European Journal of Pharmacology, 80 (1982) 93–98
Elsevier Biomedical Press

INVESTIGATION OF RELAXATIONS OF THE RABBIT ANOCOCCYGEUS MUSCLE BY NERVE STIMULATION AND ATP USING THE ATP ANTAGONIST $ANAPP_3$ *

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Received 29 September 1981, revised MS received 15 January 1982, accepted 1 February 1982

P. SNEDDON, D.P. WESTFALL and J.S. FEDAN, *Investigation of relaxations of the rabbit anococcygeus muscle by nerve stimulation and ATP using the ATP antagonist $ANAPP_3$*, European J. Pharmacol. 80 (1982) 93–98.

When tone was raised by histamine (10^{-6} M), field stimulation (0.2–8 Hz) induced relaxation of the rabbit anococcygeus muscle in the continuous presence of guanethidine (10^{-5} M) and atropine (10^{-6} M). Similar relaxations could be induced by ATP and adenosine, which were approximately equipotent, but the non-hydrolyzable analogue β-γ-methylene ATP was less potent and produced relaxations which were slower. Although PGE_2 was a potent relaxant in this muscle, release of endogenous prostaglandins does not appear to mediate the response to ATP since indomethacin (2×10^{-5} M) pretreatment did not reduce responses to ATP. The specific ATP receptor antagonist, $ANAPP_3$ (10^{-4} or 10^{-3} M) did not reduce responses to nerve stimulation and only slightly reduced those to exogenous ATP. The results indicate that responses to ATP could be mediated partly by the products of its hydrolysis and do not support the proposal that ATP is the inhibitory transmitter in this muscle.

Anococcygeus muscle Purine-receptor antagonist

1. Introduction

A non-adrenergic, non-cholinergic inhibitory innervation has been demonstrated in many types of intestinal smooth muscle (see e.g. Campbell, 1970; Furness and Costa, 1973; Campbell and Gibbons, 1979). Burnstock and others have suggested that ATP could be the inhibitory transmitter and they have provided extensive experimental evidence in support of their hypothesis in a variety of smooth muscle, including the guinea-pig taenia coli and the anococcygeus muscle of the rabbit (see Brunstock et al., 1970; Burnstock, 1972; Burnstock et al., 1978).

The general acceptance of ATP as the inhibitory transmitter in these tissues has been hindered by the lack of a specific ATP antagonist. Recently, however, work in our laboratory on a photoaffinity label, $ANAPP_3$ (arylazido aminopropionyl ATP), which is a structural analogue of ATP, has indicated that this compound can specifically antagonize responses to ATP (Hogaboom et al., 1980; Fedan et al., 1981) presumably by blocking 'P_2'-type receptors (as defined by Burnstock, 1978). Recent work on the guinea-pig taenia coli by Westfall et al. (1982) showed that ATP-induced relaxations of this muscle are blocked by $ANAPP_3$, while similar relaxations induced by field stimulation of the inhibitory nerves were unaffected. This work strongly suggests that ATP is not the inhibitory transmitter, even though the P_2-receptor is well characterized in this tissue (Brown and Burnstock, 1981b).

We have now investigated the effects of $ANAPP_3$ on relaxations of the rabbit anococcygeus muscle induced by ATP and inhibitory nerve stimulation in order to test the proposal that ATP is the inhibitory transmitter in this tissue.

* Supported in part by a grant from the National Institute of Neurological and Communicative Diseases and Stroke, NIH NS-08300.

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2. Materials and methods

2.1. General

Albino, male rabbits (1.5–3.5 kg) were killed by a blow to the head and subsequently exsanguinated. In the rabbit the anococcygeus muscles originate from the upper coccygeal vertebrae and run caudally behind the terminal colon before inserting, one on either side, into the smooth muscle of the terminal colon about a centimeter from the anal margin. When dissected and mounted the tissues were about 3–4 cm long. (For details of dissection see Creed et al., 1977).

The tissues were mounted in a jacketed organ-bath at 37°C and bubbled with 95% O_2, 5% CO_2 in a physiological salt solution of the following composition (mM); NaCl 113; KCl 4.8; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; dextrose 5.5. The bath had a volume of 1.3 ml and was continuously suffused at a rate of 3 ml/min.

The anococcygeus muscle was passed through a pair of platinum ring electrodes. One end of the muscle was fixed and the other attached to a Grass force-displacement transducer (F.T. 03) for monitoring tension. Contractile activity was recorded on a Grass polygraph. Field stimulation of the tissue was produced via pulses (0.5 ms, supramaximal voltage) from a Grass S9 stimulator for a period of 30 s. Typically, an experiment was conducted as follows: the tissue was allowed to equilibrate for 30–60 min before a frequency response curve was obtained (2–64 Hz). Muscle tone was then raised by continuous suffusion with a solution containing 10^{-6} M histamine. The solution also contained 10^{-5} M guanethidine (to block adrenergic nerve-induced responses) and 10^{-6} M atropine (to block cholinergic nerve-induced responses). Inhibitory responses were then obtained to field stimulation at various frequencies. Inhibitory responses to various doses of ATP, adenosine or β-γ-methylene ATP were obtained by adding a small volume of the drug-containing solution while suffusion of the bath was temporarily interrupted. After washing out the histamine, atropine and guanethidine, the tissue underwent its experimental treatment (e.g., $ANAPP_3$, 10^{-4} M for 30 min). The solution containing histamine, atropine and guanethidine was then reintroduced and responses to field stimulation and the purines obtained as before.

As described by Hogaboom et al. (1980), $ANAPP_3$ becomes an irreversible ATP antagonist when irradiated with visible light. In all the experiments described below, $ANAPP_3$ was added to the bath containing the tissue and irradiated with a tungsten halogen projector lamp (DVY, 650 W, 3,400°K) for 30 min.

2.2. Drugs

The drugs used in this study (and their sources) are listed below: atropine sulphate (Sigma Chemical Company, St. Louis, MO); histamine diphosphate (Schwartz-Mann Research Labs, Orangeburg, N.Y.); guanethidine sulphate (CIBA-Geigy Corp., Summit, N.J.); adenosine 5′-triphosphate, disodium salt, β-γ-methylene adenosine 5′-triphosphate, sodium salt, and adenosine hemisulphate (Sigma Chemical Company, St. Louis, MO); PGE_2 (Upjohn Company, Kalamazoo, Mich); indomethacin (Merck, Sharp and Dohme, West Point, PA). $ANAPP_3$ was synthetized according to the method of Jeng and Guillory (1975).

2.3. Calculation of results

The inhibitory responses are expressed as the reduction in tension produced by the applied stimulus as a percentage of the total tone existing in the muscle immediately before the stimulus was applied. This is expressed on graphs as '% relaxation'. The points on the graph represent means ±S.E.M.

3. Results

The anococcygeus muscle responded to field stimulation with contraction over a range of frequencies from 2–64 Hz and, after tone was raised with histamine, with relaxation over a range from a single pulse to 4 Hz in most preparations (fig. 1). These responses were completely abolished by 3×10^{-7} M tetrodotoxin. ATP also produced rapid

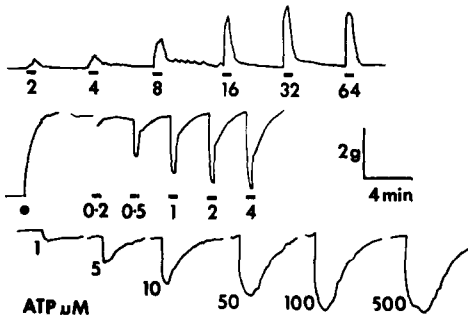


Fig. 1. Responses of the rabbit anococcygeus muscle to field stimulation and to ATP. *Top panel*: contractile responses were obtained before the introduction at (●) of histamine (10^{-6} M) guanethidine (10^{-5} M) and atrpoine (10^{-6} M). *Middle panel*: inhibitory responses could be obtained with a single pulse, and usually reached maximum at 4 Hz. *Bottom panel*: the muscle usually responded to ATP over a range from 5×10^{-7} to about 5×10^{-4} M. All responses are from the same muscle. On this, and all subsequent figures, frequency is expressed in Hz and drug concentrations in μM.

relaxations over a dose range from 5×10^{-7} to 5×10^{-4} M (fig. 1).

In preliminary experiments tissues were exposed to 10^{-4} M $ANAPP_3$ and irradiated for 3–10 min. In none of these experiments was there any noticeable antagonism of relaxations produced by field stimulation or ATP, even though such treatment blocks responses to ATP in both vas deferens and taenia coli of the guinea pig (Fedan et al., 1981; Westfall et al., 1981).

Therefore in all the experiments described below we increased the photoactivation period to 30 min to produce greater photoactivation of the compound. In none of 14 experiments performed was $ANAPP_3$ (10^{-4} M) able to antagonize responses to field stimulation. Even when the $ANAPP_3$ concentration was increased to 10^{-3} M there was no effect (figs. 2 and 3). Responses to ATP were noticeably reduced after $ANAPP_3$ in only 4 of the 14 experiments with 10^{-4} M $ANAPP_3$, and in 4 experiments with 10^{-3} M $ANAPP_3$, again only moderate antagonism was observed (figs. 2 and 4).

A possible reason for the inability of $ANAPP_3$ to block the response to ATP in this tissue could be that the response is not wholly mediated via P_2-receptors. We therefore examined the relative potency of ATP, adenosine, and β-γ-methylene ATP (an analogue of ATP which is resistant to

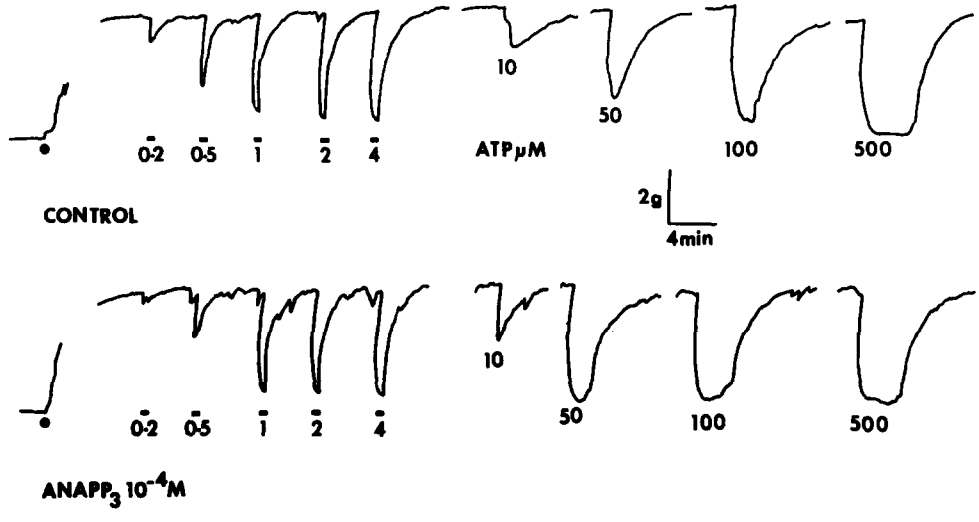


Fig. 2. The effect of $ANAPP_3$ (10^{-4} M) on inhibitory responses to ATP and nerve stimulation. Control responses (*top panel*) to nerve stimulation and ATP were obtained after histamine (10^{-6} M), guanethidine (10^{-5} M) and atropine (10^{-6} M) had been added (●). The drugs were then removed and the tissue exposed to 10^{-4} M $ANAPP_3$ and irradiated for 30 min. Histamine etc. were then reintroduced (●) and the responses to nerve stimulation and ATP repeated as before (bottom panel). All responses are from the same muscle.

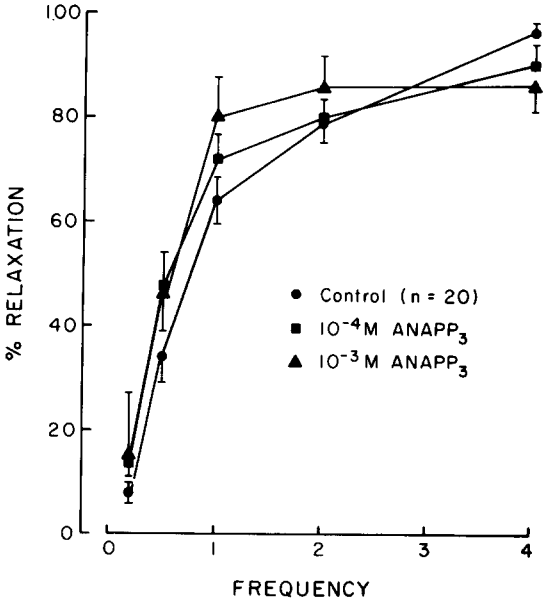
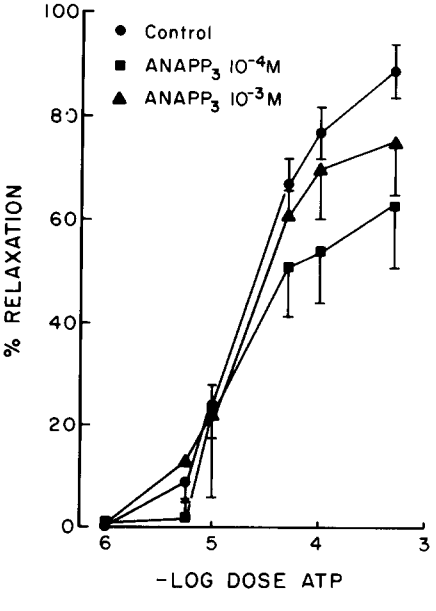


Fig. 3. The effect of ANAPP$_3$ on the frequency-response curve of the rabbit anococcygeus to field stimulation of the inhibitory nerves. Inhibitory responses were obtained to field stimulation under the conditions illustrated in fig. 2, i.e. in the presence of histamine, etc. The results are plotted as % relaxation as described in the Methods section. The results show that treatment with either 10^{-4} M ANAPP$_3$ (■, n=14), or with 10^{-3} M ANAPP$_c$ (▲, n=4) had no effect when compared with control responses (●, n=20).



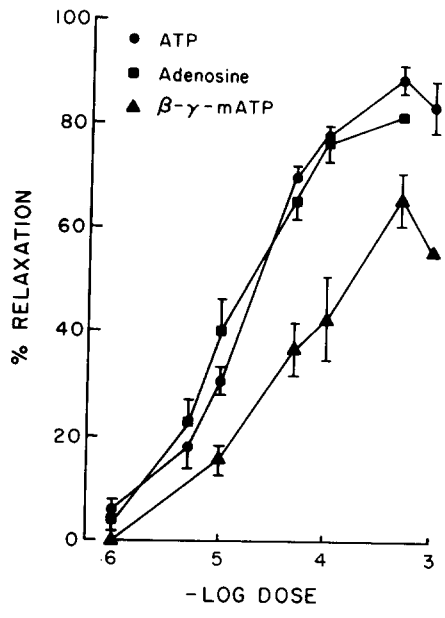


Fig. 5. Dose-response curves for ATP (●, n=18–48), adenosine (■, n=8–12) and β-γ-methylene ATP (▲, n=2–12) in rabbit anococcygeus.

hydrolysis by ATPases). Fig. 5 indicates that ATP and adenosine were, on average, approximately equipotent (although there was considerable variation between tissues), but β-γ-methylene ATP was significantly less potent. Furthermore, the response to β-γ-methylene ATP was often qualitatively different from that to ATP or adenosine, usually being much slower in its rate of relaxation and often preceded by a small contraction.

We tested the possibility that ATP might be acting via prostaglandin release, as has been demonstrated for example in tracheal smooth muscle (Brown and Burnstock, 1981a), where pretreatment with 2×10^{-5} M indomethacin completely inhibited relaxations induced by ATP. Since we found that PGE_2 was a potent relaxant in the anococcygeus muscle (fig. 6) we carried out experi-

Fig. 4. The effect of ANAPP$_3$ on the dose response-curve of the rabbit anococcygeus to ATP. Inhibitory responses were obtained to ATP under the conditions illustrated in fig. 2. The results show that treatment with either 10^{-4} M ANAPP$_3$ (■, n=14), or with 10^{-3} M ANAPP$_3$ (▲, n=4) produced only a moderate reduction in the responses to ATP when compared with control responses (●, n=20).

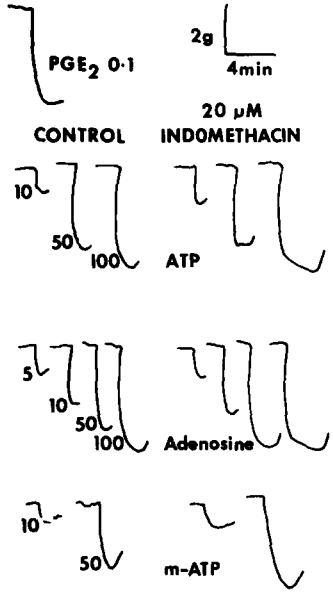


Fig. 6. The effect of idomethacin on responses of the rabbit anococcygeus to ATP. *Top panel*: PGE_2, at 0.1 μM caused complete relaxation of the rabbit anococcygeus. *Second panel*: pretreatment for approximately 30 min with indomethacin had no effect on responses subsequently obtained to ATP (on right) when compared with control responses (on left). Responses to adenosine (*third panel*) and β-γ-methylene ATP (mATP; *bottom panel*) also persisted in the presence of indomethacin 20 μM. All responses are from the same muscle.

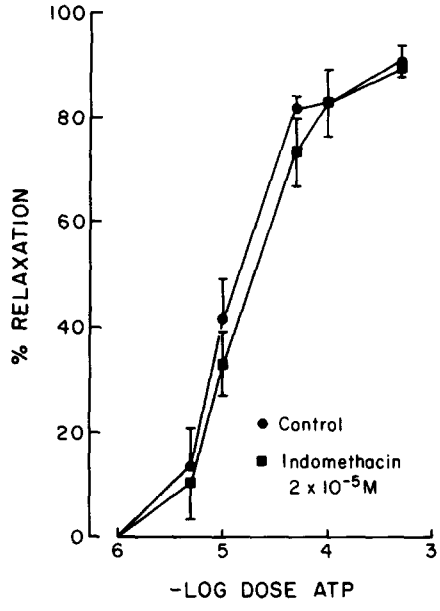


Fig. 7. The effect of indomethacin on responses of the rabbit anococcygeus to ATP. In hibitory responses to ATP were obtained as illustrated in fig. 6. Indomethacin (2×10^{-5} M) was introduced approximately 30 min before the second dose-response curve was obtained. The results indicate that pretreatment with indomethacin (■, n=10) had no effect when compared with control responses (●, n=10).

ments with indomethacin in this tissue. In none of these experiments did pretreatment with indomethacin (2×10^{-5} M) reduce ATP induced relaxations (figs. 6 and 7). Fig. 6 also indicates that indomethacin pretreatment did not antagonize responses to adenosine or β-γ-methylene ATP.

4. Discussion

The results presented indicate that the inhibitory action of ATP on histamine-induced tone of the rabbit anococcygeus muscle is unlikely to be mediated by prostaglandins, since pretreatment with indomethacin, at a dose which has been shown to be effective in other smooth muscles (see, e.g., Brown and Burnstock, 1981a), was without effect in this case.

The comparative potency of ATP, adenosine and β-γ-methylene ATP is worthy of note. The finding that in the rabbit anococcygeus ATP and adenosine are approximately equipotent, but β-γ-methylene ATP is less potent, is in striking contrast to the guinea-pig vas deferens, where β-γ-methylene ATP is 30–60 times more potent than ATP, and ATP considerably more potent than adenosine (Fedan et al., 1981). Also, in guinea-pig taenia coli ATP is at least ten times more potent than adenosine (Brown and Burnstock, 1981b). This could explain why $ANAPP_3$ is an effective antagonist to ATP in vas deferens and taenia coli but not in the rabbit anococcygeus, i.e., $ANAPP_3$ can only produce effective blockade of responses to ATP in tissues where ATP acts directly via P_2-receptors. $ANAPP_3$ has been shown to produce less antagonism of responses to adenosine than ATP in the taenia coli of the guinea pig, presumably reflecting its selectivity for the P_2- over P_1-receptor type (Westfall et al., 1981). If the inhibitory response to ATP in the anococcygeus is mediated, at least partly, by its breakdown products, e.g.

adenosine, then it might be expected to produce relaxation via P_1-receptors. In view of this hypothesis we have tried some experiments with methylaxanthines, which are, according to the classification of Burnstock (1978), able to antagonize P_1-mediated responses. However, since this treatment of itself produced some relaxation, its effect on responses to ATP etc. was equivocal.

In conclusion, the results presented indicate that ATP does not act via P_2-type, postsynaptic receptors in the rabbit anococcygeus, indicating that the model for purinergic transmission as proposed by Burnstock (1972, fig. 3c) probably does not operate in this tissue. This does not rule out the possibility that there are nerves in this muscle which release ATP. For example, it is possible that ATP (either when released from nerves or added exogenously) is broken down to adenosine, which then acts via P_1-receptors. This hypothesis can only be tested properly when a specific P_1-antagonist is available.

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