J. Physiol. (1980), **299**, *pp.* 75–83
With 4 text-figures
Printed in Great Britain

A COMPARISON OF EFFECTS EVOKED IN GUINEA-PIG TAENIA CAECUM BY PURINE NUCLEOTIDES AND BY 'PURINERGIC' NERVE STIMULATION

BY L. P. JAGER AND J. A. M. SCHEVERS

From the Department of Pharmacology, Faculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH, Utrecht, The Netherlands

(*Received 29 May 1979*)

SUMMARY

1. Effects of adenosine and the adenosine nucleotides (ATP, ADP, AMP and CoA) on the guinea-pig taenia caecum were studied by measuring simultaneously the changes in membrane potential and in contractility induced by these agents using the sucrose-gap technique.

2. In the presence of carbachol (5×10^{-8} M) the hyperpolarization and the relaxation induced by these agents was found to be closely correlated. Their different potencies suggested an action on the P_2 type purinoceptor: ATP $\geqslant$ ADP $\geqslant$ AMP > CoA > AD.

3. The transmitter released endogenously in the presence of carbachol (5×10^{-8} M) by the non-adrenergic inhibitory (n.a.i.) nerves after half-maximal stimulation induced an inhibitory junction potential (i.j.p.) which transiently counteracted the carbachol-induced depolarization. This i.j.p. was mimicked by ATP, ADP, and AMP applied exogenously in concentrations of about 3×10^{-5} M, by CoA (3×10^{-4} M) and by adenosine (3×10^{-3} M).

4. The results presented are in agreement with the hypothesis that the transmitter substance released by the n.a.i. nerves is a purine nucleotide, which in the guinea-pig taenia caecum affects the smooth muscle cell membrane via the P_2-purinoceptor. Of the putative transmitters studied, ATP, ADP and AMP seem the most likely.

INTRODUCTION

The presence of non-adrenergic inhibitory (n.a.i.) nerves within the gastro-intestinal wall has been proved beyond reasonable doubt (Bülbring & Tomita, 1967). The identity of the transmitter substance, released by these nerves (Jager & Den Hertog, 1974), is less certain. Currently only the purinergic nerve hypothesis (Satchell, Burnstock & Campbell, 1969; Burnstock, 1977) meets most of the criteria given by Eccles (1964) to the extent that the response profiles of intestinal smooth muscle to n.a.i. nerve stimulation (Den Hertog & Jager, 1975) are mimicked qualitatively by those to exogenously applied purine nucleotides (Tomita & Watanabe, 1973; Jager, 1979). However, the membrane hyperpolarization (i.j.p.) caused by K efflux, which is responsible for n.a.i. nerve-mediated relaxation response in intestinal muscle, is not evident with a dose of ATP causing relaxation (Tomita & Watanabe, 1973). This

0022-3751/80/6550-0248 $07.50

observation is corroborated by comparing the dose–response curve of ATP concerning relaxation (Satchell & Burnstock, 1975) with that concerning membrane hyperpolarization (Jager, 1974) both measured with the guinea-pig's taenia caecum. Only high doses of exogenously applied purine nucleotides above 100 μM cause membrane potential changes, comparable to those caused by the physiological n.a.i. transmitter.

Recently Burnstock (1978) proposed that there are two types of purinergic receptors, having different affinities for the adenosine moiety and for the phosphorylated group. Following a suggestion made by Tomita & Watanabe (1973) this proposition was extended by supposing that (i) the relaxation induced with adenosine and low concentrations of adenosine nucleotides is mediated via readily accessible P_1 purinoceptors, not affecting membrane hyperpolarization but increasing the cyclic AMP content of smooth muscle cells; (ii) the relaxation induced with high concentrations of adenosine nucleotides and by n.a.i. nerve stimulation is mediated via relatively inaccessible P_2 purinoceptors, hyperpolarizing the smooth muscle cell membrane (Jager, 1979).

To test this hypothesis simultaneous measurements of muscle relaxation and membrane hyperpolarization induced by n.a.i. nerve stimulation or by exogenously applied adenosine and adenosine nucleotides were made. Because coenzyme A (CoA) has also been implicated in the purinergic nerve hypothesis (Cook, Hamilton & Okwuasaba, 1978) this substituted adenosine nucleotide was included in the present study.

METHODS

Guinea-pigs (CPB-TNO, Zeist, the Netherlands) of either sex (250–300 g) were stunned and bled. Strips of the taenia caecum of about 1 mm thick and 4 cm long were used throughout.

The composition of the Locke solution used was (mM): NaCl 155·6, KCl 4, $CaCl_2$ 2·2, $NaHCO_3$ 1·8, glucose 5·6 Hepes 4, and guanethidine, $2{\cdot}5 \times 10^{-3}$. The solution was equilibrated with 100 % O_2 at 30 ± 0·2 °C and the pH of the solution was adjusted to 7·3.

The sucrose gap method was used to measure changes in membrane potential and contractility simultaneously. All experiments were carried out at 30 °C because at this temperature the preparations were generally maximally relaxed and electrically quiescent on which bursts of spontaneous electrical and mechanical activity were superposed. Furthermore, at this temperature carbachol- (5×10^{-8} M) induced depolarizations and contractions (8·1 ± 0·5 mV; 20 ± 3 mN; $n = 6$) which remained constant for minutes. Potential changes across the sucrose gap were measured by means of calomel electrodes making contact with the test solution and the reference solution (isotonic KCl). Potential changes were recorded, via a cathode follower (WPI 750), with a multichannel potentiometric recorder (W + W 314). Changes in contractility were measured isometrically, preload 5 mN, via a force transducer (Hottinger-Baldwin 0·5 W with 3080 amplifier) and recorded with the same recorder. All signals were monitored on a storage oscilloscope (Textronic 5115) to measure time courses.

Responses mediated via n.a.i. nerves were elicited by field stimulation (pulse rate 30 sec^{-1}; pulse duration 0·3 msec; supramaximal intensity: about 15 $V.mm^{-1}$).

After mounting the preparation was allowed to stabilize for an hour; it was superfused with Locke solution. Then the superfusion medium was changed to Locke solution containing carbachol. When a steady depolarization and contraction was observed (after ± 3 min) the superfusion medium was changed to Locke solution containing carbachol and the agonist. The preparation was studied during 2 min, after which superfusion was resumed with Locke solution containing carbachol. The change to Locke solution was made when the steady state contraction and depolarization was reached again (± 2 min). This sequence was then repeated after 25 min using another concentration of agonist.

Generally one preparation was used for one complete series of concentrations of an agonist, the sequence of the concentrations was chosen at random.

All data are given as mean ± standard error of the mean ($m \pm$ S.E. of mean). In the figures the S.E. of mean is only presented if it exceeds the dimensions of the symbol. Statistical analysis of the concentration–response curves was based on regression lines, calculated with the method of least squares, for that part of the concentration range where the mean response lies between 25 and 75% of the maximal response including at least three concentrations. Statistical tests were made according to the procedures given by Diem & Lentner (1969). Differences were assumed to be real when tests gave probability levels smaller than 5%.

The drugs used were: ATP (adenosine-5-triphosphate di-Na salt, Sigma); ADP (adenosine-5-diphosphate di-Na salt, Sigma); AMP (adenosine-5-monophosphate di-Na salt, Sigma); adenosine (Sigma); coenzyme A (Na salt, Sigma), carbachol (carbamoylcholine hydrochloride, Merck); guanethidine sulphate (Ciba); Hepes (2-[4-(2-hydroxy-ethyl)-1-piperacinyl]-ethanesulphonic acid, Merck). After addition of the drugs the pH of the solutions was adjusted to 7·3 with NaOH or HCl.

RESULTS

General

In order to obtain preparations with a constant contracture carbachol (5×10^{-8} M) was added to induce reproducible half-maximal contractures. The membrane depolarization evoked with carbachol was also constant throughout the experiments. The carbachol contracture and depolarization of individual experiments were used as reference and to calibrate the ordinates of the concentration–response curves made. A calibration factor of about 0·4 V.N.$^{-1}$ was found in the present experiments.

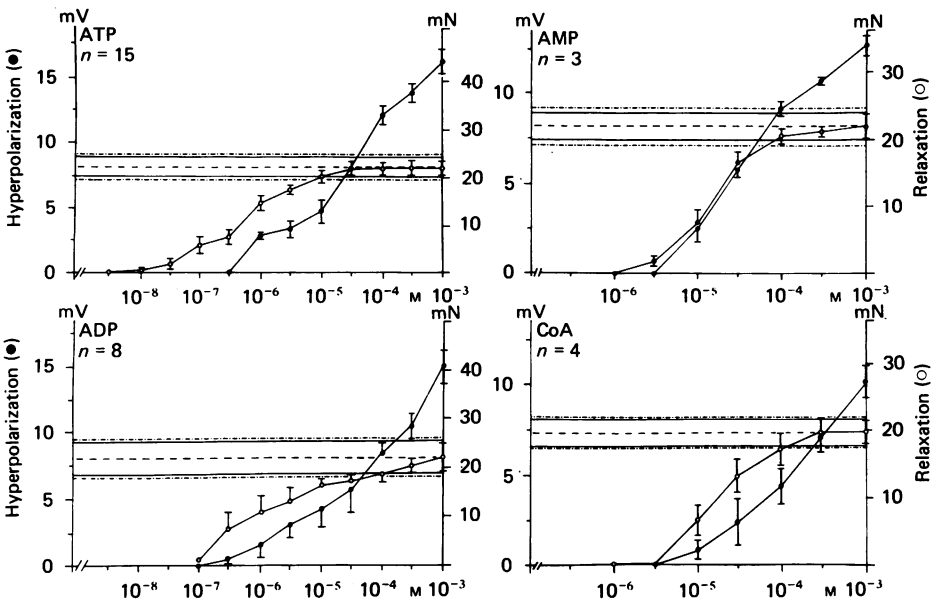

Fig. 1. The muscle relaxation (○) and the cell membrane hyperpolarization (●) induced by adenosine nucleiotides at different concentrations in the presence of carbachol (5×10^{-8} M) at 30 ± 0·2 °C. The level of the resting membrane potential and of the maximal relaxation is indicated by (— —), the S.E. of mean of the carbachol-induced depolarization is indicated by (——), the S.E. of mean of the carbachol-induced contraction by (–·–·). Each point represents the mean of values obtained from at least n preparations and is expressed ± S.E. of mean.

This made it possible to represent the relaxations and the membrane hyperpolarizations in one graph. The carbachol-induced depolarization and contracture are used to indicate in the graphs the level of the resting membrane potential and the level of maximal relaxation. Furthermore, as it is possible to induce membrane hyperpolarizations in preparations maximally relaxed, it was thus possible to take into account the 'effective' membrane hyperpolarization inducing relaxation.

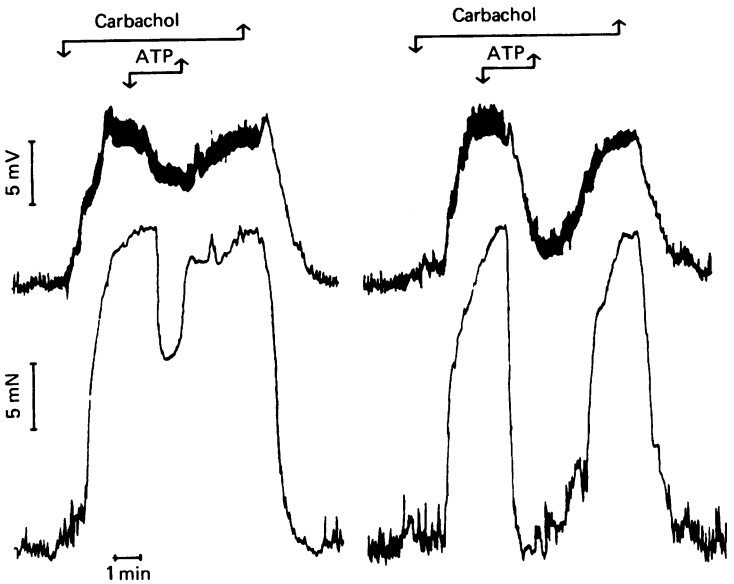

Fig. 2. Effects of ATP (left 10^{-6} M, right 3×10^{-5} M) on the membrane potential (upper trace) and on the contractility (lower trace) of the guinea-pig's caecum in the presence of carbachol (5×10^{-8} M) at $30 \pm 0{\cdot}2$ °C. Note the recovery of the contractility in the presence of 10^{-6} M-ATP.

Adenosine nucleotides

The concentration–response curves at ATP, ADP, AMP and CoA are represented in Fig. 1. With concentrations of adenosine nucleotides inducing small hyperpolarizations (on the whole below 5 mV) the preparation often recovered in the presence of the agonist (Fig. 2). The maximal relaxation measured was used for the construction of the concentration–response curves.

The membrane hyperpolarization induced with high concentrations exceeds the membrane depolarization induced with carbachol, while the relaxation never exceeds the carbachol-induced contraction. Furthermore, at low concentrations the hyperpolarization lags behind the relaxation, probably because spike activity was reduced before a detectable membrane hyperpolarization was observed. The crossing of the relaxation and hyperpolarization curves indicates a concentration of agonist which evokes a hyperpolarization inducing a just maximal relaxation. The concentration of these adenosine nucleotides which counteracted the effects of carbachol (5×10^{-8} M) is estimated as 3×10^{-5}, 5×10^{-5}, 4×10^{-5} and 3×10^{-4} M for ATP, ADP, AMP and

TABLE 1. Slopes of the assumed linear part of concentration–relaxation curves of adenosine and adenosine nucleotides. The concentration range used was estimated from Figs. 1 and 3. The mean values of all points in these graphs within this range are also presented. Slopes were calculated as the mean of those observed from individual preparations (n) of which responses were observed to at least three different concentrations of agonist within the range used. The last column (P) shows the probability that the difference between the slopes observed with ATP and the agonist indicated were due to change

Agonist	Concentration mean (range) ($-\log$ M)	Response mean (mN)	Slope mean ± S.E. of mean (mN.log^{-1} M)	n	P
ATP	6·31 (7·00–5·52)	11·6	9·65 ± 0·66	7	—
ADP	5·76 (6·52–5·00)	12·7	8·13 ± 1·33	7	0·13
AMP	4·76 (5·52–4·00)	11·6	12·63 ± 1·60	3	0·11
Co A	4·51 (5·00–4·00)	12·6	10·53 ± 1·95	4	0·56
Adenosine	3·46 (4·00–3·00)	12·9	11·95 ± 2·92	6	0·59

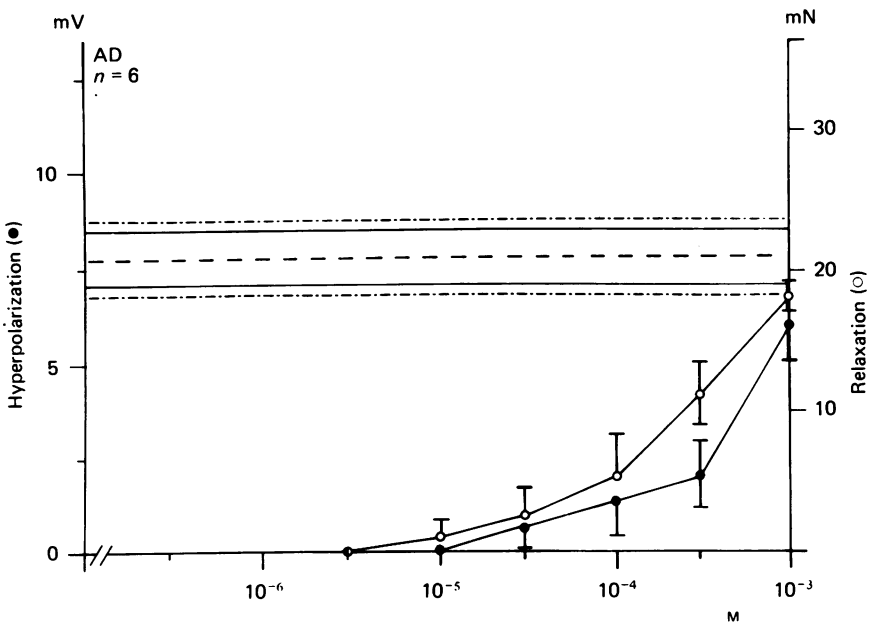

Fig. 3. The muscle relaxation (○) and the cell membrane hyperpolarization (●) induced by adenosine at different concentrations in the presence of carbachol (5×10^{-8} M) at 30 ± 0·2 °C. The level of the resting membrane potential and of the maximal relaxation is indicated by (— —); the S.E. of mean of the carbachol-induced depolarization is indicated by (——), the S.E. of mean of the carbachol-induced contraction by (· — ·).

CoA respectively (Fig. 1). It was notable that, although the potencies thus estimated for ATP, ADP and AMP were similar, the concentration–response curves of ATP and ADP started at lower concentrations. Statistical analysis of the slopes of the concentration–relaxation curves indicated only insignificant differences between the slopes for these adenosine nucleotides (Table 1).

Adenosine

The relation between the concentration of adenosine and the effects measured are presented in Fig. 3. Concentrations of adenosine which evoked small membrane hyperpolarizations generally also evoked relaxations which were only transient in spite of the continuous presence of adenosine. The concentration–response curves show a striking resemblance with those of the adenosine nucleotides. The slope of the concentration–relaxation curve did not differ significantly from that of ATP (Table 1).

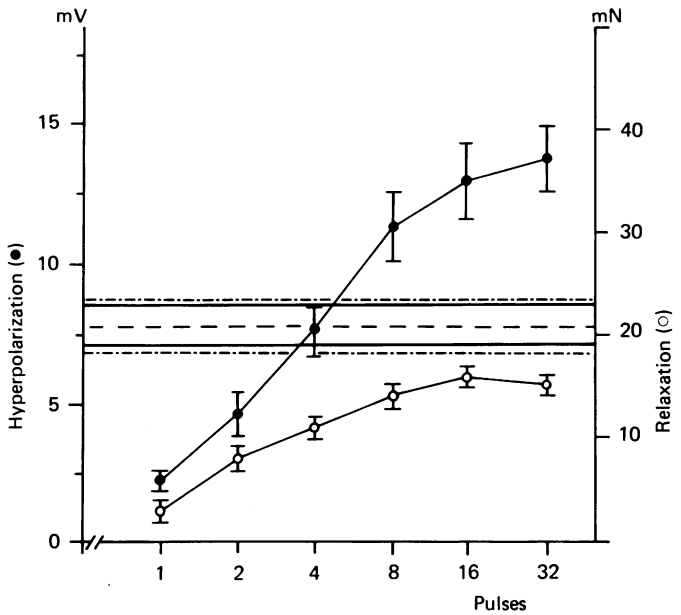

Fig. 4. The muscle relaxation (○) and the cell membrane hyperpolarization (●) induced by stimulation of the n.a.i. nerves (pulse rate 30 pulses/sec; pulse duration 0·3 msec; pulse intensity: supramaximal, ± 15 V.mm^{-1}) with different durations of the pulse train in the presence of carbachol (5×10^{-8} M) at 30 °C. The level of the resting membrane potential and of the maximal relaxation is indicated by (— —), the S.E. of mean of the carbachol-induced depolarization is indicated by (—), the S.E. of mean of the carbachol-induced contraction by (–·–). Each point represents the mean of values obtained from seventeen preparations and is expressed ± S.E. of mean.

There is a small lag of the membrane hyperpolarization with respect to the relaxation. The amplitude of the hyperpolarization induced with adenosine 3×10^{-2} M ($n = 2$) exceeds that of the depolarization evoked by carbachol, while the carbachol contracture was abolished by the accompanying relaxation. The 'cross-over' concentration was estimated to be about 2×10^{-3} M, thus the potency of adenosine to abolish the effects of carbachol (5×10^{-8} M) is less than that of the adenosine nucleotides.

N.A.I. nerve stimulation

The relation between the number of pulses used to stimulate the n.a.i. nerves and the amplitude of the i.j.p. and the amplitude of the muscle relaxation following it are presented in Fig. 4.

It is clear that now the relaxations lag behind the hyperpolarizations. Therefore, no 'cross-over' can be observed although the amplitude of the i.j.p. induced by longer stimulus trains exceeds that of the carbachol-induced depolarization. Furthermore, the muscle relaxations induced by n.a.i. nerve stimulation are always smaller than the contractures induced by carbachol.

Field stimulation during 150 msec (four pulses) induces a membrane hyperpolarization which transiently cancels the membrane depolarization induced with carbachol.

DISCUSSION

We concluded from these experiments that in the guinea-pig taenia caecum the potencies of adenosine nucleotides with regard to induction of membrane hyperpolarization do not differ from their potencies with regard to relaxation of this intestinal smooth muscle. The apparent discrepancy between previous reports (Tomita & Watanabe, 1973; Jager, 1974; Satchell & Burnstock, 1975) must be due to expression of the responses reported in units derived from the maximal response possible. Thus, maximal relaxation is determined by the difference between the tone of the muscle just before the relaxing agents are applied and the state of maximal relaxation. On the other hand maximal hyperpolarization caused by a selective increase in the potassium permeability of the smooth muscle cell (Jager, 1979) is determined by the difference between the membrane potential and the potassium equilibrium potential. However, maximal relaxation occurs at or just beyond the resting membrane potential, which is considerably less than the potassium equilibrium potential (Casteels, 1970).

Bülbring (1955) showed that the force exerted by the guinea-pig taenia caecum, measured isometrically, is inversely related to the potential difference across the cell membrane. From these data obtained with acetylcholine a 'calibration-factor' of about 1 $V.N^{-1}$ can be deduced, which is similar to that reported in the present study, using carbachol, assuming that with the sucrose-gap technique the potential changes measured are attenuated to about half their real value. Furthermore, this inverse relation, which is supposingly valid for agents causing smooth muscle contraction by inducing membrane depolarization, seems also valid for agents which relax smooth muscle by inducing membrane hyperpolarization. This to the extent that the membrane potential range for which this correlation is applicable is restricted at least on one side by the resting membrane potential, when maximal relaxation occurs.

No evidence was obtained to support the hypothesis that part of the relaxing effect of adenosine and adenosine nucleotides is due to actions not involving membrane hyperpolarizations (Tomita & Watanabe, 1973). In the taenia caecum, under the experimental conditions used, adenosine and the adenosine nucleotides all cause membrane hyperpolarization, which inhibits the generation of muscle action potentials and thus induces a relaxation of the smooth muscle, probably by increasing the membrane permeability to potassium ions (Jager, 1979).

Judging from the concentrations of adenosine and the adenosine nucleotides necessary to counteract the effects of carbachol (5×10^{-8} M) the relevant potencies of ATP, ADP and AMP are similar, whereas those of adenosine are a 100 times less

and CoA shows an intermediate potency (potency order ATP $\geqslant$ ADP $\geqslant$ AMP > CoA > adenosine). Both this potency order and the notion that agonists have the same mechanism of action indicate that all effects observed in the present study are mediated via one purinoceptor, which fits the definition of a P_2-receptor given by Burnstock (1978). The presence of a type P_1-receptor could not be demonstrated in the present study. Furthermore, although the concentration–relaxation curves presented seem to confirm the suggestion that the slopes of the ATP and ADP curves are different from those of AMP and adenosine (Satchell & Burnstock, 1975) further analysis revealed only insignificant differences. It is fair to assume that using superfusion and in the steady-state situation the concentration applied to the preparation is similar to the effective concentration at the smooth muscle cell membrane. Thus, the observation (Satchell & Burnstock, 1975) that the relative potency of AMP is similar to that of adenosine might reflect the rapid hydrolysis of AMP into adenosine using organ bath experiments.

It should be remembered in comparing the results of the agonists applied exogenously with those concerning stimulation of the n.a.i. nerves that the membrane potential changes measured with the sucrose-gap technique refer only to cells close to the boundary between test solution and sucrose solution whereas the contractions and relaxations measured refer to the whole muscle in the test solution. In the steady state, during superfusion of agonists, the cells of which the compound membrane potential is monitored, are likely to have a membrane potential similar to that of the cells elsewhere in the preparation in the test solution. Thus it seems reasonable to relate quantitatively changes in membrane potential and changes in muscle contractility observed simultaneously with each other.

However, this does not apply to the i.j.p., not only because of its transient nature but also because of the different latencies of the responses which occur throughout the muscle after nerve stimulation. This is the reason why the relaxation induced by n.a.i. nerve stimulation does not cancel the carbachol-induced contracture completely. However, hyperpolarization induced by the transmitter does exceed the carbachol-induced depolarization in the same cells. Tentatively it can be concluded that the amount of transmitter released after n.a.i. nerve stimulation which cancels the membrane depolarization induced by carbachol must be equipotent with about 3×10^{-5} M-ATP, ADP or AMP, with 3×10^{-4} M-CoA and with 3×10^{-3} M-adenosine.

With regard to the purinergic nerve hypothesis (Burnstock, 1975, 1977) the present results indicate that membrane phenomena induced by the putative neurotransmitters (ATP, ADP and AMP) occur at concentrations within the 'normal' range, such as has been established for noradrenaline. It seems unlikely that the transmitter of the n.a.i. nerves is identical with either CoA or adenosine, because that would necessitate the assumption of 'shielded' receptors, as observed in the vas deferens (Swedin, 1971).

Therefore, we concluded from our experiments that the results are in agreement with the hypothesis that the transmitter released by the n.a.i. nerves is a purine nucleotide, such as ATP, ADP, or AMP affecting the smooth muscle cell membrane via a purinoceptor of the P_2 type.

We are indebted to Dr A. den Hertog and Professor J. van Noorwijk for their advice. We wish to thank Mrs L. Chan and Mr P. W. van Dorp van Vliet for their assistance in the presentation of this paper.

REFERENCES

BÜLBRING, E. (1955). Correlation between membrane potential, spike discharge and tension in smooth muscle. *J. Physiol.* **128**, 200–221.

BÜLBRING, E. & TOMITA, T. (1967). Properties of the inhibitory potential of smooth muscle as observed in the response to field stimulation of the guinea-pig taenia coli. *J. Physiol.* **189**, 299–315.

BURNSTOCK, G. (1975). Purinergic transmission. In *Handbook of Psychopharmacology*, vol. 5, (ed: IVERSEN, L. L., IVERSEN, S. D. & SNIJDER, S. H., pp. 131–194. New York: Plenum.

BURNSTOCK, G. (1977). The purinergic nerve hypothesis. In *Purine and Pyrimidine Metabolism*, Ciba Found. Symp. 48, pp. 295–314. Amsterdam: Elsevier.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*, ed. STRAUB, R. W. & BOLIS, L., pp. 107–118. New York: Raven.

CASTEELS, R. (1970). The relation between the membrane potential and the ion distribution in smooth muscle cells. In *Smooth Muscle*, ed. BÜLBRING, E., BRADING, A. F., JONES, A. W. & TOMITA, T., pp. 70–99. London: E. Arnold Ltd.

COOK, M. A., HAMILTON, J. T. & OKWUASABA, F. K. (1978). Coenzyme A is a purine nucleotide modulator of acetylcholine output. *Nature, Lond.* **271**, 768–771.

DEN HERTOG, A. & JAGER, L. P. (1975). Ion fluxes during the inhibitory junction potential in the guinea-pig taenia coli. *J. Physiol.* **250**, 681–691.

DIEM, K. & LENTNER, C. (1969). *Wissenschaftliche Tabellen*, 7th edn. Basel: Geigy.

ECCLES, J. C. (1964). *The Physiology of Synapses*. Berlin: Springer Verlag.

JAGER, L. P. (1974). The effects of catecholamines and ATP on the smooth muscle cell membrane of the guinea-pig taenia coli, *Eur. J. Pharmacol.* **25**, 372–382.

JAGER, L. P. (1979). Effects of purinergic compounds on excitable membranes. In *Physiological and Regulatory Junctions of Adenosine and Adenine Nucleotides*, ed. BAER, H. P. & DRUMMOND, G. I. New York: Raven.

JAGER, L. P. & DEN HERTOG, A. (1974). Effect of temperature on the transmitter release from the 'purinergic' nerves in the guinea-pig taenia coli. *Eur. J. Pharmacol.* **29**, 201–205.

SATCHELL, D. G. & BURNSTOCK, G. (1975). Comparison of the inhibitory effects on the guinea-pig taenia coli of adenine nucleotides and adenosine in the presence and absence of dipyridamole. *Eur. J. Pharmacol.* **32**, 324–328.

SATCHELL, D. G., BURNSTOCK, G. & CAMPBELL, G. (1969). Evidence for a purine compound as the transmitter in non-adrenergic inhibitory neurones in the gut. *Aust. J. exp. Biol. med. Sci.* **47**, 24*P*.

SWEDIN, G. (1971). Studies on neurotransmission mechanism in the rat and guinea-pig vas deferens. *Acta physiol. scand.* suppl. **369**.

TOMITA, T. & WATANABE, H. (1973). A comparison of the effects of adenosine triphosphate with noradrenaline and with the inhibitory potential of the guinea-pig taenia coli. *J. Physiol.* **231**, 167–177.