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Receptors for ATP in rat sensory neurones: the structure-function relationship for ligands

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1 The pharmacological properties of the ATP-activated conductance in isolated sensory neurones of the rat were investigated by use of voltage clamp and concentration clamp techniques.

2 Adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), cytidine 5'-triphosphate (CTP), cytidine 5'-diphosphate (CDP) and some derivatives activate these receptors, whereas adenosine 5'-monophosphate (AMP), cytidine 5'-monophosphate (CMP) and other naturally-occurring nucleotides are competitive blockers.

3 In the sequence of substances, adenosine 5'-(β,γ-methylene)-triphosphonate (APPCP), adenosine 5'-(β,γ-difluoromethylene)- triphosphonate (APPCF2P), adenosine 5'-(β,γ-dichloromethylene)-triphosphonate (APPCC12P) and adenosine 5'-(β,γ-dibromomethylene)triphosphonate (APPCBr2P), the properties of ligands depend on the radius of the atom linked to the carbon of the diphosphonate group. Thus, APPCP is an agonist, APPCF2P is a partial agonist, while dichloromethylene and dibromomethylene analogues of adenosine 5'-(β,γ-methylene)triphosphonate demonstrate features of competitive blockers. APPCC12P is the most effective blocker of ATP-receptors (inhibition constant $K_i = 21 \pm 4\,\mu M$). An adenosyl or adenylyl radical, when connected to the terminal phosphate of ATP, converts the agonist into a partial agonist.

4 Two especially important parts of the ATP molecule are crucial for the interactions with receptors. They are: (1) the vicinity of C6 of the purine ring and (2) the polyphosphate chain. Some modifications in these regions of the molecule result in the transformation of an agonist into an antagonist.

Introduction

Adenosine 5'-triphosphate (ATP) is known to operate ionic channels via special receptors located on the cellular membrane of smooth muscles, as well as both sensory and central neurones (Burnstock, 1981; Jahr & Jessell, 1983; Krishtal *et al.*, 1983; Gordon, 1986; Marchenko *et al.*, 1987b). In the sensory neurones the receptors are activated by micromolar concentrations of ATP and also by ADP and cytidine 5'-triphosphate (Jahr & Jessell, 1983; Krishtal *et al.*, 1983; Krishtal & Marchenko, 1986). When activated, these receptors open cation-channels demonstrating low ionic selectivity and a strong inward-going rectification (Krishtal & Marchenko, 1984; Benham & Tsien, 1987; Krishtal *et al.*, 1987).

ATP is liberated from peripheral endings of non-myelinated sensory fibres (Holton & Holton, 1954) and it is found in high concentrations in synaptic vesicles of sympathetic nerves supplying a number of smooth muscle preparations (Burnstock, 1982; Sneddon & Westfall, 1984). It has been suggested that ATP might play roles as a co-transmitter, a neurotransmitter or a neuromodulator (Burnstock, 1985).

In the present paper, the structure-function relationship has been investigated for some ligands of ATP-receptors in mammalian sensory neurones.

Methods

Experiments were carried out on the neurones from rat nodose ganglia, which give large responses to ATP. Rats (of either sex) with postnatal ages from 1 to 3 weeks were killed by decapitation. The cells were isolated and put into primary culture (Marchenko *et al.*, 1987a) for 2–5 days. The experiments were performed in conditions of intracellular perfusion and voltage clamp (Hamill *et al.*, 1981; Kostyuk *et al.*, 1981). The artificial intracellular solu-

tion contained (mM): KF 110, Tris-HF 20 (pH 7.3). During special control experiments artificial intracellular solutions containing chloride or phosphate salts instead of KF were tested. Substitution of the intracellular anion did not result in any changes in the ATP-activated currents, but the cells in these cases deteriorated more rapidly.

ATP receptors demonstrate rapid desensitization and slow recovery (Krishtal & Marchenko, 1984). To avoid desensitization, cells were exposed briefly to the drugs using the concentration clamp method with 'square-pulse' application. For this, a modified version of the set-up described by Krishtal *et al.* (1983) was used (Figure 1). The rate of application of solution was such that the medium bathing the neurone was changed completely within 10 ms, as judged by control applications of 140 mM KCl. Extracellular solution contained (mM): NaCl 140, KCl 1, $MgCl_2$ 1, $CaCl_2$ 3, HEPES-NaOH 10 (pH 7.4). The experiments were carried out at room temperature (21–23°C). The holding potential was −90 mV. Dissociation and inhibition constants were calculated as the mean from the data of 3–5 experiments on different cells.

Drugs

Guanosine 5′-triphosphate (GTP), uridine 5′-triphosphate (UTP), inosine 5′-triphosphate (ITP), cytidine 5′-triphosphate and other naturally occurring 5′-nucleotides were obtained from Serva Feinbiochemica GMBH & Co. Adenosine 5′-triphosphate, adenosine 5′-triphosphate 2′,3′-acyclic dialcohol (acyclic ATP), 8-bromoadenosine 5′-triphosphate (8-Br-ATP), 1,N^6-ethenoadenosine 5′-triphosphate (ethenoATP), HEPES and collagenase (Type IV) were all obtained from Sigma Chemical Company. APPCF2P and APPCC12P were synthesized by G.M. Blackburn (The University of Sheffield). Purine riboside 5′-triphosphate (PuTP), ribavirin 5′-triphosphate (RTP) and other analogues of ATP and AMP were synthesized by A.A. Krayevsky (Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow). *bis*(Adenosyl-5′) tetraphosphate (AP4A), *bis*(adenosyl-5′)pentaphosphate (AP5A) with derivatives and also APPCBr2P, adenosine 5′-(β,γ-monobromomethylene)triphosphonate (APPCHBrP) and adenosine 5′-(α,β-monobromomethylene)triphosphonate (APCHBrPP) were synthesized by N.B. Tarussova from the same Institute. As AP4A and AP5A are comparatively unstable, they were purified before the experiments using a FPLC System (Farmacia) on Mono Q HR 5/5 column. Dinucleotides were eluted by the linear gradient of KCl (0–1 M) in triethanolamine-HCl buffer (20 mM, pH 7.7).

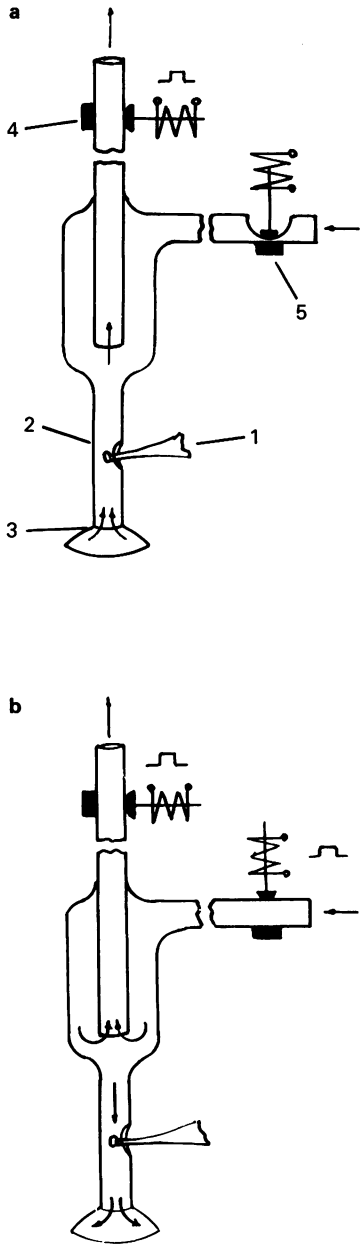


Figure 1 Diagram of set-up for 'square pulse' concentration clamp. (a) The perfusion pipette with a cell in the pore (1) is put into a side hole in the polyethylene tube (2) filled with extracellular solution. The solution containing agonist is sucked into the tube via the opening (3) after the valve (4) is open. The initial saline is returned via the valve (5), The movement of saline during application and washing out is indicated in (a) and (b), respectively.

Results

Pharmacological properties of naturally occurring nucleotides

As mentioned above, ATP, ADP and CTP activate the receptors (Figure 2a) while AMP acts as a competitive blocker. Among other naturally occurring ribonucleotides only CDP was found to be an agonist when the following were tested in millimolar concentrations: CMP, CDP, GTP, GDP, GMP, ITP, IDP, IMP, UTP, UDP, UMP. Apart from CDP, all of these inhibited the response to ATP in a reversible and competitive manner (e.g. GTP, Figure 3). This blocking action diminished in the sequence GTP > UTP > ITP and also with a decrease in the number of phosphate groups in the nucleotide molecule. GTP blocked ATP responses with an inhibition constant of 0.2 ± 0.03 mM. The latter value was calculated from the change in the apparent dissociation constant (K_d) for ATP (Figure 4a).

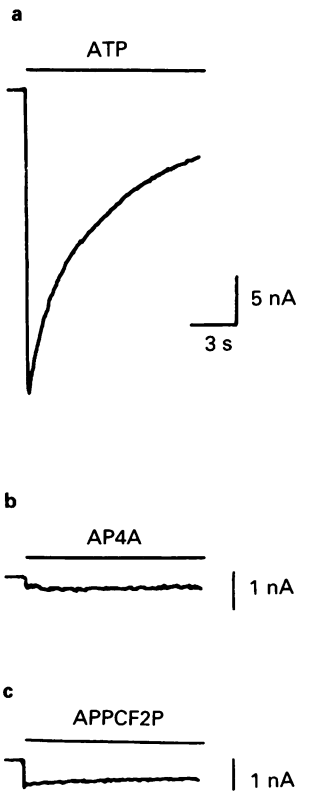


Figure 2 Inward currents activated by externally applied ATP (0.1 mM, a), AP4A (0.5 mM, b) and APPCF2P (0.4 mM, c). Ligands were applied during the time indicated by horizontal bar.

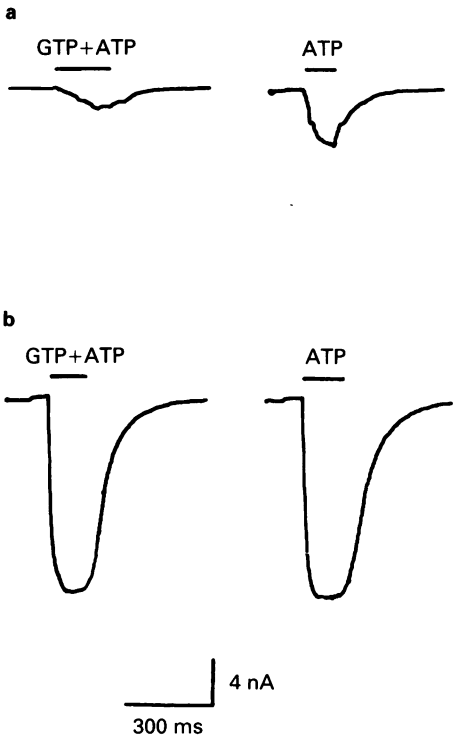


Figure 3 Inward currents activated by ATP at a concentration of 1.25 μM (a) and 50 μM (b). The recordings were made in the presence of 0.5 mM GTP (left traces) and in its absence (right traces). Horizontal bar indicates the duration of the application.

Pharmacological properties of ATP and AMP analogues

The structures of the analogues of AMP that were tested are shown in Table 1. Like AMP, its derivatives demonstrate an ability to block the response to ATP in the following sequence AMP (K_i = 1 mM) ≅ AMP(3' - OPhef) ≅ AMP(3' - OIlef ≫ AMP-(3'-Of). Substitution of the 3'-OH-group of AMP by an amino group decreased the blocking effectiveness by about an order of magnitude. A similar result was obtained when the OH-group was substituted by an azido group. Introduction of amino acid derivatives as substitutes for the amino group increased the blocking potency so that the following sequence was observed: AMP ≅ AMP(3'-NHMetf) > AMP(3'-NHGlyf) > AMP(3'-NH2).

ATP-derivatives with modified base and carbohydrate residue can act as agonists. These are: PuTP, RTP, ethenoATP, APCHBrPP, 8-Br-ATP, acyclic ATP, 3'-amino-3'-deoxyadenosine 5'-triphosphate (ATP(3'-NH2)), 3'-azido-3'-deoxyadenosine 5'-triphosphate (ATP(3'-N3)), 3'-N-

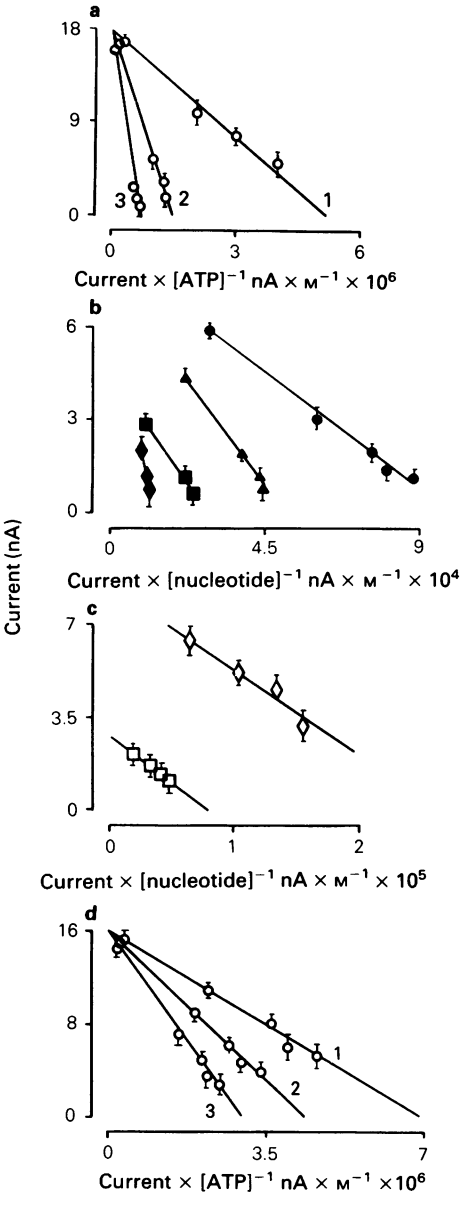


Figure 4 Dose-response relationships obtained for various agonists (Eadie-Hofstee plots). Note: open circles were used in all cases in this figure when ATP was the agonist. (a) Dose-response relationships for ATP in control (1), in the presence of 0.5 mM GTP (2) and 0.1 mM APPCC12P (3) (the data obtained on the same cell). (b) Dose-response relationships for PuTP (●), ethenoATP (▲), APCHBrPP (■), and RTP (◆); (c) the same plot, but for partial agonists: AP4A (□) and APPCF2P (◇) (the data obtained on different cells). (d) Dose-response relationships for ATP in control (1), in the presence of 20 μM APPCF2P (2) and 50 μM AP4A (3) (the data obtained on the same cell). Each point is the mean of 3–5 experiments. Vertical bars denote s.e.mean where larger than symbols.

Table 1 Abbreviated formulae of the AMP analogues tested

Abbreviation	*Structure of substitutes*
AMP(3'-Of)	$-O-CH=O$
AMP(3'-OIlef)	$-O-CO-CH(NH-CH=O)-CH(CH_3)C_2H_5$
AMP(3'OPhef)	$-O-CO-CH(NH-CH=O)-CH_2-C_2H_5$
AMP(3'-NH2)	$-NH_2$
AMP(3'-N3)	$-N_3$
AMP(3'-NHGlyf)	$-NH-CO-CH_2-NH-CH=O$
AMP(3'-NHMetf)	$-NH-CO-CH(NH-CH=O)-CH_2-CH_2-S-CH_3$

(biotinylamino-3'-deoxyadenosine 5'-triphosphates (ATP(3'-NHbio)), 3'-fluorescaminylimino-3'-deoxyadenosine 5'-triphosphate (ATP(3'-NHfluo)). Complete cross-desensitization of the neuronal responses to these substances with the responses to ATP proved that the same system of receptors was activated.

Dose-response relationships were obtained for all ATP analogues that demonstrated agonistic properties. These were linear when presented as Eadie-Hofstee plots. The agonist potency of these substances declines in the following sequence: ATP ($K_d = 1.2 \pm 0.2$ μM) > ATP(3'-NHbio) ($K_d = 5 \pm 0.7$ μM) > ATP(3'-NH2) ($K_d = 18 \pm 3$ μM) > ATP(3'-N3) ($K_d = 51 \pm 6$ μM) > PuTP ($K_d = 0.08 \pm 0.01$ mM) > ethenoATP ($K_d = 0.13 \pm 0.03$ mM) > APCHBrPP ($K_d = 0.2 \pm 0.02$ mM) > 8-Br-ATP ($K_d = 0.3 \pm 0.04$ mM) > RTP ($K_d = 0.5 \pm 0.03$ mM) > ATP(3'-NHfluo) ($K_d > 1$ mM) > acyclic ATP. Some dose-response relationships are shown in Figure 4b.

In contrast to APCHBrPP, APPCHBrP was found to be a competitive blocker of the ATP receptors ($K_i = 0.1 \pm 0.05$ mM). In view of this observation we investigated the effects of several dihalogen derivatives of APPCP. It was found that APPCF2P when applied in saturating concentration (40 mM) elicited responses that desensitized very slowly and had amplitudes almost one order of magnitude smaller than the amplitudes of the ATP-activated currents (Figure 2c, 4). There was no response to APPCF2P after desensitization caused by ATP. In contrast to APPCF2P, dibrom- and dichlor-analogues were found to be competitive blockers of the ATP receptors. APPCC12P seems to be the most effective blocker known for this type of receptor ($K_i = 21 \pm 4$ μM, Figure 4a).

Pharmacological activity of bis(adenosyl-5') oligophosphates

ATP-sensitive neurones also show responses to AP4A. When compared in the same cell, the maximum amplitude of the AP4A-activated current was 10–15% of the maximum amplitude of the ATP-activated current (Figure 2b). An approximate estimate of the K_d for AP4A is $\sim 40\,\mu M$ (Figure 4c). Desensitization produced by AP4A developed much more slowly than for ATP, and was only evident about 1 min after the start of application. Desensitization to AP4A was accompanied by a decrease in the ATP-activated current. Similarly, responses to AP4A disappeared in parallel with desensization to ATP. Thus ATP and AP4A activate the same receptors.

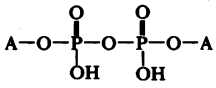
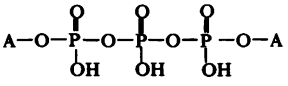
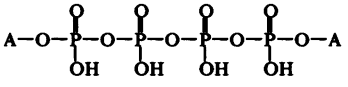
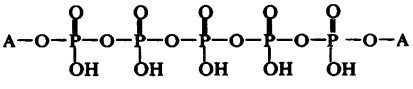
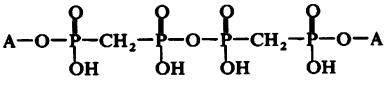
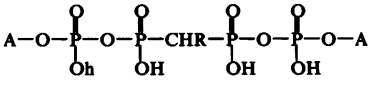
When applied together with ATP, AP4A reversibly decreased the amplitude of the ATP-activated response. Figure 4d demonstrates that this effect is due to the increase in the apparent K_d value for ATP. AP5A demonstrated similar properties. Non-hydrolysable analogues of AP4A (Table 2) had negligible agonistic activity and were much less effective as antagonists. The antagonistic capability of the tested dinucleotides diminished in the following order: AP4A ($K_i = 38 \pm 5\,\mu M$) = AP5A > APP(CH2)PPA > APP(CHCOCH3)PPA > AP(CH2)PP(CH2)PA > AP3A > AP2A > APP(CHCOOC2H5)PPA.

Discussion

All nucleoside monophosphates tested (both naturally occurring and synthetic) were competitive blockers of ATP receptors. Thus, the nucleotide should contain the β- or γ-phosphates in order to be an agonist of the receptors. Nucleotide triphosphates containing a heterocyclic base differing from adenine or cytosine did not activate ATP receptors. Therefore, the presence of the terminal phosphates represents a necessary but not sufficient condition for receptor activation.

Judging from the base selectivity and from the importance of length of the phosphate chain it seems possible to indicate two especially 'important points' in the ATP molecule that are crucial for the ligand properties. They are: (1) position 6 of the purine ring, since nucleoside triphosphates containing oxygen at the C6 of the base (e.g. GTP and ITP) are competi-

Table 2 Abbreviated formulae of the tested dinucleotides (A = adenosyl)

Abbreviation	*Structure of a analogue*
AP2A	
AP3A	
AP4A	
AP5A	
AP(CH2)PP(CH2)PA	
	
APP(CH2)PPA	R = H
APP(CHBr)PPA	Br
APP(CHCOCH3)PPA	$COCH_3$
APP(CHCOOC2H5)PPA	$CHCOOC_2H_5$

tive blockers; (2) the β- and γ-phosphates of polyphosphate chain, since the introduction of bulky groups into this chain or a decrease in the number of phosphate groups transforms an agonist into a partial agonist or into a competitive blocker (e.g. APPCC12P). The changes in the ribose moiety lead to changes in the apparent affinity. It seems likely that the carbohydrate residue is responsible for the orientation of both the heterocyclic base and the polyphosphate at the receptor since adenosine 5'-triphosphate 2',3'-acyclic dialcohol is an especially weak agonist.

Thus the data obtained demonstrate that the activity of nucleotides as agonists and antagonists of the ATP receptors in the membrane of sensory neurones depends on the structure of nucleotide base and phosphate chain.

The most effective receptor blocker among those tested in the present investigation was APPCC12P. This observation is in contrast to the data of Cusack *et al.* (1987). These authors have shown that APPCC12P mimics the effects of ATP in a number of smooth muscle preparations. It is of interest that a similar discrepancy exists for AMP, which is an antagonist in single smooth muscle cells from the guinea-pig urinary bladder (Marchenko *et al.*, 1987b) but an agonist in multicellular bladder preparations (Burnstock, 1978).

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