NICOTINIC ACETYLCHOLINE RECEPTORS SUBSERVING NOCICEPTION IN THE DOG HINDLIMB

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ARMSTRONG et al. (1953) showed that when applied to the human exposed blister base acetylcholine excites cutaneous pain receptors, and KEELE and ARMSTRONG (1965) further demonstrated that such pain receptors are nicotinic. JANCSÓ et al. (1961) showed that nociceptors of the conjunctival sac of rats or guinea pigs and those of the nasal mucosa of guinea pigs are also nicotinic. However, no information is available as to whether the nociceptors in deep structures beneath the skin or mucosa are nicotinic. On the other hand, it has been demonstrated that in conscious or lightly anesthetized dogs injection of acetylcholine into the femoral artery causes the nociceptive response consisting of vocalization, withdrawal or shaking of the hindlimb of the injected side (target limb), biting and struggling (GUZMAN et al., 1962, 1964; HIRATA et al., 1966; TAIRA et al., 1968a). By this method of application acetylcholine presumably excites not only the nociceptors of the skin but those of deep structures in the hindlimb (GUZMAN et al., 1962, 1964).

In an attempt to elucidate whether the somatic nociceptors of deep structures in the hindlimb are nicotinic like those of the skin, we compared the algogenic potency of acetylcholine and nicotinic compounds with that of muscarinic ones by injecting them into the femoral artery of dogs. We also studied the effects of nicotinic and muscarinic receptor blocking agents on the acetylcholine-induced nociceptive response. Vocalization was chosen as a principal measure of nociception, because it was shown that in dogs vocalization in response to noxious stimuli is the most reliable indication of nociception (GUZMAN et al., 1962, 1964; TAIRA et al., 1968a). A part of the results of the present experiments was published as a short report (TAIRA et al., 1968b)
METHODS

Seventy-five experiments were conducted on 17 immature mongrel dogs with an average body weight (±S.D.) of 5.8±0.9 kg (range 3.5-7.0 kg). Three or four days prior to the experiment, under pentobarbital anesthesia (30 mg/kg, i.v.), a fine polyvinyl catheter was implanted aseptically in the femoral artery of either side in the following way. One end of polyvinyl tubing of 0.8 mm in O.D. and 0.5 mm I.D. was tapered down to a tip of about 0.3 mm in O.D., and a polyvinyl cuff with a fine thread attached was cemented with a drop of cyclohexanone to the tubing about 1.5 cm away from the tip according to the technique of Herd and Barger (1964). The tubing was filled with whale heparin (Taiyo Fishery Co.) solution and the other end was plugged with a piece of 23-gauge stainless steel tubing crushed at one end. While blood flow was interrupted with two pairs of hemonstats, a small pin hole was made through the wall of the femoral artery by means of a 22-gauge hypodermic needle, and the tip of the catheter was thrust into the arterial lumen through the hole up to the cuff. After release of the hemostats, the catheter was tied loosely to the artery with the thread attached to the cuff, and a tiny amount of a bioadhesive (Alon Alpha A) was mounted around the hole. The other end of the catheter was led outside at the nape through the subcutaneous tissue. Another cuff with a thread attached was cemented to the catheter about 5 cm away from the end. The catheter was fixed to the skin of the nape with the thread attached to the cuff. The catheter was flushed with whale heparin after each experiment. Sodium whale heparin was dissolved in 0.9% saline to give a concentration of 1000 U/ml and sterilized.

Experimental procedures were essentially the same as those described in a previous paper (Taira et al., 1968a). The animals were loosely restrained with a hammock. Vocalization was picked up with a non-directional microphone (Toshiba, OB-1419B) and recorded once on magnetic tapes by means of a tape recorder (TEAC, R-314). Throughout the experiment vocalization was simultaneously displayed as an integrated trace on an ink-writing oscillograph and served as a monitor. The time constant of an integration circuit was 2 sec. After each experiment tapes were played back and vocalization responses were reproduced as integrated traces on the chart so that the biggest response was displayed just in the full range of pen excursion. All measurements of latency and duration of vocalization were made on these traces. Experiments on one animal were conducted at intervals of at least one day for a span of two or three weeks. Each experiment ran usually for 2 to 3 hr.

Compounds used were as follows: Acetylcholine chloride, nicotine base, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, Aldrich), methacholine chloride (Merck), betahanechol chloride, 4-(m-chlorophenylcharbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343, McNeil Laboratories), hexamethonium bromide, tetraethylammonium bromide (TEA), l-hyoscyamine sulfate, histamine dihydrochloride and bradykinin (Sandoz). These compounds except for bradykinin were dissolved in sterilized 0.9% saline. All of the drug solutions were diluted with sterilized 0.9% saline. A constant volume of 0.1 ml of drug solution freshly prepared was injected into the catheter. Injected materials were flushed in with 0.4 ml of sterilized and warmed 0.9% saline at a rate of 0.1 ml/sec, since the dead space of the catheter was about 0.3 ml.

Statistical significances of the differences between means were determined by Student's t test.
RESULTS

Ability of nicotinic compounds to produce the nociceptive response

Acetylcholine. In accord with results of previous experiments (Guzman et al., 1962, 1964; Hirata et al., 1966; Taira et al., 1968a) in all 14 dogs investigated, injections of acetylcholine into the femoral artery caused the nociceptive response consisting of vocalization, withdrawal or shaking of the target limb, biting and struggling. Of these the vocalization response increased with increasing doses in the range of 0.3 to 10 μmol. The upper tracings of Fig. 1 are typical of results of 27 such experiments. The average threshold dose (±S.E.) of acetylcholine for vocalization obtained on the 14 dogs were 1.2±0.3 μmol (Table 1). The average latency and duration (±S.E.) of vocalization in response to acetylcholine in doses of 3 to 10 μmol, 3 to 10 times threshold dose, were 3.6±0.2 sec and 11.1±0.9 sec respectively (Table 2).

Acetylcholine in large doses (3 to 10 μmol) produced transient systemic effects such as cough and respiratory excitation in 12 out of the 14 dogs.

Nicotine. In all eight dogs nicotine given into the femoral artery elicited essentially the same nociceptive response as acetylcholine. In the range of 0.06 to 3 μmol of nicotine the vocalization response was dose-dependent. The lower tracings of Fig. 1 are representative of such relations obtained in 10 experiments on the eight dogs. As clearly seen in this figure, nicotine was about 10 times as potent as acetylcholine. The average threshold dose (±S.E.) of nicotine producing vocalization was 0.14±0.04 μmol (Table 1). The average latency and duration (±S.E.) of the vocalization responses to nicotine in doses of 0.3 to 3 μmol, 3 to 10 times threshold dose, were 3.4±0.2 sec and 10.9±1.9 sec respectively (Table 2), approximating those produced by equi-effective doses of acetylcholine.

Respiratory excitation in response to large doses (1 to 3 μmol) of nicotine was the only systemic effect observed.

DMPP. DMPP, a nicotinic agent, administered into the femoral artery elicited the nociceptive response in six out of eight dogs, although all of them responded to either acetylcholine or nicotine as usual. In the six dogs DMPP produced short-latency vocalization (Fig. 2, Table 2) and other somatic nociceptive responses resembling those elicited by either acetylcholine or nicotine. However, this short-latency vocalization response, unlike that to acetylcholine or nicotine, was so inconsistent that sigmoid dose-response relations were scarcely obtainable with DMPP (Fig. 2). The average threshold dose (±S.E.) of DMPP for vocalization determined on the six dogs was 1.1±0.4 μmol (Table 1).

In five of the six dogs, besides the short-latency vocalization response, DMPP in medium doses (about 3 μmol) again produced vocalization with a latency of 28 to 32 sec (Fig. 2), which was regularly associated with respira-
FIG. 1. Integrated vocalization responses of a dog to increasing doses of acetylcholine (ACh) (upper tracings) and to nicotine (lower tracings) administered into the femoral artery. Time in 10 sec. Rectangular blocks on time marks indicate periods of flushing for 4 sec.

**Table 1.**
Threshold doses for vocalization of nicotinic and muscarinic compounds, bradykinin, and histamine administered into the femoral artery of dogs.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>N^a</th>
<th>Threshold dose (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.E.</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>14</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Nicotine</td>
<td>8</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>DMPP</td>
<td>6</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Bethanechol</td>
<td>8</td>
<td>&gt;6.8 ± 1.1^b</td>
</tr>
<tr>
<td>Methacholine</td>
<td>8</td>
<td>&gt;5.1 ± 1.0^d</td>
</tr>
<tr>
<td>McN-A-343</td>
<td>8</td>
<td>&gt;8.5 ± 0.7^b</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>6</td>
<td>0.9 ± 0.2^e</td>
</tr>
<tr>
<td>Histamine</td>
<td>5</td>
<td>1.3 ± 0.4</td>
</tr>
</tbody>
</table>

^a Number of dogs.
^b Significantly different from any of the average threshold doses of acetylcholine, nicotine and DMPP (P < .001).
^c The largest doses tested.
^d Significantly different from any of the average threshold doses of acetylcholine, nicotine (P < .001) and DMPP (P < .01).
^e Doses in nmol.
TABLE 2.
Latency and duration of the vocalization response to doses of 3 to 10 times threshold dose of nicotinic compounds, bradykinin, and histamine administered into the femoral artery of dogs.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>N</th>
<th>Absolute dose (µmol)</th>
<th>Latency (sec)</th>
<th>Duration (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>14</td>
<td>6.0±2.9</td>
<td>3.6±0.2</td>
<td>11.1±0.9</td>
</tr>
<tr>
<td>Nicotine</td>
<td>8</td>
<td>1.3±1.1</td>
<td>3.4±0.2</td>
<td>10.9±1.9</td>
</tr>
<tr>
<td>DMPP</td>
<td>6</td>
<td>3.8±3.2</td>
<td>4.8±0.2</td>
<td>5.8±1.4</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>6</td>
<td>5.1±2.8</td>
<td>14.7±0.6e</td>
<td>20.8±2.4e</td>
</tr>
<tr>
<td>Histamine</td>
<td>5</td>
<td>6.2±2.5</td>
<td>7.5±1.9</td>
<td>10.9±2.0</td>
</tr>
</tbody>
</table>

a Number of dogs.
b All values are mean±S.D.
c All values are mean±S.E.
d Doses in nmol.
e Significantly different from corresponding values obtained with acetylcholine (P<.001).

Dog 16, Exp. 80

Fig. 2. Vocalization responses of the dog shown in Fig. 1 to increasing doses of DMPP injected into the femoral artery. Otherwise, the same as in Fig. 1.

In short, when injected into the femoral artery, the two nicotinic agents, nicotine and DMPP, and acetylcholine, known to have a nicotinic in addition to a muscarinic action, were able to produce the characteristic nociceptive response which originates in the hindlimb. For all the three agents, the

...tory excitation, momentary stretching of four extremities and abrupt urination. With increasing doses (up to 6 µmol), the latency of the long-latency vocalization response was shortened to an extent of 11 to 14 sec so as to occur immediately following the short-latency response (Fig. 2). Salivary discharge, relaxation of the nictitating membrane and injection of the conjunctival vessels were usually observed with medium and large doses of DMPP. The relaxation of the nictitating membrane and the injection of the conjunctival vessels wore off in 3 to 5 min.

In short, when injected into the femoral artery, the two nicotinic agents, nicotine and DMPP, and acetylcholine, known to have a nicotinic in addition to a muscarinic action, were able to produce the characteristic nociceptive response which originates in the hindlimb. For all the three agents, the
threshold dose producing only withdrawal or shaking of the target limb was about 1/3 to 1/10 that producing vocalization.

Inability of muscarinic compounds to induce the nociceptive response from the target limb

**Bethanechol.** Bethanechol is known to stimulate preferentially the muscarinic receptors at the musculature of the gastrointestinal tract and the urinary bladder (KOELLE, 1965). Even when given bethanechol in doses as large as 3 to 10 μmol, all eight dogs which responded as usual to both acetylcholine and nicotine, showed no nociceptive sign, and instead were wagging their tails in a friendly manner for 1 to 2 min after the administration. However, 1 to 2 min later, all the dogs showed signs of generalized parasympathetic excitation, viz., coughing, salivary discharge, defecation, retching and vomiting. **Table 1** shows the mean value of the largest doses of bethanechol tested that were ineffective in producing the nociceptive response characteristic for having its origin in the target limb. Severe signs of parasympathetic excitation did not permit the investigation of whether bethanechol in still larger doses has an algogenic action in the hindlimb.

**Methacholine.** Methacholine is a potent muscarinic agent with a weak nicotinic action (KOELLE, 1965). In seven out of eight dogs, which responded well to either acetylcholine or nicotine, methacholine failed to produce any sign indicative of nociception in the target limb even with doses (1 to 10 μmol) producing severe parasympathomimetic effects systemically. In only one dog which was very sensitive to the algogenic action of acetylcholine, 0.3 to 1 μmol of methacholine caused gentle flexion of the target limb but failed to elicit short-latency vocalization associated with flexion of the target limb. **Table 1** presents the mean value of the largest doses of methacholine injected that were ineffective in causing the short-latency vocalization.

As compared with bethanechol, methacholine was extremely potent in producing the parasympathomimetic effect. When given into the femoral artery, methacholine in doses as small as 0.3 to 0.6 μmol produced lacrimation, profuse salivation, defecation, urination and retching. With 3 to 10 μmol of methacholine all the eight dogs vocalized after a latency of 20 to 30 sec (Fig. 3), bending the trunk toward the abdomen and discharging watery feces, and appeared as if they were suffering from abdominal pain. With increasing doses, the long-latency vocalization response, like that to DMPP, had a progressively shorter latency down to 16–17 sec, but was never accompanied by withdrawal or shaking of the target limb.

**McN-A-343.** When injected into the femoral artery, McN-A-343, said to stimulate preferentially the muscarinic receptors at sympathetic ganglia (ROSZKOWSKI, 1961), failed to cause any sign of nociception in doses of 6 to 10 μmol in all eight dogs which vocalized in response to either acetylcholine
**FIG. 3.** Vocalization responses of a dog elicited by injection of methacholine (MeCh) into the femoral artery (lower tracings). Compare the latency of the methacholine-induced vocalization response with that of the acetylcholine-induced one (upper tracings).

or nicotine (Table 1). However, in the same doses it caused lacrimation, salivation or vomiting in all eight dogs.

In short, when administered into the femoral artery, all of the three muscarinic compounds, bethanechol, methacholine and McN-A-343, even in doses producing generalized parasympathomimetic effects failed to cause the nociceptive response originating in the target limb.

**Specific block by hexamethonium and tetraethylammonium of the acetylcholine-induced vocalization response**

Since acetylcholine in doses of 6 to 10 μmol, 6 to 10 times threshold dose, elicited consistently the vocalization response, the antagonistic effect of hexamethonium to these doses of acetylcholine was examined. In all five dogs investigated, intra-arterial administration of hexamethonium 1 to 2-min prior to equal doses of acetylcholine was able to prevent the vocalization as well as other somatic nociceptive responses to acetylcholine. The upper tracings of Fig. 4 are typical of results obtained from such five experiments. Although the blocking effect of hexamethonium was so transient as to wear off in about 10 min, the block was almost complete. With tetraethylammonium (TEA) in doses equal to those of acetylcholine similar results were obtained (Fig. 5). However, the blocking activity of TEA was somewhat weaker than that of hexamethonium, i.e., of five dogs, TEA caused complete block of the
FIG. 4. Specific block by hexamethonium (C6) of the vocalization response to acetylcholine. Intra-arterial hexamethonium caused complete block of the vocalization response to acetylcholine (upper tracings) but failed to affect the vocalization responses to histamine (lower left) and to bradykinin (lower right).

FIG. 5. Specific block by TEA of the vocalization response to acetylcholine.

Acetylcholine-induced nociceptive response in three, partial block in one and no block at all in one.

When given into the femoral artery, histamine was similar to acetylcholine in algogenic action (Tables 1 and 2, Fig. 4). In all six dogs, bradykinin administered into the femoral artery was about 1000 times more potent than acetylcholine and histamine in producing the vocalization response (Table 1). However, in comparison with that caused by equi-effective doses of acetylcholine, the vocalization response to bradykinin was definitely (P<.001) slow
to develop and lasted significantly (P<.001) longer (FIGS. 4 and 5, TABLE 2). In three dogs in which hexamethonium or TEA was effective in blocking the nociceptive response to acetylcholine, the specificity of the blocking action was investigated by using histamine and bradykinin as control algogenic agents. FIG. 4 depicts typical results obtained in three successive experiments on the same dog. Hexamethonium at a dose of 6 μmol, which was just sufficient to prevent the vocalization response to 6 μmol of acetylcholine, failed to affect those of comparable amplitude caused by 6 μmol of histamine and 3 nmol of bradykinin. Essentially identical results were obtained in five experiments on two dogs. This indicates clearly that the blocking action of hexamethonium is specific for acetylcholine. The specificity of the blocking action of TEA for acetylcholine was also confirmed in four experiments on two dogs. Fig. 5 is representative of such experiments.

Specific block by DMPP of the acetylcholine-induced vocalization response

In addition to its direct algogenic action, intra-arterial DMPP produced long-lasting block of the vocalization and other somatic nociceptive responses to acetylcholine. The result shown in Fig. 6 is typical of five such experiments on three dogs. The duration of block by DMPP was very long. With 6 to 10 nmol of DMPP, it took about 2 hr for complete recovery of the acetylcholine-induced vocalization response. In contrast, the vocalization response to bradykinin or histamine was never altered by the blocking action of DMPP. In this respect the blocking action of DMPP was specific for the nicotinic action of acetylcholine.

**FIG. 6.** Specific block by DMPP of the vocalization response to acetylcholine.
Failure of block by l-hyoscyamine of the acetylcholine-induced vocalization response

In seven experiments conducted on five dogs, intra-arterial l-hyoscyamine in doses of 1, 3 and 6 µmol failed to affect the vocalization response to equal doses of acetylcholine, as illustrated in Fig. 7.

DISCUSSION

In the present experiments, nicotine, acetylcholine and DMPP administered into the femoral artery were able to produce the nociceptive response consisting of vocalization, withdrawal or shaking of the target limb, biting and struggling. Withdrawal or shaking of the target limb indicates conclusively that the nociceptive response originates in the target limb. In contrast to these three agents, bethanechol, methacholine and McN-A-343, known as muscarinic agents, failed to produce the characteristic nociceptive response originating in the target limb even with doses producing severe signs of generalized parasympathetic excitation. The more than 4-fold difference in threshold doses for the algogenic action between the two nicotinic agents, nicotine and DMPP, on one hand and the three muscarinic agents on the other (Table 1), indicates that the afferent neuroreceptors of the hindlimb whose excitation by acetylcholine elicits the nociceptive response are nicotinic in nature. In fact, in the present experiments, 3 to 10 µmol of methacholine produced the vocalization response with a latency of 16 to 30 sec, which approximates that caused by bradykinin. However, the vocalization response to methacholine occurred without any sign of nociception in the target limb such as withdrawal or shaking of the target limb, and instead was invariably accompanied by abrupt defecation and bending of the trunk toward the abdomen, as if the animals were suffering from abdominal pain. Therefore, the possibility has to be considered that the vocalization response to large doses of methacholine administered into the femoral artery may be due to the algogenic effect on some visceral organs of the redistributed methacholine.
In support of this view, the authors have found that methacholine injected into the superior mesenteric artery was most potent in producing the nociceptive response. Moreover, seven dogs, the mean threshold dose for vocalization was about 0.13 μmol (unpublished results).

Prevention of the acetylcholine-induced nociceptive response by hexamethonium or TEA and lack of any blocking effect of l-hyoscyamine also support the view that the acetylcholine receptors subserving nociception in the hindlimb are nicotinic in type. It is rather surprising that in the present experiments the algogenic action of acetylcholine was antagonized by hexamethonium in doses equal to those of acetylcholine, in view of the observation that more hexamethonium was needed to antagonize the algogenic action of acetylcholine applied to the human exposed blister base (Keele and Armstrong, 1964).

In the present experiments DMPP caused long-lasting block of the algogenic action of acetylcholine. Since such blocking effect of DMPP was not exerted toward bradykinin and histamine, it must be considered to be specific for acetylcholine. Leach (1957) described that DMPP blocks ganglionic transmission in a manner similar to that of TEA or large doses of acetylcholine. In other words, DMPP behaves like a nicotinic blocking agent. Specific block by DMPP of the algogenic action of acetylcholine as observed in the present experiments may be ascribed to a mechanism similar to that operating in autonomic ganglia. If so, sigmoid dose-response relations for vocalization would scarcely be obtainable with DMPP, because it is highly probable that DMPP blocks itself.

In the present experiments afferent neuroreceptors responsible for induction of the nociceptive response characteristic for having its origin in the hindlimb probably involve not only the nociceptors of the skin but also those of deep structures (Guzman et al., 1962, 1964). Their pharmacological properties revealed in the present experiments can be characterized as nicotinic. This indicates that the somatic nociceptors in deep structures have the same pharmacological properties as those of the skin (Keele and Armstrong, 1964) and of the conjunctiva and the nasal mucosa (Jancsó et al., 1961). The present results are also in accords with those on the pharmacological properties of nerve endings of cutaneous afferent Aδ and C fibers revealed by electrophysiological methods (Brown and Gray, 1948; Douglas and Ritchie, 1957, 1960).

**SUMMARY**

Properties of nociceptors excited by acetylcholine in the hindlimb were investigated on conscious dogs utilizing vocalization as a measure of nociception. All compounds were administered into the femoral artery through
a chronically indwelling catheter. Acetylcholine and two nicotinic compounds, nicotine and DMPP, produced vocalization, withdrawal or shaking of the hindlimb, biting and struggling. Three muscarinic compounds, bethanechol, methacholine and McN-A-343, failed to cause any sign indicative of nociception in the hindlimb even with large doses producing severe signs of generalized parasympathetic excitation. The nociceptive responses to acetylcholine were blocked by prior (1 to 2 min) administration of hexamethonium, tetraethylammonium, or DMPP in doses equal to those of acetylcholine, but not by comparable doses of l-hyoscymine. The nociceptive responses to equi-effective doses of histamine or bradykinin were not affected either by any of these blockers. These results indicate that the afferent neuroreceptors which give rise to the nociceptive responses to acetylcholine administered into the femoral artery are nicotinic in nature.

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