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INHIBITION BY SUBSTANCE P OF SOME PERIPHERAL ACTIONS OF ACETYLCHOLINE IN THE CAT

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1 The effect of substance P on contractions of the nictitating membrane and pressor responses to acetylcholine (ACh) and dimethylphenyl-piperazinium (DMPP) which were mediated via nocotinic receptors was studied in cats anaesthetized with chloralose.

2 Substance P (2–20 nmol) injected into the lingual artery giving estimated concentrations in arterial blood of 10^{-6} to 10^{-5} M, or intravenously giving estimated concentrations in blood of 10^{-8} to 10^{-7} M, reduced hexamethonium-sensitive but not atropine-sensitive responses.

3 The pressor effects of ACh and DMPP injected intra-arterially in atropinized and non-atropinized cats respectively were consistently attenuated by substance P given intra-arterially or intravenously.

4 The contractile effect of ACh in atropinized and of DMPP in non-atropinized cats was attenuated by substance P injected intra-arterially but only rarely when the polypeptide was injected intravenously.

5 The depressor effects of substance P *per se* were variable in magnitude and duration as were the inhibitory effects upon nicotinic receptors. The depressor and inhibitory effects of substance P were unrelated.

6 There was desensitization to all of these effects of substance P which probably contributed to the variation in the magnitude of the effects observed.

7 Substance P had no effect on muscarinic actions of acetyl-β-methylcholine on the nictitating membrane or blood pressure.

8 The results are discussed in relation to the ubiquity of the modulatory actions of substance P on nicotinic receptors and in relation to the possible physiological significance of the action.

Introduction

Substance P is found in neurones in the central and peripheral nervous systems (Hökfelt, Johansson, Kellerth, Ljungdahl, Nilsson, Nygards & Pernow, 1977; Cuello & Kanazawa, 1978; Chan-Palay, 1979a). It is particularly associated with primary afferent fibres and with sensory fibres in peripheral autonomic nerves. It may also co-exist with 5-hydroxytryptamine in fibres descending the spinal cord from supraspinal structures (Hökfelt, Ljungdahl, Steinbusch, Verhofstad, Nilsson, Brodin, Pernow & Goldstein, 1978; Chan-Palay, Jonsson & Palay, 1978; Chan-Palay, 1979b). In sympathetic ganglia, substance P is located in sensory fibres and is also found to a variable degree in postganglionic noradrenergic neurones (Hökfelt, Elfvin, Schultzberg, Goldstein & Nilsson, 1977; Robinson, Schwartz & Costa, 1980; Kessler, Adler, Bohn & Black, 1981). Substance P has several different effects. It usually excites central neurones (Otsuka & Konishi, 1977; Krnjević, 1977; Nowak & MacDonald, 1981) and in spinal motoneurones a large component of the depolarization is attributable to a decrease in potassium conductance (Krnjević, 1977; Nowak & MacDonald, 1981). However, there is another component to the depolarization which is potentiated by the cholinesterase inhibitor, edrophonium (Otsuka, 1982), which could be the same as that which causes tetrodotoxin-resistant burst discharges *in vitro* (Nowak & MacDonald, 1981). These effects may therefore be mediated indirectly by the release of acetylcholine (ACh) from cholinergic nerve terminals.

On spinal Renshaw cells, substance P occasionally causes excitation which is blocked by the nicotinic receptor antagonist, dihydro-β-erythroidine (Belcher & Ryall, 1977; Ryall & Belcher, 1977) but more consistently antagonizes the excitatory effect of ACh, leaving excitation by acidic amino acids unchanged (Belcher & Ryall, 1977; Ryall & Belcher, 1977; Krnjević & Lekić, 1977). Furthermore, the inhibition is selective for nicotinic ACh receptors because responses to acetyl-β-methylcholine, acting

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via muscarinic receptors, are not changed (Belcher & Ryall, 1977; Ryall & Belcher, 1977). Steinacker & Highstein (1976) have shown that substance P has a postsynaptic depressant effect in the hatchet fish, which could also be due to ACh antagonism. On other central neurones on which the effect of ACh is mediated predominantly by muscarinic receptors, substance P has no inhibitory effect (Krnjević & Lekić, 1977).

We have therefore studied the effects of substance P on the contractions of the nictitating membrane in response to intra-arterial injections of ACh to the superior cervical ganglion in atropinized cats, and also on the blood pressure responses to such injections in order to determine whether the modulatory effects demonstrated on Renshaw cells may be characteristic of its effects at nicotinic receptors at other sites.

A brief account of some of these data has been presented (Ryall, 1982).

Methods

Experiments were carried out on cats of either sex weighing between 2.1–4.1 kg, anaesthetized with α-chloralose after induction with halothane. Chloralose was given in an initial dose of 80 mg/kg intravenously, supplemented by further injections of 20–30 mg/kg as required. In some, but not all experiments, the animals were paralysed by periodic injections of gallamine triethiode and in these experiments end-tidal CO_2 was maintained between 3.5–4.5% by artificial ventilation. The cephalic veins in the fore-limbs were cannulated for intravenous drug administration. Arterial blood pressure was recorded from a femoral artery. Retrograde intra-arterial injections were made through a cannula inserted into the lingual artery. When injections were routed through the superior cervical ganglion the external carotid artery was temporarily occluded by a ligature placed around it (Trendelenburg, 1956; Beleslin, Radmanović, & Varagić, 1960). For injections to be routed mainly via the nictitating membrane the external carotid was left patent.

All intra-arterial injections were made in volumes of 0.2 ml over periods of 2–4 s and were not usually washed in. After preliminary observations were made some cats were atropinized by the intravenous injection of atropine sulphate in amounts up to 2 mg/kg, until depressor responses to ACh were abolished. In later experiments, after an initial priming dose of atropine had been given, the antagonist was infused at a rate of 1–2 mg kg^{-1} h^{-1} to maintain a stable blockade of the muscarinic receptors for ACh.

Drugs used were: α-chloralose (BDH); ACh bromide (mol.wt. 226.1; Sigma); dimethylphenylpiperazinium iodide (DMPP), (mol.wt. 318.2; Ralph, Emanuel); atropine sulphate (mol.wt. 694.9; Cal-Biochem); hexamethonium bromide (mol.wt 362.2; Koch Light) and substance P (mol.wt. 1348; Protein Research Foundation); acetyl-β-methylcholine bromide (mol.wt. 240.2; Cal-Biochem). All solutions were made up in isotonic saline. Substance P was usually injected in amounts ranging from 2 to 20 nmol (2.7 to 27 μg) but in one experiment up to 80 nmol (108 μg) was injected.

The preganglionic cervical sympathetic nerve was transected bilaterally and the contractions of both decentralized nictitating membranes were recorded by means of force-displacement transducers. The preganglionic nerves were stimulated with rectangular constant voltage pulses 0.2 ms in duration at frequencies of 0.5–15 Hz. Submaximal contractions were evoked by continuous stimulation at 0.5 Hz or intermittent periods of stimulation at 1–1.5 Hz for 15 s.

Results

Effects of acetylcholine, dimethylphenylpiperazinium, acetyl-β-methylcholine, atropine and hexamethonium

In the absence of antagonists, contractions of the nictitating membrane could be evoked by small doses of ACh or acetyl-β-methylcholine injected into the lingual artery so as to reach either the superior cervical ganglion or the nictitating membrane (Figure 1a, b). Since contractions in response to ACh or acetyl-β-methylcholine, by either route, were blocked by intravenous atropine they were due to interactions with muscarinic receptors in the membrane or the ganglion (Trendelenburg, 1966). DMPP caused contractions only when injected to the ganglion (Figure 1c) but these were not blocked by the intravenous injections of atropine (Figure 1). Atropine also blocked the depressor effects of ACh and acetyl-β-methylcholine but not the pressor effect evoked by DMPP. Larger amounts of acetylcholine, but not of acetyl-β-methylcholine, again caused contractions (Figure 1) which were reduced by intravenously injected hexamethonium (Figure 2b) and were therefore due to activation of nicotinic receptors.

Pressor responses obtained with ACh after the injection of atropine were blocked by hexamethonium.

In one experiment, large doses of acetyl-β-methylcholine injected to the membrane were required to elicit contractions in the absence of atropine. These contractions were almost completely blocked by hexamethonium and the residual contraction was abolished by atropine. This suggests that

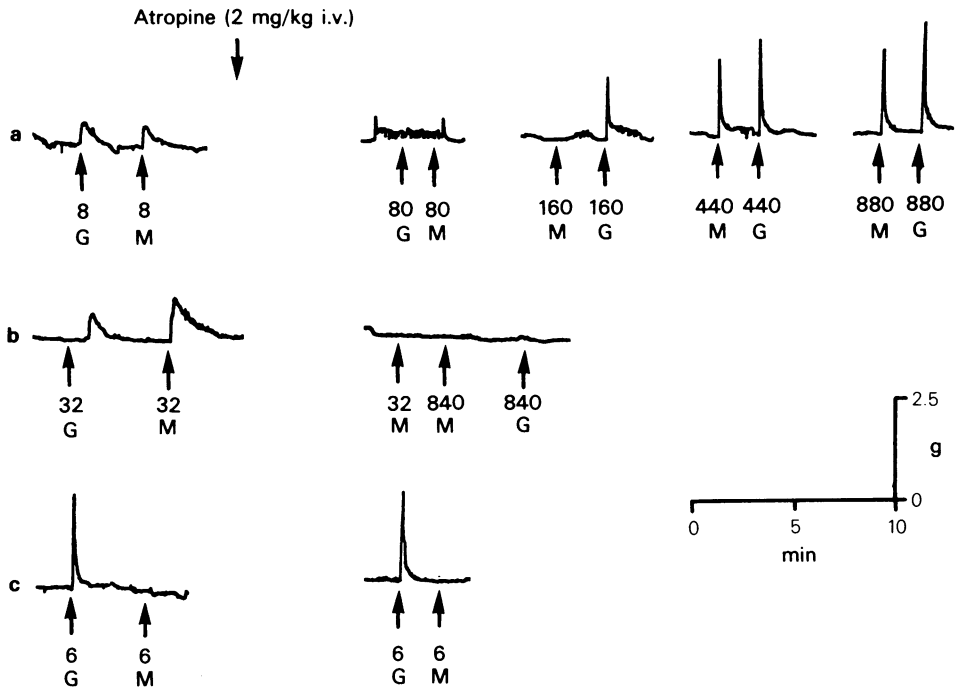

Figure 1 Effect of atropine (2 mg/kg i.v.) on contractions of the nictitating membrane to acetylcholine ACh (a), acetyl-β-methylcholine (b) and dimethylphenyl-piperazinium (DMPP) (c) injected intra-arterially to either the superior cervical ganglion (G) or to the membrane (M). Control records are shown in the left hand column. Quantities of agonists are shown below each record and are expressed as the total amount injected (nmol in a volume of 0.2 ml). Calibrations are time in min and change in tension (g).

there was a predominantly nicotinic response to acetyl-β-methylcholine in this experiment. The receptors may have been located either in the ganglion or on noradrenergic terminals in the membrane (Thompson, 1958).

Hexamethonium in doses that abolished the contractions to ACh in atropinized cats reduced the contractions evoked by maximal or submaximal stimulation of the preganglionic cervical sympathetic nerve (Figure 2b).

Effects of substance P on the blood pressure

In every animal (12 experiments) the intravenous or intra-arterial injection of substance P in amounts ranging from 5–20 nmol caused an initial fall in arterial blood pressure with a duration of about 15 s to about 6 min in different animals (Figures 3 and 4). Subsequent injections even at long intervals up to 1 h usually caused smaller and briefer effects. In 5 of the 12 cats, the initial fall in blood pressure was succeeded, after recovery to near control values, by a secondary fall lasting from 20 min to more than 60 min. The secondary depressor action of substance P was not observed with subsequent injections of substance P, except in one cat in which both phases were evident with each of three injections of substance P at 1 h intervals.

Effects of substance P on pressor effects of acetylcholine and dimethylphenyl-piperazinium

Substance P reduced the pressor effects of ACh or DMPP, injected to the ganglion, in 8 of 9 atropinized cats and in 2 non-atropinized cats respectively. In 6 of these cats substance P was injected intravenously in amounts ranging from 5–20 nmol and in only one cat was there no effect on pressor responses to ACh. In the remaining 5 cats, the substance P was injected intra-arterially to the ganglion and nearby structures in amounts similar to those employed intravenously and in every instance the pressor effect of ACh or DMPP was reduced or abolished.

The attenuation of the pressor effects of ACh or DMPP lasted for periods ranging from 6 min to about 2 h but the effect was variable in both duration and

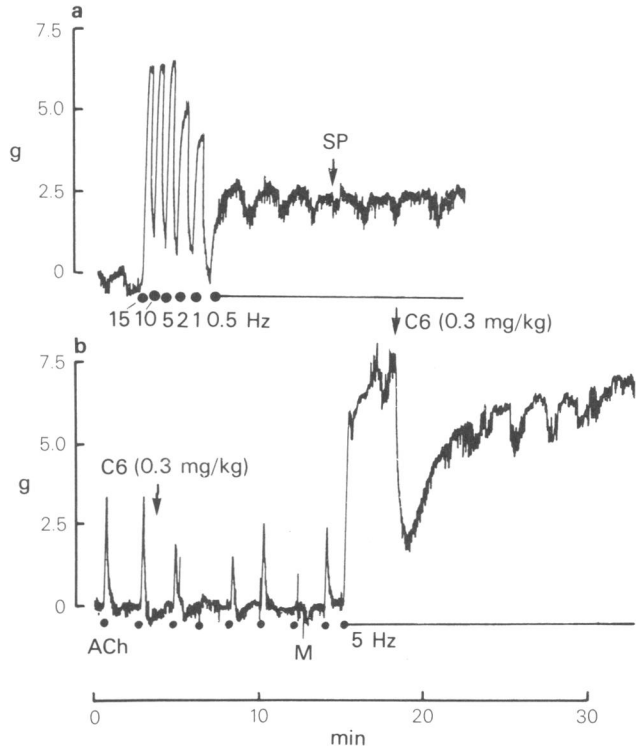

Figure 2 Atropinized cats. (a) Lack of effect of substance P (SP, 20 nmol, 27 μg, in 0.2 ml injected to the ganglion) on submaximal contraction of the nictitating membrane to continuous stimulation of the preganglionic nerve at 0.5 Hz: a maximal contraction was produced at 5 Hz. (b) Block by hexamethonium (C6, 0.3 mg/kg i.v.) of contractions of nictitating membrane to acetylcholine (ACh, ●, 880 nmol, 200 μg, injected to the ganglion) and to stimulation of the preganglionic nerve at 5 Hz. At M, ACh was injected to the nictitating membrane.

extent, even in the same animal. This was attributed in part to desensitization (Figure 4) of substance P responses but the extent of desensitization was also quite variable because it was possible to reproduce effects at relatively short intervals in some cats but in others intervals as long as 1.5 to 2 h seemed inadequate to prevent desensitization. The depression of the pressor responses was unrelated to either the extent or duration of the depressor action of substance P.

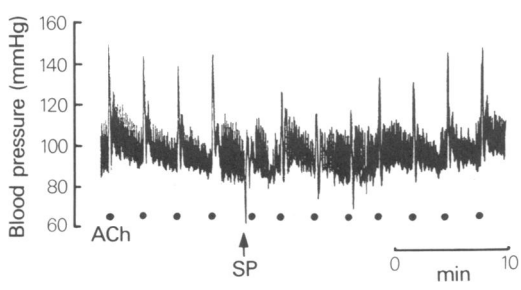

Figure 3 Atropinized cat. Effect of substance P (SP, 5 nmol, 6.8 μg, i.v.) on pressor response to acetylcholine (ACh, ●, 220 nmol, 50 μg, injected to the ganglion).

Effects of substance P on the contractions of the nictitating membrane

Intravenous or intra-arterial injections of substance P did not evoke contractions of the unstimulated nictitating membrane and usually (8 experiments) had no effect on the contractions produced by intermittent or continuous submaximal stimulation of the preganglionic sympathetic nerve (Figure 2a). In two experiments there was a small (10–20%) reduction in the amplitude of the contractions with intravenous injections of 4 or 20 nmol respectively, but these small effects were not reproducible. They were observed on only one occasion in each animal after several preceding injections in the same animals had no effect.

In 6 atropinized cats the intravenous injection of

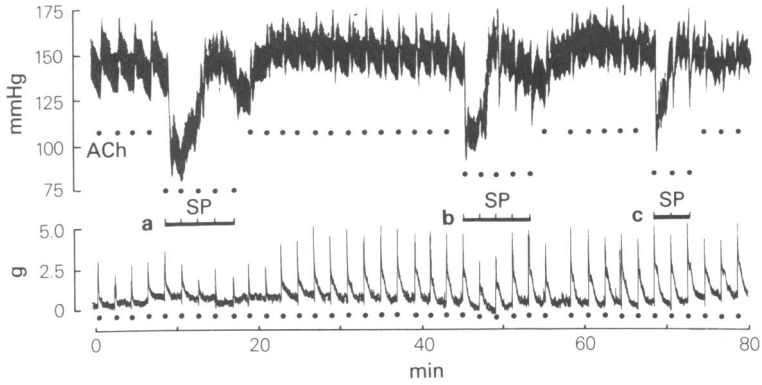

Figure 4 Atropinized cat. Effect of repeated intra-arterial injections of substance P (SP, 18 nmol, 24 μg, injected to the ganglion) on the blood pressure and nictitating membrane and responses to acetylcholine (ACh ●, 440 nmol, 100 μg, injected to the ganglion). A series of five injections (a,b) or three injections (c) of substance P were given as indicated.

substance P either had no effect on the contractions evoked by intra-arterial injections of ACh to the ganglion (4 experiments) or caused a 10–50% attenuation of the response in the remaining 2 cats. In both of these animals the magnitude and duration of the effect was not constant with repeated injections.

The intra-arterial injection of 20 nmol of substance P reduced the contractions evoked by either ACh (3 experiments in atropinized cats; Figures 4 and 5) or DMPP (2 experiments in non-atropinized cats; Figure 5) in all 5 animals. The magnitude of the inhibitory effect was quite variable and ranged from only 10% inhibition (one experiment) to complete inhibition (one experiment) with 30–60% inhibition in 3 experiments. The duration of the inhibitory effect was also quite variable even in the same animal: in one instance the inhibition exceeded 100 min in duration but it was more usually 3–10 min in duration. With repeated injections of substance P at short intervals, desensitization to the inhibitory effect was clearly evident (Figure 4).

The effect of intra-arterial injections of substance P on contractions to acetyl-β-methylcholine was investigated in 3 non-atropinized cats. In these experi-

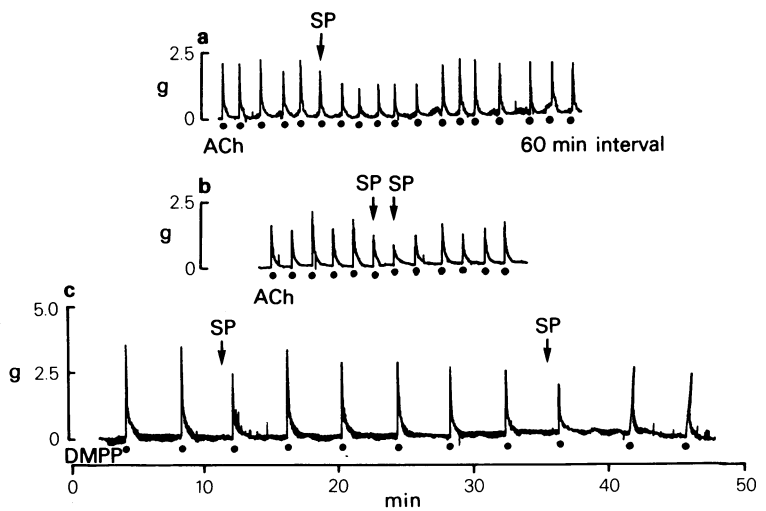

Figure 5 (a an b) Inhibitory effect of substance P (SP, 20 nmol, 27 μg, injected to the ganglion) on contractions of the nictitating membrane to acetylcholine (ACh, ●, 400 nmol, 91 μg, injected to the ganglion) in an atropinized cat: 60 min interval between records (a) and (b). (c) Results from a different experiment in which dimethylphenylpiperazinium (DMPP, ●, 6 nmol, 1.9 μg, injected to the ganglion) was used as the agonist in a non-atropinized cat.

ments both substance P and acetyl-β-methylcholine injections were made with the external carotid artery patent to direct the injections to the nictitating membrane, rather than towards the ganglion. In 2 of these experiments the contractions evoked by acetyl-β-methylcholine were unaffected by substance P (Figure 6) but in the third experiment they were almost completely abolished with recovery occurring in 1 h. In this experiment, the contractions were only produced by large amounts (250 nmol) of the agonist and were reduced to a similar extent by the intravenous injection of hexamethonium, the residual contraction being completely inhibited by atropine.

Substance P had no effect on the depressor actions of acetyl-β-methylcholine in 3 experiments in which both substances were given intra-arterially or in one experiment in which they were injected intravenously. In only one of these experiments was the dose of acetyl-β-methylcholine submaximal.

Discussion

This study has shown that synthetic substance P reduces hexamethonium-sensitive contractions of the nictitating membrane and also reduces pressor responses evoked by the intra-arterial injection of ACh in atropinized animals or of DMPP in non-atropinized animals.

In low doses injected intra-arterially to the nictitating membrane, ACh or acetyl-β-methylcholine, but not DMPP, caused contractions and depressor responses that were completely blocked by small intravenous injections of atropine. These contractions and depressor responses were therefore due to an activation of muscarinic receptors. In higher doses in atropinized cats the intra-arterial injection of ACh again caused contractions that were larger when the external carotid artery was occluded than when it was patent. This observation, together with the fact that the contractions evoked by ACh were unaffected by larger doses of atropine but were completely blocked by hexamethonium shows that they were due to the activation of nicotinic receptors in the ganglion.

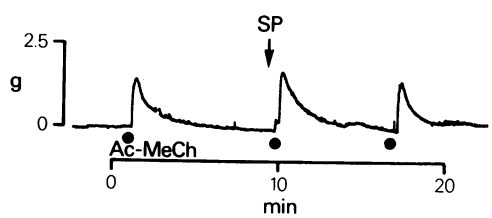

Figure 6 Non-atropinized cat. Lack of effect of substance P (SP, 20 nmol, 27 μg, injected to the membrane) on contractions of the nictitating membrane to acetyl-β-methylcholine (●, AcMeCh; 32 nmol, 7.7 μg, injected to the membrane).

Although in some experiments in atropinized cats contractions to large doses of ACh were obtained when the external carotid artery was patent, the contractions were still abolished by hexamethonium. They were therefore due to activation of nicotinic receptors and were probably related to the ability of some of the injected ACh to reach the superior cervical ganglion, even when the external carotid was not occluded, or to the presence of nicotinic receptors on noradrenergic terminals in the membrane (Thompson, 1958).

The pressor responses to ACh in atropinized cats and to DMPP in non-atropinized or atropinized animals were blocked by hexamethonium and so were also due to the activation of nicotinic receptors.

The inhibitory effect of substance P on the nictitating membrane was best demonstrated with intra-arterial injections and was only rarely observed after intravenous injections of the same amounts. Only a minimal or more usually no effect was observed on the contractions produced by submaximal stimulation of the preganglionic nerve. The resistance of preganglionic nerve stimulation in atropinized cats to substance P is unexplained but may be due to the higher subsynaptic concentrations of ACh achieved relative to the concentrations of ACh which were injected: hexamethonium in a dose which completely blocked the response to injected ACh caused only a partial block of the response to nerve stimulation (Figure 2b). Similarly, on Renshaw cells, substance P produced only a small attenuation of cholinergic nerve excitation but completely blocked the effects of administered ACh (Belcher & Ryall, 1977).

The effects of substance P were quite variable in extent and duration in different animals and even within the same animal. This was shown to be due in part to desensitization. The half life of substance P in the circulation of the cat is unknown, but the long duration of some of the effects observed suggests that the half life could be as long as 30 min, at least in some animals.

Although slow infusion of known concentrations intra-arterially may have made inhibitory effects of substance P more consistent at low concentrations, this was not studied because it was considered that desensitization would have been even more marked under these conditions. Although desensitization to substance P probably accounted for much of the variability in the response obtained, other factors such as sex and uncontrolled variations in experimental conditions cannot be excluded.

Substance P was injected intra-arterially in a volume of 0.2 ml in 2–4 s at a concentration of 10^{-5} or 10^{-4}M. The actual concentration in the arterial blood, assuming a carotid blood flow rate of

0.2–0.5 ml/s, was certainly less than this by a factor of 3–10 because of dilution in the arterial blood. Nevertheless, such concentrations seem superficially to be too high to be of physiological significance although if substance P were to be released from nerve terminals in close proximity to the receptors then high local concentrations might well be achieved. These concentrations are of the same order of magnitude as those required *in vitro* for substance P to block the release of noradrenaline from adrenal medullary neurones by ACh acting on nicotinic receptors (Mizobe, Kosousek, Dean & Livett, 1979).

Intra-arterial injections of substance P also reduced the pressor effects of intra-arterially injected ACh in atropinized cats. Since the pressor effects, like the contractions of the nictitating membrane, were abolished by hexamethonium they are also due to activation of nicotinic receptors. McQueen (1980) has shown, by direct recordings of chemoreceptor discharges, that intra-arterial injections of ACh, in amounts similar to those used in the present study activate cat carotid chemoreceptors and that the activation is reduced by the intra-arterial injection of 10 μg of substance P. Therefore, it seems likely that the pressor actions of ACh which we have recorded were due to activation of chemoreceptors in the carotid region and that the inhibitory effects of substance P were due to an interaction with nicotinic receptors in this region rather than with the release of noradrenaline from nerve terminals elsewhere. This conclusion is supported by observations which show that substance P enhances, rather than decreases transmitter release from noradrenergic terminals (Zetler, 1977).

The pressor effects of intra-arterially injected ACh, unlike the effects on the nictitating membrane, were quite regularly reduced by the intravenous injection of substance P. Assuming a simple distribution of substance P in the blood volume, the concentrations achieved by intravenous injections were of the order of 10^{-8} to 10^{-7}M and are therefore lower than those probably achieved with intra-arterial administration (10^{-6} to 10^{-5}M).

Thus, the modulatory action of substance P, as seen on the nicotinic actions of ACh on Renshaw cells (Belcher & Ryall, 1977; Ryall & Belcher, 1977; Krnjević & Lekić, 1977) is also exerted at several other sites at which the effects of ACh are mediated via nicotinic receptors. Effects of ACh which are mediated via muscarinic receptors, either on Renshaw cells, on the nictitating membrane or on the blood pressure, are not reduced by substance P. Further evidence of the selectivity in this inhibitory effect of substance P is that the polypeptide does not reduce neuronal excitation by acidic amino acids (Belcher & Ryall, 1977; Krnjević & Lekić, 1977) or the potassium or veratridine-evoked release of noradrenaline from adrenal neurones (Mizobe *et al.*, 1977; Dean & Livett, 1980) or the excitatory effects of sodium cyanide or 5-hydroxytryptamine on carotid chemoreceptors (McQueen, 1980).

These effects of substance P are also produced by other polypeptides on adrenal neurones (Mizobe *et al.*, 1979; Mizobe, Dean & Livett, 1979) but Met-enkephalin has only minimal effects on the excitatory action of ACh on carotid chemoreceptors (McQueen & Riveiro, 1980, although it does have marked effects on spontaneous chemoreceptor discharges.

In other experiments we have found no evidence of antagonism by substance P of the nicotinic excitatory effects of ACh at the neuromuscular junction, either on the rectus abdominis muscle of the frog (Ryall & Belcher, 1977) or on the biventer cervicis muscle of the chick (R.N.R. Wallace and R.W. Ryall, unpublished data). Nevertheless, substance P does have a presynaptic facilitatory effect on ACh release at the neuromuscular junction (Steinacker, 1977) as well as on central neurones (see Introduction).

In conclusion, a modulatory effect of substance P on nicotinic receptors has now been demonstrated at a number of central and peripheral sites including Renshaw cells, sympathetic ganglia, adrenal neurones and carotid chemoreceptors, but not at the neuromuscular junction. Whether any or all of these effects are related to a physiological function of substance P must await the demonstration that neurally released substance P has an inhibitory effect on cholinergic transmission at these sites. In this respect, the actions of new substance P antagonists which are more selective than those currently available may provide some interesting data.

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