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Indirect evidence that purinergic modulation of perivascular adrenergic neurotransmission in the portal vein is a physiological process

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1 The effects of adenine nucleotides and nucleosides on the contractile response to perivascular nerve stimulation were compared in the isolated portal vein of rabbit, rat and guinea-pig.

2 2-Chloroadenosine was more potent than adenosine and ATP, which were equipotent in producing inhibition of neurogenic contractions in the *rabbit* and *rat* via prejunctional P_1-purinoceptors.

3 In contrast, neurogenic contractions of the *guinea-pig* portal vein were not inhibited by adenosine and were potentiated by 2-chloroadenosine and, to a lesser extent, by ATP.

4 Fluorescence histochemical localization of quinacrine, which binds to high levels of ATP, revealed a dense perivascular nerve plexus in the portal vein of rabbit and rat but not of guinea-pig.

5 After chemical sympathectomy, quinacrine-positive nerves persisted in the rabbit (supporting other evidence for the presence of purinergic nerves) but not in the rat (supporting other evidence for ATP as a cotransmitter in adrenergic nerves).

6 It is concluded that a prejunctional purinergic modulatory mechanism operates in adrenergic neurotransmission in the portal vein of rabbit and rat but not guinea-pig, and it is suggested that this indicates a physiological mechanism.

Introduction

It is widely claimed that adrenergic neurotransmission can be modulated prejunctionally by negative feedback inhibition by endogenous noradrenaline (NA) (see Langer, 1981). Furthermore, adrenergic neurotransmission has been shown to be modulated by a number of other pharmacological agents including adenine nucleosides and nucleotides. Adenosine 5′-triphosphate (ATP) and adenosine selectively depress the response to sympathetic nerve stimulation in several isolated blood vessels including rabbit portal vein (Su, 1978; Brown *et al.*, 1982) and rat portal vein (Enero & Saidman, 1977; Wakade & Wakade, 1978; Moylan & Westfall, 1979; Enero, 1981). These compounds also inhibit sympathetic neurotransmission in various visceral preparations (see Burnstock & Brown, 1981).

The portal vein receives a dense adrenergic innervation in the rabbit, rat and guinea-pig (Burnstock *et al.*, 1979; Johansson *et al.*, 1970). Non-adrenergic, non-cholinergic inhibitory nerves have also been found in the rabbit portal vein (Hughes & Vane, 1967), where evidence has been presented to support the involvement of ATP or a related purine as the neurotransmitter (Burnstock *et al.*, 1979; Hung & Su, 1982). However, non-adrenergic, non-cholinergic inhibitory nerves have not been found in the guinea-pig portal vein (Burnstock *et al.*, 1979).

The aim of this study was to compare the actions of various purines and purine analogues on adrenergic neurotransmission in the portal veins of rabbit, rat and guinea-pig and to consider these actions with reference to the histochemical localization in these vessels of quinacrine, which is known to bind to high levels of ATP (Irvin & Irvin, 1954; Olson *et al.*, 1976). A preliminary communication about this work has been presented (Török & Burnstock, 1982).

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Methods

The experiments were performed on isolated portal veins of male New Zealand White rabbits (2.5–3.5 kg), male Wistar rats (300–400 g) and male albino guinea-pigs (400–500 g). The animals were killed by a blow to the head and exsanguination.

Pharmacology

A section of portal vein was removed and placed in Krebs solution gassed with 95% O_2, 5% CO_2. Rabbit portal veins were cut into two equal longitudinal strips, whereas rat and guinea-pig portal veins were used unopened. The strips were mounted vertically under isometric conditions in 12 ml organ baths. Contractions of the longitudinal smooth muscle were recorded using a Grass FT03 transducer and a Grass polygraph (Model 79).

The preparations were allowed to equilibrate for at least 90 min under a resting tension of 2 g (rabbits) or 1 g (rats and guinea-pigs). During this period, the veins were washed with fresh Krebs solution every 20 min. Modified Krebs solution had the following composition (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1.2, K_2HPO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaNa_2$ EDTA 0.032, ascorbic acid 0.55; (pH 7.3–7.4). Intramural nerves were stimulated using two platinum ring electrodes separated by a distance of 1 cm. Pulses of supramaximal voltage, 0.7 ms duration, at a frequency of 8 Hz, were applied for 10 s at 5 min intervals using a Grass S44 stimulator. Exogenous NA (2×10^{-7} M) was added to the organ bath for 45 s, during which time a maximum response was attained, and was then washed out by overflow. ATP, adenosine or 2-chloroadenosine were added to the bath either cumulatively, in preparations stimulated electrically, or immediately after washout in preparations to which exogenous NA had been added. Contractions obtained in the presence of the purines were expressed as a percentage of 3 initial contractions obtained in their absence. All results have been expressed as the mean ± s.e.mean and were analysed using Student's paired and non-paired t tests. A probability of 0.05 or less was considered significant.

Histochemistry

Quinacrine fluorescence Portal veins dissected from rabbits ($n = 6$), guinea-pigs ($n = 6$) and rats ($n = 6$) were washed in modified Krebs solution (see Burnstock *et al.*, 1979) at room temperature and were then incubated for 1 h in Krebs solution containing 5×10^{-7} M quinacrine dihydrochloride and continually gassed with 95% O_2, 5% CO_2. At the end of the incubation period they were washed for a further 20–30 min with quinacrine-free Krebs solution.

After removing fat and connective tissue, the portal veins were slit open longitudinally, stretched on glass slides (with the adventitial side uppermost) and air dried until they appeared translucent. They were mounted in liquid paraffin and were viewed with a Zeiss photomicroscope fitted with an epifluorescence condenser III RS. A high pressure mercury light source was used (Osram HB50) with excitation filters (BP 436/8), barrier filter (LP 470) and dichroic mirror (FT 460). Selected areas were photographed on Ilford HP5 film.

Chemical sympathectomy Rabbits ($n = 4$), rats ($n = 4$) and guinea-pigs ($n = 4$) were injected intraperitoneally with 6-hydroxydopamine (6-OHDA) dissolved in sterile saline solution (0.9% w/v NaCl solution) containing 0.2% ascorbic acid. The animals were injected with 6-OHDA (125 mg kg^{-1}) twice with an interval of 48 h between injections and were killed 4 days after the start of the treatment. The portal veins from these animals were then treated with quinacrine as described above.

Drugs

Adenosine 5′-triphosphate (ATP); adenosine 5′-diphosphate (ADP); adenosine 5′-monophosphate (AMP); adenosine; 2-chloroadenosine; 6-hydroxydopamine (6-OHDA); noradrenaline bitartrate (NA); quinacrine dihydrochloride; (all obtained from Sigma Chemical Company); 8-phenyltheophylline (8-PT) and tetrodotoxin (TTX); (Calbiochem); guanethidine sulphate (Ismelin, CIBA).

Results

Effects of purines and analogues on spontaneous activity and basal tension

In the rabbit and rat portal veins, ATP and ADP (3×10^{-5} M and above) caused a transient increase in basal tension and a decrease in the amplitude of spontaneous activity. Adenosine and AMP (10^{-4} M and above) and 2-chloroadenosine (10^{-6} M and above) reduced the amplitude of spontaneous activity without altering basal tension. In the guinea-pig portal vein, ATP and ADP (10^{-6} M and above) increased the frequency but decreased the amplitude of spontaneous activity. Adenosine and AMP (10^{-5} M and above) increased both the amplitude and the frequency of spontaneous activity. 2-Chloroadenosine (10^{-6}–3×10^{-5} M) increased the amplitude of spontaneous activity, but in higher concentrations (10^{-4}–3×10^{-4} M) it decreased both amplitude and frequency.

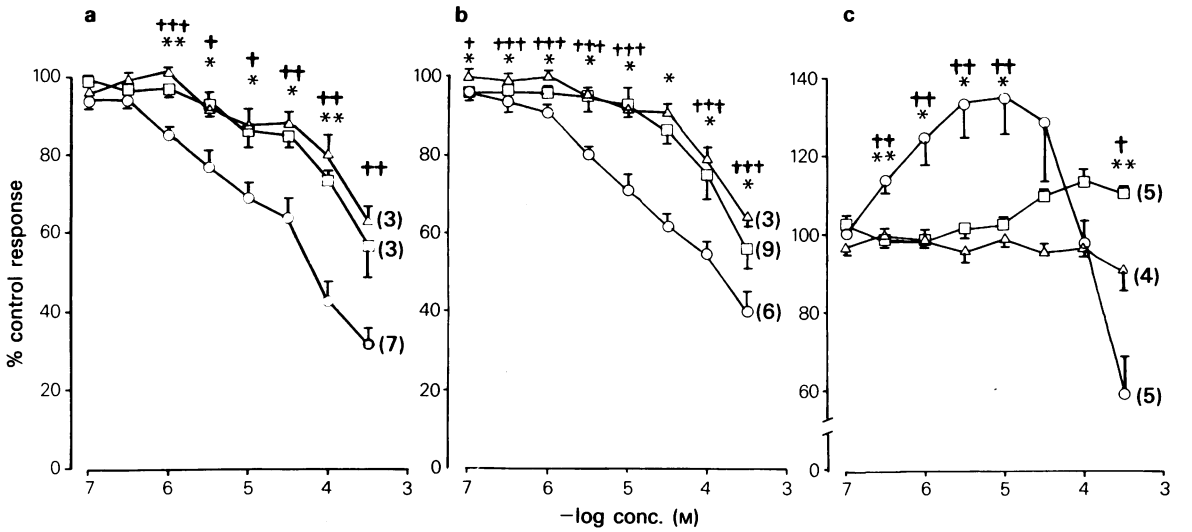

Figure 1 The effect of 2-chloroadenosine (○), ATP (□) and adenosine (△) on neurogenic contractions of the portal vein of (a) rabbit (b) rat and (c) guinea-pig. Each point is the mean of observations from the number of preparations indicated in parentheses. Vertical bars show the s.e.mean. Statistically significant differences between 2-chloroadenosine and ATP: $*P<0.05$, $**P<0.01$, between 2-chloroadenosine and adenosine: $\dagger P<0.05$, $\dagger\dagger P<0.01$, $\dagger\dagger\dagger P<0.001$.

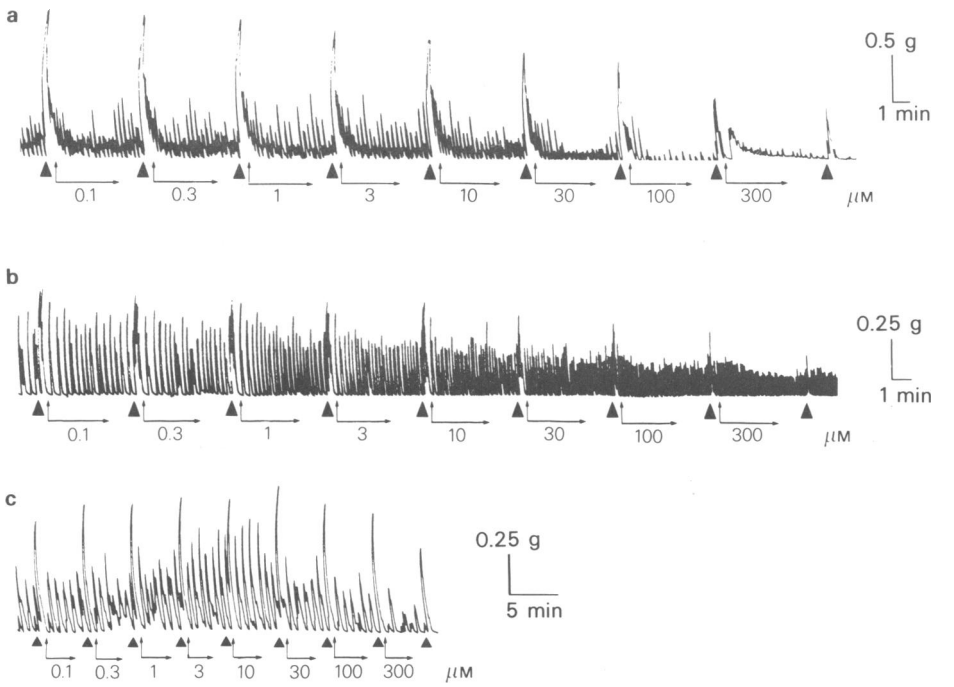

Figure 2 The effect of 2-chloroadenosine on neurogenic contractions in the portal vein of (a) rabbit (b) rat and (c) guinea-pig. (▲) Indicates intramural nerve stimulation at 8 Hz, 0.7 ms pulse duration, at supramaximal voltage for 10 s. Figures refer to the cumulative concentration of 2-chloroadenosine present in the bathing solution.

Effects of ATP, adenosine and 2-chloroadenosine on neurogenic contractions

The contractions produced in response to transmural stimulation of perivascular nerves were completely abolished by guanethidine (10^{-6} M) or TTX (3×10^{-6} M) in all three species. ATP (10^{-7}–3×10^{-4} M), adenosine (10^{-7}–3×10^{-4} M) and 2-chloroadenosine (10^{-7}–3×10^{-4} M) each caused an inhibition of the neurogenic contraction in the rabbit and rat portal vein (Figures 1a and b, 2a and b and 3). In both species 2-chloroadenosine was significantly more potent than ATP and adenosine ($P<0.05$), which were equipotent. There was no significant difference in the potency of 2-chloroadenosine between the two species.

In the guinea-pig portal vein ATP (3×10^{-5}–3×10^{-4} M) potentiated the neurogenic response but adenosine (10^{-7}–3×10^{-4} M) had little effect (Figure 1c). 2-Chloroadenosine potentiated the neurogenic response at low concentrations (10^{-7}–3×10^{-5} M); higher concentrations (10^{-4}–3×10^{-4} M) caused inhibition (Figures 1c, 2c and 3). In each species the effects of ATP, adenosine and 2-chloroadenosine were fully reversible on washout.

These results indicate that 2-chloroadenosine is significantly more potent than ATP and adenosine. Metabolic breakdown and uptake appear to limit the action of ATP and adenosine but not that of 2-chloroadenosine (see Muller & Paton, 1979). 2-Chloroadenosine was therefore used in preference to ATP and adenosine in further experiments.

Effects of 2-chloroadenosine on contractions due to exogenous noradrenaline (NA)

Since postjunctional purinoceptors are present in each of the three preparations (Sjöberg & Wahlström, 1975; Burnstock *et al.*, 1979; Ishii & Shimo, 1983) the postjunctional actions of 2-chloroadenosine were also considered. NA, 2×10^{-7} M, elicits a contraction similar in magnitude to that caused by nervous stimulation at the parameters used above. In rabbit and rat portal vein, 2-chloroadenosine was significantly more potent at inhibiting neurogenic contractions than those due to exogenous NA (2×10^{-7} M) ($P<0.05$) (Figure 4a and b). At concentrations less than 10^{-5} M, 2-chloroadenosine had a preferential prejunctional action. At higher concentrations a postjunctional inhibitory effect was also seen.

In the guinea-pig portal vein there was no difference between the actions of 2-chloroadenosine (10^{-7}–3×10^{-4} M) on neurogenic contractions and on those due to exogenous NA (2×10^{-7} M) (Figure 4c). This suggests that 2-chloroadenosine acts entirely postjunctionally, with no prejunctional actions.

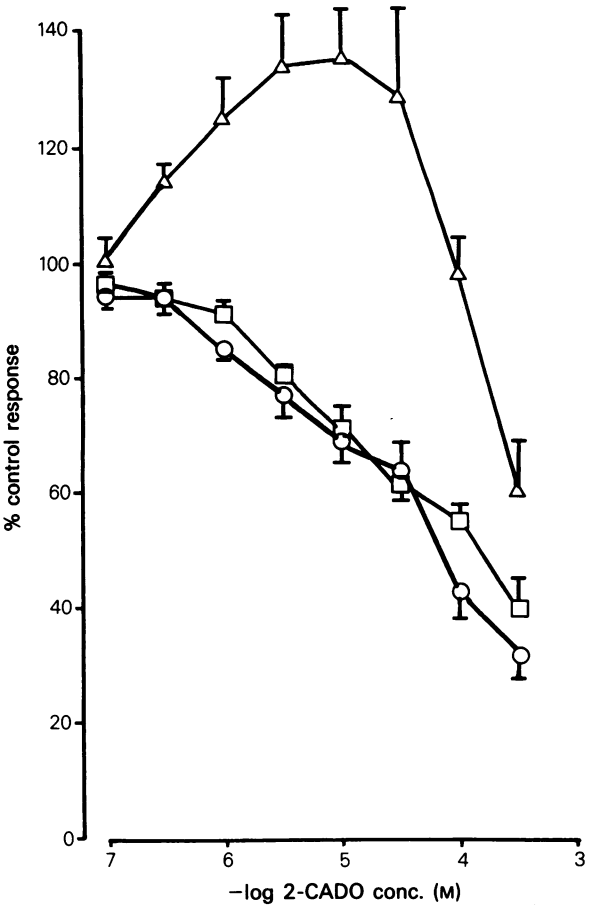

Figure 3 The effect of 2-chloroadenosine (2-CADO) on neurogenic contractions in rabbit (○) ($n=7$), rat (□) ($n=6$), and guinea-pig (△) ($n=5$) portal veins. The results are plotted as percentage of control response (ordinate) against the − logarithm of the concentration of 2-chloroadenosine. Vertical bars show the s.e.mean.

The effect of 8-phenyltheophylline (8-PT) upon the actions of 2-chloroadenosine

Incubation of the tissues with 8-PT (10^{-5} M), a potent P_1-purinoceptor antagonist (Smellie *et al.*, 1979; Griffith *et al.*, 1981) decreased the amplitude of spontaneous activity and also slightly decreased the amplitude of noradrenergic contractions.

In the rabbit portal vein both the prejunctional and the postjunctional concentration-response curves were reversibly shifted to the right (Figure 5a). However, this was only significant at concentrations of 2-chloroadenosine of 10^{-6}–10^{-5} M, acting prejunctionally ($P<0.05$). Likewise, in the rat portal vein, 8-PT (10^{-5} M) reversibly antagonized both prejunctional and postjunctional actions of 2-

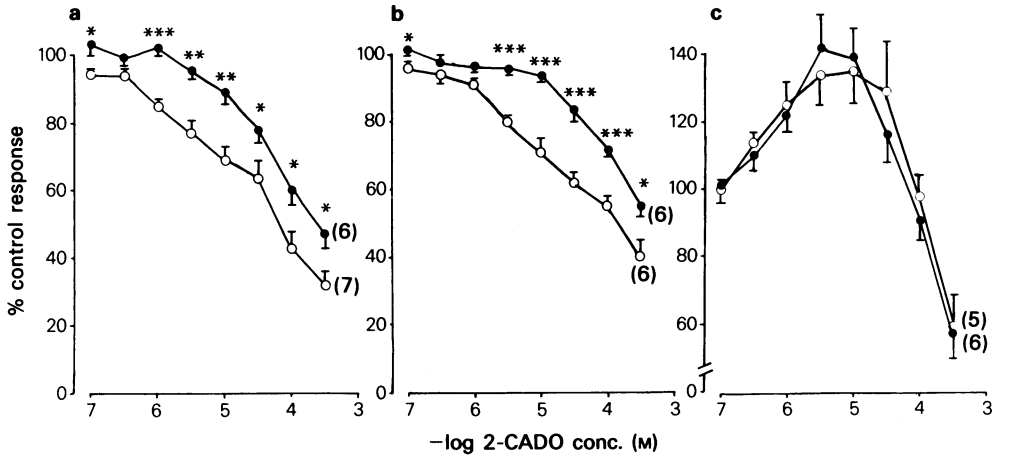

Figure 4 The effect of 2-chloroadenosine (2-CADO) on neurogenic contractions (○) and on contractions due to exogenous noradrenaline (NA) (2×10^{-7} M) (●) in the portal vein of (a) rabbit (b) rat and (c) guinea-pig. Each point is the mean of observations from the number of preparations indicated in parentheses. Vertical bars show the s.e.mean. Statistically significant differences are denoted by asterisks; $*P<0.05$, $**P<0.01$, $***P<0.001$.

chloroadenosine, but whereas antagonism of prejunctional responses was significant at 2-chloroadenosine concentrations of 3×10^{-6}–10^{-5} M, postjunctional antagonism was only significant at a concentration of 3×10^{-4} M ($P<0.05$) (Figure 5b). The low solubility of 8-PT in aqueous solution precluded the use of concentrations higher than 10 μM.

In the guinea-pig portal vein, 8-PT (10^{-5} M) caused significant rightward shifts of the concentration-response curves for 2-chloroadenosine against both neurogenic contractions and contractions due to exogenous NA (2×10^{-7} M) ($P<0.05$) (Figure 5c). This was fully reversible upon washout.

Histochemistry

In the rabbit portal vein, quinacrine fluorescence histochemistry revealed a dense plexus of varicose nerve fibres on the adventitial-medial border running largely parallel to the vessel axis (Figure 6a). The

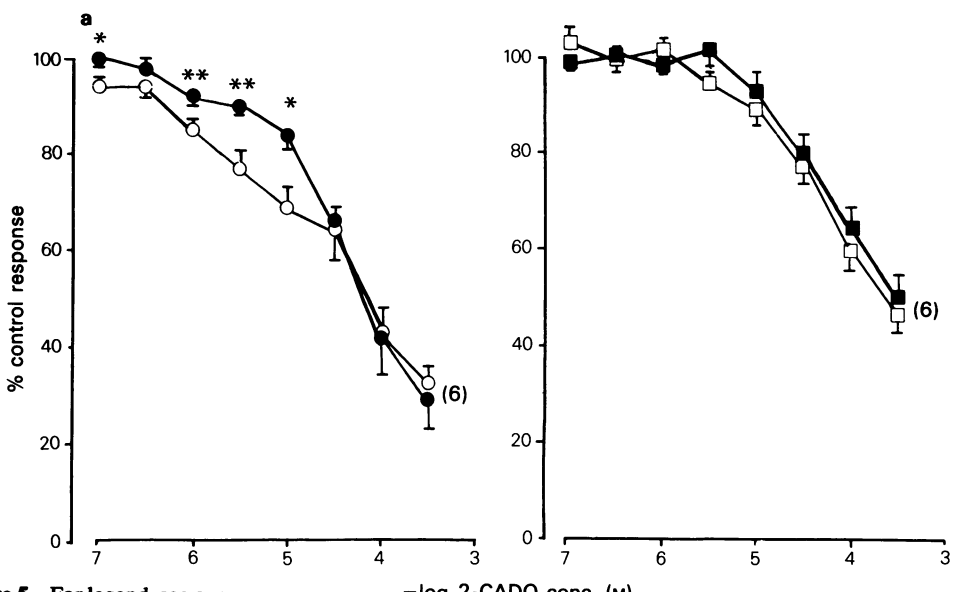

Figure 5 For legend, see over.

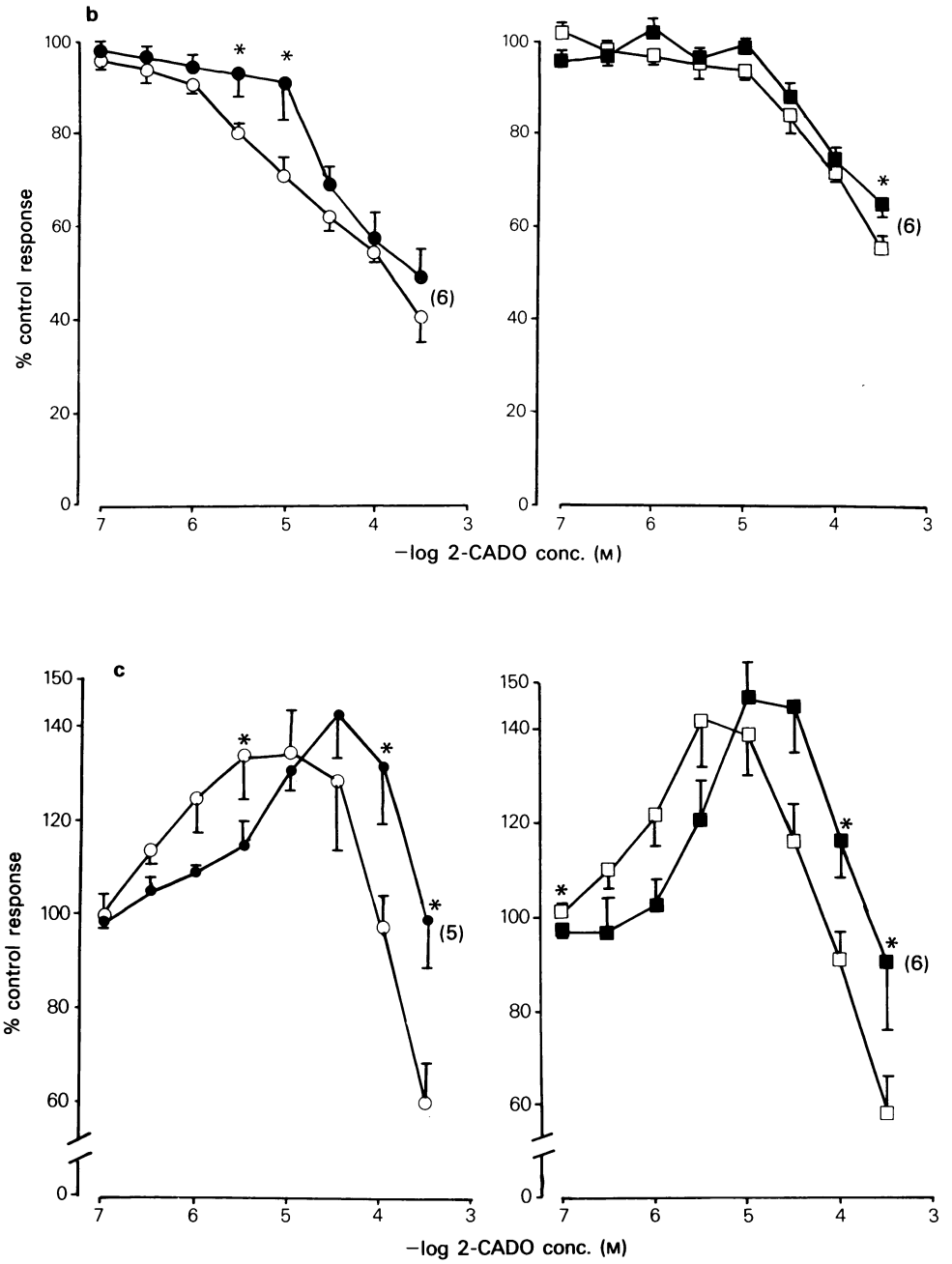

Figure 5 The effect of 2-chloroadenosine (2-CADO) on contractions of the portal vein of (a) rabbit, (b) rat and (c) guinea-pig, due to nerve stimulation in the absence (○) and in the presence (●) of 8-phenyltheophylline (8-PT) (10^{-5} M) and on those due to exogenous noradrenaline (2×10^{-7} M) in the absence (□) and in the presence (■) of 8-PT (10^{-5} M). Each point is the mean of observations from the number of preparations indicated in parentheses. The results are plotted as percentage of control response (ordinate scale) against the − logarithm of the concentration of 2-chloroadenosine. Vertical bars show the s.e.mean. Statistically significant differences are denoted by asterisks; $*P<0.05$, $**P<0.01$.

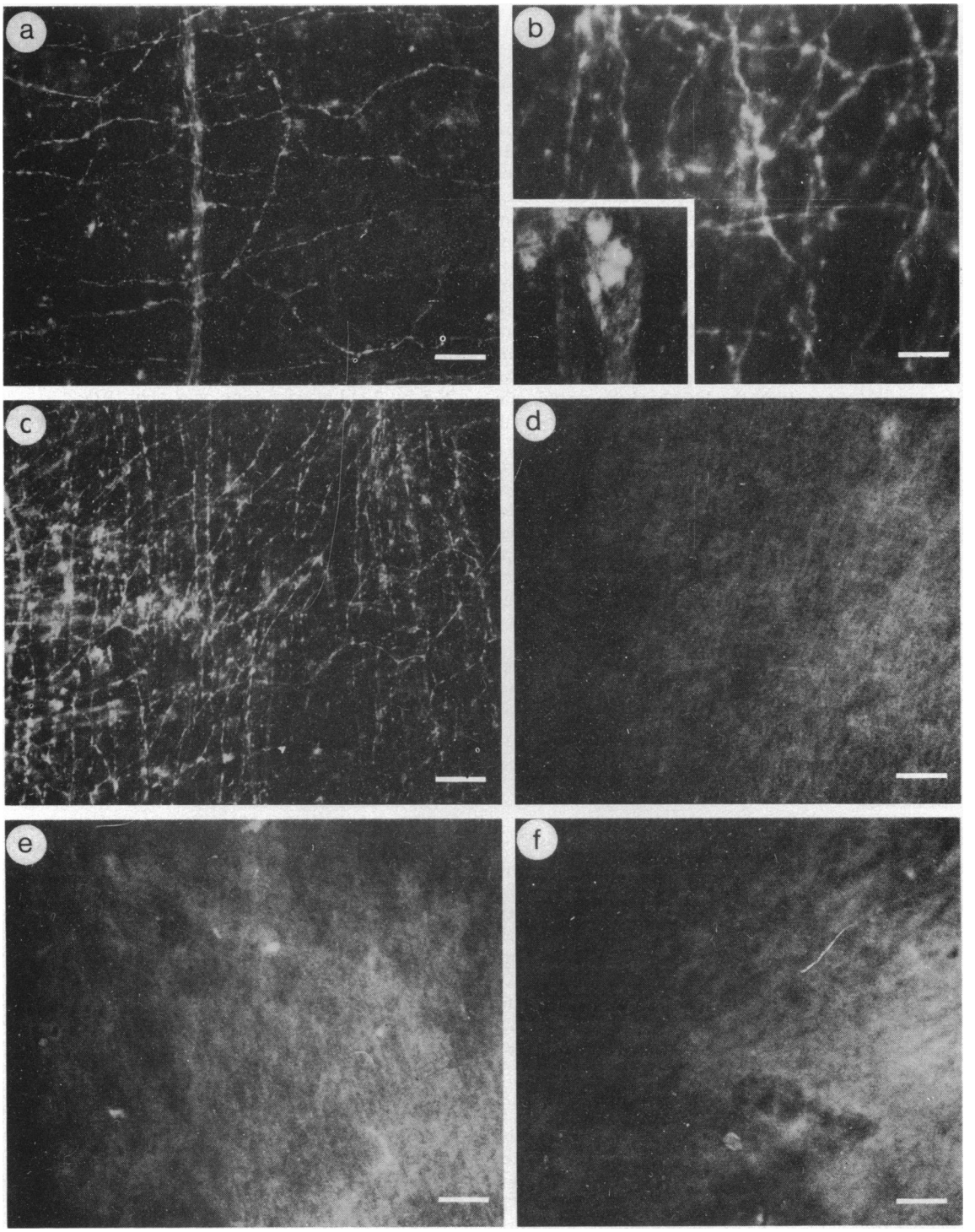

Figure 6 Quinacrine fluorescence histochemistry on rabbit, rat and guinea-pig portal veins. (a) and (b) Rabbit portal vein. (a) A plexus of fine varicose fibres is seen. Many of the fibres are orientated parallel to the vessel axis (calibration bar = 100 μm). (b) After chemical sympathectomy, the varicose nerve fibre plexus persists; insert: intensely stained cell bodies with unstained nuclei are seen associated with a nerve bundle (calibration bar = 50 μm). (c) and (d) Rat portal vein. (c) A dense quinacrine-positive nerve plexus is seen (calibration bar = 100 μm). (d) After chemical sympathectomy, the nerve plexus is abolished (calibration bar = 100 μm). (e) and (f) Guinea-pig portal vein. No quinacrine-positive elements are seen before (e) and after (f) chemical sympathectomy (calibration bar = 100 μm).

nerve plexus consisted predominantly of single fibres although some were found grouped together to form bundles. The varicosities appeared as brightly fluorescent swellings along the nerve fibres (10 per 100 μm of terminal fibres) or were sometimes grouped together; some fluorescence was also seen in intervaricose regions. Intramural ganglia (4–6 per 1–1.5 cm length tissue) containing brightly fluorescent spherical nerve cell bodies (diam. 20–40 μm) were also observed (Figure 6b insert). These were occasionally associated with weakly fluorescent nerve fibre bundles (20–30 μm in diameter) containing preterminal fibres. This pattern of innervation was found throughout the length of the portal vein and was unaffected by chemical sympathectomy with 6-OHDA (Figure 6b).

In the rat portal vein, quinacrine treatment revealed a dense network of fine varicose nerve fibres on the adventitial-medial border. In this tissue, the nerve fibres were not orientated in any preferential direction (Figure 6c), and the plexus density appeared greater than that seen in the rabbit portal vein. No intramural ganglia were seen. The quinacrine-positive nerve fibres were abolished by pretreatment with 6-OHDA (Figure 6d).

In the guinea-pig portal vein neither quinacrine-positive nerve fibres nor nerve cell bodies were seen before or after chemical sympathectomy (Figure 6e and f).

Discussion

The results in the present study clearly demonstrate that ATP, adenosine and 2-chloroadenosine inhibit neurogenic contractions of the portal vein of the rabbit and rat in a concentration-dependent manner, confirming results from other groups where inhibition of [^{3}H]-NA release was studied (see Introduction). At concentrations of up to 10^{-5} M, 2-chloroadenosine had a preferential prejunctional inhibitory action. At higher concentrations a postjunctional inhibitory effect was also seen. The postjunctional actions of the purines and analogues were similar to those reported elsewhere (Sjöberg & Wahlström, 1975; Burnstock *et al.*, 1979; Ishii & Shimo, 1983). Preliminary studies suggested that the degree of prejunctional inhibition was inversely related to the frequency of electrical stimulation. This is in accordance with previous findings (Enero, 1981) where inhibition of [^{3}H]-NA release by adenosine was highest at low frequencies.

2-Chloroadenosine was significantly more potent than ATP and adenosine. Metabolic breakdown and uptake appear to limit the action of ATP and adenosine, but not that of 2-chloroadenosine (see Muller & Paton, 1979). 2-Chloroadenosine was equipotent on the rabbit and rat portal vein. In the present study ATP and adenosine were equipotent in inhibiting the neurogenic contractions of the rabbit and rat portal veins, suggesting that ATP may be acting at a P_1-purinoceptor (Burnstock, 1978) following breakdown to adenosine (De Mey *et al.*, 1979; Willemot & Paton, 1981; Moody & Burnstock, 1982).

In contrast to the rabbit and rat portal vein, no evidence was found to suggest the presence of prejunctional inhibitory purinoceptors in the guinea-pig portal vein in the present study. Instead, a postjunctional potentiation of exogenous and endogenous NA by 2-chloroadenosine, was seen. Adenine nucleosides and nucleotides have been shown to interact with NA postjunctionally *in vitro* in the guinea-pig seminal vesicle (Nakanishi & Takeda, 1973), rabbit kidney (Hedqvist & Fredholm, 1976), guinea-pig vas deferens (Holck & Marks, 1978; Sakai *et al.*, 1979; Kazić & Milosavljević, 1980) and rabbit mesenteric artery (Krishnamurty & Kadowitz, 1982). The mechanism of this potentiation is as yet unclear.

The potentiating action of 2-chloroadenosine on the guinea-pig portal vein was reversibly antagonized by 8-PT (10 μM), a selective P_1-purinoceptor antagonist (Smellie *et al.*, 1979; Griffith *et al.*, 1981). Thus it was surprising that the inhibitory actions of 2-chloroadenosine in the rabbit and rat portal veins were only slightly antagonized. The reason for this is not known. However, 8-PT (10 μM) has some phosphodiesterase inhibitory activity (Smellie *et al.*, 1979), which may be involved.

Pharmacological and histochemical differences between the portal veins of the three species suggests that each has a distinct pattern of innervation which may be physiologically relevant. Coexistence of NA and high levels of ATP seems to occur in the sympathetic neurones supplying the rat portal vein since, in the present study, chemical sympathectomy abolished quinacrine fluorescence in this tissue. Quinacrine is a fluorescent compound which binds to ATP (Irvin & Irvin, 1954; Olson *et al.*, 1976) and which has been used to demonstrate histochemically fluorescent nerve cell bodies and fibres in a variety of autonomic tissues claimed to be innervated by purinergic nerves (see Burnstock, 1981). In contrast to the rat portal vein, separate purinergic nerves are present in the rabbit portal vein in addition to the coexistence of NA and ATP in noradrenergic nerves. Indirect evidence for a physiological role for purines in adrenergic neurotransmission has been found in spontaneously hypertensive rats where a diminished purinergic modulation of vascular adrenergic neurotransmission was observed (Kamikawa *et al.*, 1980; Kubo & Su, 1983). In the guinea-pig portal vein neither prejunctional inhibitory purinoceptors nor quinacrine fluorescence is seen, suggesting that

physiological purinergic inhibition of noradrenergic neurotransmission does not occur in this tissue.

The concept that each nerve cell can synthesize, store and release only one neurotransmitter (Dale's principle) has been challenged in recent years (Burnstock, 1976; Cuello, 1982). NA and ATP appear to be stored together in sympathetic neurones in various ratios (see Burnstock, 1982). Adenine nucleoside and nucleotide release has been measured following stimulation of noradrenergic neurones both in visceral organs and in blood vessels (Su *et al.*, 1971; Su, 1975; Westfall *et al.*, 1978; Katsuragi & Su, 1980; 1982; Levitt & Westfall, 1982). It has also been suggested that the residual response to nerve stimulation in the cat nictitating membrane after pretreatment with reserpine is due to ATP (Langer & Pinto, 1976) and the initial phasic contraction during nervous stimulation of the guinea-pig vas deferens is due to ATP (Fedan *et al.*, 1981).

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