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Pilocarpine-Induced Reciprocal Hindlimb Scratching in Mice

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SCOTT, R. W., T. L. GOODE AND R. B. RAFFA. *Pilocarpine-induced reciprocal hindlimb scratching in mice.* PHARMACOL BIOCHEM BEHAV **26**(2) 327–331, 1987.—Intrathecal (IT) administration of pilocarpine (0.25–2.0 μg) to mice produced a vigorous and dose-related reciprocal hindlimb scratching response that lasted for 10–15 minutes. Neither the intracerebroventricular administration of pilocarpine at up to 10 times the intrathecal ED90 dose nor the subcutaneous administration of 10 mg/kg pilocarpine caused as robust an effect as IT administration. The reciprocal hindlimb scratching produced by the ED90 dose of pilocarpine (2 μg, IT) was antagonized in a dose-related manner by simultaneous IT administration of atropine (ID50=0.002 μg), methysergide (ID50=1.89 μg), the substance P antagonist [D-Pro^2,D-Trp^{7,9}]-SP (ID50=4.94 μg), and the putative neurokinin B antagonist [D-Pro^2,D-Trp^{6,8},Nle^{10}]-NK (ID50=3.33 μg), but not by yohimbine (5 μg), phentolamine (2 μg), or naloxone (2.5 μg). These results suggest (a) that pilocarpine-induced reciprocal hindlimb scratching is mediated spinally, (b) that the effect is produced by an action of pilocarpine on muscarinic receptors in the spinal cord, and (c) that neurokinin, and perhaps 5-HT, mechanisms might also be involved.

Pilocarpine Behavior Scratching Grooming Mice Intrathecal Neurokinins

DURING the course of studies on the antinociceptive properties of the muscarinic agonist pilocarpine (manuscript in preparation), we observed that intrathecal (IT) administration of the compound to mice produced a series of behaviors that included excessive grooming and reciprocal hindlimb scratching (alternating scratching first with one hindlimb, then the other). Several pharmacologic agents are known to induce grooming-related activities in mice and rats [1–5, 7, 9–11, 15, 16, 33, 35], but reciprocal hindlimb scratching is not a common drug-induced behavior. We found the reciprocal hindlimb scratching produced by IT pilocarpine to be rapid in onset and relatively long lasting. The response was robust, consistent, and easy to score. This behavioral effect of pilocarpine is quantified in the present communication.

In an effort to uncover the mechanism by which reciprocal hindlimb scratching was produced by pilocarpine, various antagonists were co-administered intrathecally with the compound. These included atropine, methysergide, phentolamine, yohimbine, and naloxone, compounds having varying degrees of selectivity for muscarinic, serotonergic, alpha$_1$-adrenoceptor, alpha$_2$-adrenoceptor, and opioid receptors, respectively. In addition, the substance P antagonist [D-Pro^2,D-Trp^{7,9}]-SP [6] and the putative neurokinin B antagonist [D-Pro^2,D-Trp^{6,8},Nle^{10}]-NK [29, 34, 37] were used.

METHOD

Animals and Conditions

Male, Swiss CD-1 (Charles River Laboratories, Kingston Facility, Stoneridge, NY), pathogen-free mice (18–24 g) were used in all experiments. The animals were housed in groups of 8–10 in plastic cages and were maintained on a 12 hr light-dark cycle, with the light period starting at 06.00 hr. Food and water were available ad lib up to the time of the test.

Drugs and Drug Administration

The intrathecal administration of compounds into the subarachnoid space followed the technique of Hylden and Wilcox [17]. It consisted of direct puncture of the subvertebral space between L5 and L6 using a disposable 30-gauge needle (B-D Yale, Rutherford, NJ) mated to a 10 μl Luer's tip syringe (Hamilton, Reno, NV).

The intracerebroventricular (ICV) administration of compounds to conscious mice followed the procedure described by Haley and McCormick [13]. The mice were grasped firmly by the loose skin behind the head and the skin over the scalp was pulled taut. A 0.25 in., 27 gauge hypodermic needle mated to a 25 μl Luer's tip syringe (Hamilton, Reno, NV) was fitted with a rubber stopper to leave 3/8 in. of the needle exposed. The needle was inserted perpendicularly through the skull 2 mm from the midline, on a line just anterior to the base of the ears. Proper placement was verified (in pilot studies) using dye.

All compounds were dissolved in distilled water except for [D-Pro^2,D-Trp^{7,9}]-SP and [D-Pro^2,D-Trp^{6,8},Nle^{10}]-NK, which were dissolved in 0.01 N acetic acid. Intrathecal injection of vehicle was devoid of any noticeable behavioral effect.

All intrathecal injections were made in a volume of 5 μl.

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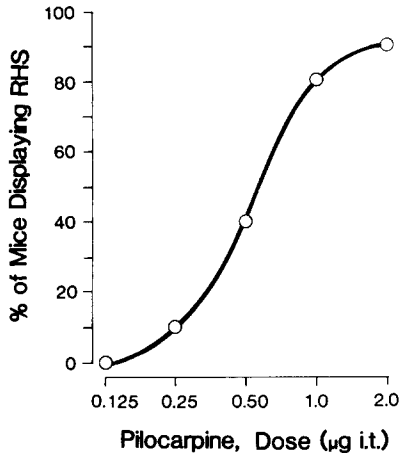

FIG. 1. Dose-response curve of pilocarpine-induced reciprocal hindlimb scratching (RHS) in mice. Pilocarpine was administered IT (5 μl) and the mice were observed for the subsequent 5 min for signs of RHS. The percentage of animals that displayed RHS within the 5 min observation period following IT injection of pilocarpine is indicated on the ordinate. N=10 at each dose.

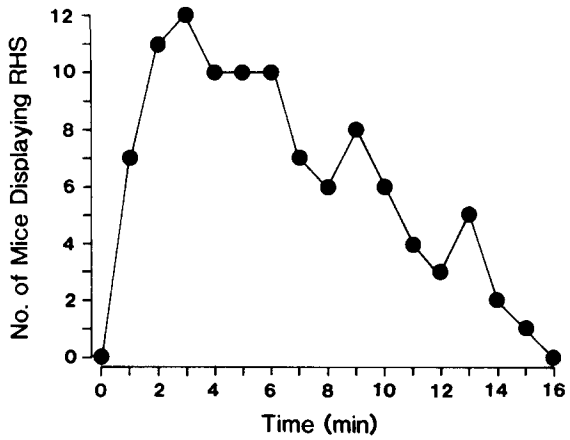

FIG. 2. Time course of pilocarpine (2 μg, IT) induced RHS in mice. The number of mice displaying RHS is shown on the ordinate. N=12.

When antagonists were tested, they were co-injected with pilocarpine in a total volume of 5 μl.

[D-Pro^2,D-Trp^{7,9}]-SP was purchased from Peninsula Laboratories, Inc. (Belmont, CA). [D-Pro^2,D-Trp^{6,8},Nle^{10}]-NK was synthesized and purified by Dr. David Wright, Department of Chemical Research, McNeil Pharmaceutical.

Experimental Design and Scoring

The procedure for monitoring and quantitating reciprocal hindlimb scratching activity consisted of placing the treated mice into clear plastic containers (36.8×17.8 cm) immediately after administration of compound or vehicle and observing their behavior for the subsequent 5 minutes. The results are expressed as the percentage of animals that demonstrated reciprocal hindlimb scratching within this 5 min observation period.

The data for the time course of reciprocal hindlimb scratching behavior was obtained using a technique similar to the one described by Gmerek and Cowan [12] and Murray *et al.* [25]. Testing took place between 09.00 and 16.00 hr. For each experiment, groups of 6 mice were injected IT or ICV with vehicle, pilocarpine or pilocarpine plus antagonist and placed singly in large cylindrical glass containers (15.3 cm, ID) that were lined with ground wood chip bedding. Each mouse was observed for 10 sec of each minute, over a 15 min period, starting immediately after the injection. The occurrence of reciprocal hindlimb scratching during the 10 sec observation period generated a positive score for the period. The absence of such behavior during the observation period resulted in no score. Reciprocal hindlimb scratching occurring outside the observation period and other types of grooming behavior were not scored. Hence, over the total 15 min period, the maximum achievable score for each mouse was 15.

ED50 and ID50 values were determined from linear regression analysis of probit plots and are presented together with the 95% fiducial limits.

RESULTS

Saline-injected (IT) control mice displayed normal grooming-related activities, but not reciprocal hindlimb scratching. Mice injected intrathecally with pilocarpine, in contrast, displayed dose-related reciprocal hindlimb behavior (alternating scratching first with one hindlimb, then the other) over the range of pilocarpine doses 0.25 μg to 2.0 μg (Fig. 1). The scratching was primarily directed at the head, usually to an area directly behind the ears. The ED50 (dose of pilocarpine that elicited reciprocal hindlimb scratching in 50% of the mice) for this behavioral response was 0.59 (0.39–0.91) μg. As the effect of the drug wore off, and at doses lower than those that produced reciprocal hindlimb scratching, one-sided scratching was sometimes observed and excessive grooming was usually seen.

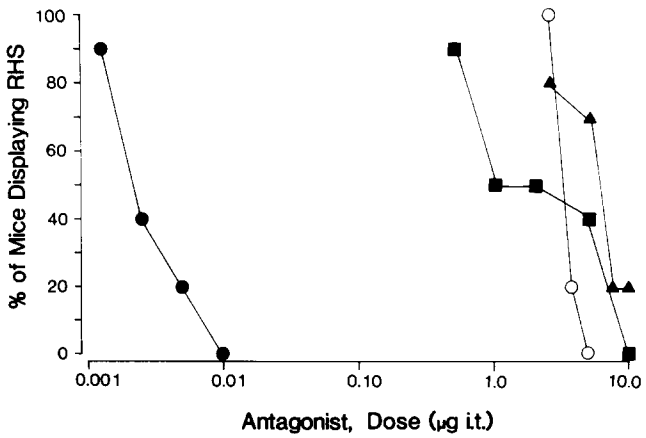

FIG. 3. The effect of simultaneous IT injection of antagonists on the RHS produced by pilocarpine (2 μg, IT). The percentage of animals displaying RHS within 5 min after drug administration is shown on the ordinate. The antagonists are atropine (●), methysergide (■), [D-Pro^2,D-Trp^{7,9}]-SP (▲), and [D-Pro^2,D-Trp^{6,8},Nle^{10}]-NK (○). N=10 at each dose.

The duration of the reciprocal hindlimb scratching response to intrathecal pilocarpine was of relatively short onset (1–3 min) and lasted approximately 10–15 min (Fig. 2). Intracerebroventricular administration of 20 μg of pilocarpine induced minimal reciprocal hindlimb scratching and

TABLE 1

ANTAGONISM OF RECIPROCAL HINDLIMB SCRATCHING (RHS) INDUCED BY INTRATHECAL PILOCARPINE EXPRESSED AS THE ID50 (μg) OF THE ANTAGONIST OR, WHERE THE RESPONSE WAS NOT SIGNIFICANTLY DIFFERENT FROM PILOCARPINE-TREATED CONTROLS ($p<0.05$), BY THE % RHS

	ID50 (μg)*	MW†	ID50/MW‡
Atropine	0.002 (0.001–0.004)	347	0.006
Methysergide	1.89 (0.93–3.56)	470	4.02
[D-Pro^2,D-Trp^{7,9}]-SP	5.31 (3.37–7.59)	1516	3.26
[D-Pro^2,D-Trp^{6,8},Nle^{10}]-NK	3.33 (2.82–3.82)	1645	2.02
	% RHS§		
Pilocarpine (2 μg, IT)	90		
Pilocarpine + Yohimbine (5 μg, IT)	80		
Pilocarpine + Phentolamine (2 μg, IT)	70		
Pilocarpine + Naloxone (1.0 μg, IT)	80		
(2.5 μg, IT)	90		

*ID50 (μg): the dose of antagonist that reduced pilocarpine-induced RHS to 50%. Values in parentheses represent 95% confidence limits. N=10 at each dose. †MW: molecular weight (daltons). ‡ID50/MW: μg/daltons ($\times 10^{-3}$). §% RHS: percent of animals displaying RHS when treated with antagonist simultaneously with pilocarpine. N=10.

grooming behavior. Pilocarpine injected subcutaneously (10 mg/kg) did not induce noticeable scratching or excessive grooming.

Atropine (0.00125–0.01 μg, IT), given simultaneously with pilocarpine (2 μg, IT), produced a dose-related inhibition of pilocarpine-induced reciprocal hindlimb scratching (Fig. 3). The behavior was nearly totally blocked by 0.01 μg, IT of atropine. The ID50 (dose of antagonist that reduced pilocarpine-induced reciprocal hindlimb scratching by 50%) for this inhibition was 0.002 (0.001–0.004) μg.

Methysergide (0.5–10 μg, IT), the substance P antagonist [D-Pro^2,D-Trp^{7,9}]-SP, and the neuromedin K antagonist [D-Pro^2,D-Trp^{6,8},Nle^{10}]-NK given with pilocarpine (2 μg, IT), also produced dose-related inhibition of reciprocal hindlimb scratching, but were less potent (even when adjusted for molecular weight differences) than atropine in inhibiting this effect of pilocarpine (Fig. 3). The ID50 values for these three antagonists are given in Table 1.

The reciprocal hindlimb scratching induced by pilocarpine (2 μg, IT) was not blocked by simultaneous IT administration of 5 μg of yohimbine, 2 μg of pentolamine, or 1.0–2.5 μg of naloxone (Table 1).

DISCUSSION

The observation of reciprocal hindlimb scratching in mice administered pilocarpine intrathecally was a surprising finding. Reciprocal hindlimb scratching is not a common drug-induced effect and, to our knowledge, has not previously been reported for pilocarpine.

Behavioral effects other than reciprocal hindlimb scratching have been previously noted by a number of investigators to be consequences of pilocarpine administration. For example, Marini [22] reported that 0.25–1.0 mg/kg of pilocarpine injected IP into cats elicited, in addition to a variety of parasympathomimetic effects, a significantly increased frequency of occurrence of grooming and "limb flicking." This behavior is seen in normal cats as a response to foreign substances, such as water, on their paws and is also observed as a behavioral effect of LSD in cats [18, 19, 30]. At relatively low doses (3–12 mg/kg), pilocarpine administered peripherally to rats elicits yawning [32] and a decrease in motor activity [23]. Pilocarpine also produces antinociception and, at higher doses, catalepsy (16 mg/kg, SC) and seizures (300 mg/kg, IP) in mice and rats [8, 31, 36]. Chronic treatment with high doses of pilocarpine (25 mg/kg/day) has been reported to stimulate mouse-killing behavior in rats [24].

Atropine was the most potent inhibitor of pilocarpine-induced reciprocal hindlimb scratching of the compounds investigated, with an ID50 value in the nanogram range. The other compounds that blocked the response did so in the microgram range. Based on this finding, it seems reasonable to presume that the initial event in pilocarpine-induced reciprocal hindlimb scratching is activation of muscarinic receptors in the spinal cord and that reciprocal hindlimb scratching follows directly or indirectly. If other steps are involved, they may include (based on the results with methysergide, [D-Pro^2,D-Trp^{7,9}]-SP and [D-Pro^2,D-Trp^{6,8},Nle^{10}]-NK) 5-HT and neurokinin pathways. It should be mentioned, however, that the antagonists, particularly methysergide, may have non-specific actions.

That the neurokinins might be involved in the scratching behavior is not surprising. Katz [20,21] described grooming behavior produced by ICV substance P and other tachykinins in mice. Dobry *et al.* [3] demonstrated that reciprocal hindlimb scratching was induced by ICV substance P (as first described by Rackham and Share [27], and by the C-terminal SP hexapeptide (SP 6–11). It would be of interest to see if age and strain differences reported for substance P induced grooming [14] are the same in pilocarpine-induced reciprocal hindlimb scratching.

The fact that neither phentolamine nor yohimbine blocked the reciprocal hindlimb scratching even at relatively high doses (2 μg and 5 μg IT, respectively), suggests that adrenergic mechanisms are not significantly involved in

mediating this effect of pilocarpine, even by an indirect action.

The scratching reflex may be mediated by more than one neurotransmitter, since this reflex not only involves elaborate tonic adjustments to attain the required posture, but also several neuronal circuits to co-ordinate the reciprocal movements of this behavior. It is even possible to consider that pilocarpine could be working by release of reciprocal inhibition of the reflex.

We have previously reported a grooming behavior brought about by intrathecal administration of the tetrapeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) to mice [28]. The behavior induced by pilocarpine was quite distinct from that produced by FMRFamide in that the primary behavior observed with pilocarpine, reciprocal hindlimb scratching, was not observed in mice administered FMRFamide. Also unlike FMRFamide-induced grooming, pilocarpine-induced behavior was blocked by several antagonists.

Likewise, pilocarpine-induced reciprocal hindlimb scratching is distinct from bombesin- and somatostatin-induced grooming and scratching both in the nature of the behavior [1,3] and in the fact that these effects of bombesin and somatostatin, unlike that of pilocarpine, are not blocked by [D-Pro^2,D-$Trp^{7,9}$]-SP and [D-Pro^2,D-$Trp^{6,8}$,Nle^{10}]-NK [34].

Hence, pilocarpine, substance P and 5-HT represent a relatively limited number of compounds that produce similar scratching behaviors. Whether these compounds induce these behaviors through independent pathways or whether one or more of the steps are common to two or more of the compounds, as suggested by the present results, is presently being investigated in our laboratory. In addition, the use of more selective muscarinic agents will determine if reciprocal hindlimb scratching behavior can serve as an in vivo endpoint for the discrimination of M_1 and M_2 agonists and/or antagonists.

In summary, our findings demonstrate that pilocarpine administered intrathecally to mice causes reciprocal hindlimb scratching that is rapid in onset and is relatively long lasting. The response is robust, reliably reproducible, and easy to quantitate. The mechanisms underlying this action appear to involve muscarinic, neurokinin, and possibly 5-HT, pathways, but details remain unclear.

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