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RELAXATION OF GUINEA-PIG FUNDIC STRIP BY ADENOSINE, ADENOSINE TRIPHOSPHATE AND ELECTRICAL STIMULATION: LACK OF ANTAGONISM BY THEOPHYLLINE OR ATP TREATMENT

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1 Theophylline relaxed isolated strips of guinea-pig stomach fundus in a dose-dependent manner; above 50 to 100 μM responses showed no fade for up to 90 min.

2 Relaxant responses to adenosine, adenosine triphosphate (ATP), noradrenaline, and to electric field stimulation of non-adrenergic inhibitory nerves were not affected in a significant manner in the presence of 50 μM theophylline.

3 In tissues which showed complete fade of initial responses in the continued presence of 50 μM ATP, the effects of stimulation of non-adrenergic inhibitory nerves remained unaltered, suggesting that the ATP receptor has no function in non-adrenergic inhibitory transmission in this tissue.

4 These findings are opposite to those of Okwuasaba, Hamilton & Cook (1977), who claimed that 50 μM theophylline almost fully inhibited relaxation induced by adenosine, ATP and nerve stimulation and that ATP-induced fade also abolished sensitivity to inhibitory nerve stimulation.

Introduction

In 1970 it was suggested by Burnstock, Campbell, Satchell & Smythe that the transmitter substance of non-adrenergic, non-cholinergic inhibitory (NAI) nerves present in various gastrointestinal and other smooth muscle systems may be adenosine triphosphate (ATP) or a related purine substance. This suggestion was supported by the similarity of *in vitro* relaxation response profiles to nerve stimulation and to exogeneous ATP, enhanced release of purine substances from electrically stimulated preparations as well as a variety of circumstantial observations and claims (Burnstock, 1972).

This attractive 'purinergic nerve' hypothesis has since led to a number of studies in various laboratories, attempting to establish its validity in several smooth muscle systems and species. No conclusive proof of the hypothesis has been obtained as yet. It has become clear that the use of specific inhibitory substances of either the physiological transmitter or exogenous ATP could allow a differentiation between these two stimuli, or proof of their identity.

In adenosine and ATP-sensitive systems such as the coronary vasculature, theophylline has been found to function as an inhibitor (Afonso, 1970; Schaumann, Juhran & Dietmann, 1970), and it has been suggested that it acts as a competitive antagonist at adenosine receptors (Bünger, Haddy & Gerlach, 1975; Ally & Nakatsu, 1976). Unfortunately, this drug is known to cause direct effects on smooth muscle, usually relaxation, which may be related to its ability to interfere with Ca binding or movement and to inhibit cyclic nucleotide phosphodiesterase. Thus, while theophylline may indeed function as a competitive antagonist of adenosine or ATP receptors, these additional direct effects on contractile activity in smooth muscle have complicated or discouraged its use as a tool to probe the validity of the 'purinergic nerve' hypothesis. In this laboratory, theophylline could not be used in preparations such as the guinea-pig taenia coli, which has been used extensively as a model system of a 'purinergically' innervated tissue, because of its inability to antagonize adenosine-induced relaxation at concentrations not causing a direct relaxation.

In view of the foregoing, we noted with interest a recent study by Okwuasaba, Hamilton & Cook (1977) in which it was shown that theophylline caused effective antagonism of both ATP-induced and nerve-mediated relaxations of isolated strips from guinea-pig stomach fundus, thus providing rather strong support for the validity of the 'purinergic nerve' hypothesis in this system. Considering the importance of this

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finding, we have attempted to duplicate these studies. The following is an account of our inability to substantiate and reproduce essential parts of the work of the above authors.

Methods

Adult guinea-pigs of either sex were stunned and bled and their stomachs removed. Muscle strips (25 × 2 mm) were prepared from the fundic portion and partially freed of mucosa by careful trimming. The strips were suspended in 15 ml organ baths in Krebs-Henseleit buffer of the following composition (mM): NaCl 116, KCl 5.4, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 22 and D-glucose 11.2. The baths were gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. Atropine sulphate (0.5 μM) was routinely included.

Contractile activity was recorded isometrically with force displacement transducers (Grass FT03C) and a Beckmann Type RB Dynograph recorder. Tissues were placed initially under a load of 1 g and equilibrated for 60 min before drug testing.

Relaxant agonists were administered to the baths in a cumulative manner, each dose being left in the bath for 1 to 2 min and allowed to reach a plateau response before addition of the next dose. Following the initial dose or frequency-response study, one of a pair of tissues was treated with 50 μM theophylline for 30 min, the other serving as a control, and after washing with Krebs-Henseleit buffer (with and without theophylline, respectively), dose- or frequency-response studies were repeated. All inhibitory responses were expressed as percentages of the relaxation obtained with 15 mM $NaNO_2$ at the end of the experiment.

Transmural electric field stimulation was carried out on tissues treated with 4 μM guanethidine by means of a pair of shielded platinum ring electrodes and a Grass SD9 stimulator. Voltage sensitivity was determined for each tissue by stimulating at 5 Hz starting at 15 V and increasing in 5 V increments until maximum relaxation was obtained. A voltage of about 25% above maximum was then used. Frequency-responses were obtained in the range of 0.2 to 10 Hz (1 ms for 30 s), allowing 5 min recovery periods between stimulations. Significance of differences in experimental results were assumed if $P < 0.05$ in paired t test calculations.

The effect of theophylline on tissue responses to agonists or nerve stimulation was studied in a manner ensuring that time- and tissue-dependent variability were eliminated as error sources. The experimental design is outlined in Figure 1.

Thus, two strips from one animal were studied in parallel, one being exposed to the inhibitory drug, the other serving as a time control. Dose-response or frequency-response curves were obtained in both strips before and after theophylline treatment of the experimental strip. Most meaningfully, dose-response curves 2 and 4 (cf. Figure 1) were compared and are shown in all of the following figures. However, it was found that comparison of response curves 1 and 3 as well as response curves 1 and 2 indicated that no significant difference in these curves appeared, i.e., the number of animals (6 to 8) used in each study was adequate and the effects of experimental time were likewise insignificant. It was observed consistently, that in time (ca. 30 min, comparing response curves 1 and 2) a trend towards higher maximal response levels occurred with adenosine, ATP or nerve stimulation (cf. Figures 4, 5 and 7), while the opposite occurred with noradrenaline (cf. Figure 6), although the differences between results at any given dose level were insignificant ($P > 0.05$) in all cases.

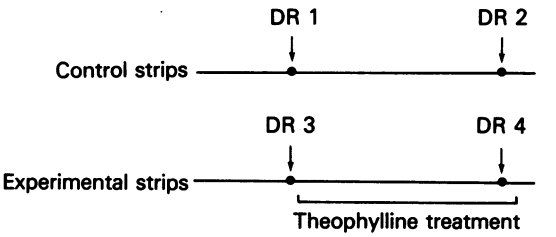

Figure 1 Experimental design of dose-response studies: following an equilibration period, cumulative dose-response (DR) studies with adenosine, ATP or noradrenaline, or frequency-response studies, were performed in control strips (DR 1) and experimental strips (DR 3). Following treatment of experimental strips with 50 μM theophylline for 30 min, dose or frequency-responses were repeated (DR 2 and DR 4) in the continued presence of theophylline.

The following drugs and suppliers were used: adenosine, ATP, (—)-noradrenaline bitartrate, propranolol hydrochloride, atropine sulphate, tetrodotoxin (Sigma); phentolamine hydrochloride (Mount Royal Chemicals); guanethidine sulphate (Ciba-Geigy); 6-(2-hydroxy-5-nitrobenzyl)-thioguanosine (HNBTG) (kindly donated by Dr A. R. P. Paterson, Cancer Research Unit, University of Alberta). Stock solutions (10 mM) were prepared in H_2O and stored frozen; 10 mM HNBTG was prepared in dimethylsulphoxide.

Results

General observations

Individual tissues exhibited different levels of tone

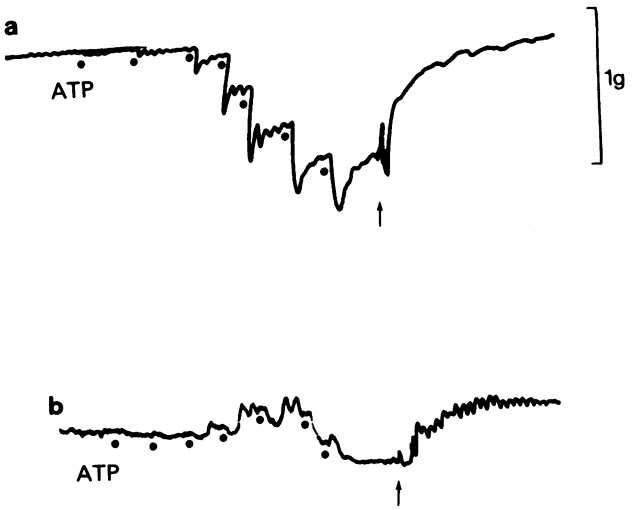

Figure 2 Typical response patterns to ATP: cumulative dose-response studies on individual strips of high tone (a) and low tone (b). Dots indicate additions of ATP to achieve concentrations of 0.3, 1, 3, 10, 30, 100 and 300 μM; arrows indicate wash out (2 min intervals between additions). Maximal responses following drug additions were used to construct dose-response curves.

and spontaneous activity even when obtained from one animal. High tone preparations showed less spontaneous activity than low tone preparations. Since inhibitory responses were studied, we have used only strips with high or moderate levels of tone. In the case of studies with ATP as the relaxant agonist, further selection of tissues was required since in some strips predominantly contractile activity was obtained. Thus, only those strips producing pure relaxation or predominantly relaxation were used. In the latter case quantification was carried out as indicated in Figure 2.

In the presence of 0.5 μM atropine and after treatment with 4 μM guanethidine for 15 min, fundic strips relaxed in response to electric field stimulation; the threshold frequency was 0.2 Hz and maximal relaxation occurred at 5 to 10 Hz. Inhibitory responses to nerve stimulation were not modified by α- and β-adrenoceptor blocking drugs such as phentolamine (1.6 μM) or propranolol (7 μM) but were fully eliminated by tetrodotoxin (0.6 μM) as tested at 10 Hz.

Studies of direct effects of theophylline

As is frequently observed with other smooth muscle preparations, guinea-pig fundic strips were relaxed by theophylline as shown in Figure 3. At the lower doses used, tissues, when left exposed to theophylline, redeveloped tone to within 10% of the original level within 30 to 45 min. However, at doses above 50

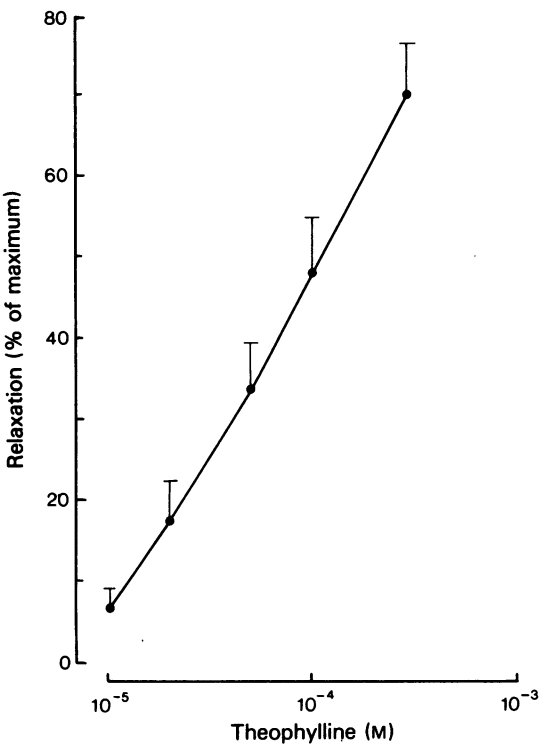

Figure 3 Dose-response curve to theophylline (molar concentrations). See text for details. Six strips from six animals were used. Vertical lines indicate s.e.

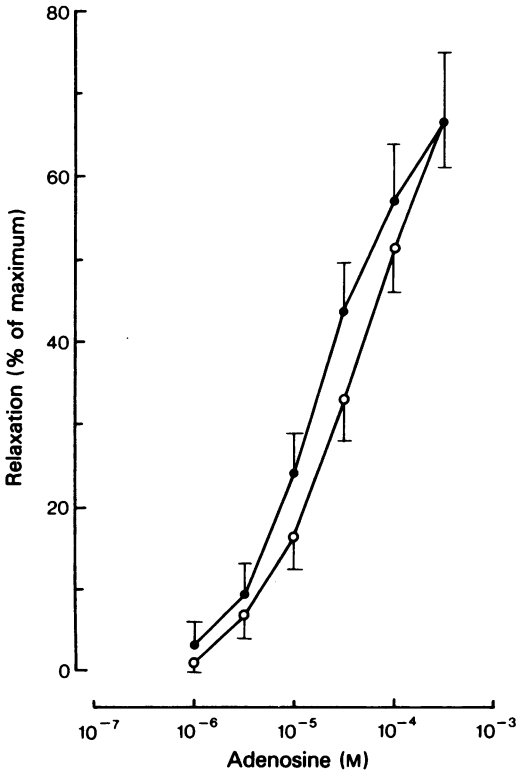

Figure 4 Dose-response curves to adenosine in the absence (●) and presence (○) of 50 μM theophylline. Details in text. Six strips from six animals were used for each curve. The individual responses with and without theophylline were not significantly different (paired *t* test; $P > 0.05$). Vertical lines indicate s.e.

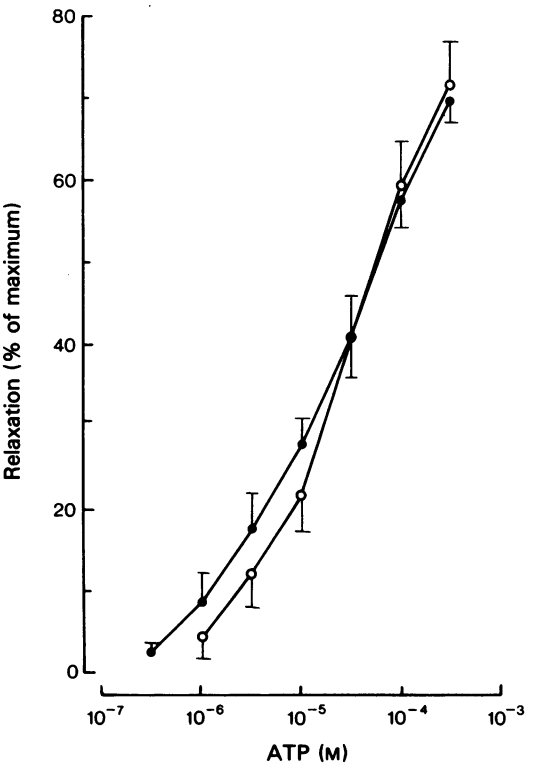

Figure 5 Dose-response curves to ATP in the absence (●) and presence (○) of 50 μM theophylline. Further details as in the legend of Figure 4.

to 100 μM, inhibition persisted. Accordingly, a dose of 50 μM theophylline could not be exceeded to study its possible antagonism to adenosine, ATP or nerve stimulation, in agreement with Okwuasaba *et al.* (1977).

Effects of theophylline on drug-induced relaxations

The effect of 50 μM theophylline on the response curves to adenosine, ATP, or noradrenaline was determined. It is evident from Figures 4, 5 and 6 that the methylxanthine did not affect the responsiveness of the tissue to adenosine, ATP or noradrenaline in a significant manner. With adenosine, a possible trend to a shift of the dose-response curve to the right is evident while a shift to the left may occur with noradrenaline.

Neither adenosine nor ATP produced a plateau response at 300 μM, accordingly no determination of ED_{50} value and quantitative comparison of their means was possible. However, considering that none of the responses at individual agonist concentrations was significantly different in the absence and presence of theophylline, no significant differences in ED_{50} values would be expected.

We have also measured dose-response curves to adenosine in the presence of 10 μM HNBTG, an inhibitor of adenosine uptake (Paterson, Kim, Bernhard & Cass, 1975). However, in the presence of HNBTG no change of the dose-response curve to adenosine in the presence of theophylline was observed (data not shown).

Effect of theophylline on frequency-response curves

Frequency-response curves obtained in the absence and presence of theophylline are shown in Figure 7. It is apparent that again no significant changes in

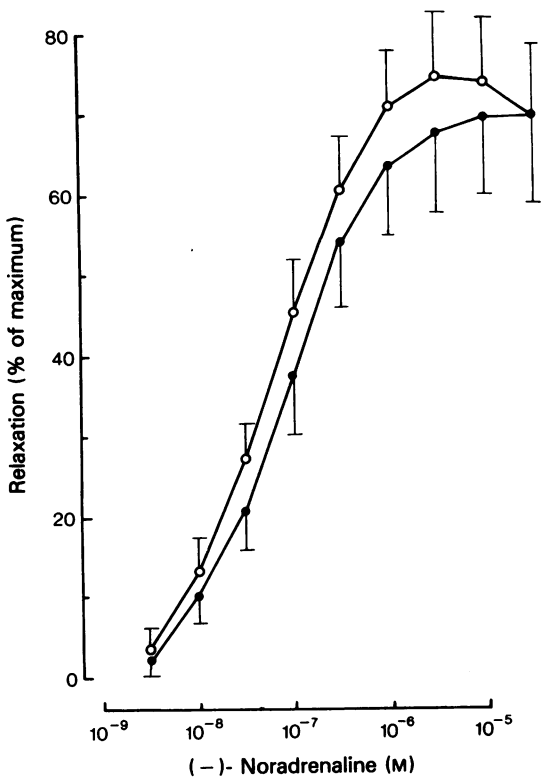

Figure 6 Dose-response curves to noradrenaline in the absence (●) and presence (○) of 50 μM theophylline. Further details as in the legend of Figure 4.

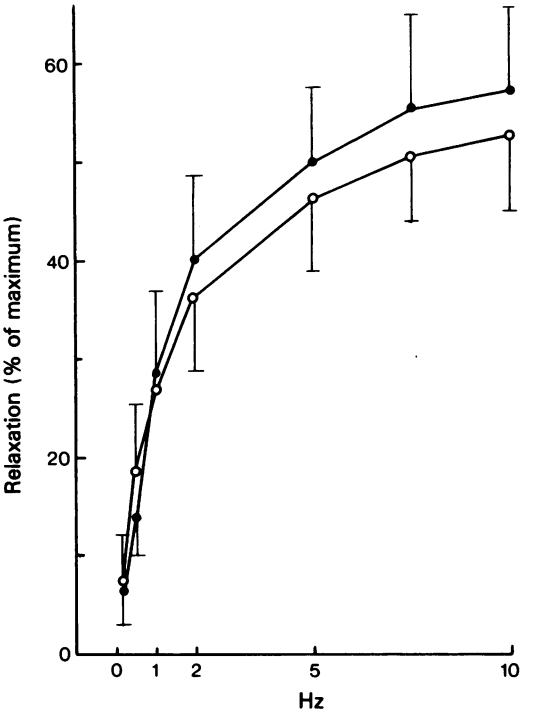

Figure 7 Frequency-response curves in the absence (●) and presence (○) of 50 μM theophylline, both atropine (0.5 μM) and guanethidine (4 μM) being present throughout. Eight pairs of strips from eight animals were used; further details as in the legend of Figure 4.

tissue responses are caused by the presence of theophylline.

Responsiveness to nerve stimulation in ATP-treated tissues

When tissues were exposed to 50 μM ATP for extended time periods to cause fade of responses, variable contractile patterns were observed. Five out of eleven preparations showed a relaxant response which persisted over 60 to 90 min; accordingly these could not be used in fade studies. The remaining tissues responded with an initial relaxation of varying magnitude followed by a contractile phase within 1 min which faded to control levels within 60 to 90 min. In these tissues, frequency-response curves had been measured before treatment with ATP and were repeated after the response had faded, i.e., when base lines were re-established. It is evident (Figure 8) that the continued presence of 50 μM ATP did not modify the responsiveness of the tissues to NAI nerve stimulation.

To assess any breakdown of ATP by phosphorylytic ecto-enzymes, which would be expected to lead to adenosine formation followed by rapid cellular uptake of the latter and deamination to inosine, aliquots from tissue baths were taken at the beginning and the end of the ATP treatment period and u.v. spectra recorded. No detectable change in the maxima (260 nm) and shape of the spectra occurred suggesting that the nucleotide remained stable throughout this period of incubation with fundic muscle strips.

Discussion

The present investigation has shown that theophylline at doses which caused no persistent relaxation of fundic strips from guinea-pig stomachs has no significant effects on the responsiveness of the tissue to adenosine, ATP, noradrenaline or NAI nerve stimulation. There is a possible trend to a shift of the response

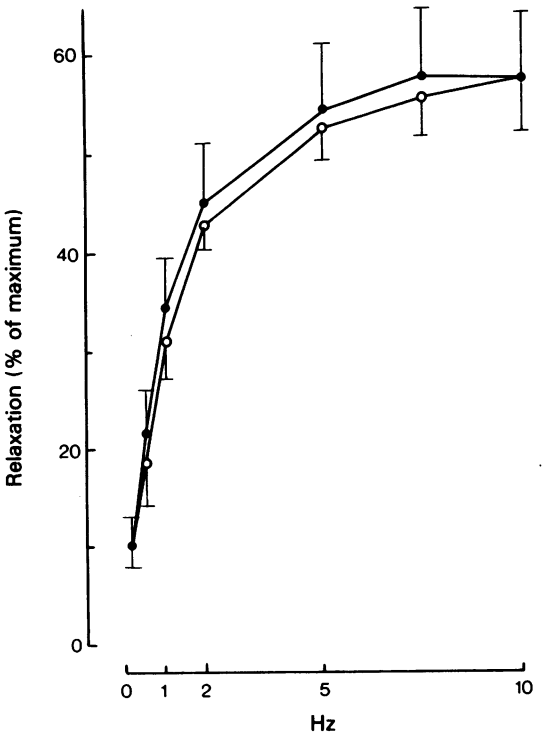

Figure 8 Frequency-response curves in tissues before (●) and after (○) treatment with, and in the continued presence of, 50 μM ATP. Atropine (0.5 μM) and guanethidine (4 μM) were present throughout. Six strips from six animals were used; further details in the text.

curve of adenosine to the right, i.e., indicating a possible blocking action of the methylxanthine. However, when adenosine uptake was inhibited by HNBTG there was still no change in the responsiveness of the tissue to adenosine in the presence of theophylline. In other systems, uptake of agonist has been shown to limit the potency and obscure the nature of antagonism of a competitive blocking agent (Furchgott, 1972; Humphrey, 1978), and this has recently been demonstrated to apply to adenosine and theophylline action as well (Clanachan, personal communication). Thus it is clear that 50 μM theophylline has no blocking action on the relaxant stimuli investigated; higher concentrations were not tested due to the irreversible, direct inhibitory effect of the drug. The possibility that theophylline binds to adenosine receptors at higher concentrations has not been excluded.

Thus our data conflict with those recently presented by Okwuasaba *et al.* (1977) who reported that 50 μM theophylline blocked almost fully the responsiveness of the tissue to adenosine, ATP and NAI nerve stimulation. These authors concluded that their work strongly supported the purinergic nerve hypothesis of Burnstock (1972).

In regard to the existing discrepancy, it should be emphasized that our experimental conditions were essentially identical to those used by Okwuasaba *et al.* (1977). The only apparent difference is that we have used isometric force transducers for recording contractile activity rather than auxotonic levers. In our initial studies, we attempted to use isotonic recording. Under these conditions we observed that most tissue preparations exhibited low tone, leading to only small relaxant responses to various stimuli. In a few preparations which exhibited high tone, 50 μM theophylline caused a persistent (75 min) relaxation of about 50% relative to 15 mM $NaNO_2$. Obviously, additional relaxant responses caused by adenosine, ATP or NAI nerve stimulation were then depressed relative to the respective base lines before and after theophylline treatment. However, the magnitude of responses to $NaNO_2$ under these conditions was similarly reduced.

We can thus only speculate that the discrepancy between the results of this study and those of Okwuasaba *et al.* (1977) is due to the latter authors, neglecting to assure the presence of a proper base-line for quantitative comparison, ignoring the direct and persistent relaxant action of theophylline. It is clear on the basis of the present results that the use of theophylline allows no conclusions regarding the 'purinergic nerve' hypothesis. Our data are in agreement with a similar investigation by Small & Weston (1979). Further, Cook & Hamilton (personal communication) have been unable to reproduce their previous results as reported by Okwuasaba *et al.* (1977).

With respect to fade studies, involving the measurement of tissue responsiveness in the continued presence of high doses of an agonist, it is evident from our data that under conditions where ATP shows a fade of its relaxation/contraction response, NAI nerve-stimulated responses occur without a change of the threshold frequency level or the shape and position of the frequency-response curve. A shift would be expected if the transmitter was indeed ATP or a compound acting upon the ATP receptor. However, it should be recognized that even if a significant change of the frequency-response curve following ATP treatment were observable, conclusions regarding the transmitter role of a purine compound could not be made with certainty since conceivably a presynaptic inhibitory action could exist. For example, adenosine reduces noradrenaline release presynaptically, the physiological significance of this action not being clear (Clanachan, 1979).

The latter results again contrast with those of Okwuasaba *et al.* (1977), in which the complexity of ATP treatment of fundic strips has not been discussed

and where it is claimed that responsiveness of the tissue to NAI nerve stimulation is fully eliminated following treatment of tissues with, and in the continued presence of, 50 μM ATP. Contrary to the conclusions of the latter authors, the results of the present fade studies with ATP suggest that the ATP receptor system plays no role in NAI nerve transmission in guinea-pig fundic muscle.

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