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Absence of 5-HT_3 and cholinergic mechanisms in improgan antinociception

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Abstract

Improgan, an analgesic derived from histamine antagonists, acts in the brain stem to activate descending non-opioid, pain-relieving circuits, but the mechanism of action of this drug remains elusive. Because improgan has a moderate affinity for 5-HT_3 receptors, and, since cholinergic and serotonergic drugs can modulate descending analgesic circuits, roles for 5-HT_3, nicotinic and muscarinic receptors in improgan antinociception were presently investigated in rats. Improgan (80 μg, icv) induced nearly maximal inhibition of hot plate and tail flick nociceptive responses, and these actions we unaffected by antagonists of muscarinic (atropine, 5.9 mg/kg, i.p.) and nicotinic (mecamylamine, 2 mg/kg, i.p.) receptors. Control experiments verified that these antagonist treatments were maximally effective against muscarinic and nicotinic antinociception in both tests. In addition, improgan antinociception was unaffected by icv pretreatment with a 5-HT_3 antagonist (ondansetron, 20 μg). When given alone, icv treatment with neither this antagonist nor a 5-HT_3 agonist (*m*-chlorophenylbiguanide, 1000 nmol, icv) modified thermal nociceptive latencies. These results show no role for supraspinal cholinergic and 5-HT_3 receptors in improgan antinociception. The findings help to narrow the search for the relevant mediators of the action of this novel analgesic agent.
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Improgan is a member of a new group of non-opioid analgesics that are chemically related to histamine (Hough et al., 2001a). Intracerebroventricular (icv) administration of improgan reduces thermal and mechanical nociception in rodents (Li et al., 1996, 1997), yet does not impair rotorod performance, alter locomotor activity, or produce tolerance with daily dosing (Li et al., 1997; Bannoura et al., 1998). Recent mapping studies show that administration of improgan directly into pain-related nuclei in the brain stem (i.e. periaqueductal grey [PAG] and rostral ventral medulla [RVM]) produces significant antinociception (Nalwalk et al., 2004). Known opioid and histamine receptors play no role in improgan antinociception (Li et al., 1997; Hough et al., 2000b; Mobarakeh et al., 2003). In addition, improgan has little or no affinity at over 60 G protein-coupled receptor sites and ion channels, although the drug did show a moderate affinity for the 5-HT_3 receptor (Hough et al., 2000a). Thus, improgan is a highly effective, non-opioid pain reliever which acts in the brain stem by an unknown molecular mechanism.

There is evidence that both muscarinic cholinergic and nicotinic cholinergic receptors can participate in brain stem pain-modulating pathways (Iwamoto and Marion, 1994; Rao et al., 1996; Bitner et al., 2000). Activation of muscarinic receptors (primarily M_2 and M_4 (Duttaroy et al., 2002)) produces non-opioid antinociception following systemic or CNS administration of agonists (Swedberg et al., 1997; Bartolini et al., 1992). Like improgan (Nalwalk et al., 2004), these drugs are highly effective against thermal nociception after direct microinjection into the RVM (Iwamoto and Marion, 1994). However, binding studies

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found that improgan has no significant affinity for M_1–M_5 muscarinic receptors (Hough et al., 2000a). Taken together, these data suggest that improgan does not directly activate muscarinic receptors, but might indirectly utilize muscarinic antinociceptive mechanisms.

Cholinergic antinociception is also produced by nicotinic agonists administered systemically or centrally. In the RVM, nicotinic agonists reduce thermal nociception, and these effects are fully antagonized by the nicotinic antagonist mecamylamine, but not by opioid antagonists (Iwamoto, 1991; Bannon et al., 1998). Alpha$_2$ and alpha$_4$ subunits of the receptor are most relevant for these responses (Marubio et al., 1999). Similar to the case with muscarinic receptors, improgan did not show significant binding affinity at four isoforms of nicotinic receptors containing either the alpha$_2$ or alpha$_4$ subunits (Hough et al., 2000a). However, these radioligand binding experiments do not rule out a direct nicotinic mechanism for improgan action, because allosteric modulation of nicotinic channel function is possible. Thus, nicotinic cholinergic mechanisms (either direct or indirect) could also be important for improgan's pain-relieving actions.

Serotonergic mechanisms have long been suspected of participating in pain modulation, and these effects occur through a number of different serotonin receptors throughout the brain and spinal cord (Fields et al., in press). The 5-HT$_3$ receptor has been reported to function in both pain-facilatory and pain-inhibitory circuits, depending on the receptor location and the nature of the nociceptive stimulus (Zeitz et al., 2002). Most relevant presently is a report that microinjections of the 5-HT$_3$ antagonist tropisetron into the RVM blocked thermal antinociception induced by intra-PAG morphine (Kiefel et al., 1992), implying that activation of brain stem 5-HT$_3$ receptors causes analgesia. Although this hypothesis has not been further investigated, the moderate affinity of improgan for 5-HT$_3$ receptors (Hough et al., 2000a) led us to suspect that improgan could be activating brain stem 5-HT$_3$ receptors to produce antinociception. Presently, in vivo experiments were performed to test the hypothesis that muscarinic, nicotinic and/or 5-HT$_3$ receptors in the brain play a role in improgan antinociception.

1. Materials and methods

1.1. Animals

Male Sprague–Dawley rats (250–350 g, Taconic Farms, Germantown, NY) were maintained on a 12-h light/dark cycle (lights on from 0700 to 1900) with food and water ad libitum. Animals were housed in groups of three or four until the time of surgery and were housed separately thereafter. All animal experiments were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

1.2. Drugs and solutions

Improgan, synthesized as described (Hough et al., 2000b), was dissolved in 1 N HCl, titrated to a pH between 5.5–6.5, and diluted with saline. Ondansetron hydrochloride (Zofran®, GlaxoSmithKline, Research Triangle Park, NC) was available in a citrate-buffered, injectable solution, 2 mg/ml). The ondansetron vehicle consisted of 9 mg of sodium chloride and 0.96 mg of citric acid trisodium dihydrate per ml of deionized water. All other drugs were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Methylcarbamylcholine chloride (MCC) and (−) nicotine hydrogen tartrate were dissolved in saline and titrated to a pH between 5.5 and 6.5. 1-(3-Chlorophenyl)biguanide hydrochloride (*m*-CPBG) was dissolved in deionized water. Atropine was dissolved in a minimal amount of dimethylsulfoxide and then further diluted with deionized water. Mecamylamine hydrochloride was dissolved in saline. A 50% saline–50% deionized water vehicle control was used for all systemically administered drug studies. Doses reported for ondansetron, *m*-CPBG and mecamylamine are given as salts, and for all other drugs are given as base. All systemic (i.p.) treatments were administered in a volume of 1 ml/kg.

1.3. Intracerebral surgery

Rats were anesthetized with pentobarbital (25 mg/kg, i.p.) and supplemented with isofluorane. Chronic cannulas were stereotaxically implanted into the left lateral ventricle and anchored to the skull using three stainless steel screws and dental cement (Crane and Glick, 1979). Stereotaxic coordinates (millimeters from bregma) for placements of the guide cannulas were: −0.8 AP, 1.5 ML, −3.3 DV (Paxinos and Watson, 1986). Following surgery, the animals were individually housed and allowed to recover for 5–7 days before testing. Each animal was used for a single experiment.

1.4. Injections and nociceptive testing

Two nociceptive tests were used. For the hot plate test (Eddy and Leimbach, 1953), animals were placed on a 52 °C surface and the latency to hind paw lift or lick was recorded with a maximal exposure of 60 s. Baseline latencies were 10–15 s. For the tail flick test (D'Amour and Smith, 1941), the ventral surface of the tail (a randomly selected location 2–5 cm from the tip) was exposed to radiant heat, and the latency for tail movement was recorded. The heat source was set so that baseline latencies were generally between 3 and 4 s with a 15-s cutoff; the heat source was not adjusted for individual animals.

Subjects were tested with a single, baseline hot plate test, followed by three tail flick tests performed at 1 min intervals, with the third test used as the baseline score. Animals were then gently secured by wrapping with a laboratory pad and received either an i.p. or an icv

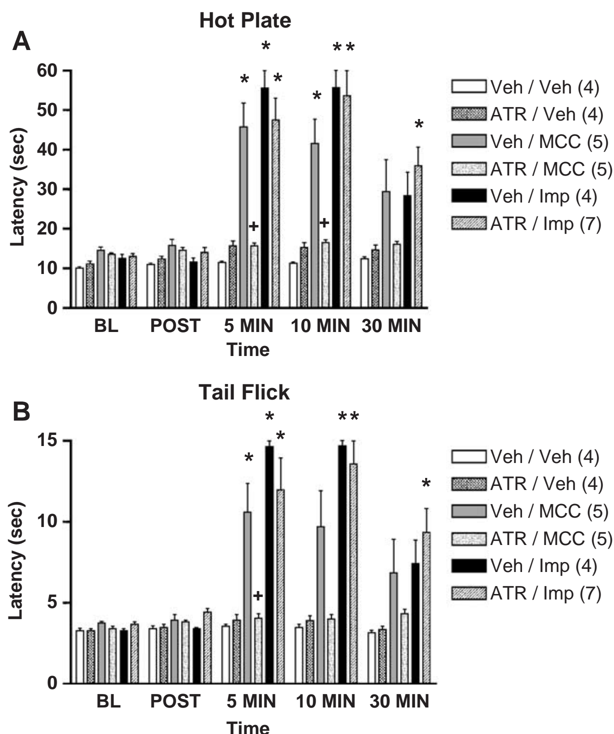

Fig. 1. Effects of the muscarinic antagonist atropine on improgan antinociception. Rats (cannulated as described in Materials and methods) were tested for baseline nociceptive response (BL) with both the hot plate (A) and tail flick (B) assays, then received an i.p. injection of either vehicle (Veh) or atropine (ATR, 5.9 mg/kg). Animals were tested again 20 min later (POST), then received an icv injection of saline (Veh), methyl carbamylcholine (MCC, 50 μg) or improgan (Imp, 80 μg); the icv injection was completed 30 min after the initial i.p. injection. Animals were retested at the times shown (min, abscissa) after the icv injection. Nociceptive latencies (s, ordinate, mean±SEM, n values in parentheses) for each combined treatment group are shown. *P<0.05 vs. Veh/Veh at the same time; ^{+}P<0.05 compared to Veh/MCC at the same time. Data from the Veh/Veh and Veh/Imp groups are also plotted in Fig. 2.

injection. For icv injections, the guide stylet was removed, and the injection cannula (which extended 1 mm beyond the guide to penetrate the ventricle) was inserted. Icv injections were delivered in a volume of 5 μl over 5 min unless noted otherwise. One min after the end of the icv injection, wire cutters were used to cut off and seal the injection cannula approximately 2 mm above the juncture with the guide cannula. Successful icv injections were assured by following the movement of an air bubble in the tubing between the syringe and the cannula and by the absence of leakage. Following the initial treatment, animals were retested with a single hot plate and tail flick test then received an additional icv injection. At the specified times after the second treatment, subjects were again retested and hot plate and tail flick latencies recorded. At the end of testing, animals received an overdose of pentobarbital sodium (100 mg/kg, i.p.) followed by icv injection (5 ul) of India Ink in order to verify cannula placement in the lateral ventricle. Brains were removed and distribution of the ink in the ventricular space was used to confirm successful cannulation. Data from animals with unsuccessful injections were excluded.

1.5. Data analysis

Results are expressed as latencies (s, mean±SEM) and repeated measures ANOVA was used to assess the significance of drug treatments. If indicated, Bonferroni post-hoc analyses were performed to determine significant differences between groups. Statistical analysis was performed with Prism Vers 4.0 (GraphPad Software, San Diego, CA).

2. Results

Baseline latencies ranged from 10.1 to 15.1 s (hot plate, Figs. 1–3) and 3.2–4.1 s (tail flick, Figs. 1–3). There were no differences in baseline latencies between any of the groups. In all cases, animals appeared alert and showed normal locomotion and righting reflexes during nociceptive testing.

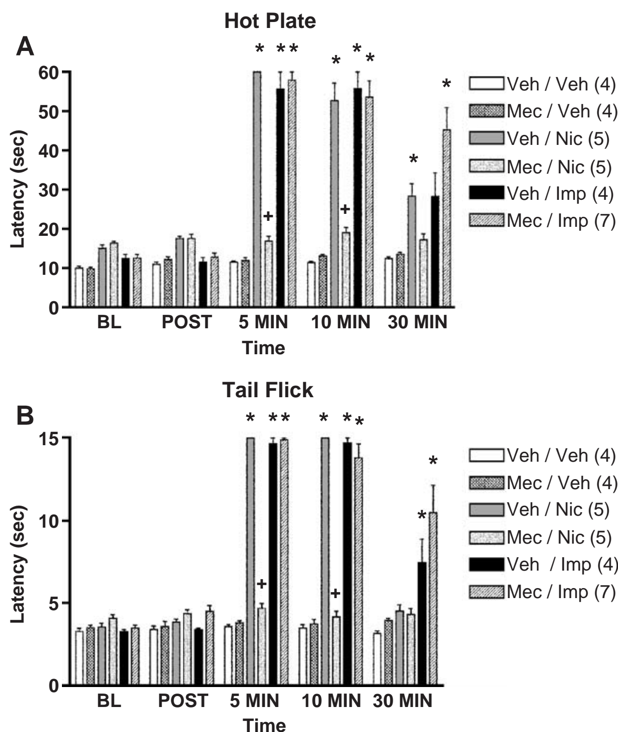

Fig. 2. Effects of the nicotinic antagonist mecamylamine on improgan antinociception. Rats were tested as described in Fig. 1 on both the hot plate (A) and tail flick (B) assays. Subjects were tested for baseline response (BL), received an i.p. injection of either vehicle (Veh) or mecamylamine (Mec, 2 mg/kg), were tested again (POST), and then received an icv injection of saline (Veh), nicotine (Nic, 65 μg) or improgan (Imp, 80 μg). Animals were retested at the times shown (min, abscissa) after the icv injection. Nociceptive latencies (s, ordinate, mean±SEM, n values in parentheses) for each combined treatment group are shown. *P<0.05 vs. Veh/Veh at the same time; ^{+}P<0.05 vs. Veh/Nic at the same time. Data for the Veh/Veh and Veh/Imp groups are the same as in Fig. 1.

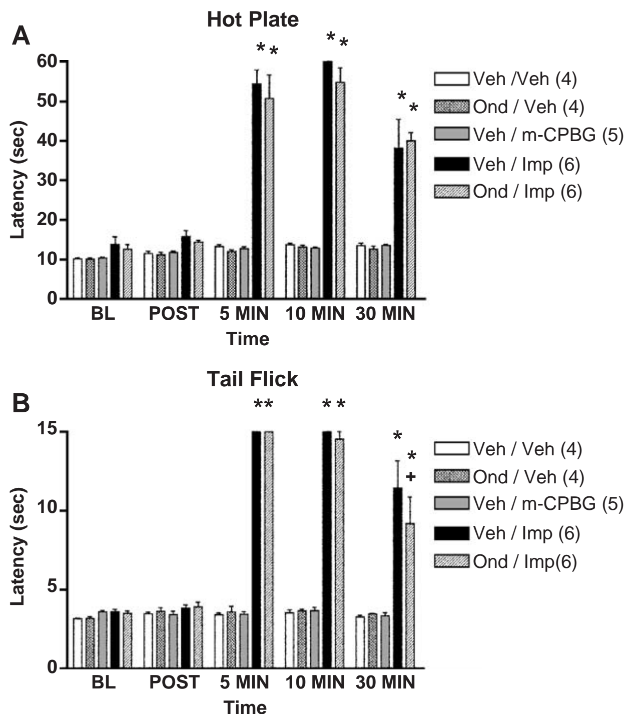

Fig. 3. Effects of a 5-HT_3 receptor agonist and antagonist on nociceptive responses in the presence and absence of improgan. Rats were tested as described in Fig. 1, on both the hot plate (A) and tail flick (B) assays. Subjects were tested for baseline response (BL), received an icv injection of either vehicle (Veh) or the 5-HT_3 antagonist ondansetron (Ond, 20 μg, 10 μl in 5 min) and were tested again 6 min later (POST). A second icv injection of either saline (Veh), the 5-HT3 agonist m-CPBG (1000 nmol) or improgan (Imp, 80 μg) was completed 15 min after the initial icv injection. Animals were retested at the times shown (min, abscissa) after the second injection. Nociceptive latencies (s, ordinate, mean±SEM, n values in parentheses) for each combined treatment group are shown. *$P<0.05$ vs. Veh/Veh at the same time; $^{+}P<0.05$ Veh/Imp vs. Ond/Imp at the same time.

On both hot plate and tail flick tests, improgan produced >90% of maximal antinociceptive responses 5 and 10 min after icv administration (Figs. 1–3). Treatment with the muscarinic antagonist atropine alone had no significant effect on baseline latencies, and this treatment also had no effect on improgan antinociception in either test (Fig. 1). In contrast, the cholinergic agent MCC produced significant antinociception on both the hot plate (5 and 10 min) and tail flick (5 min) tests; these effects were fully inhibited by atropine pretreatment (Fig. 1). ANOVA (between groups—1: i.p., 2: icv; within groups—3: time) of the hot plate data in Fig. 1 found highly significant effects between icv treatments (F=28.5, df=2, $p<0.00001$), time (F=59.0, df=4, $p<0.00001$) and a significant icv by i.p. by time interaction (F=3.3, df=8, $p<0.01$).

In experiments from Fig. 2, improgan antinociception was not altered by pretreatment with the nicotinic antagonist mecamylamine. The mecamylamine treatment alone did not alter nociceptive responses (Fig. 2). However, centrally-administered nicotine significantly increased nociceptive thresholds on both hot plate (5, 10 and 30 min) and tail flick (5 and 10 min) tests, and these effects were completely inhibited by mecamylamine (Fig. 2). ANOVA (between groups—1: i.p., 2: icv; within groups—3: time) of the hot plate data in Fig. 2 found highly significant effects between icv treatments (F=85.5, df=2, $p<0.00001$), time (F=124.3, df=4, $p<0.00001$) and a significant icv by i.p. by time interaction (F=13.3, df=8, $p<0.00001$).

In experiments from Fig. 3, improgan antinociception was unchanged by icv pretreatment with the 5-HT_3 antagonist ondansetron. In the absence of improgan, neither m-CPBG (the 5-HT_3 agonist), nor ondansetron (the antagonist) altered baseline antinoceptive responses when administered alone (Fig. 3). ANOVA (between groups—1: drug, within groups—2: time) on the hot plate data of Fig. 3 found highly significant effects of drug (F=72.6, df=4, $p<0.00001$), time (F=58.7, df=4, $p<0.00001$) and a drug by time interaction (F=18.2, df=2, p<0.00001).

3. Discussion

The present results find no evidence to suggest a direct or indirect mechanistic role for muscarinic cholinergic, nicotinic cholinergic, or 5-HT_3 serotonergic receptors in the antinociceptive actions of improgan. As discussed, similarities in the characteristics of cholinergic antinociception and improgan antinociception led us to consider both of the cholinergic receptor types as possibly significant for improgan action. Results (Fig. 1) showing that a large dose of the muscarinic antagonist atropine has no effect on improgan antinociception in two assays seem to exclude muscarinic involvement. The finding that MCC antinociception was completely blocked by atropine (Fig. 1) verifies the adequacy of the atropine treatment and confirms literature reports (Rao et al., 1996) that MCC induces atropine-sensitive antinociception. Similarly, present results showing that nicotine-induced (but not improgan-induced) antinociception is blocked by the nicotinic antagonist mecamylamine (Fig. 2) are convincing that nicotinic analgesic mechanisms are not significant for improgan's effects.

A study reporting the ability of the 5-HT_3 antagonist tropisetron (given into the RVM) to block morphine antinociception suggested that RVM 5-HT_3 receptors might be part of a supraspinal analgesic circuit (Kiefel et al., 1992). Although this has not been confirmed or pursued in other published studies, the report was of interest because improgan has measurable affinity at the 5-HT_3 receptor (Hough et al., 2000a), and because improgan acts in the RVM (Nalwalk et al., 2004). Our present results (Fig. 3), showing that a large icv dose of ondansetron neither altered nociceptive thresholds nor affected improgan antinociception, suggest that brain 5-HT_3 receptors do not have a mechanistic role in improgan action. Attempts to verify that the ondansetron treatment adequately blocked brain 5-HT_3 receptors failed, however, since administration of the 5-HT_3 agonist m-CPBG did not modify nociceptive thresholds

(Fig. 3). Nevertheless, it is likely that the presently-used ondansetron dose (20 μg, Fig. 3) was adequate to block brain 5-HT_3 receptors, since this dose is 80-fold larger than the intracerebral dose which was effective against morphine (0.25 μg) (Kiefel et al., 1992). This conclusion seems sound even when considering that tropisetron is about two-fold more potent than ondansetron (Macor et al., 2001), and that icv doses are generally 5- to 10-fold larger than intracerebral doses of the same drugs.

In contrast to the hypothesis of Kiefel et al., 1992, the lack of antinociceptive activity of a large icv dose of the 5-HT_3 agonist *m*-CPBG found presently (Fig. 3) suggests that activation of brain 5-HT_3 receptors does not directly reduce thermal nociception. If these receptors participate in morphine signaling, then they may play a permissive, rather than a primary signaling role, as suggested to occur for other serotonin receptors in the spinal cord (Gao et al., 1998). It is likely that the dose of *m*-CPBG used presently (1000 nmol, icv) is more than sufficient to activate brain 5-HT_3 receptors, since it is 10–20 times larger than intrathecal doses of this drug which effectively reduced nociceptive responses (Bardin et al., 1997).

Both in vitro and in vivo studies have been performed to search for the mechanism of antinociceptive action of improgan. Because of improgan's chemical similarities to the H_2 antagonist cimetidine, histamine receptors have been examined closely for potential involvement. The drug has little or no measurable affinity for the four known histamine receptors (Hough, 2001; Li et al., 1996, 1997) Furthermore, neither histamine receptor agonists nor antagonists reduce improgan effects, and the drug's antinociceptive properties are not reduced in H_1, H_2, or H_3 knock-out mice (Li et al., 1997; Mobarakeh et al., 2003). Similarly, in vitro studies of opioid receptor affinities (Hough et al., 2001a), and in vivo work with opioid antagonists and opioid receptor knockout mice have excluded known opioid receptor involvement (Hough et al., 2000b).

Despite a lack of knowledge of improgan's receptor, there is progress on the neural structures, circuits and transmitters utilized by improgan. Improgan acts directly in the PAG and RVM (Nalwalk et al., 2004), two highly connected brain stem regions known to participate in both opioid and non-opioid analgesic mechanisms (Fields et al., in press). Neuronal activation of these spinally-projecting brain stem circuits is likely following improgan. Even though supraspinal $GABA_A$ (Hough et al., 2001b), supraspinal cannabinoid (Hough et al., 2002), and spinal alpha adrenergic receptors (Svokos et al., 2001) are likely to be indirectly involved in improgan action, the drug does not act directly at any of these receptors (Hough et al., 2002; Cannon et al., 2004). The present results, showing no role for cholinergic or 5-HT_3 mechanisms in the action of this non-opioid pain reliever, help narrow the search for the relevant non-opioid mediators. Studies to find the direct and indirect messengers of improgan antinociception are continuing.

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