLC profiles obtained at increasing digestion times. The disappearance of the Ip_3I is concomitant with the appearance of peaks of their degradation products (IMP and IDP). B, Characterization of Ip_4I through digestion with phosphodiesterase. Putative Ip_4I was analyzed as described in A for Ip_3I. The consecutive chromatograms show the formation of ITP and IMP from Ip_4I through incubation at increasing times with phosphodiesterase. C, Characterization of Ip_5I through digestion with phosphodiesterase. Putative Ip_5I was treated in the manner described for Ip_3I and Ip_4I. Digestion of Ip_4I peak with phosphodiesterase yields IMP and another metabolite with a retention time higher than that for ITP. This compound has been putatively identified as inosine-5′-tetraphosphate.

as a consequence of receptor activation (Fig. 3A). Ap_5A assayed at a concentration of 100 μM elicited a Ca^{2+} increase of 30.51 ± 3.56 nM, as shown in Fig. 3A (basal synaptosomal Ca^{2+} concentration before the agonist application, 115.08 ± 5.12 nM).

Because the Ip_nIs did not exert any agonistic effect on the rat brain synaptic terminals (Fig. 3A), their capacity to act as a possible antagonists was tested. The incubation of the three Ip_nIs at a final concentration of 100 μM at 2 min before the Ap_5A application reduced the Ca^{2+} signal elicited by the Ap_nA (Fig. 3B). The treatment of synaptic terminals with other purinergic antagonists is shown in Fig. 3B. Suramin and PPADS, also at final concentrations of 100 μM, did not mimic the effect of Ip_nI. Suramin did not significantly modify the Ca^{2+} entry induced through the P_4 purinoceptors, and PPADS surprisingly induced an enhancement of the signal compared with control (Fig. 3B). Similar behavior to that exhibited by PPADS was shown by DPCPX (250 nM), which is known to antagonize adenosine receptors.

Antagonistic effect of Ip_nIs on the P_4 purinoceptor. Because Ip_nIs behaved as antagonists on the dinucleotide receptor, they were preincubated in a wide range of concentrations in the presence of 100 μM Ap_5A. The Ca^{2+} responses elicited by Ap_5A were attenuated by all Ip_nI to various degrees depending on the concentration and the diinosine compound assayed. An example of the antagonistic effect of one of these dinucleotides, Ip_5I, is shown in Fig. 4A, in which a gradual attenuation of the Ca^{2+} signal by higher doses of the Ip_5I can be observed. Following a similar protocol inhibition, curves were obtained and analyzed to determined IC_{50} val-

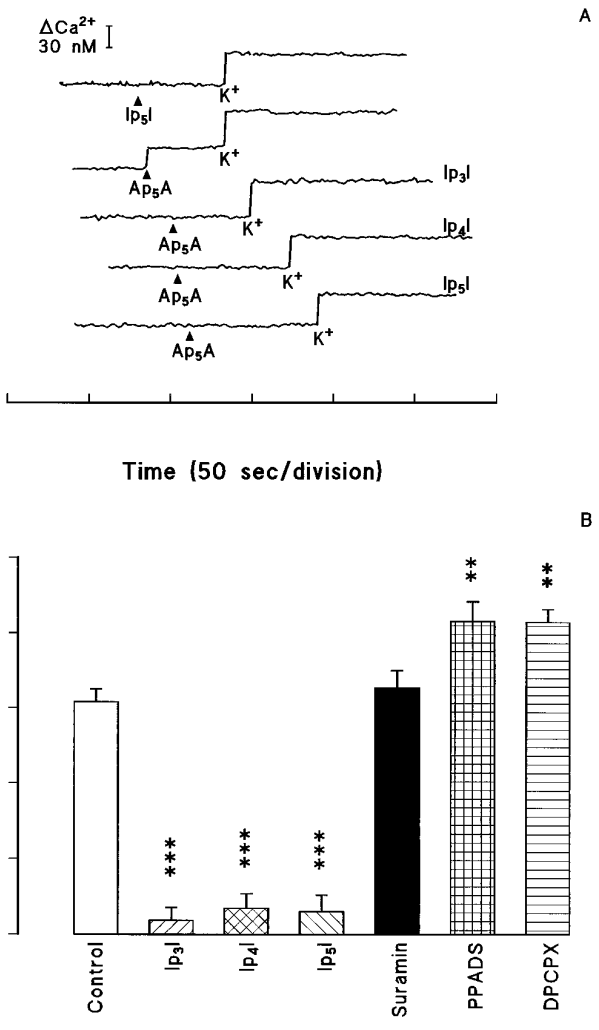


Fig. 3. Antagonistic effect of Ip_nIs and purinergic antagonists on P_4 purinoceptors present in rat brain synaptic terminals. A, Effects of Ip_3I, Ip_4I, and Ip_5I on calcium entry into rat brain synaptosomes evoked by 100 μM Ap_5A. Effects of Ip_5I and Ap_5A on Ca^{2+} responses induced through the dinucleotide receptor (*top two traces*). The effect of Ap_5A was blocked by the three Ip_nIs applied at a final concentration of 100 μM (*bottom three traces*). B, Comparative behavior of Ip_nIs and purinergic antagonists. All compounds were assayed at a final concentration of 100 μM, except DPCPX, which was applied at a concentration of 250 nM, modifying the Ap_5A response in a different way. All experiments represent the mean ± standard error of four different experiment performed in duplicate. **, $p < 0.01$ versus control; ***, $p < 0.001$ versus control.

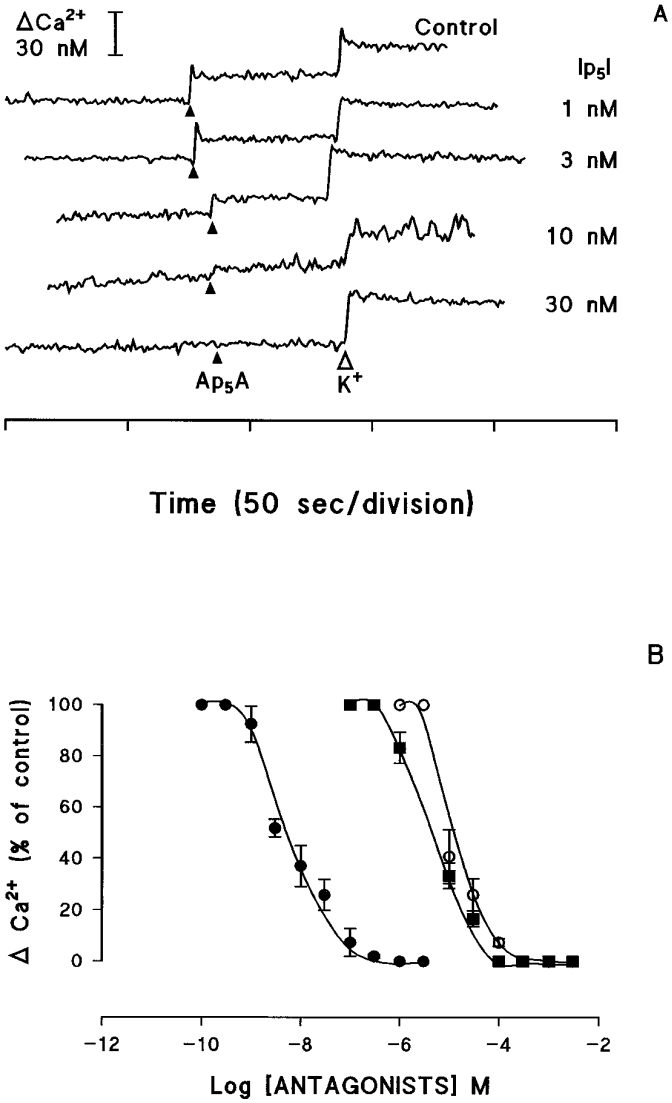


Fig. 4. Effects of Ip_nIs on the responses elicited by 100 μM Ap_5A. A, Increasing doses of Ip_5I produced a gradual blocking of the responses elicited by Ap_5A. A 30 mM K^+ pulse is given at the end of each record as a control of the synaptosomal functional response. B, Effect of (■) Ip_3I, (○) Ip_4I, and (●) Ip_5I on the Ca^{2+} transients elicited by 100 μM Ap_5A. Graded concentrations of the Ip_nI were added to obtain the corresponding responses. *Ordinates*, Ca^{2+} transients as a percentage of the control value for 100 μM Ap_5A in the absence of any substance. Values are mean ± standard error from four experiments performed in duplicate.

ues for the three compounds (Table 1). It was noteworthy that Ip_5I was the most potent antagonist for the Ap_5A effect, with an IC_{50} value in the nanomolar range (Table 1). Ip_3I and Ip_4I had IC_{50} values in the micromolar range (Fig. 4B).

Various Ap_5A series of dose-response curves were assayed in the presence of different concentrations of Ip_5I to obtain the inhibition constant (K_B) of the antagonist for the receptor. The concentration-response analyses for Ap_5A were performed in both the absence and the presence of the following concentrations: 4, 40, and 400 nM Ip_5I (equivalent to IC_{50}, 10-fold IC_{50}, and 100-fold IC_{50} value, respectively). As displayed in Fig. 5A, there was a gradual displacement of the dose-response curves to the right that was directly related to the increase in the antagonist concentration. EC_{50} values were analyzed according to the Stone and Angus equation and represented as a Clark plot (Fig. 5, B and C). The results obtained after the analysis yielded a K_B value for Ip_5I of 78.9 ± 9.9 nM and a slope of 1.0 ± 0.1, indicating the existence of a competitive antagonist mechanism.

Effects of Ip_nIs on ATP responses. The antagonistic properties of the Ip_nIs were also assayed for the ATP responses in isolated rat brain synaptic terminals. ATP (100 μM) elicited a Ca^{2+} increase of 25 ± 2.7 nM (basal synaptosomal Ca^{2+} concentration before the agonist application, 115.08 ± 5.12 nM).

Ip_3I, Ip_4I, and Ip_5I antagonized the responses elicited by ATP, with IC_{50} values in the micromolar range (Fig. 6 and Table 1). Ip_5I antagonized the Ca^{2+} transients elicited by ATP, but the concentration required to attenuate the response compared with Ap_5A was 6000 times higher, indicating that this Ip_nI behaves as a good tool with which to discriminate between P_4 and P_2 receptors in rat brain synaptic terminals (Fig. 6A).

Inosine mononucleotide effect on Ap_5As and ATP Ca^{2+} transients. To find out whether inosine mononucleotides could mimic the effect of Ip_nIs on both the dinucleotide and ATP receptor, ITP, IDP, and IMP were assayed as agonists/antagonists on the Ca^{2+} transients elicited by 100 μM Ap_5A and ATP. ITP induced a Ca^{2+} transient that did not abolish a secondary Ca^{2+} response triggered by Ap_5A. This result suggested that both nucleotides activate different receptors and that the antagonistic effect of the Ip_nI compounds is indeed caused by them rather than by their degradation products (Fig. 7A). None of the other inosine mononucleotides affected the responses elicited by Ap_5A (Fig. 7B). The application of ITP to the synaptosomal preparation before ATP produced a Ca^{2+} increase that blocked the responses induced by ATP, suggesting that ITP and ATP share a purinergic receptor (Fig. 7A). The other inosine mononucleotides, IDP and IMP, also exhibited direct effects on the Ca^{2+} transients, and both attenuated the responses elicited by ATP (Fig. 7B).

TABLE 1

IC_{50} values of Ip_nIs for the Ca^{2+} responses elicited by Ap_5A and ATP

Ap_5A and ATP were assayed at a final concentration of 100 μM in the presence of graded concentrations of Ip_nIs to elaborate dose-response curves. IC_{50} values represent the concentration of the antagonist producing an inhibition of 50% of the maximal effect induced by the agonists. Values are mean ± standard error.

	IC_{50}	
	Ap_5A response	ATP response
Ip_3I	4.90 ± 0.10 μM	100.08 ± 5.72 μM
Ip_4I	8.33 ± 0.22 μM	29.51 ± 1.40 μM
Ip_5I	4.23 ± 0.12 nM	27.75 ± 1.65 μM

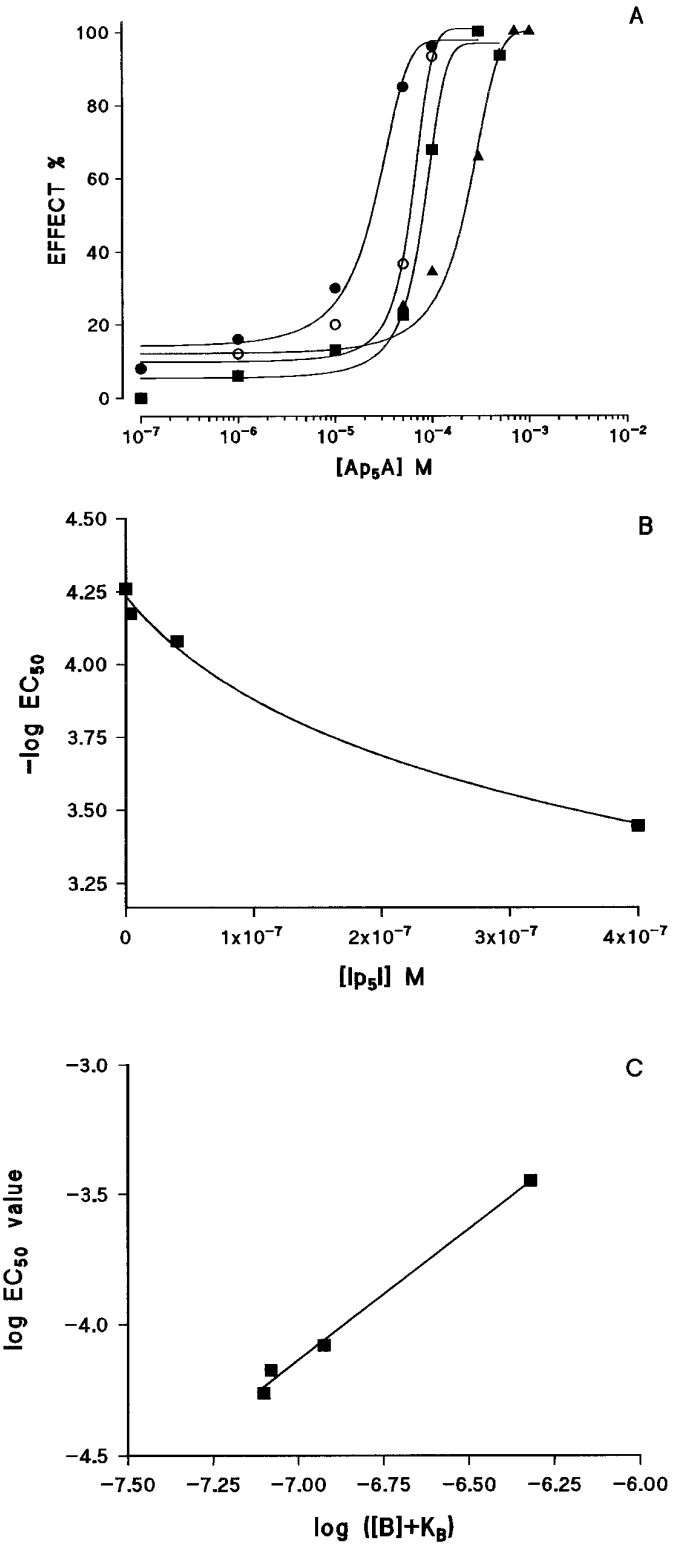
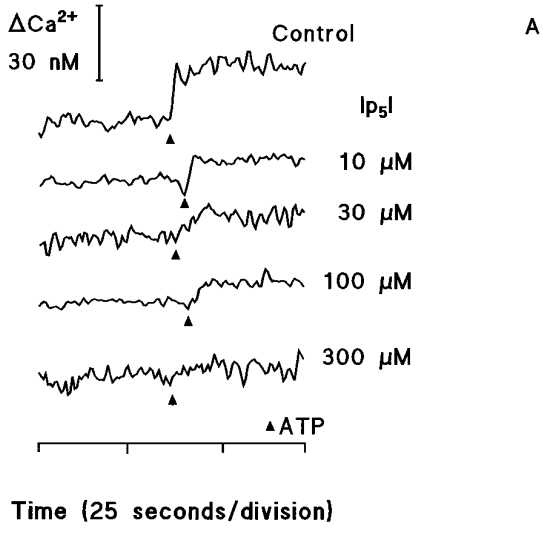
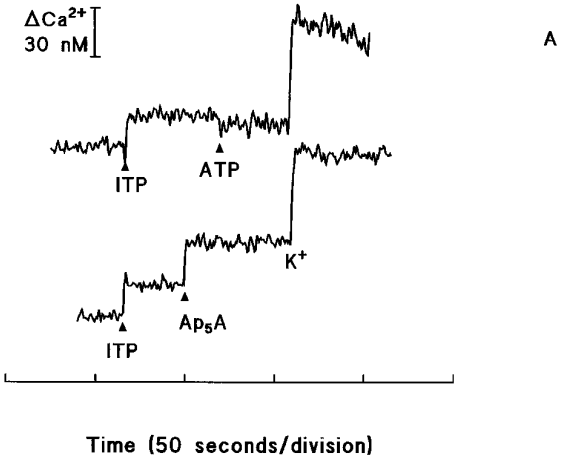
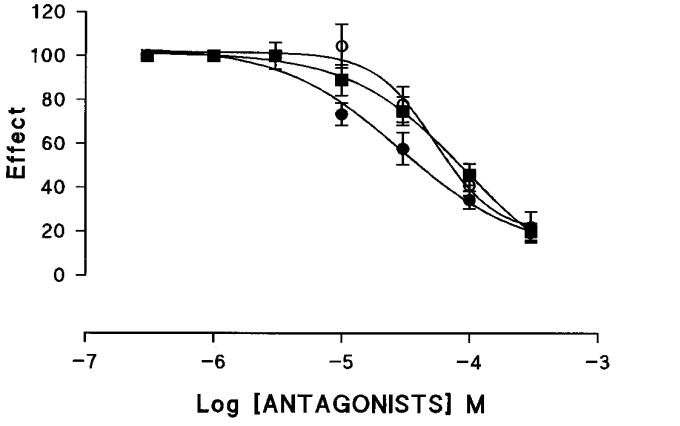


Fig. 5. Effect of different Ip_5I concentrations on Ap_5A dose-response curve. A, Effect of Ip_5I (●, 0 nM; ○, 4 nM; ■, 40 nM, and ▲, 400 nM) on Ap_5A Ca^{2+} transients. Data are fitted to logistic curves. B, Effect of increasing concentrations of Ip_5I on Ap_5A pEC_{50} value. Agonist potency data were analyzed using nonlinear regression to yield the pK_B estimate. C, Clark plot displaying the effect of Ip_5I on Ap_5A pEC_{50} value.







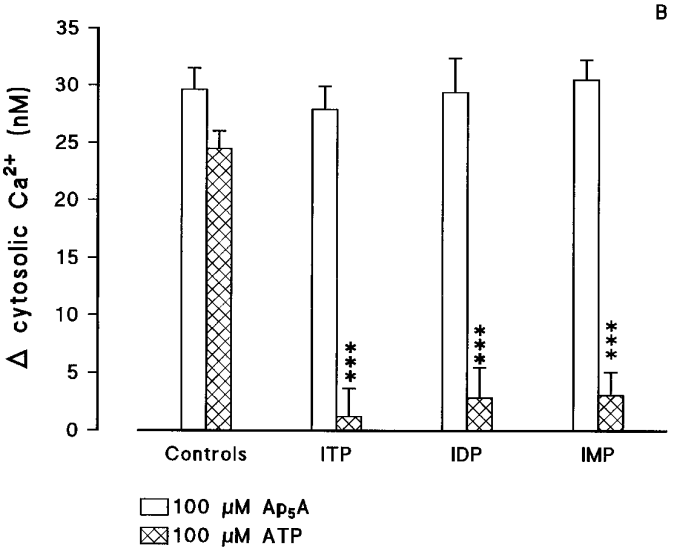


Fig. 6. Antagonistic effect of Ip_nI on the responses induced by ATP. A, Responses to ATP (100 μM) in the presence of graded concentrations of Ip_5I. The antagonist was preincubated for 2 min before the application of the agonist, as described in Experimental Procedures. B, Inhibitory effect of Ip_nIs (■), Ip_3I; (○), Ip_4I; (●, Ip_5I) on the Ca^{2+} transients elicited by 100 μM ATP. Graded concentrations of the Ip_nIs were added to obtain the corresponding responses, which were attenuated in the presence of higher doses of the antagonists. *Ordinates*, Ca^{2+} transients as a percentage of the control value for 100 μM ATP in the absence of any substance. Values are mean ± standard error from four experiments performed in duplicate.

Fig. 7. Competition studies of inosine mononucleotides on P_4 and P_2 purinoceptors present in rat brain synaptosomes. A, Cross-desensitization study between ITP and ATP, both at a final concentration of 100 μM (*top*). Application of ITP and an additional application of Ap_5A (both at a final concentration of 100 μM) were used as a cross-desensitization study (*bottom*). B, Effects of IMP, IDP, and ITP on the Ca^{2+} responses elicited by both Ap_5A and ATP (both 100 μM). Data are mean ± standard error of four experiments performed in duplicate. ***, $p < 0.001$ versus control.

Discussion

The results that we present describe a simple method to synthesize Ip_nI by starting with Ap_nA. The synthesis procedure is similar to that described by Guranowski *et al.* (11), but a commercial 5′ adenylic acid deaminase from *Aspergillus* sp. is used instead one from the snail *Helix pomatia*. The effects of Ip_3I, Ip_4I, and Ip_5I were tested on rat brain synaptic terminals, either alone or in the presence of Ap_5A and ATP.

The lack of an action on the Ca^{2+} transients by the three compounds suggested to us that they be studied as antagonists on the purinoceptors already characterized in rat brain synaptosomes (5). The three Ip_nIs completely blocked the effect of the best P_4 purinoceptor agonist (Ap_5A). Ip_3I and Ip_4I presented a quite similar IC_{50} value, in the micromolar range, that was higher than that exhibited by Ip_5I, which was in the nanomolar range. This fact indicates some degree of structural requirement for the dinucleotide receptor, which is fulfilled by Ip_5I (the closer structural antagonists compared with the best agonist, Ap_5A) but not so well by the other antagonists. Extremely important is the IC_{50} value for Ip_5I, which was ~3 orders of magnitude lower than the micromolar values presented by the other diinosine compounds. Actions of Ap_nA in the nanomolar range have been previously reported in neural models. For example, the neu-

ral P_{2X2} purinoceptor expressed in *Xenopus laevis* oocytes is positively modulated by Ap_5A. This dinucleotide allosterically potentiates the ATP response with an EC_{50} value of 2.95 nM (15). Autoradiographic studies carried out with [^{3}H]Ap_4A at a concentration of 1 nM demonstrated specific distribution of binding sites on rat enkephalon for this dinucleotide (16). These results correspond very closely to those obtained previously during experiments performed in rat brain synaptosomes in which Ap_nAs had K_d values in the low nanomolar range (17).

Suramin and PPADS did not follow the antagonistic behavior displayed by the Ip_nI on the P_4 purinoceptor. These results clearly contrast with those described in guinea pig urinary bladder, in which the effect of Ap_4A is inhibited by suramin and especially by PPADS (18). In this model and in the vas deferens, Ap_nAs seem to activate a P_{2X} purinoceptor (19, 20), explaining why both suramin and PPADS antagonized the effect of Ap_4A.

The effect of Ap_5A was not blocked by the adenosine receptor antagonist DPCPX. This is an interesting point because in some central locations, such as the cerebral cortex and the hippocampus, the effect of Ap_nA can be blocked by methylxanthines (2, 3). Nevertheless, a more detailed study on single CA3 hippocampal neurons revealed that extracellular Ap_5A induced a Ca^{2+} inward current that was not blocked by A_1- and A_2-specific antagonists. CA3 neurons possess dinucleotide receptors that have the same features as the purinoceptors characterized in this study (21). The question of whether P_4 purinoceptors in CA3 neurons are also antagonized by Ip_nIs is a topic to be investigated.

The Ip_nIs did antagonize the responses elicited by ATP through other purinergic receptors different from the dinucleotide receptor. In this case, Ip_3I, Ip_4I, and Ip_5I attenuated the Ca^{2+} response induced by ATP with IC_{50} values in the micromolar range. Although Ip_3I and Ip_4I were not very useful in discriminating between the P_4 and P_2 purinoceptors, this was not the case for Ip_5I. Ip_5I seems to be a good candidate for a dinucleotide receptor antagonist because it is 6000 times more effective on the P_4 purinoceptor than on the ATP receptor. This result could be at the origin of new pharmacological tools in the nucleotide receptor field. It is, nevertheless, necessary to take into consideration the features of the presynaptic model in which these experiments were performed.

Ap_nA activity on metabotropic P_2 purinoceptors have been reported in many tissues (4, 22). The human P_{2U} purinoceptor is extremely sensitive to Ap_4A, and this dinucleotide has been suggested to be a physiological regulator of this receptor (23). Also, receptors for Ap_nAs described in heart (24–26) and mouse brain (6, 21) would be good models in which Ip_nI compounds could be assayed to establish pharmacological criteria for the classification of the dinucleotide receptors.

It is well known that Ap_nAs are inactivated by the action of high affinity ectodinucleotide hydrolases (27, 28). Based on the results of the current study, possible Ip_nI formation at the extracellular level by an unidentified adenylate deaminase cannot be ruled out. Further studies are necessary to demonstrate this possibility and its physiological relevance.

In summary, deamination products of Ap_nAs should be considered when studying the nucleotide receptors. The selectivity of Ip_5I in antagonizing the dinucleotide receptor suggests that this substance could be an appropriate compound with which to identify P_4 purinoceptors in biological systems.

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