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Adenosine-modulation of cholinergic and non-adrenergic non-cholinergic neurotransmission in the rabbit iris sphincter

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1 The characteristics of smooth muscle responses to transmural nerve stimulation in the rabbit iris sphincter were examined.

2 Transmural stimulation elicited a composite contractile response that could be divided in two phases. Atropine abolished the phase I contraction and inhibited the phase II contraction. The atropine-resistant component of the phase II contraction which was unaltered by sympathetic denervation, was mimicked by substance P and abolished by capsaicin.

3 Adenosine inhibited the phase I contraction. The adenosine analogue L-N^6-phenylisopropyladenosine (L-PIA) was more potent than 5'-N-ethylcarboxamideadenosine (NECA) in mimicking this adenosine effect.

4 By contrast, adenosine enhanced the phase II contraction in non-pretreated preparations, as well as the atropine-resistant capsaicin-sensitive part of this contraction. Here, NECA was more potent than L-PIA.

5 Adenosine, NECA, L-PIA and D-PIA also enhanced the atropine-sensitive component of the phase II contraction, as well as the contractile response to exogenous acetylcholine or carbachol, but not to exogenous substance P. In this respect, L-PIA was the most powerful adenosine analogue with at least 10 fold higher potency than D-PIA.

6 The adenosine antagonist 8-*p*-sulphophenyltheophylline enhanced the phase I contraction and decreased the capsaicin-sensitive non-adrenergic non-cholinergic component of the phase II contraction.

7 We conclude that adenosine inhibited the nerve-induced cholinergic twitch (phase I) responses by action at prejunctional A_1-receptors. Furthermore, adenosine enhanced the phase II contractile responses via postjunctional enhancement of the cholinergic transmission by action at A_1-receptors, and via enhancement of the non-adrenergic non-cholinergic transmission by action at presumably prejunctional A_2 receptors.

Introduction

Transmural nerve stimulation of the rabbit iris sphincter elicits a contractile response that is partly of cholinergic and partly of non-adrenergic non-cholinergic origin (Ueda *et al.*, 1981; Tornqvist *et al.*, 1982; Gustafsson, 1982; Björkroth, 1983; Zhang, *et al.*, 1984). It has been suggested that the non-adrenergic non-cholinergic component is mediated by substance P or a similar substance (Ueda *et al.*, 1981; Tornqvist *et al.*, 1982; Björkroth, 1983; Zhang *et al.*, 1984), and also that substance P is the neurogenic factor responsible for the miosis of the nerve-mediated response of the eye to irritation (Bill *et al.*, 1979; Butler & Hammond, 1980; Soloway *et al.*, 1981; Mandahl & Bill, 1981).

Adenosine and adenine nucleotides are released during nerve stimulation and can inhibit transmitter release from both adrenergic and cholinergic nerves (cf. Fredholm *et al.*, 1983). Adenosine can also influence the effector response to the neurotransmitter and may thus function as a postjunctional modulator (cf. Fredholm & Hedqvist, 1980). We therefore have investigated the capacity of adenosine to modulate the cholinergic and the non-adrenergic non-cholinergic neurotransmission in the rabbit iris sphincter, with the

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aim of characterizing the adenosine receptor types involved. Ligand binding studies have shown that adenosine actions are mediated via at least two surface receptor types: A_1- and A_2-receptors (cf. Daly, 1982). The subclasses of adenosine receptors may be distinguished by the relative agonist potencies of certain adenosine analogues (cf. Daly, 1982). At the adenosine A_2-receptor, 5'-N-ethylcarboxamideadenosine (NECA) is more potent than L-N^6-phenylisopropyladenosine (L-PIA), whereas L-PIA is equi- or more potent than NECA at the A_1-receptor. L-PIA is 50–100 times more potent than its stereoisomer D-PIA at the A_1-receptor. The difference in potency between L-PIA and D-PIA at the A_2-receptor is at most 5 fold (Bruns *et al.*, 1980). Both receptor subtypes are blocked by methylxanthines although at present no receptor-selective antagonist is available. We have used 8-*p*-sulphophenyltheophylline, as an adenosine receptor antagonist, since it has recently been identified as a competitive adenosine antagonist without direct inhibitory actions on smooth muscle (Gustafsson 1984).

Methods

General procedure

New Zealand White rabbits of either sex were stunned and bled. The eyes were rapidly enucleated and placed on ice. The cornea was removed by cutting along the limbus, after which the iris sphincter loop was excised and suspended vertically in 6 ml organ bath containing Tyrode solution (composition, mM: Na 149, K 4.8, Ca 2.5, Mg 0.5, Cl 147, HCO_3 11.9, H_2PO_4 0.4 and glucose 5.5) kept at 37°C and continuously aerated with 5% CO_2 in O_2. The preparations were given an initial tension of 2.5–5 mN and were allowed to equilibrate for 1 h before experiments started.

Contractile responses were measured isometrically by means of a Grass FT03C force-displacement transducer and a Grass Polygraph. Transmural stimulation was applied through a set of electrodes in parallel with the tissue, 10 mm apart and 10 mm long, placed opposite each other in the bath walls, and connected to either a Grass S88 or a Somedic AB stimulator. Specific nerve stimulation was elicited by monophasic pulses at 3–10 Hz, 0.3–1 ms pulse duration, 30–300 pulses at 1–3 min intervals. Direct smooth muscle stimulation was performed at a pulse duration of 5 ms in the presence of tetrodotoxin 3×10^{-7}-10^{-6} M.

In 6 animals the iris of the right eye was sympathetically denervated by means of extirpation of the superior cervical ganglion under pentobarbitone anaesthesia. In each experiment complete denervation was confirmed by treating dried stretch preparations of iris segments from control and denervated sides with paraformaldehyde gas, and examining them under the fluorescence microscope.

Drugs

Acetylcholine chloride, adenosine, atropine sulphate, capsaicin, carbamylcholine chloride (carbachol), 2-chloroadenosine, substance P and tetrodotoxin were purchased from Sigma Co. (St. Louis, U.S.A.). Other drugs used were guanethidine sulphate (Ciba-Geigy AG, Basel, Switzerland), L-PIA (L-N^6-phenylisopropyladenosine), its (+)-stereoisomer D-PIA (Boehringer Mannheim Gmbh, Mannheim, West Germany) and NECA (5'-N-ethylcarboxamideadenosine; Byk-Gulden Lomberg Chem. Fabr., Konstanz, Germany). 8-*p*-Sulphophenyltheophylline was synth-

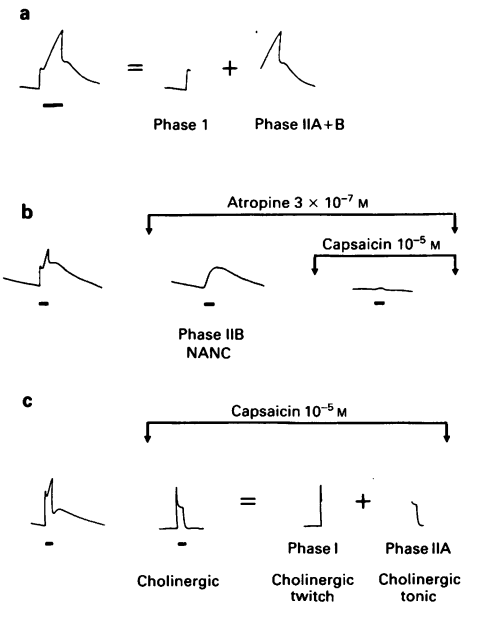

Figure 1 Contractile responses to transmural nerve stimulation (10 Hz, 0.5 ms, 200 pulses at 2 min intervals) in three preparations of rabbit iris sphincter muscle. Horizontal markers indicate application of transmural stimulation. (a) Separation of the contractile response into a twitch (phase I) and a tonic (phase IIA and IIB) response. (b) Effect of atropine 3×10^{-7} M and capsaicin 10^{-5} M. Note the abolition of phase I and IIA by atropine and the abolition of the non-adrenergic, non-cholinergic (NANC) phase IIB during subsequent application of capsaicin. (c) Effect of capsaicin 10^{-5} M on the rabbit isolated iris sphincter. Note the attenuation of the phase II contraction due to abolition of phase IIB, and thus showing the distinction between the two cholinergic components, phase I and phase IIA.

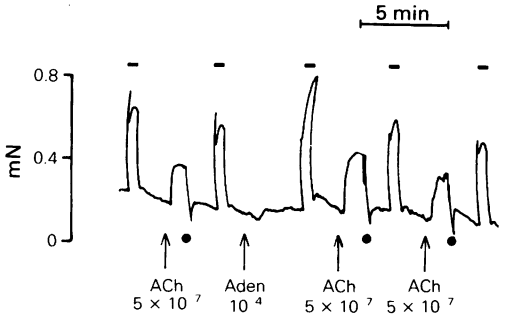

Figure 2 Alternating contractile responses of rabbit isolated iris sphincter to either transmural stimulation (horizontal marker; 10 Hz, 1 ms, 300 pulses at 5 min intervals) or to application of acetylcholine (ACh) 5×10^{-7} M. Wash at dots. Note enhancing effect of adenosine (Aden) 10^{-4} M on both types of responses.

esized according to Daly *et al.* (1985), and was pure as analyzed by h.p.l.c., using as reference a sample kindly provided by Dr R.F. Bruns (Warner-Lambert Co, Ann Arbor, Michigan, U.S.A.).

Statistics

Experimental data are expressed as mean ± s.e.mean of n values. Statistical significance was tested according to Student's t test for unpaired or paired variables. $P < 0.05$ was considered to be significant.

Results

The rabbit iris sphincter muscle responded to transmural nerve stimulation (3–10 Hz, 0.5–1 ms, 30–300 pulses at 3 min intervals) with a biphasic contraction, an initial twitch and a delayed 'hump' (in this paper referred to as 'phase I' and 'phase II', respectively) (see Figure 1a). The response to transmural nerve stimulation was abolished by tetrodotoxin 3×10^{-7} M. Guanethidine (3×10^{-6} M) did not modify this response, but acetylcholine mimicked it, and application of atropine (3×10^{-7}–10^{-6} M) abolished the phase I contraction and also inhibited the phase II contraction (Figures 1b and 3b). Thirty min application of capsaicin (10^{-5} M) only transiently increased tone and then inhibited the phase II contraction. Capsaicin did not affect the phase I contraction (Figure 1c, 3a). Furthermore, the part of the phase II contraction which remained in preparations treated with atropine was abolished by application of capsaicin at 3×10^{-6}–3×10^{-5} M (Figures 1b, 3) or tetrodotoxin at 3×10^{-7} M (not shown). Indeed, application of capsaicin sometimes caused an initial enhancement of the phase II contraction both in untreated preparations and after application of atropine (3×10^{-7} M), before its effect developed into inhibition. The capsaicin-sensitive part of the phase II contraction was mimicked by application of substance P (10^{-9}–10^{-7} M). Taken together, this suggests that the phase I contraction was cholinergic in origin and that the phase II contraction had a cholinergic and a capsaicin-sensitive non-adrenergic, non-cholinergic component. In most preparations the two components

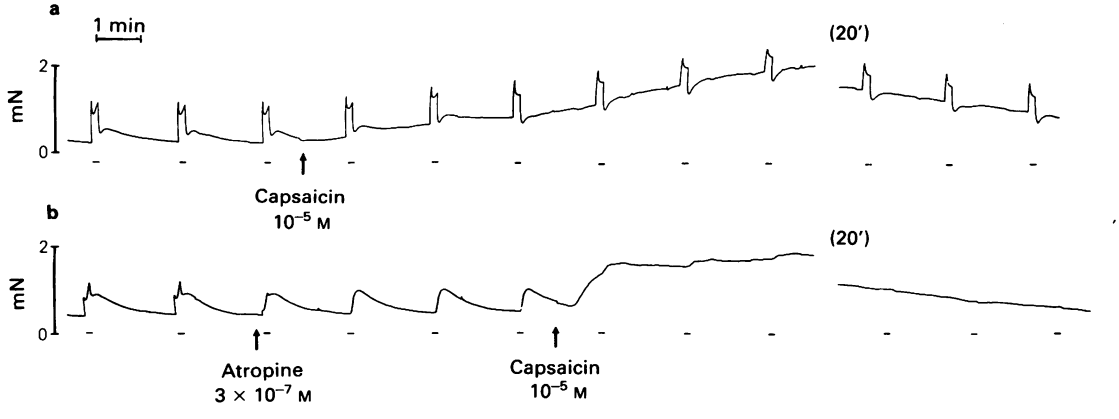

Figure 3 Contractile responses to transmural stimulation (10 Hz, 0.5 ms, 200 pulses at 2 min intervals) in two preparations of rabbit isolated iris sphincter: 20 min of experimental recordings omitted as indicated by (20'). (a) Effect of capsaicin 10^{-5} M. Note increase in tonus and the abolition of the slow component of the phase II contraction (phase IIB). (b) Effect of atropine 3×10^{-7} M and capsaicin 10^{-5} M. Note abolition of phase I and phase IIA contractions after atropine and the transient increase in tonus and abolition of phase IIB contractions after capsaicin.

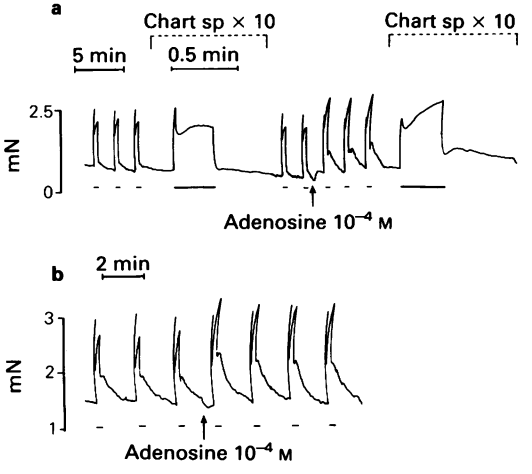

Figure 4 Effect of adenosine 10^{-4} M on contractile responses to transmural nerve stimulation (10 Hz, 1 ms, 200 pulses at 2 min intervals) in two preparations of rabbit isolated iris sphincter muscle. Stimulation applied as indicated by horizontal markers. During two periods in the experiment in (a) the recording chart speed was increased 10 fold as indicated by broken horizontal brackets. (a) Adenosine inhibition of the phase I contraction and enhancement of the phase II contraction as a whole. (b) Adenosine enhancement of both components of the phase II contraction, but no effect on or slight enhancement of the phase I contraction.

of the phase II contraction could clearly be distinguished, the cholinergic tonic response having a shorter decay time and being superimposed on the non-adrenergic, non-cholinergic response (Figures 1, 3, 4 and 5). Henceforth for convenience, the atropine-sensitive part of the phase II contraction is referred to as phase IIA, and the capsaicin-sensitive part of the phase II contraction is referred to as phase IIB, although it should be pointed out that the two components, albeit regularly observed, are not distinguishable at all times in the sphincter preparation.

Adenosine (10^{-6}–10^{-4} M) concentration-dependently and reversibly inhibited the phase I contraction (Figures 2, 4a, 5 and 6), but concomitantly enhanced the phase II contraction (Figures 2, 4, 5 and 7). However, in some preparations adenosine showed only a weak inhibitory effect on or even slightly enhanced the phase I contraction, and meanwhile showed strong enhancement of the phase II contraction (Figure 4b). In order to study whether adrenergic mechanisms were involved in contractile responses or in the adenosine effects, we performed surgical denervation of one eye in each of 6 rabbits, the other eye being used as control. However, neither the contractile response to nerve stimulation, nor the effect of adenosine were affected by the denervation (Figure 5). Furthermore, application of guanethidine (3×10^{-6} M) did not modify the effects of adenosine.

The receptor type involved in effects of adenosine was studied by means of adenosine analogues with receptor selective action. Thus, L-PIA, 2-chloroadenosine or NECA (10^{-8}–10^{-6} M) all inhibited the phase I contraction. L-PIA (10^{-7}–10^{-6} M, $n = 8$–9) was significantly more potent than NECA (10^{-7}–10^{-6} M, $n = 7$; $P < 0.05$). The rank order of potency was L-PIA > 2-chloroadenosine > NECA (Figure 6). On the other hand, NECA (10^{-6} M, $n = 7$) was more potent than L-PIA (10^{-6} M, $n = 9$) in enhancing the phase II contraction ($P < 0.05$), and here the rank order of potency was NECA > 2-chloroadenosine > L-PIA (Figure 7).

We also studied the effect of adenosine and adenosine analogues on the two components of the phase II contraction. The residual contractile response to transmural nerve stimulation after application of atropine (3×10^{-7} M) (the phase IIB contraction) was enhanced by adenosine (10^{-6}–10^{-4} M), NECA (10^{-8}–10^{-6} M) and L-PIA (10^{-7}–10^{-6} M) (Figure 8 and 9). At 10^{-8}–10^{-6} M, NECA was significantly more potent than L-PIA (Figure 9). Adenosine, L-PIA

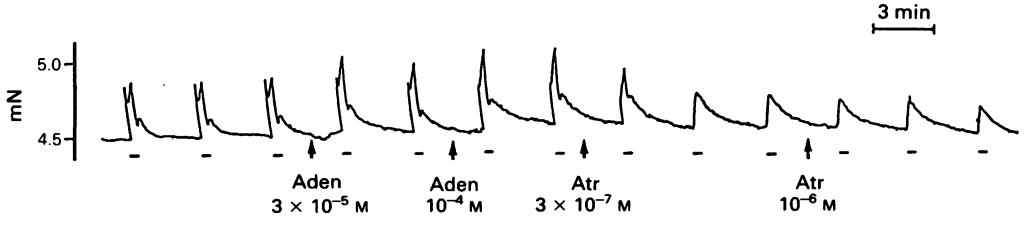

Figure 5 Contractile responses during transmural nerve stimulation (10 Hz, 1 ms, 200 pulses at 3 min intervals) of rabbit isolated iris sphincter muscle. Preparation obtained from eye sympathectomized by ganglionectomy (verified by fluorescence microscopy) one week before experiment. Note conformity with responses in non-denervated preparations. Also note enhancement of both components of the phase II contraction during adenosine (Aden) administratioin. Atropine (Atr) spares only the slow component of the phase II contraction (phase IIB), indicating that the enhanced phase IIA was also cholinergic in origin.

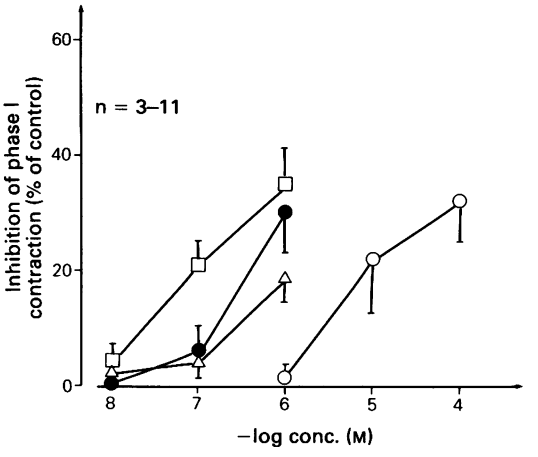

Figure 6 Rabbit isolated iris sphincter preparations: inhibition of the phase I contractile response to transmural nerve stimulation (10 Hz, 1 ms, 100–200 pulses at 2 min intervals) by adenosine (○), 2-chloroadenosine (●), L-PIA (□) and NECA (△). Means with s.e.mean, n = number of observations. L-PIA at 10^{-7} and 10^{-6} M (n = 8 and 9) was significantly more potent than NECA 10^{-7} and 10^{-6} M (n = 7 and 7; $P < 0.05$, Student's t test for unpaired variables).

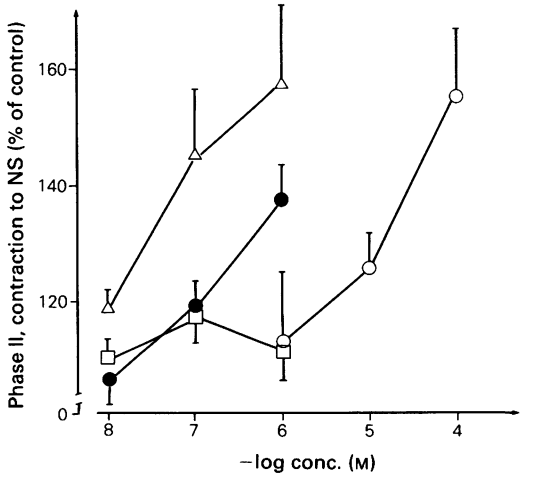

Figure 7 Rabbit isolated iris sphincter preparations: enhancement of the phase II contractile response to transmural nerve stimulation (10 Hz, 1 ms, 100–200 pulses at 2 min intervals) by adenosine (○), 2-chloroadenosine (●), L-PIA (□) and NECA (△). Means with s.e.; n = number of observations. NECA 10^{-6} M (n = 7) was significantly more potent than L-PIA 10^{-6} M (n = 9; $P < 0.05$, Student's t test for unpaired variables).

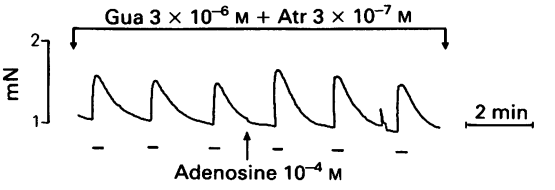

Figure 8 Contractile responses to transmural nerve stimulation (10 Hz, 0.5 ms, 200 pulses at 2 min intervals) in an isolated iris sphincter muscle preparation. Effect of adenosine 10^{-4} M in the presence of atropine (Atr) 3×10^{-7} M and guanethidine (Gua) 3×10^{-6} M, suggesting enhancement of phase IIB contractile responses does not require adrenergic or cholinergic mechanisms.

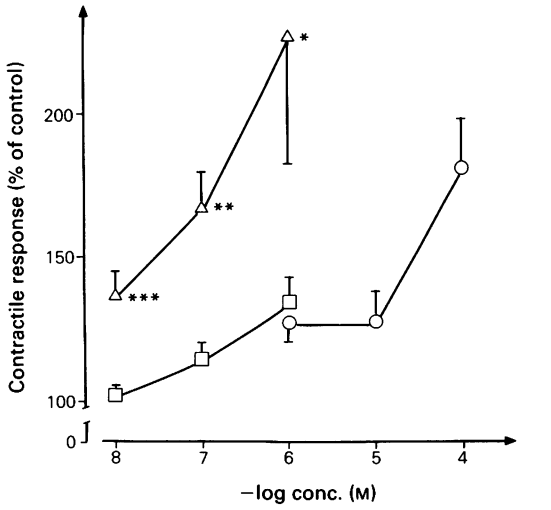

Figure 9 Rabbit isolated iris sphincter preparations pretreated with atropine 3×10^{-7} M. Enhancement of contractile responses to transmural nerve stimulation (10 Hz, 1 ms, 100–200 pulses at 2 min intervals) by adenosine (○), L-PIA (□) and NECA (△). Means with s.e., n = number of observations. NECA was at all concentrations significantly more potent than L-PIA or adenosine. Level of significance in comparison between NECA and L-PIA denoted by $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$, respectively. Student's t test for unpaired variables.

and NECA also enhanced the cholinergic component of the phase II contraction (phase IIA) (Figures 2, 4 and 5). Thus, L-PIA 10^{-6} and 10^{-7} M enhanced the phase IIA contraction to 122 ± 5 and 131 ± 5% of control (n = 5 and 9), and NECA 10^{-6} and 10^{-7} M enhanced the phase IIA contractions to 112 ± 3% and 135 ± 7% of control (n = 7 and 11), respectively. By contrast D-PIA 10^{-7} and 10^{-6} M had only a weak enhancing effect on the phase IIA contractions

(106 ± 3 and 108 ± 3% of control, $n = 4$ and 6, respectively). Thus, there was a more than 100 fold difference in potency between L-PIA and D-PIA in enhancing the phase IIA contractions ($P < 0.05$, Student's t test for unpaired variables).

The adenosine receptor antagonist 8-*p*-sulphophenyltheophylline (10^{-5}–3×10^{-4} M) dose-dependently and reversibly counteracted both the inhibitory and stimulatory effects of adenosine and the analogues (data not shown).

To study whether the effects of adenosine and the analogues were pre- or postjunctional, their effects on contractile responses to exogenous acetylcholine, carbachol and substance P were studied. Adenosine (10^{-4} M) enhanced the contractile response to acetylcholine, 5×10^{-7} M (Figure 2), and L-PIA (10^{-7}–10^{-6} M), NECA (10^{-7}–10^{-6} M) and D-PIA (10^{-6} M) enhanced the response to 10^{-6} M carbachol (Figure 10). L-PIA (10^{-7} M) was significantly more potent than D-PIA (10^{-7}–10^{-6} M) ($P < 0.01$). There was also a tendency for L-PIA to be more potent than NECA although this was not statistically significant (Figure 10). However, adenosine and its analogues did not enhance the contractile response to electrical direct smooth muscle stimulation (10 Hz, 5 ms, 200 pulses at 2 min intervals) in the presence of tetrodotoxin (3×10^{-7} M).

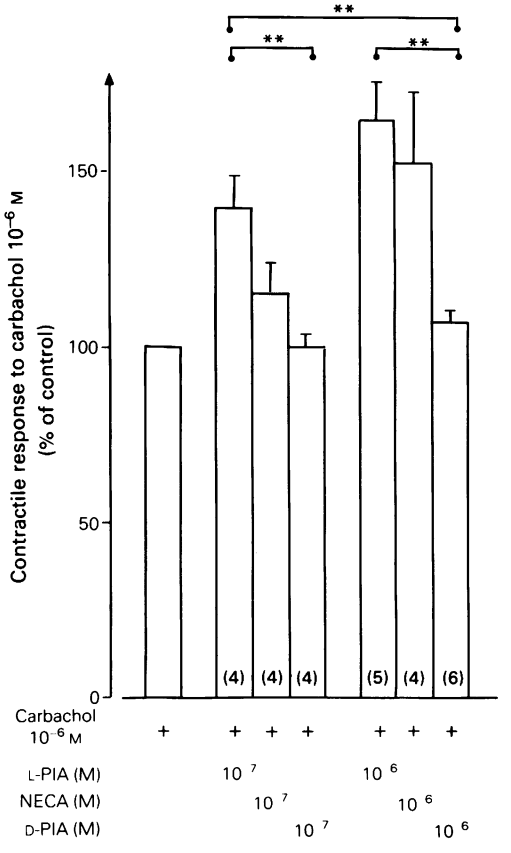

Figure 10 Rabbit isolated iris sphincter preparations. Contractile responses to repeated applications of carbachol 10^{-6} M. Enhancement of the contractile response by L-PIA (10^{-7}–10^{-6} M), NECA (10^{-7}–10^{-6} M) and D-PIA (10^{-6} M). Means wtih s.e., number of observations within parentheses. L-PIA 10^{-6} M was more potent than D-PIA 10^{-7}–10^{-6} M, Student's t test for unpaired data (significance denoted by $^{**}P < 0.01$).

We also studied the effect of NECA (10^{-6} M) on submaximal contractile responses to substance P. Thus, the contractile effect of 10^{-6} M substance P was 433 ± 57% ($n = 8$) of the control phase I contraction induced by transmural nerve stimulation. During subsequent application of NECA the contractile response to substance P became 429 ± 27% ($n = 8$) of the nerve-induced phase I contraction obtained before application of NECA. Thus, a significant change was not seen during the adenosine analogue administration.

In order to study whether endogenous purines could modulate contractile responses to transmural nerve stimulation (10 Hz, 0.5 ms, 200 pulses at 2 min intervals), the adenosine antagonist 8-*p*-sulphophenyltheophylline (3×10^{-4} M) was added to the iris muscle preparation. This increased the phase I contraction to 139 ± 8% ($P < 0.01$, $n = 7$), whereas the non-adrenergic, non-cholinergic component decreased to 75 ± 4% ($P < 0.001$, $n = 7$) of the control contraction, respectively.

Discussion

Transmural stimulation of the rabbit iris sphincter elicited a biphasic contractile response. The phase I and part of the phase II contraction (phase IIA) were probably due to release of acetylcholine since they were mimicked by acetylcholine and blocked by atropine. The atropine-resistant part of the phase II contraction (phase IIB) was, after an initial enhancement of the contractions, abolished by capsaicin. This is in agreement with studies by Ueda *et al.* (1981), Tornqvist *et al.* (1982), Björkroth (1983) and Zhang (1984), suggesting that the non-adrenergic, non-cholinergic nerve-induced contraction in the rabbit iris may be due to release of substance P. Thus, our results clearly indicate that in addition to the peptidergic response, both the twitch (phase I) and the tonic (phase II) response have a strong cholinergic component, and the following discussion will illustrate that effects of adenosine and derivatives exert discrete actions on the different parts of the iris' contractile response.

The present study demonstrates that adenosine can

inhibit the nerve-induced phase I contraction in the rabbit iris sphincter. Since the order of potency for the analogues was L-PIA > 2-chloroadenosine > NECA, it suggests action at inhibitory A_1-adenosine receptors. On the other hand, adenosine enhanced the nerve-induced phase II contraction, and the rank order of potency for the adenosine analogues was NECA > 2-chloroadenosine > L-PIA; this suggests action at stimulatory A_2-adenosine receptors. Adenosine also enhanced the nerve induced non-adrenergic, non-cholinergic contraction after atropine (phase IIB contractions), and since NECA was more potent than L-PIA this also suggests action at stimulatory A_2-adenosine receptors. Furthermore, adenosine, L-PIA, and D-PIA enhanced the cholinergic component of the phase II contraction (phase IIA contractions) and also enhanced contractile responses to exogenously applied acetylcholine or carbachol. Here there was a more than 10 fold difference in potency between L-PIA and D-PIA. This suggests a postjunctional enhancement of the cholinergic transmission via A_1-adenosine receptors. Furthermore, these results also favour the notion that the A_1-adenosine receptor-mediated inhibition of the phase I contraction was exerted prejunctionally, which is in agreement with our previous studies (Gustafsson *et al.*, 1985a) showing that the adenosine inhibition of acetylcholine release in guinea-pig ileum is mediated via A_1-adenosine receptors. The observation is also in agreement with studies on effects of adenosine analogues on effector responses in guinea-pig ileum (Paton, 1981). Unfortunately, acetylcholine release in rabbit iris tissue has been found to be below measurable levels (Gustafsson *et al.*, 1980 and unpublished). We suggest that the failure of adenosine to inhibit the phase I contraction in some preparations was probably due to concomitant postjunctional enhancement of the cholinergic transmission.

In addition to postjunctional enhancement of the cholinergic transmission at A_1-adenosine receptors, there was also an A_2-adenosine receptor-mediated enhancement of the phase II contraction. This enhancement was probably of greater significance since the enhancement of the phase II contraction *in toto* showed characteristics of A_2-receptor activation (Figure 7). The same apparently applied to the effect of adenosine analogues on the non-adrenergic, non-cholinergic IIB contractile response (Figure 9). The level of this A_2 effect may be prejunctional, since adenosine or NECA did not enhance the contractile response to exogenous substance P, and since smooth muscle contractile responses to electrical stimulation in the presence of tetrodotoxin were not affected by adenosine or adenosine analogues.

Previously it has been shown that adenosine can enhance cholinergic contractile responses in rabbit bronchi via action at A_2-adenosine receptors (Gustafsson *et al.*, 1985b). In the bronchi, adenosine and NECA were unable to enhance the contractile response to exogenous carbachol or acetylcholine and they did not enhance contractile responses to direct smooth muscle stimulation. Thus, the A_2-adenosine receptor-mediated enhancement of cholinergic transmission in the rabbit bronchi resembles the A_2-adenosine receptor-mediated enhancement of the non-adrenergic, non-cholinergic transmission in the rabbit iris. Taken together, these findings suggest that adenosine might act at prejunctional A_2-receptors to enhance transmitter release both in the rabbit iris and bronchi.

The adenosine enhancement of the non-adrenergic, non-cholinergic transmission can be of importance since substance P is a candidate as a neurogenic factor released from sensory nerve fibres during noxious stimuli to the eye. Application of susbtance P can induce miosis and other inflammatory responses (Bill *et al.*, 1979; Butler & Hammond, 1980; Mandahl & Bill, 1981), and it can also be released into the aqueous humor (Bill *et al.*, 1979). Furthermore, substance P antagonists may suppress inflammation in the eye (Holmdahl *et al.*, 1981). Since adenosine was found to enhance the non-adrenergic, non-cholinergic transmission, the possibility exists that adenosine enhances the inflammatory response in the eye.

The finding that the adenosine receptor antagonist 8-*p*-sulphophenyltheophylline enhanced the phase I contraction, and inhibited the non-adrenergic, non-cholinergic componenet of the phase II contractile response provides at least circumstantial evidence for the presence of endogenous purines in amounts sufficient for modulation of neurotransmission in the rabbit iris sphincter.

In conclusion, adenosine inhibited the nerve-induced cholinergic twitch contraction, but enhanced the nerve-induced slow cholinergic tonic and non-adrenergic, non-cholinergic contraction in the rabbit iris sphincter. The inhibitory effect was probably exerted at prejunctional A_1-adenosine receptors. The enhancing effect was probably due to enhancement of the non-adrenergic, non-cholinergic transmission at A_2-adenosine receptors, possibly prejunctionally, and by postjunctional enhancement of the cholinergic transmission at A_1-receptors. The results indicate a role for adenosine as modulator of neurotransmission and might indicate a role for adenosine in the inflammatory responses of the eye.

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