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Drug receptor sites in the rabbit saphenous nerve

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Summary

1. The actions of acetylcholine, carbachol, methacholine, pilocarpine, nicotine, tetramethylammonium, 1,1-dimethyl-4-phenylpiperazinium, histamine, 5-hydroxytryptamine, angiotensin, bradykinin and potassium chloride were investigated on the sensory endings of the rabbit saphenous nerve.

2. By means of specific antagonists the presence of separate receptor sites sensitive to acetylcholine, histamine, 5-hydroxytryptamine and bradykinin were demonstrated.

3. The cholinoceptive receptors were shown to be predominantly nicotinic, but there was evidence of the existence of at least a small population of muscarinic receptors.

4. Catecholamines of both α and β types exhibited some stimulatory activity on administration. It is unlikely that this was due to either vasomotor effects or to any action involving the arrectores pilorum muscles.

5. The α-receptor stimulants modified the response to acetylcholine in a biphasic way. There was a brief enhancement of effect followed by a more prolonged depression. β-receptor stimulation only produced a prolonged enhancement of acetylcholine-evoked activity.

6. The discharge generated by acetylcholine was augmented by anticholinesterases and blocked by ganglion blocking agents. None of these drugs in reasonable doses affected the response to stroking the fur. The results therefore support earlier evidence against a sensory cholinergic synaptic link.

Introduction

Evidence is accumulating that " muscarinic " receptors exist, together with " nicotinic " receptors, at several sites classically believed to be exclusively nicotinic. In cats, muscarinic receptors have been found in the superior cervical ganglion (Ambache, Perry & Robertson, 1956 ; Roszkowski, 1961 ; Pappano & Volle, 1963 ; Volle, 1967 ; Gebber & Snyder, 1968), in the thermal receptors of the tongue (Dodt, Skouby & Zotterman, 1953), and on the Renshaw cells (Curtis & Eccles, 1958 ; Curtis & Ryall, 1966a, b) ; in dogs, they have been described in the stellate ganglion (Flacke & Gillis, 1968 ; Fleisch, Flacke & Gillis, 1969) and in the adrenal medulla (Kayaalp & Türker, 1969 ; Kayaalp & McIsaac, 1969).

In the experiments described in this paper the actions of several drugs, including " muscarinic " and " nicotinic " agonists, have been studied in the rabbit saphenous nerve *in situ*.

Methods

Albino rabbits of either sex were anaesthetized with intravenous urethane (6 ml/kg of a 25% solution). The method of recording from the saphenous nerve, and for injecting drugs intra-arterially, was similar to that used by Douglas & Ritchie (1960) in the cat, and identical with that described by Watson (1967).

The saphenous nerve was exposed, and either cut or crushed high in the thigh. Bipolar platinum electrodes were placed on the branch innervating the skin over the knee. The leg was immobilized by a drill through the lower end of the femur and by a clamp upon the ankle joint. The shank of the drill was connected to earth. The electrodes were immersed in a pool containing mineral oil (heavy liquid paraffin, B.P.) which was formed by raising the skin flaps with crocodile clips.

Intra-arterial injections were made retrogradely through a fine polythene cannula tied into the cut central end of the saphenous artery at a point peripheral to the branches supplying the skin in the region of the knee. Temporary occlusion of the main femoral artery at the time of injection was effected by raising a loose ligature previously placed around the artery, thus ensuring that the injected fluid was forced into the branches supplying the skin over the knee. Systemic drug injections were made through a cannula in the jugular vein.

After differential amplification by a Grass P5 pre-amplifier, the nerve action potentials were displayed on a Tektronix 502A oscilloscope and recorded on 35 mm film.

The drugs used were acetylcholine chloride (ACh), acetyl β-methylcholine chloride (methacholine), adrenaline acid tartrate, ambenonium chloride, angiotensin amide, acetylsalicylic acid, atropine sulphate, bradykinin, carbachol chloride, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), hexamethonium iodide, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate (5-HT), hyoscine hydrobromide, isoprenaline sulphate, lignocaine hydrochloride, mecamylamine hydrochloride, mepyramine maleate, methysergide maleate, neostigmine methyl sulphate, nicotine hydrogen tartrate, (−)-noradrenaline bitartrate, phentolamine methane sulphonate, phenylbutazone, physostigmine salicylate, pilocarpine nitrate, procaine hydrochloride, propanidid, pronethalol hydrochloride, tetraethylammonium chloride (TEA), tetramethylammonium chloride (TMA), (+)-tubocurarine chloride.

The drugs were dissolved in 0·9% w/v NaCl, and the doses quoted refer to the salts. When given intra-arterially the volume of solution did not exceed 0·3 ml.

Results

Lightly stroking the skin over the knee and the leading aspect of the lower leg, or the intra-arterial injection of acetylcholine (ACh) in doses of 5–10 μg and above, produced a brisk afferent discharge in the saphenous nerve. It has been previously shown (Watson, 1967) by the use of the collision technique of Douglas & Ritchie (1960) that at least part of this activity is carried in C fibres. Control injections of 0·9% NaCl solution, or of distilled water, were without effect. Touching the skin, after cohesion of the fur with liquid paraffin, failed to evoke any afferent discharge, as also did pinching the skin or application of heat.

Responses to stroking and to ACh (20 μg) were elicited in all experiments ; each of the other drugs was tested in three to eight rabbits.

The first response to acetylcholine in each preparation was often the greatest: smaller but approximately constant responses were obtained thereafter when ACh (20 μg) was injected at intervals of 15 min (Fig. 1).

Other stimulant drugs

Table 1 lists the other drugs tested. All of them were effective in eliciting an afferent discharge, and the table gives the approximate equiactive doses, both on a weight and a molar basis. In this table are also included the approximate dose ratios (giving ACh the value of 1) calculated on a molar basis.

The stable "nicotinic" drugs (carbachol, nicotine, DMPP and TMA) were qualitatively similar to ACh in their actions in that they were always effective at their appropriate dose level and that the onset of their action was rapid. They differed from ACh in that a longer dose interval was necessary between succeeding doses in order to obtain constant responses. Tachyphylaxis and cross-tachyphylaxis occurred within this group when doses were given at intervals of 30 min or less. The "muscarinic" drugs methacholine and pilocarpine were less consistent in their actions.

TABLE 1

Drug	Approx. equiv. effective doses (μg)	Approx. equiv. effective doses (μM)	Dose ratio on molar basis (ACh = 1)
Acetylcholine	20	0·11	1
Carbachol	30	0·16	1·5
Methacholine	200	1·02	9·3
Pilocarpine	200	0·74	6·7
Nicotine	10	0·02	0·2
TMA	200	1·82	16·6
DMPP	100	0·34	3·1
Histamine	20	0·07	0·6
5-HT	40	0·10	0·9
Angiotensin	20	<0·02	<0·2
Bradykinin	10	<0·01	<0·1
KCl	1,800	24·13	219·2

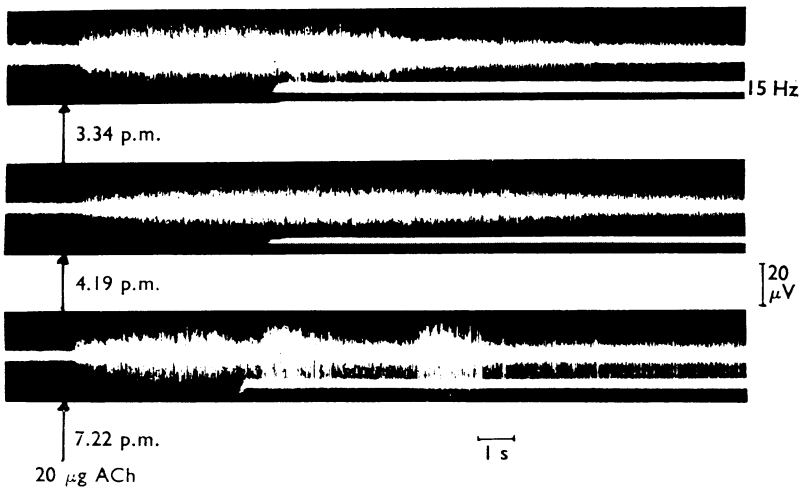

FIG. 1. Responses to acetylcholine (20 μg) were evoked after 15 min recovery periods. Three such responses, taken over an experimental period of 228 min, illustrate the brisk and continuing reactivity.

In two rabbits, 80 μg of methacholine was the smallest effective dose, while in a third, 120 μg was necessary. In a series of five rabbits, 200 μg doses of methacholine or pilocarpine were always effective. Methacholine and pilocarpine differed from the nicotinic group in that there was a latent period of about 5 s between injection and the onset of the afferent discharge.

Histamine, in a dose of 20 μg, evoked an afferent discharge in six out of eight rabbits. It also potentiated ACh when the latter was injected within 5–15 min after histamine, and this occurred even when histamine itself was apparently without effect.

When injected at intervals of 15 min or less, tachyphylaxis to histamine rapidly developed, but there was no cross-tachyphylaxis to ACh. Tachyphylaxis to histamine has also been reported in the cat superior cervical ganglion (Trendelenburg, 1956).

In doses above 40 μg, 5-HT was regularly effective, but tachyphylaxis developed when the interval between doses was less than 45–60 min. Tachyphylaxis to 5-HT has also been demonstrated in the fibres from the cat aortic arch (Ginzel & Kottegoda, 1954) and in the cat superior cervical ganglion (Robertson, 1954; Trendelenburg, 1954). Responses to histamine and 5-HT were rapid in onset, although a considerable latent period has been reported in experiments on isolated receptors supplied by fibres of the cat saphenous nerve (Fjällbrant & Iggo, 1961; Keele, 1962).

Bradykinin was the most potent drug studied (Table 1) and was always effective. No tachyphylaxis to repeated doses was evident.

Angiotensin was effective at a dose level of 20 μg in two out of four rabbits. In one of the others the drug was without effect even with doses of 50 and 75 μg. When effective, there was a latent period of 15–30 s between the injection and onset of response.

Isotonic KCl was occasionally administered intra-arterially as a non-specific stimulant in the sense that its action does not involve specific drug receptors, but presumably depends simply on its ability to depolarize excitable membranes. Injections at intervals of less than 10 min exhibited tachyphylaxis. Doses of 10 mg or above had a pronounced depressant effect on both the responses to stroking and to ACh for up to 30 min or more after injection. None of the other stimulant drugs in the doses used affected responses to stroking in any way.

Anticholinesterase drugs

Physostigmine (20–40 μg), neostigmine (10–40 μg) and ambenonium (5 μg) injected intra-arterially were without effect by themselves, and did not modify the response to stroking. All three drugs potentiated ACh, the augmentation lasting for about 30 min after neostigmine and ambenonium, and for about 45 min after physostigmine. Responses to nicotine, methacholine, histamine or bradykinin were not changed after injection of these anticholinesterase drugs. The absence of effect on responses to methacholine, which is a substrate for acetylcholinesterase, suggests that the effect of an intra-arterial injection of this drug is largely terminated by diffusion and redistribution rather than through hydrolysis by acetylcholinesterase.

Antagonist drugs

Hexamethonium (1–2 mg intra-arterially or 3–5 mg/kg intravenously), (+)-tubocurarine (1 mg intra-arterially), mecamylamine (0·2 mg intra-arterially) or tetraethylammonium (5 mg intra-arterially) blocked responses to acetylcholine (20 μg), nicotine (20 μg), carbachol (30 μg), tetramethylammonium (20 μg) and 1,1-dimethyl-4-phenylpiperazinium (100 μg), but did not affect responses to the other agents, even when the dose of antagonist was increased 2 or 3-fold. The blocking effect of TEA against ACh lasted about 20 min, those of hexamethonium and tubocurarine about 60 min, and that of mecamylamine lasted about 90 min. Responses to carbachol, nicotine and DMPP returned to normal somewhat more quickly than those to ACh.

A surprising observation was that bradykinin (20 μg) hastened the recovery of ACh sensitivity, reducing the time of blockade in the same animal to about one-half of that in its absence.

In doses below the blocking level, TEA (0·25 μg) itself produced a weak afferent discharge, which was antagonized by hexamethonium. TEA has been reported to produce a tingling sensation in the hands and feet of human subjects (Laurence, 1966) and the weak afferent discharge in the present preparation is probably a reflection of this effect.

At no time or dose level did these antagonist drugs affect the response to stroking in this preparation.

Atropine or hyoscine, in doses of 2 mg/kg intravenously or 20 μg intra-arterially, blocked the responses to methacholine or pilocarpine, but were without effect on responses to the usual doses of all the other stimulant agents. However, the responses to large doses of ACh (200 μg intra-arterially) were slightly reduced by these doses of atropine or hyoscine. Even massive doses of atropine or hyoscine (1–2 mg), injected intra-arterially, failed to affect the response to stroking, suggesting that the doses used were well below the local anaesthetic level. With doses of 200 μg of atropine intra-arterially, responses to ACh (20 μg), carbachol (30 μg), DMPP (150 μg) and nicotine (25 μg) were completely blocked for about 15 min, and then gradually returned to control levels during the next 15 min. These observations suggest that these large doses of atropine were producing a transient block of nicotinic receptors, because the responses to bradykinin (20 μg) or to KCl (10 mg) were unaltered during the time that the responses to nicotinic agents remained completely blocked.

Mepyramine in doses of 0·5–1 mg intra-arterially selectively reduced or abolished the responses to histamine. Larger doses of mepyramine (2 mg and above, intra-arterially) also blocked responses to ACh (20 μg). Methysergide in doses of 10 μg intra-arterially selectively blocked responses to 5-HT, and the block persisted for about 2 h. During this period the responses to ACh were augmented and prolonged by 50–100%. Larger doses of methysergide (up to 200 μg intra-arterially) caused a longer lasting block of 5-HT responses, but still augmented and prolonged the responses to ACh.

Antagonism of bradykinin by acetylsalicylic acid and phenylbutazone has been reported in the splenic artery preparations of dogs (Guzman, Braun, Lim, Potter & Rodgers, 1964 ; Lim, Guzman, Rodgers, Goto, Braun, Dickerson & Engle, 1964) and against guinea-pig bronchoconstriction (Lewis, 1961). Phenylbutazone, in doses up

to 2 mg intra-arterially, had no effect on responses to ACh in the present experiments, but transiently depressed activity induced by bradykinin.

Considerable difficulty was experienced with acetylsalicylic acid in obtaining a solubilizing agent which was without interfering stimulant effect, but ultimately 2% potassium citrate was used. In this solution 3 mg acetylsalicylic acid intra-arterially markedly depressed, but did not abolish, the bradykinin reaction. However, this dose of acetylsalicylic acid completely blocked the response to ACh for up to 45 min, and recovery was still incomplete after 2 h.

The local anaesthetic drugs lignocaine (2 mg) and procaine (3 mg) abolished responses to all chemical agents as well as to stroking, and similar properties were displayed by propanidid (10 mg), a short-acting general anaesthetic with local anaesthetic properties (Wirth & Hoffmeister, 1965). The local anaesthetics were the only drugs that abolished responses to KCl and to stroking.

Catecholamines

In doses of 10–20 μg injected intra-arterially, adrenaline and noradrenaline produced a short-lasting and weak afferent discharge in the saphenous nerve. In smaller doses (2 μg and above) adrenaline transiently (up to 2–3 min after injection) and slightly depressed the response to stroking, which then returned to its normal level. This effect was not obvious with noradrenaline. Both amines, in doses of 10–20 μg, produced a biphasic effect on the response to ACh. For up to 2–3 min after catecholamine injection the response to ACh was clearly augmented, but during the subsequent 30–45 min it was abolished or depressed. Noradrenaline was slightly less active than adrenaline in all cases, though their effects were qualitatively similar.

In doses of 0·5–1 mg intra-arterially, phentolamine itself depressed responses to ACh for up to 45 min. In doses of 250 μg this effect was negligible, and this dose was used in subsequent experiments involving sympathomimetic amines. After the injection of phentolamine, adrenaline and noradrenaline (20 μg) no longer produced an afferent discharge, nor did they produce the biphasic change in the ACh response described above.

Pronethalol, in doses of 250 μg intra-arterially, did not alter any of the responses to adrenaline or noradrenaline. At this dose level pronethalol was without effect on responses to stroking or to ACh, but in doses of 1 mg and above intra-arterially there was a definite depressant effect, resembling that of the local anaesthetics.

Isoprenaline, in doses of 20–200 μg intra-arterially, occasionally produced a transient, weak afferent discharge, but was without effect on the response to stroking. When tested 2–15 min after the administration of isoprenaline, responses to ACh were prolonged by about 40%, returning to normal within 30–40 min. Isoprenaline, unlike adrenaline and noradrenaline, did not depress the responses to ACh. In doses of 250 μg, phentolamine did not affect the responses to isoprenaline, but pronethalol (250 μg) abolished the effects of isoprenaline and prevented its interaction with ACh.

Lignocaine was also tested three times for its influence on the adrenaline-acetylcholine interaction. The maximum dose used (350 μg) was more potent in its local anaesthetic action than the selected dose of pronethalol, as confirmed by the guinea-pig wheal test (Bülbring & Wajda, 1945). In the presence of this dose

of lignocaine there was no change in the modified ACh response following adrenaline, indicating that the effect of pronethalol was independent of its local anaesthetic activity.

Discussion

Conclusions about drug receptor sites in experiments involving intravascular injection are complicated by lack of knowledge about diffusion barriers which may differentially influence the penetration of drugs to their sites of action. Thus some drugs, although potentially effective, may have a slower onset of action than others, and after intra-arterial injection pass into the venous return without reaching effective concentrations in the region of the receptor sites. Moreover, some drugs may affect skin blood flow, thus altering their penetration to, and time of contact with, receptor sites.

The sensory receptors of the rabbit saphenous nerve, presumably the basket-like nerve networks around hair follicles, were activated by stroking the fur, and it is therefore possible that drugs which stimulate the smooth arrectores pilorum muscles cause an afferent discharge as a secondary effect of hair movement rather than by a direct action on the sensory neurone. This possibility has been discussed by others (Brown & Gray, 1948 ; Douglas & Gray, 1953 ; Douglas & Ritchie, 1959 ; Paintal, 1964), and it is difficult to exclude it entirely with some drugs, for example adrenaline. However, this is unlikely because with the recording system used it was necessary, when stroking, to produce a gross movement of the hairs to generate an afferent discharge, but close observation failed to detect any hair movement following drug injection. Nevertheless, these limitations of the present method should be borne in mind when assessing the validity of the results obtained.

The results recorded with agonists and their appropriate specific antagonists demonstrated the presence of separate receptor sites sensitive to ACh, histamine, 5-hydroxytryptamine and bradykinin. The cholinoceptive receptors, activated by relatively small doses of ACh and carbachol, were mainly of the nicotinic type, since responses to these agonists were blocked by hexamethonium, mecamylamine, TEA and tubocurarine, and unaffected by small intra-arterial doses of atropine or hyoscine, or even by large intravenous doses of these two drugs. Moreover, nicotine, DMPP and TMA produced an afferent discharge resembling that produced by acetylcholine. Gray (1959) concluded that the cholinoceptive receptors in the cat saphenous nerve were also essentially nicotinic.

Large doses of atropine injected intra-arterially did briefly block responses to the above " nicotinic " agents. This block was specific in the sense that responses to KCl or bradykinin were not depressed. Large doses of atropine have been shown to block the actions of nicotinic drugs at other sites and to produce ganglion block (Feldberg & Vartiainen, 1934 ; Marrazzi, 1939 ; Abdon, 1940 ; Dutta, 1949 ; Konzett & Rothlin, 1949 ; Fink & Cervoni, 1953 ; Giotti, 1954 ; Bainbridge & Brown, 1960 ; Quilliam & Shand, 1964).

The results also indicated the presence of muscarinic cholinoceptive sites. Large doses of methacholine and pilocarpine produced an afferent discharge which was unaltered by hexamethonium or mecamylamine, but was blocked by atropine or hyoscine in doses too small to abolish responses to nicotinic agents. The size of the doses of methacholine and pilocarpine required suggest that these muscarinic

sites are few in number or relatively inaccessible. Brown & Gray (1948) failed to detect muscarinic sites in the cat saphenous nerve, possibly because the doses they used were below the threshold level for such receptors, but Widdicombe (1954) reported responses from pulmonary stretch receptors with large doses of pilocarpine. Presumably large doses of ACh will excite both nicotinic and muscarinic receptors, but the more pronounced nicotinic effects will predominate. The reactions to methacholine and pilocarpine were unlikely to be secondary to the contraction of the arrectores pilorum muscles since, (*a*) there was no obvious hair movement, (*b*) these muscles are adrenergically innervated, (*c*) there is no evidence that they will respond to muscarinic agents.

Responses to angiotensin were relatively weak, and it is impossible to exclude the possibility that its effects were secondary to some other action. In any case, the weakness and variability of the responses indicate that any receptor sites present in the nerve endings must be sparse and that they play little part in modulating sensory perception involving mechanoreceptors of the rabbit saphenous nerve.

Catecholamine effects mediated by both α- and β-adrenoceptors were demonstrated. While it is feasible that these effects, particularly the initial stimulatory effect produced by adrenaline and noradrenaline, were secondary to actions on smooth muscle, it nevertheless remains possible to explain them in terms of their known actions on nerve. It seems unlikely that their interaction with ACh could be related to vascular changes, since both adrenaline and noradrenaline constrict skin vessels, and this should hinder the access of ACh to the sensory nerve endings, and therefore reduce the afferent discharge produced. In fact, the opposite proved to be the case, the ACh-induced discharge being enhanced by these amines. Adrenaline and noradrenaline have been shown to lower the threshold of excitation of nerve (Bülbring & Whitteridge, 1941 ; Goffart & Holmes, 1962) and a similar effect on the saphenous nerve could account for the initial enhancement of the discharge produced by ACh. Surprisingly, the membrane potential of nerve is raised at the same time (Goffart & Holmes, 1962), and if this hyperpolarization develops more gradually and outlasts the earlier threshold-lowering effect, it might account for the secondary depression of the response to ACh. The present experiments showed that these actions of adrenaline and noradrenaline were mediated by α-adrenoceptors. Isoprenaline prolonged the duration of the ACh-induced discharge, and it is possible that this effect was secondary to its vasodilator action. However, the potentiation by isoprenaline was slow in onset (2 min) and lasted for 30–40 min, and it is unlikely that its vasodilator activity would last as long. Isoprenaline has been shown to depolarize ganglion cells (De Groat & Volle, 1965 ; Volle, 1966) and a similar depolarizing action on sensory nerve endings might explain its ability to augment the response to ACh. This effect was shown to be mediated by β-adrenoceptors.

It was found that, although the sensory discharge produced by ACh was augmented by prior treatment with anticholinesterase drugs, and was abolished by hexamethonium, mecamylamine, TEA and tubocurarine, none of these drugs modified the response to stroking the fur. Paintal (1964) has reviewed the evidence for and against the existence of a cholinergic link in the initiation of sensory impulses from the skin, and the present results therefore support the latter view. A similar argument against an essential chemical link involving histamine, 5-HT or bradykinin may also be advanced, since the relevant antagonists abolished the effects of these

substances without modifying the responses to stroking. Nevertheless, naturally occurring substances such as ACh, histamine, 5-HT, bradykinin or potassium ions, all of which have been shown to be capable of initiating or modifying sensory discharges, may well be concerned with the modulation of sensory perception in life, or even with the initiation of painful stimuli as a result of injury or disease (Rosenthal, 1964). Bradykinin, the most potent substance examined in the present tests, may be particularly important in this respect. The possible role of such substances in the mediation of painful stimuli has been fully discussed by Keele & Armstrong (1964) and by Jancsó and his co-workers (see Jancsó, 1960; Jancsó, Jancsó-Gábor & Szolcsányi, 1968).

Although it was found possible to inhibit the action of bradykinin on the saphenous nerve by prior treatment with acetylsalicylic acid and related substances, this effect was found to be non-specific because responses to ACh were also inhibited, and to a more pronounced degree. This finding raises the question as to whether the antagonistic action of acetylsalicylic acid on painful stimuli evoked from the spleen by bradykinin (Guzman *et al.*, 1964; Lim *et al.*, 1964) is as specific as has been thought to be the case. Similar doubts have been expressed by Aarsen (1966) as a result of his studies of bradykinin on guinea-pig lungs, and by Gryglewski, Panczenko, Górka, Chytkowski & Zmuda (1969) following their investigations of a wide variety of anti-inflammatory drugs.

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