Br. J. Pharmac. (1986), **87**, 409–416

α_2-Adrenergic hyperpolarization is not involved in slow synaptic inhibition in amphibian sympathetic ganglia

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1 The adrenaline-induced hyperpolarization (Ad_H), slow inhibitory postsynaptic potential (slow i.p.s.p.) and hyperpolarizing phase of the response to methacholine (MCh_H) in *Rana pipiens* sympathetic ganglia were studied by means of the sucrose-gap technique.

2 Desmethylimipramine (DMI, 0.5 μM) lowered the EC_{50} for adrenaline from 1.65 μM (1.23–2.21 μM, $n = 10$) to 0.30 μM (0.21–0.41 μM, $n = 8$). DMI did not potentiate the slow i.p.s.p. or the MCh_H.

3 Propranolol, sotalol or prazosin (1 μM) did not antagonize the Ad_H. The response was antagonised by phentolamine (IC_{50} = 0.53 μM), yohimbine (IC_{50} = 6.2 nM) and idazoxan (IC_{50} = 0.59 μM). Yohimbine (0.1 μM) did not reduce the amplitude of the slow i.p.s.p. or the MCh_H.

4 The slow i.p.s.p. was eliminated in Ringer solution containing Cd^{2+} (100 μM). This concentration of Cd^{2+} did not reduce the amplitude of the MCh_H.

5 α-Methylnoradrenaline produced a concentration-dependent hyperpolarization with an EC_{50} of 0.31 μM (0.13–0.73 μM, n = 5), in the presence of DMI (0.5 μM).

6 These results are consistent with the hypothesis that the Ad_H may be generated by activation of a receptor similar to the mammalian α_2-adrenoceptor. No evidence was found in support of the hypothesis that an adrenergic interneurone is involved in the synaptic pathway for the slow i.p.s.p.

Introduction

Several kinds of synaptic potentials may be recorded in amphibian sympathetic ganglia. These are (i) the classical, fast, nicotinic e.p.s.p. (excitatory postsynaptic potential); (ii) the slow e.p.s.p.; (iii) the late slow e.p.s.p; and (iv) the slow i.p.s.p. (inhibitory postsynaptic potential) (for reviews see Kuba & Koketsu, 1978; Weight *et al.*, 1979; Weight, 1983). Although it is generally accepted that the slow e.p.s.p. is a muscarinic cholinergic response (Tosaka *et al.*, 1968; Koketsu, 1969; Adams & Brown, 1982) and the late slow e.p.s.p. is mediated by a peptide similar to luteinizing hormone releasing hormone (Jan *et al.*, 1979; Jones *et al.*, 1984), there is still some controversy with regard to the synaptic pathway and neurotransmitter mediating the slow i.p.s.p. Tosaka *et al.* (1968) suggested that an adrenergic interneurone might be involved and proposed that acetylcholine (ACh) released from preganglionic fibres activated muscarinic receptors on interneurones. It was proposed that these cells, later defined as SIF (small intensely fluorescent) cells, released a catecholamine which hyperpolarized ganglionic neurones and thereby generated the slow i.p.s.p. When Weight & Padjen (1973) re-examined the slow i.p.s.p. in amphibian sympathetic ganglia, their results suggested generation by a direct, muscarinic hyperpolarizing action of ACh on ganglionic C cells. Subsequently, Libet & Kobayashi (1974) presented the following lines of experimental evidence in support of the adrenergic interneurone hypothesis: (i) the slow i.p.s.p. was antagonized by the non-selective α-adrenoceptor antagonists, phentolamine (400 or 200 μM) and dihydroergotamine (40 μM); (ii) the slow i.p.s.p. was potentiated by 3',4' dihydroxy-2-methylpropriophenone (U-0521, Upjohn), an inhibitor of catechol-*O*-methyl transferase (COMT); (iii) the hyperpolarizing response to muscarinic agonists was reduced when transmitter release from interneurones was blocked with low Ca^{2+}, high Mg^{2+} Ringer and (iv) this response was also reduced by dihydroergotamine (40 μM) and blocked by phentolamine (200 μM). The evidence from more recently publised reports indicates that the slow i.p.s.p. is a direct muscarinic response (Weight & Weitsen, 1977; Weight & Smith, 1980; Horn & Dodd, 1981; Yavari & Weight, 1981; Dodd & Horn, 1983; Weight, 1983).

In mammalian ganglia, adrenergic hyperpolariza-

tion is mediated via an α_2-adrenoceptor (Brown & Caulfield, 1979; Cole & Shinnick-Gallagher, 1981) and catecholamines are not appreciably metabolized by COMT (Adler-Graschinski *et al.*, 1984) but are removed by re-uptake (Hanbauer *et al.*, 1972).

In the light of these findings, we thought it worthwhile to repeat the experiments of Libet & Kobayashi (1974) using more selective and appropriate pharmacological agents. The results obtained argue against the hypothesis that a SIF cell may be involved and support the idea that the slow i.p.s.p. may be generated by the direct muscarinic action of ACh on ganglion cell bodies.

Methods

Drug responses and the slow i.p.s.p. were recorded from the IXth or Xth paravertebral sympathetic ganglia of *Rana pipiens* by the sucrose-gap technique (Smith, 1984) and displayed on a rectilinear pen recorder (Gould Brush Model 2400, bandwidth; d.c. to 5 Hz, except where noted in Figure legends). The slow i.p.s.p. was evoked by tetanic stimulation of the VIIIth spinal nerve with 1 ms pulses at 10 Hz for 1 s (cf. Weight & Smith, 1980).

A system of three-way taps was fitted to the inlet of the recording chamber to allow the addition of drugs by superfusion. The composition of the Ringer solution used for all experiments was (mM): NaCl 100, KCl 2, $CaCl_2$ 1.8, tris (hydroxymethyl) aminomethane-HCl buffer (pH 7.2) 16 and D-glucose 10. In most experiments, 0.5 μM desmethylimipramine (DMI) was included in the Ringer solution. In those experiments where the slow i.p.s.p.. was studied or where methacholine (MCh) was used to evoke muscarinic responses, 70 μM (+)-tubocurarine chloride (Tc) was included in the Ringer solution to limit activation of nicotinic receptors.

All graphical data are presented as mean ± s.e.mean. Several log-concentration-effect curves for each agonist were obtained from 5 or more preparations and EC_{50} values determined using linear regression analysis on the straight line portion of each curve (approximately 20–80% of maximum response). The mean EC_{50} value (for n experiments) for each agonist was expressed as a geometric mean with 95% confidence limits. Drugs were purchased from the Sigma chemical company, St. Louis, MO, USA, except for DMI (Geigy Pharmaceuticals, Canada), prazosin (Pfizer Co. Ltd., Canada), clonidine (Boehringer Ingelheim (Canada) Ltd.), methoxamine (Burroughs Wellcome Ltd., Canada), α-methylnoradrenaline (Sterling Drug Co., Canada) and idazoxan (RX 781094) (Reckitt & Colman Pharmaceuticals).

Results

Adrenaline-induced hyperpolarization

The adrenaline-induced hyperpolarization (Ad_H) of bullfrog (*Rana catesbeiana*) sympathetic ganglion cells has been described by several authors (Libet & Kobayashi, 1974; Koketsu & Nakamura, 1976; Weight & Smith, 1980; Rafuse & Smith, 1982). In the present experiments however, we used paravertebral sympathetic ganglia from *Rana pipiens* since the Ad_H in this tissue is larger and less prone to desensitization than that recorded from bullfrog ganglia (Smith, 1984). Although depolarizing responses to high concentrations of adrenaline (> 20 μM) have occasionally been observed in *Rana catesbeiana* ganglia (Koketsu & Nakamura, 1976; Rafuse & Smith, 1982) we always observed membrane hyperpolarization in the present experiments.

Figure 1 illustrates the log concentration-effect curve for adrenaline. The EC_{50} which was calculated as described in the methods section was 1.65 μM (1.23–2.21 μM, $n = 10$).

[^{3}H]-noradrenaline uptake by rat superior cervical ganglia was reduced to 54% of control by 0.01 μM DMI (Hanbauer *et al.*, 1972). If an uptake system exists in frog ganglia, the Ad_H should be potentiated by uptake blockers. In agreement with this possibility,

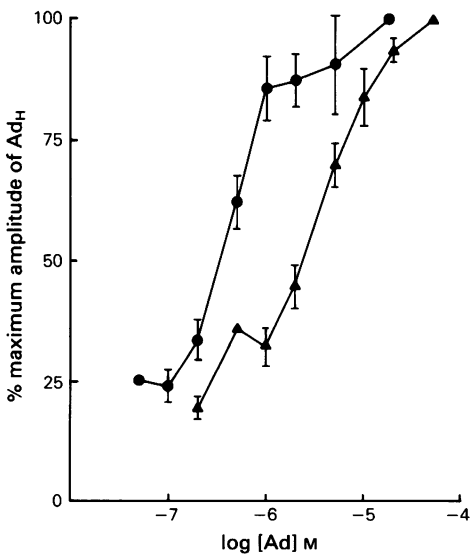

Figure 1 Log concentration-effect curve for adrenaline-induced hyperpolarization (Ad_H) of *Rana pipiens* sympathetic ganglia. (●) Ad_H in presence of 0.5 μM desmethylimipramine (DMI, 8 determinations on 5 preparations); (▲) Ad_H in absence of DMI (10 determinations on 5 different preparations).

we found that 0.5 μM DMI potentiated the Ad_H and lowered the EC_{50} for adrenaline to 0.30 μM (0.21–0.41, $n = 8$) (Figure 1). Since 1 μM adrenaline (in the presence of DMI) produced a large but submaximal response, this concentration was used in all subsequent experiments and produced responses ranging from 500 μV to 3 mV.

Receptor dependence of the adrenaline-induced hyperpolarization

In an attempt to determine the type of adrenoceptor mediating catcholamine induced-hyperpolarization in amphibian sympathetic ganglia, we tested the effects of various antagonists on the Ad_H, evoked by 1 μM adrenaline in the presence of 0.5 μM DMI. The Ad_H was not antagonized by the non-selective β-blockers propranolol ($n = 5$) or sotalol ($n = 3$) in concentrations up to 1 μM, nor by the selective α_1-blocker, prazosin (1 μM, $n = 3$). However, the Ad_H was reduced by the non-selective α-blocker, phentolamine (IC_{50} = 0.53 μM), by the α_2-selective antagonist, idazoxan (Doxey *et al.*, 1983) (IC_{50} = 0.59 μM) and by yohimbine (IC_{50} = 6.2 nM) (Figures 2 and 4). During superfusion of idazoxan, it was noted that a small transient depolarization occurred but this had entirely decayed before the application of adrenaline. No changes in membrane potential were seen with any of the other antagonists used.

Effects of other agonists

The above data suggest that the Ad_H in frog sympathetic ganglia may be generated via a receptor similar to the mammalian α_2-adrenoceptor. We therefore examined the effect of the potent α_2-receptor agonist, α-methylnoradrenaline (Berthelsen & Pettinger, 1977). This produced membrane hyperpolarization of similar amplitude and time course to the Ad_H. The EC_{50} was 0.31 μM (0.13–0.73 μM, $n = 5$). The response to 1 μM α-methylnoradrenaline was reduced to 11% of control by idazoxan 0.5 μM. Although clonidine has also been reported to act selectively at α_2-adrenoceptors (Berthelsen & Pettinger, 1977; but see also Bousquet *et al.*, 1984), only one out of six preparations tested exhibited clear hyperpolarizing responses to this substance (0.1–10 μM in presence or absence of DMI). The α_1-selective agonist, methoxamine (1 μM–1 mM) failed to produce hyperpolarization in any of 3 preparations tested.

Brown & Caulfield (1979) noted that dopamine and isoprenaline could promote hyperpolarization of rat superior cervical ganglia. We also observed hyperpolarizing responses to these substances in frog ganglia but these were smaller and generally more susceptible to desensitization than the Ad_H. We tested the effect of chlorpromazine (1 μM) as a dopamine receptor antagonist, on the dopamine-induced hyperpolarization (DA_H). Although the response became smaller in the presence of this antagonist, we were not confident that this effect did not reflect receptor desensitization rather than antagonism. On the other hand, in two experiments we were able to antagonize selectively the Ad_H but not the DA_H with yohimbine (0.01 μM).

Effect of desmethylimipramine on the slow i.p.s.p. and the methacholine induced hyperpolarization

Since the Ad_H was potentiated by DMI, this substance should also potentiate the slow i.p.s.p. if it is mediated

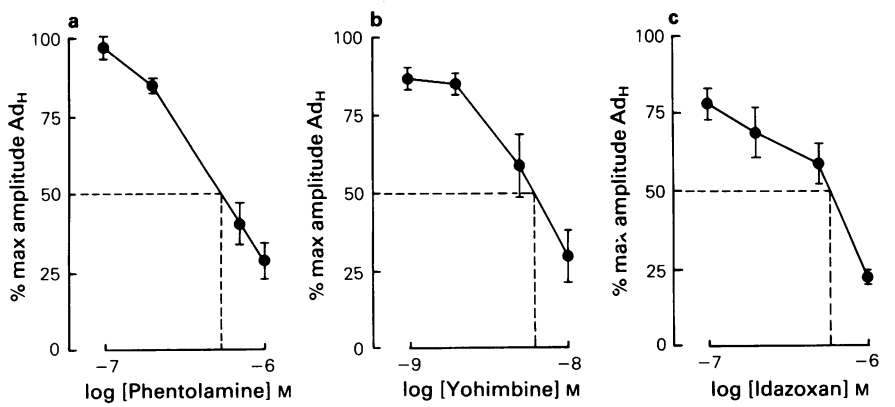

Figure 2 Log concentration-effect curves for antagonism of the hyperpolarization induced by 1 μM adrenaline (Ad_H) in the presence of 0.5 μM desmethylimipramine (DMI): (a) Phentolamine; (b) yohimbine and (c) idazoxan. IC_{50} values were calculated from these graphs by estimating the antagonist concentration required to promote a 50% depression of the control Ad_H.

via an adrenergic interneurone (Libet & Kobayashi, 1974). However, DMI (0.5 μM) failed to potentiate the slow i.p.s.p. in any of 4 preparations tested (Figure 3). If, as postulated by Libet & Kobayashi (1974), the hyperpolarizing effect of MCh is mediated via the release of an adrenergic neurotransmitter, the MCh_H, like the slow i.p.s.p., should be potentiated by DMI. In 4 experiments however, DMI (0.5 μM) was completely ineffective in potentiating the MCh_H (Figure 3).

Effect of yohimbine on the slow i.p.s.p. and MCh_H

With an IC_{50} of 6.2 nM, yohimbine was by far the most effective antagonist of the Ad_H. If an adrenergic interneurone is involved in the synaptic pathway for the slow i.p.s.p. (Libet & Kobayahsi, 1974) then both this response and the MCh_H should be antagonized by yohimbine. However, superfusion of yohimbine for periods of 45 min or more at 0.1 μM, failed to promote any antagonism of either the slow i.p.s.p. (2 experiments) or the MCh_H (4 experiments) (See Figure 4). We also noted that 1 μM chlorpromazine failed to reduce the amplitude of the MCh_H in any of 4 preparations tested. Also, 0.5 μM idazoxan failed to antagonize the slow i.p.s.p. recorded from bullfrog sympathetic ganglia ($n = 6$).

Effect of cadmium on the MCh_H

If an interneurone is involved in the slow i.p.s.p. pathway, Ca^{2+}-dependent release of a neurotransmitter should be required for the production of the MCh_H. In 3 experiments, the highly selective Ca^{2+} channel blocker, Cd^{2+} (100 μM) (Cooper & Manalis, 1984), not only failed to antagonize the MCh_H but slightly enhanced its amplitude (see Figue 5). This concentration of Cd^{2+} was effective in blocking transmitter release, since it eliminated the slow i.p.s.p. after about 15 min superfusion. The amplitude of the slow i.p.s.p. returned to control following 25 min washing with normal Ringer's solution (Figure 5).

Discussion

Pharmacology of the Ad_H

The responses to adrenaline recorded in *Rana pipiens*

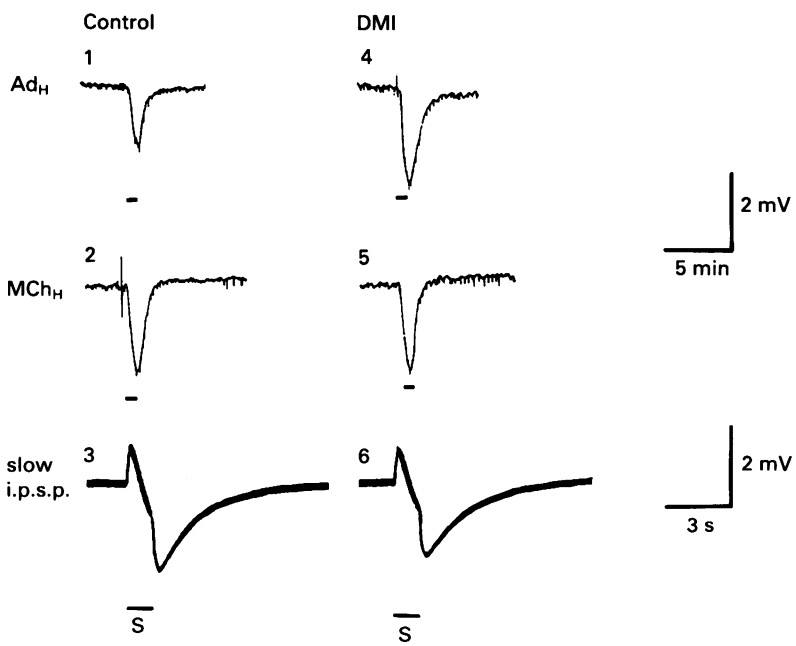

Figure 3 Effect of desmethylimipramine (DMI) on the Ad_H, the MCh_H and the slow i.p.s.p. (1,2 and 3). Control responses recorded in Ringer solution containing 70 μM (+)-tubocurarine (Tc). (4) Ad_H produced by 30 s superfusion of 1 μM adrenaline recorded after 10 min superfusion with Tc Ringer plus 0.5 μM DMI. (5) MCh_H evoked by a 30 s superfusion of 10 μM methacholine after 20 min in Tc/DMI Ringer solution. (6) slow i.p.s.p. evoked by tetanic stimulation of VIIIth spinal nerve (1 ms pulses, 1 s, 10 Hz) after 20 min superfusion with Tc/DMI Ringer. Note potentiation of Ad_H but not the slow i.p.s.p. or the MCh_H. Traces (1), (2), (4) and (5) from the same preparation; traces (3) and (6) from another preparation. The 2 mV/5 min calibration bar applies to all drug responses. S indicates period of stimulation for slow i.p.s.p. Records from rectilinear pen recorder, bandwidth d.c. to 5 Hz for drug responses and d.c. to 15 Hz for slow i.p.s.p.

were considerably larger, and less susceptible to desensitization than those recorded in *Rana catesbeiana* (Rafuse & Smith, 1982). This presumably reflects the presence of considerable diffusion barriers in the bullfrog. Such barriers would both limit access of agonist to the receptor and limit the diffusion of the agonist away from the receptor, the latter perhaps increasing receptor desensitization.

The antagonism of the Ad_H by phentolamine, but not by propranolol or sotalol, indicates that the response may be generated via an α-adrenoceptor. Its antagonism by yohimbine and idazoxan but not by 1 μM prazosin suggests that this receptor is of the α_2-subtype. The ability of α-methylnoradrenaline, but not the α_1-agonist, methoxamine, to produce membrane hyperpolarization is also consistent with this possibility. In addition clonidine, dopamine and isoprenaline produced membrane hyperpolarization and all three responses desensitized much more rapidly than adrenaline responses evoked in the same preparation. Although it is assumed that clonidine exerts its effects through α_2-adrenoceptors (Berthelsen & Pettinger, 1977), it is possible there are 'imidazoline receptors' which are activated by this substance (Bousquet *et al.*, 1984). Similarly, dopamine could act on a dopamine receptor (Dun *et al.*, 1977; but see also Brown & Caulfield, 1979) and isoprenaline on a β-receptor (but see Suzuki & Volle, 1978). The lack of cross-desensitization between adrenaline and clonidine, dopamine or isoprenaline is consistent with the possibility that different receptor sites exist for each substance. Alternatively, these agonists could bind at a different site from adrenaline on the α_2-receptor as suggested by Brown & Caulfield (1979) for rat superior cervical ganglion. Unfortunately, the rapid desensitization seen with clonidine and isoprenaline precluded our testing the effects of idazoxan, yohimbine or propranolol. Since it was possible, in a few experiments, to observe antagonism of the Ad_H by yohimbine at a time when the DA_H was unchanged (cf. Brown & Caulfield, 1979, it is likely that these two agonists act at different receptors. Unfortunately, the rapid desensitization

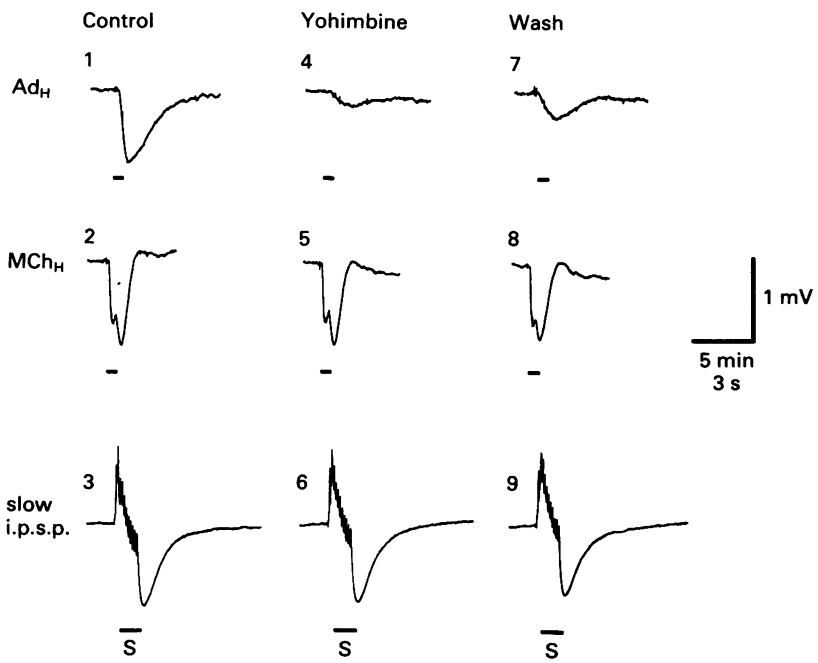

Figure 4 Effect of yohimbine of Ad_H, MCh_H and slow i.p.s.p. All responses recorded from the same preparation in Ringer solution containing 0.5 μM desmethylimipramine (DMI) and 70 μM (+)-tubocurarine (Tc). (1) Control response to 1 μM adrenaline (30 s superfusion); (2) control response to 1 mM MCh (30 s superfusion); (3) control slow i.p.s.p. evoked by tetanic stimulation of VIIIth spinal nerve (1 s, 10 Hz, 1 ms pulse width); (4) Ad_H recorded after 35 min in 0.1 μM yohimbine; (5) MCh_H recorded after 50 min in 0.1 μM yohimbine; (6) slow i.p.s.p. recorded after 15 min in 0.1 μM yohimbine. Note that Ad_H is almsot blocked whilst the MCh_H and slow i.p.s.p. are not attenuated. (7), (8) and (9) responses recorded 35, 20 and 45 min, respectively, after resuming superfusion with DMI/Tc Ringer solution. 3 s calibration refers to slow i.p.s.p. records only. Responses recorded from rectilinear pen recorder, bandwidth d.c. to 5 Hz for drug responses and d.c. to 15 Hz for slow i.p.s.p.

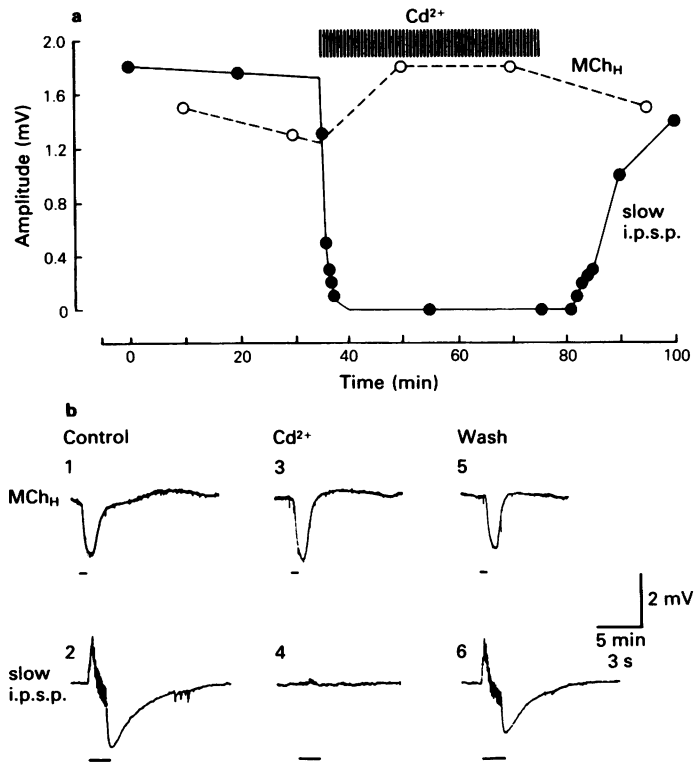

Figure 5 Effect of 100 μM Cd^{2+} on the MCh_H and synaptic transmission (slow i.p.s.p.) in curarized *Rana pipiens* sympathetic ganglia. (a) Time course of effect of 100 μM Cd^{2+} on slow i.p.s.p. and MCh_H. (b) Records from same experiment as (a). (b1) response to 30 s superfusion of 10 μM MCh; (b2) slow i.p.s.p. evoked by tetanic stimulation of VIIIth spinal nerve (1 s, 10 Hz, 1 ms pulse width); (b3) MCh response 35 min after superfusion with (+)-tubocurarine (Tc) Ringer solution containing 100 μM Cd^{2+}; (b4) effect of tetanic stimulation of VIIIth spinal nerve after 20 min superfusion with Ringer solution containing 100 μM Cd^{2+}. Note blockade of slow i.p.s.p. and slight enhancement of MCh_H. (b5) and (b6) MCh response and slow i.p.s.p. recorded 25 and 20 min after resuming superfusion with normal Tc Ringer solution; 5 min calibration refers to MCh response. 3 s refers to slow i.p.s.p. Traces from rectilinear pen recorder, bandwidth d.c. to 5 Hz for MCh responses and d.c. to 15 Hz for slow i.p.s.p.

usually seen with dopamine precluded a meaningful evaluation of the effect of the dopamine antagonist, chlorpromazine.

In terms of sensitivity to antagonists and agonists, the adrenoceptors involved in the generation of the Ad_H in *Rana pipiens* sympathetic ganglia resemble those in mammalian superior cervical ganglia and can therefore be tentatively assigned to the α_2-category. However, our IC_{50} values for antagonists differed from those obtained for mammalian neuronal α_2-receptors. For example, North & Surprenant (1985) reported IC_{50} values of 62 nM, 5.7 nM and 4.7 nM for yohimbine, idazoxan and phentolamine, respectively. Our values for the same three drugs acting at amphibian adrenoceptors were 6.2 nM, 0.59 μM and 0.53 μM, respectively. This suggests some differences between mammalian α_2-receptors and the receptors mediating the Ad_H in amphibian sympathetic ganglia. Also, in contrast to results on rat ganglia obtained by Brown & Dunn (1983), we have no evidence for depolarizing β-receptors in *Rana pipiens* ganglia.

Adrenergic interneurone hypothesis

Our results using selective and appropriate pharmacological agents are completely at odds with the hypothesis that interneurones are involved in the slow i.p.s.p. (Libet & Kobayashi, 1974). Firstly, prolonged superfusions with yohimbine (0.1 μM, 16 × the IC_{50} for antagonism of the Ad_H) caused no reduction in the amplitude of the slow i.p.s.p. or the MCh_H. If an adrenergic interneurone were involved, these responses should have been antagonized. Secondly, DMI (0.5 μM), which caused a five fold decrease in the ED_{50}

for adrenaline, failed to potentiate either the MCh_H or the slow i.p.s.p. Since uptake seems to be a major mechanism for inactivation of catecholamines in ganglia (Hanbauer *et al.*, 1972; Adler-Graschinsky *et al.*, 1984), DMI should have potentiated these responses if an adrenergic interneurone were involved in their electrogenesis. Thirdly, Cd^{2+} (100 μM), which was effective in blocking synaptic transmission, failed to antagonize the MCh_H. If muscarinic hyperpolarization requries neurotransmitter release from an interneurone, the response should have been blocked. We therefore conclude, in agreement with Weight & Padjen (1973), Weight & Weitsen (1977), Weight & Smith (1980), Horn & Dodd (1981), Yavari & Weight (1981), Dodd & Horn (1983) and Weight (1983), that the slow i.p.s.p. is generated by the direct muscarinic action of ACh on ganglionic neurones.

The antagonism of the slow i.p.s.p. by high concentrations of α-blockers used by Libet & Kobayahsi (1974) is probably due to non-specific blockade of synaptic transmission by these substances (Yavari & Weight, 1981); they may block Ca^{2+} channels in cultured neuroblastoma-glioma hybrid cells (Atlas & Adler, 1981). The reduction fo the MCh_H by dihydroergotamine (40 μM) and its blockade by phentolamine (200 μM) reported by Libert & Kobayashi (1974) probably reflect non-specific effects of these high concentrations. The potentiation of the slow i.p.s.p. by U-0521 which was described by Libet & Kobayashi (1974) has not been observed by others (P. Yavari & F.F. Weight-personal communication).

The blockade of muscarinic hyperpolarization by low Ca^{2+} high Mg^{2+} Ringer reported by Libet & Kobayashi (1974) has not been observed by other workers (Weight & Smith, 1980; Horn & Dodd, 1981). It may have resulted from changes in membrane responsiveness following 1 h of superfusion with high Mg^{2+}, low Ca^{2+} Ringer.

The significance of ganglionic adrenergic interneurones was originally advanced for rabbit superior cervical ganglia by Eccles & Libet (1961). However, several recent investigations on mammalian sympathetic ganglia provide no (Gallagher *et al.*, 1980; Cole & Shinnick-Gallagher, 1980; 1981; 1984) or only qualified support for their presence (Dun, 1980; Dun & Karczmar, 1980; Ivanov & Skok, 1980). More recently, Ashe & Libet (1982) have reported antagonism of the slow i.p.s.p. in rabbit superior cervical ganglion with yohimbine (5 μM). Since the IC_{50} for antagonism of adrenergic synaptic inhibition in other neuronal systems is 43 nM (North & Surprenant, 1985), any antagonism of the slow i.p.s.p. in sympathetic ganglia by micromolar doses of yohimbine may reflect a non-specific drug effect. We therefore conclude that the available evidence lends little support to the idea that adrenergic interneurones are involved in the generation of slow synaptic inhibition in either mammalian or amphibian sympathetic ganglia.

Supported by grants from the Medical Research Council of Canada and The Alberta Mental Health Advisory Council. A studentship for P.E.R. and a scholarship for P.A.S. from the Alberta Heritage Foundation for Medical Research are also gratefully acknowledged. We thank Ms Jacquie Tucker for typing, Mr Gus Duchon for graphics and Dr David Cook for reading an early version of the manuscript.

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(Received May 23, 1985.
Revised October 2, 1985.
Accepted October 22, 1985.)