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[^{3}H]-adenosine nucleotide and [^{3}H]-noradrenaline uptake by cold stored guinea-pig taenia caecum; mechanical effects and release of [^{3}H]-adenosine nucleotide by noradrenaline, papaverine, and nitroglycerine

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Summary

1. Cold storage (2° C) treatment progressively reduced noradrenaline uptake by the taenia caecum of the guinea-pig. After 15 days of cold treatment, [^{3}H]-noradrenaline uptake by tissue was reduced to about 10% of control. On the other hand, prolonged cold storage failed to decrease [^{3}H]-adenosine uptake by the taenia caecum.

2. Cocaine (10 μM) inhibited noradrenaline uptake by about 82% but nucleoside uptake was inhibited by 20%. Cocaine treatment failed to decrease the residual noradrenaline uptake in the cold stored strips (more than 10 days). Phenoxybenzamine (1 μM) or oligomycin (1 μg/ml) treatment decreased the tissue adenosine uptake to about 34% and 28% of the respective controls.

3. Based on thin layer chromatography, it was estimated that approximately 68% of [^{3}H]-adenosine was converted into and retained as [^{3}H]-ATP in the fresh tissues, a small fraction was accountable as [^{3}H]-adenosine (18%) but virtually no [^{3}H]-AMP, [^{3}H]-cyclic AMP or [^{3}H]-ADP was detected. Similar distribution of radioactivity of nucleotides was observed in tissues cold stored for 8 days.

4. The inhibition of the mechanical activity of taenia by noradrenaline (10 μM), papaverine (100 μM) and nitroglycerine (100 μM) was accompanied by [^{3}H]-adenine nucleotide but not [^{3}H]-noradrenaline release. Treatment with phentolamine and propranolol (both 1 μM) had no effect on the adenine nucleotide release elicited by nicotine and electrical field stimulation, whereas such treatment reduced the inhibitory action of both stimuli.

5. These results suggest that the nucleotide release after application of electrical and chemical stimulation may be from an extraneurogenic source. Thus, we conclude that ATP or a related nucleotide is not the chemical transmitter of the non-adrenergic inhibition in the taenia caecum of the guinea-pig.

Introduction

It has been proposed that the smooth muscle in the mammalian gastrointestinal tract is subserved by two distinct inhibitory systems (Burnstock, 1972). One is adrenergic, conveyed through the postganglionic sympathetic nerves and in the taenia caecum activated by stimulation of the periarterial nerves innervating the

organ. The neuronal network of the second, on the other hand, is non-adrenergic, is intramural and is activated by electric field stimulation applied directly across the tissue. Whereas the anatomy, physiology and pharmacology of the adrenergic system is well established, comparatively little is known concerning the non-adrenergic innervation. Even the neurohumoral transmitter for this inhibition, although it has been postulated, has yet to be conclusively defined.

Burnstock, Campbell & Rand (1966) and Burnstock, Campbell, Satchell & Smythe (1970) have shown that the mechanisms underlying the relaxation of the guinea-pig taenia caecum produced by perivascular nerve stimulation and by transmural stimulation, differ physiologically as well as pharmacologically. These investigators also demonstrated that the taenia caecum can readily incorporate and accumulate adenosine from the bathing medium and in response to electric field stimulation of the taenia caecum release the newly synthesized adenine nucleotides. Based on these observations they suggest that the mediator substance of the non-adrenergic inhibitory innervation is ATP or a closely related nucleotide (Su, Bevan & Burnstock, 1971 ; Burnstock *et al.*, 1970 ; Satchell & Burnstock, 1971). On the other hand as shown in a previous paper (Kuchii, Miyahara & Shibata, 1973) the inhibitory action of nicotine and electrical field stimulation in the guinea-pig taenia caecum is not causally related with [^{3}H]-adenine nucleotide release.

The primary purpose of the present investigation was to compare in the isolated taenia caecum of the guinea-pig the uptake of adenosine and noradrenaline and to measure the release of adenine nucleotides and noradrenaline upon application of electrical and chemical stimulation. Because of the comparative nature of the study, a major portion of the work is concerned with whether procedures known to affect adrenergic transmitter reuptake, its storage and receptors have similar effects on the non-adrenergic inhibitory system. For this purpose the effects of several pharmacological agents and those produced by prolonged cold storage were examined.

Methods

Dorsal and ventral taenia caeca dissected from guinea-pigs of either sex weighing between 300 to 600 g were used in this study. Before initiating the experiment the taenia caeca were cut into 1·0 to 1·5 cm segments and kept in a 50 ml tissue bath containing Krebs bicarbonate buffer solution of the following composition (mM): NaCl, 120·3 ; KCl, 4·8 ; $CaCl_2$, 1·2 ; $MgSO_4$-$7H_2O$, 1·3 ; KH_2PO_4, 1·2 ; $NaHCO_3$, 24·2 ; and glucose, 5·5 ; bubbled with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37° C ; the pH was 7·4.

For uptake studies, the taenia caeca were cut into strips about 1 cm in length and allowed to equilibrate in the Krebs solution for 1 hour. These strips were then transferred into the incubation medium containing either (−)[7, ^{3}H]-noradrenaline (6·6 Ci/mmol, Amersham/Searle Corp.) or [^{3}H]-adenosine (G) (12 Ci/mmol, Amersham/Searle Corp.) at 0·1 μM for 1 hour. With incubation of [^{3}H]-noradrenaline the medium also contained 0·1 mg/ml of ascorbic acid and 1·5 μg/ml of the sodium salt of ethylene diamine tetra-acetic acid (EDTA) to prevent the autooxidation of noradrenaline. Determination of uptake was made radioisotopically after the taenia strips were prepared in the following manner:

the strips were rinsed three times in non-isotopic solution, blotted dry on filter paper, weighed, and digested in 0·5 ml of Soluene-100 (Packard) in polyethylene vials for 24 hours. Fifteen ml of scintillation flued (5 g POP, 0·05 g dimethyl POPOP in 1 l of toluene) was added to the tissue digest and the radioactive content determined on a Packard liquid scintillation counter. The uptake of [^{3}H]-noradrenaline or [^{3}H]-adenosine was expressed as ml of media fluid cleared per g of tissue.

The release of [^{3}H]-noradrenaline and [^{3}H]-adenosine nucleotide in the perfusate, and the tension changes after application of electrical field stimulation (60V, 30 Hz, 10 s) and nicotine (100 μM) were measured according to the method described in a previous paper (Kuchii *et al.*, 1973).

For cold storage treatment, the taenia caecum was placed in 250 ml Krebs solution and stored in a refrigerator set at $2 \pm 0{\cdot}5°$ C for the prescribed days as previously described (Fukuda & Shibata, 1972). After the cold treatment, the taenia strip was allowed to equilibrate in aerated Krebs Ringer (95% O_2–5% CO_2) at room temperature for 1 hour. The taenia strip was then transferred into the warm Ringer medium (37° C) for an additional 2 h before the experimental procedure was started.

Tissue synthesis of adenine nucleotide was determined by the following method. The taenia strip was incubated in [^{3}H]-adenosine for 1 h and homogenized in 10 volumes of ice cold 0·4 N perchloric acid solution. The perchlorate homogenate was then centrifuged at 18,000 x *g* for 15 min and the supernatant (10 μl) analyzed on a thin-layer paper chromatogram (ITLC type SAF Gelman Company) with a mixture of *n*-butanol, acetic acid, water (7:1:5) serving as the solvent front. A mixture of authentic adenosine, ATP, ADP, AMP and cyclic AMP was also applied to run concurrently on the same chromatogram. After separation and detection of the nucleotides on the basis of their R_F values and development by an ultraviolet lamp, the corresponding bands were cut out and extracted in 2 ml methanol in a 20 ml polyethylene vial for 24 hours. After this procedure the scintillator was added to the extraction solution and the radioactivity counted spectrometrically to determine the level of tritium content in each adenine nucleotide band.

All drug solutions were prepared from aqueous stock solutions and the requisite dilutions made with the Krebs Ringer. The following drugs and compounds were used: (−)-noradrenaline bitartrate, papavarine, nitroglycerine, acetylcholine chloride, potassium chloride, oligomycin, phenoxybenzamine hydrochloride, cocaine hydrochloride, phentolamine hydrochloride and propranolol hydrochloride (Smith-Kline & French Labs.). The concentrations of drugs tested are expressed as final concentration in the tissue bath.

Results

Uptake studies

When the freshly dissected taenia strips were incubated with tritiated noradrenaline or adenosine (both 0·1 μM) for 1 h, the tissues incorporated the nucleoside more readily than noradrenaline (Figure 1). The uptake for [^{3}H]-adenosine and [^{3}H]-noradrenaline, expressed as millilitres of incubation fluid

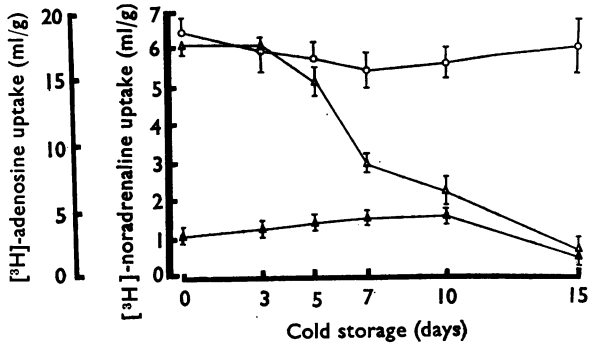

FIG. 1 Effect of cold storage treatment on the [³H]-adenosine (○——○) and [³H]-noradrenaline uptake (△——△) of taenia strips and the effect of cocaine (▲——▲) (10 μM) on the [³H]-noradrenaline uptake of fresh and cold stored taenia. Ordinates show the uptake of [³H]-adenosine and [³H]-noradrenaline; abscissae indicate the days of the cold storage treatment. Vertical lines show ± S.E. mean of 7 experiments.

cleared per g of tissue, was 18·0 and 6·3 times the tissue volume, respectively. As shown in this figure the uptake process for these substances was differentially affected by cold storage treatment and by cocaine (10 μM). Even in the strips stored for 15 days, uptake of [³H]-adenosine was not significantly affected. Preincubation for 20 min in cocaine caused only a slight decrease (19·9%) in adenosine uptake in the fresh preparations (Table 1). In contrast, cold storage treatment and cocaine had a marked effect on the uptake of [³H]-noradrenaline by the taenia strips. The tissue [³H]-noradrenaline uptake remained unaffected up to about the 4th day of cold storage, but after 5 days of treatment and thereafter

TABLE 1. *Effect of cocaine, phenoxybenzamine and oligomycin on the [³H]-adenosine uptake by taenia caecum of guinea-pig*

[³H]-Adenosine (ml/g)			
Control	Cocaine	Phenoxybenzamine	Oligomycin
18·1±1·0	14·5±0·8	6·3±0·68	5·0±0·6

Each value indicates mean±S.E.M. of 5 experiments. Tissues were pretreated with cocaine (10 μM), phenoxybenzamine (5 μM) or oligomycin (1 μg/ml) for 20 minutes.

the ability of the taenia to concentrate noradrenaline sharply declined. By about the 15th day of cold treatment noradrenaline clearance was reduced to a fairly constant level which was not alterable by further treatment. After cocaine, on the other hand, tissue noradrenaline uptake in the fresh taenia preparation was consistently decreased by 75–80%; and this residual uptake, resistant to cocaine action, remained functional almost throughout the course of the cold storage treatment. About the 15th day of cold treatment the tissue [³H]-noradrenaline content was still detectable over background, indicating the existence of some residual uptake. At this point noradrenaline uptake after the cocaine plus cold storage treatment was not appreciably different from that after the cold treatment alone.

The effects of phenoxybenzamine (10 μM), an agent known to affect noradrenaline uptake, and oligomycin (1 μg/ml), an inhibitor of ATP synthesis, were tested on tissue [³H]-adenosine uptake (Table 1). The net uptake of [³H]-adenosine after phenoxybenzamine and oligomycin was 34·2% and 27·8% of control, respectively.

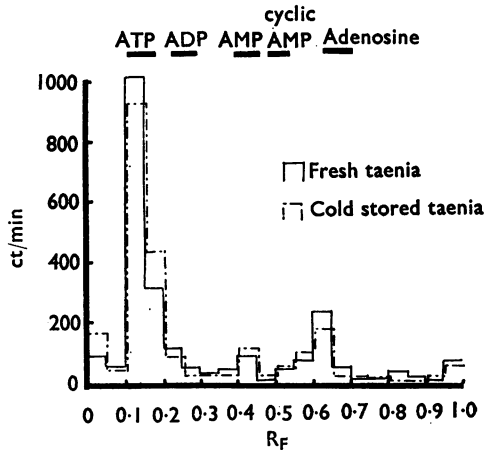

FIG. 2. Radioactivity distribution of nucleotides extracted from fresh (continuous lines) and 8 days cold stored (broken lines) taenia caeca (see text). Solid black bars indicate and the postion of marker spot for ATP and related nucleotides (ADP, AMP, cyclic AMP and adenosine).

Biotransformation studies

Although the taenia strip incorporates and concentrates [^{3}H]-adenosine from the bathing medium through an uptake system, the immediate question concerns the ultimate biotransformation product of adenosine. In the chromatogram in Fig. 2 are shown the location and identification of the tritiated products when the extract of the [^{3}H]-adenosine-incubated taenia strips was subjected to thin-layer chromatography. From the radioactivity content of the bands and from the R_F values, it was shown that ^{3}H activity was contained mostly in the ATP band (68%); the remainder was distributed as adenosine (18%) with only traces of AMP, cyclic AMP and ADP (less than 5%). This indicates that the predominant biotransformation product of administered [^{3}H]-adenosine in the taenia was [^{3}H]-ATP. These results corroborate those presented by Su *et al.* (1971). Although attempts were made to separate and identify the nucleotides released in the perfusate by nicotine and electrical stimulation, the results were inconclusive presumably because of the low radioactivity in the separate fractions and perhaps the chemical alteration of released nucleotides.

Effects of noradrenaline, papaverine and nitroglycerine

Since adenine nucleotide release accompanied the muscle relaxation elicited by electrical stimulation, several pharmacological agents, capable of inducing muscle relaxation, were tested on this release of [^{3}H]-nucleotide (Figure 3). The agents used were noradrenaline (1 μM) papaverine (200 μM) and nitroglycerine (200 μM). As may be seen, at the doses selected each agent produced comparable muscular relaxation. For the same degree of relaxation the release of [^{3}H]-nucleotide was almost equivalent for noradrenaline and papaverine and comparable to transmural stimulation (Kuchii *et al.*, 1973) but for nitroglycerine the release was less marked. Although not presented in the figure, similar experiments conducted on [^{3}H]-noradrenaline incubated taenia strips showed no release of [^{3}H]-noradrenaline.

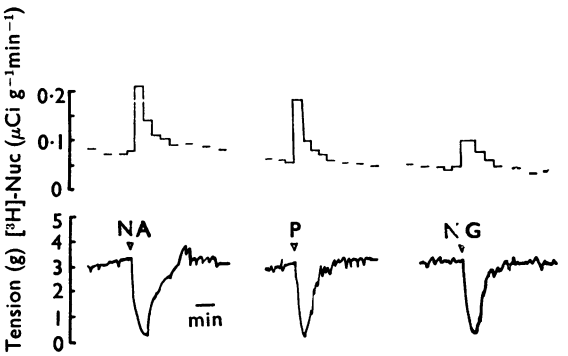

FIG. 3. Effect of the application of noradrenaline (NA, 10 μM), papaverine (P, 100 μM) and nitroglycerine (NG, 100 μM) on [³H]-adenine nucleotide ([³H]-Nuc) release and on the mechanical activity of the taenia caecum.

Effect of adrenoceptor blocking agents

Since the application of exogenous noradrenaline to the taenia facilitated the release of adenine nucleotide, some of the nucleotide release might be mediated through activation of adrenoceptors. Thus, the effects of adrenoceptor blocking agents on the tritiated nucleotide and noradrenaline release were studied.

The combined effects of phentolamine and propranolol (both 1 μM) on [³H]-nucleotide and [³H]-noradrenaline release and on the inhibitory responses to nicotine and electrical field stimulation are shown in Figs. 4 and 5, respectively. Inhibitory activity of both nicotine (100 μM) and electrical stimulation (60V, 30 Hz, 10 s) was associated with [³H]-nucleotide and [³H]-noradrenaline release.

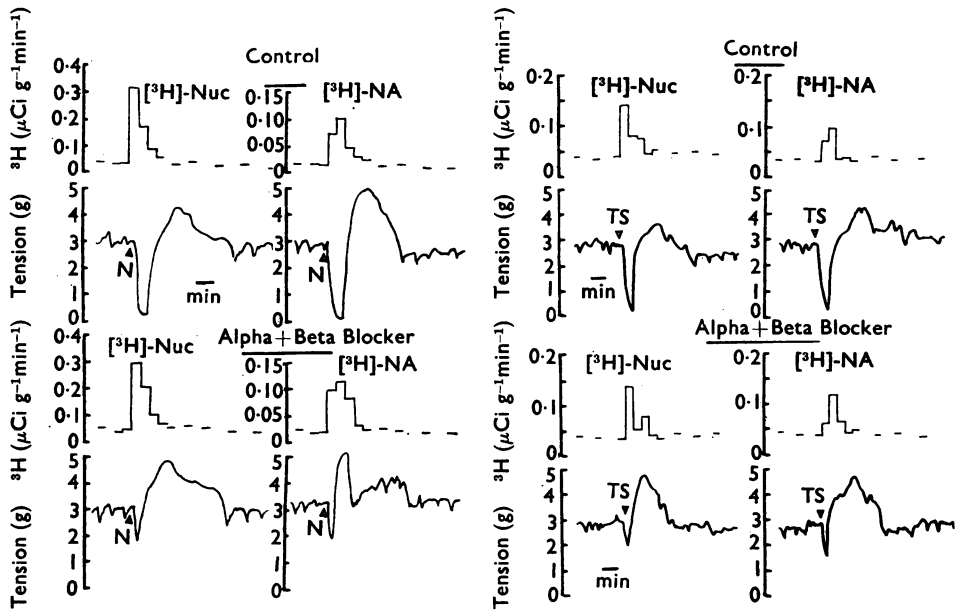

FIG. 4. Effects of treatment with phentolamine and propranolol (both 1 μM) on the [³H]-adenine nucleotide ([³H]-Nuc) and [³H]-noradrenaline ([³H]-NA) release and on the inhibitory action evoked by nicotine (N, 100 μM).

FIG. 5. (Above right.) Effect of treatment with phentolamine and propranolol (both 1 μM) on the [³H]-adenine nucleotide ([³H]-Nuc) and [³H]-noradrenaline ([³H]-NA) release and on the inhibitory action of electrical field stimulation (60V, 30 Hz, 10 s).

However, after treatment with the adrenoceptor blocking agents the inhibitory action was diminished, but not abolished, without any significant change in the release of both nucleotide and noradrenaline.

Discussion

The present investigation has confirmed the observation that the isolated taenia caecum of the guinea-pig actively accumulates adenosine from the bathing medium, converts adenosine to adenine nucleotide and releases the formed adenine nucleotides upon adequate stimulation (Su *et al.*, 1971). From the results of our radioisotope studies it would appear that, in contrast to noradrenaline uptake, the mechanism for adenosine was more effective and more resistant to alteration by chemical and physical changes. The taenia uptake process for adenosine, in contrast to that for noradrenaline, was not depressed throughout the course of the cold storage treatment; after 15 days of treatment the uptake of [^{3}H]-adenosine by the cold stored taenia was not significantly different from the fresh strip. This finding is especially significant in light of the demonstration that both adrenergic and cholinergic nerve fibres in the taenia caecum undergo degeneration subsequent to such prolonged cold treatment (Hattori, Kurahashi, Mori & Shibata, 1972).

It is well established that there are two sites where uptake of noradrenaline can occur: at the presynaptic or adrenergic nerve terminal and at the postsynaptic or the neuroeffector organ (Iversen, 1971). Although there are two functionally distinct sites, the former is thought to be the principal site of uptake inasmuch as tissue incorporation of noradrenaline is severely affected when neuronal uptake is inhibited. Our results on the effects of drugs and of cold storage on noradrenaline uptake are in accord with this postulate in that noradrenaline uptake was markedly reduced by cocaine and that once neuronal uptake was inhibited by the cold treatment, this drug had no effect. Moreover, the effect of cold storage on noradrenaline uptake was coincident with the appearance of histochemical signs of degeneration of adrenergic nerves in the taenia (Hattori *et al.*, 1972). A similar inhibitory action of cold storage was observed in the adrenergic nerve terminals in vascular smooth muscle (Shibata, Hattori, Sakurai, Mori & Fujiwara, 1971; Shibata, Kuchii, Hattori & Fujiwara, 1972).

The strong inhibitory action of phenoxybenzamine, in comparison to cocaine, on adenosine uptake, may indicate that exogenous adenosine is taken up by non-neural tissue. In addition, the decrease in [^{3}H]-adenosine uptake by oligomycin treatment leads us to speculate that the uptake process of adenosine is energy dependent.

Burnstock (1972) and co-workers proposed that in addition to the classical adrenergic and cholinergic components in the autonomic nervous system there also exist 'purinergic' nerves. In proposing their purinergic nerve hypothesis they presented a cogent argument favouring ATP as the transmitter substance at the synapse of non-adrenergic inhibitory nerves while discounting any extra-neuronal site or other possible source of the released ATP. In our experiments the taenia strips readily incorporated adenosine from the medium; however, we suggest that our results cannot be interpreted solely on the basis of neurogenic uptake. These data are more compatible with the view that active uptake of adenosine is carried out extraneuronally, primarily at the muscle membrane site. After prolonged cold storage, at a time when noradrenaline uptake was reduced

to the low basal level, the taenia strips were still capable of concentrating adenosine as in the fresh preparations. We believe that it is likely that, after prolonged cold-storage, all neuronal tissues have undergone degeneration while only the muscle fibres are functionally intact (Shibata, Hattori & Timmerman, 1970 ; Hattori *et al.*, 1972; Fukuda & Shibata, 1972). The site of biotransformation of the incorporated adenosine to ATP presents no problem and does not conflict with our postulation since enzyme conversion of such an ubiquitous compound as ATP can take place in muscle cells as well as in neurones. The immediate question then concerns the source of the released ATP. Burnstock and co-workers demonstrated in various preparations and in a variety of species that ATP release is evoked by stimulation of the non-adrenergic inhibitory nerves. For the taenia caecum of the guinea-pig these workers concluded that these neurones are intramural without extrinsic connection but can be stimulated selectively by transmural stimulation and by ganglion stimulating agents. In our previous paper (Kuchii *et al.*, 1973), the relaxation of the taenia strip by perivascular nerve stimulation was associated mainly with efflux of noradrenaline but also a trace of adenine nucleotide, whereas that produced by transmural stimulation and by application of nicotine caused the release of both noradrenaline and adenine nucleotide. Since adenine nucleotide can still be released by electric field stimulation from taenia strips subjected to prolonged cold treatment in which only muscle contraction or excitation occurred, and since this releasing effect is shared by drugs, such as noradrenaline, papaverine and nitroglycerine that act directly on smooth muscle, the adenine nucleotide release is related more to the mechanical response than to neuronal activity. Furthermore, since adrenoceptor blocking agents had no effect on the adenine nucleotide release by noradrenaline, this nucleotide release is not mediated by the stimulation of adrenoceptors.

Our results support the theory that the neuroeffector organ is the most likely site of adenosine uptake and the principle source of the released ATP.

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