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Effects of Forskolin and Phosphodiesterase Inhibitors on Spinal Antinociception by Morphine

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NICHOLSON, D., A. REID AND J. SAWYNOK. *Effects of forskolin and phosphodiesterase inhibitors on spinal antinociception by morphine*. PHARMACOL BIOCHEM BEHAV **38**(4) 753–758, 1991.—The effect of intrathecal pretreatment with forskolin and the phosphodiesterase inhibitors Ro 20-1724, rolipram and 3-isobutyl-1-methylxanthine (IBMX) on the antinociceptive action of morphine administered intrathecally was examined using the rat tail-flick test to determine whether inhibition of adenylate cyclase contributed to spinal antinociception. Intrathecal pretreatment with forskolin (10 μg), Ro 20-1724 (15 μg) and IBMX (10 μg) inhibited the action of morphine in the tail-flick test. However, pretreatment with Ro 20-1724 (30 μg), rolipram (10 and 30 μg) and IBMX (30 μg) increased the action of morphine. These agents were devoid of intrinsic antinociceptive activity. Inhibition of spinal antinociception by morphine with agents which increase cyclic AMP levels in biochemical experiments is consistent with the hypothesis that some opiate actions are due to inhibition of adenylate cyclase. However, in view of the consistent increase in the effect of morphine with phosphodiesterase inhibitors at higher doses, this hypothesis may be insufficient to account for opiate interactions with the adenylate cyclase system in the spinal cord. Some effects on spinal antinociception also may be due to additional pharmacological actions of the agents used.

Morphine Antinociception Forskolin Ro 20-1724 Rolipram 3-Isobutyl-1-methylxanthine

THE spinal administration of opioids produces antinociception by interactions with μ-, δ- and κ-opioid receptors [reviewed (56,57)]. A number of cellular mechanisms have been implicated in spinal opioid actions, but the biochemical mediators of antinociception are not well characterized. Opiates and opioids have been shown to inhibit adenylate cyclase activity and the production of cyclic adenosine 3′,5′-monophosphate (AMP) in cultured neuroblastoma × glioma hybrid cells (43,49) and in various regions of the brain (7, 8, 27, 50, 52), and this mechanism has been proposed to account for a number of opioid actions (6). Within the spinal cord, inhibition of adenylate cyclase activity by opioids has been demonstrated in cultured dorsal root ganglion and spinal cord preparations (3,28) and in membranes from adult spinal cord (3). In an earlier electrophysiological study in the adult spinal cord, no evidence to support an involvement of changes in cyclic AMP production in the action of morphine was found (16). However, more recently, in spinal cord ganglion explants, inhibitory electrophysiological effects of opioids have been attributed to inhibition of adenylate cyclase as such effects were blocked by forskolin, lipid soluble analogs of cyclic AMP (10) and pertussis toxin (9). This latter agent ADP ribosylates and inactivates G_i which mediates opioid inhibition of adenylate cyclase (25).

Recently, it was demonstrated that intrathecal (IT) pretreatment with pertussis toxin inhibits the spinal antinociceptive action of morphine (20) and other opioids selective for μ-, δ- and κ-receptors (33). However, because pertussis toxin interacts with a family of G_i-proteins which may be linked to multiple effector systems including ion channels, and G_o which is linked to Ca^{2+} channels (35), additional approaches are required before inhibition of adenylate cyclase can be implicated in spinal antinociception by morphine. In this study, we have determined the effects of forskolin, a direct stimulant of adenylate cyclase (42) and the phosphodiesterase inhibitors Ro 20-1724 (40), rolipram (41) and 3-isobutyl-1-methylxanthine (IBMX) (45) on spinal antinociception by morphine to determine whether an inhibition of cyclic AMP production was a major mechanism involved in this action of morphine. The underlying hypothesis was that if inhibition of adenylate cyclase was involved in antinociception, elevating levels of cyclic AMP with forskolin and phosphodiesterase inhibitors would reduce the antinociceptive effect of morphine.

METHOD

Intrathecal Cannulation and Injections

Male rats (Sprague-Dawley 325–350 g, from Canadian Hybrid Farms) were implanted with chronic indwelling IT cannulas (7.5

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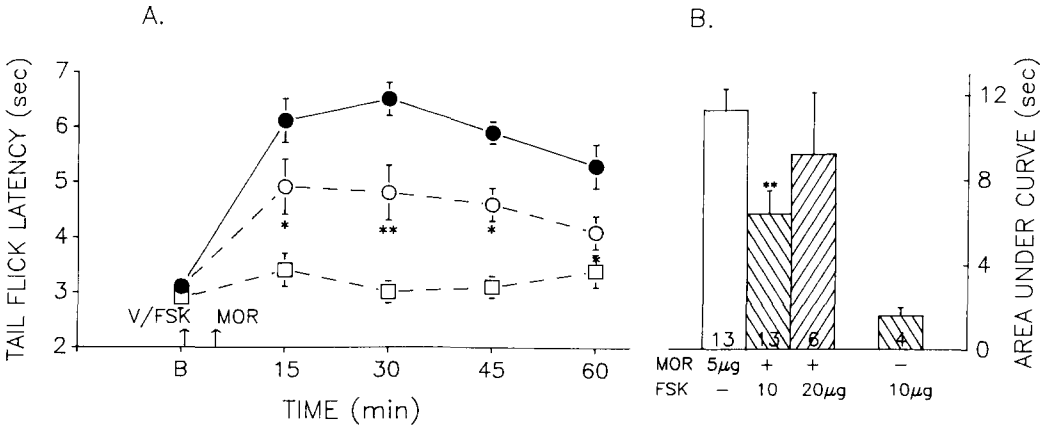

FIG. 1. Effect of forskolin on spinal antinociception by morphine in the rat tail-flick (TF) test. (A) Time course of interaction. The vehicle (V) 50% ethanol/saline (●) or forskolin (FSK) 10 μg (○) was injected intrathecally following a baseline determination (B), and this was followed 15 min later by an IT injection of morphine (MOR). The control curve for forskolin 10 μg followed by saline also is indicated (□). (B) Area under the curve summary data. Data represents mean ± s.e.m. for number indicated in columns. *$p<0.05$, **$p<0.01$ compared to vehicle-pretreated rats.

cm, to the lumbar enlargement) under halothane anaesthesia as described previously (20). Rats were tested following a 7-day recovery period. In Figs. 3B and 4B, rats also were tested 5–7 days later with the highest dose of the phosphodiesterase inhibitor. The control response to morphine was similar in both trials (Fig. 3C).

For the IT injections, rats were restrained briefly in a container which allowed access to the cannula. All injections were in a volume of 10 μl, and this was followed by a 10 μl flush with saline to ensure complete drug delivery from the cannula (volume 8 μl). Morphine and IBMX (1–10 μg) were dissolved in 0.9% saline, IBMX (30 μg) in 50% dimethylsulfoxide/saline, and forskolin, Ro 20-1724 and rolipram in 50% ethanol/saline. Control rats were pretreated with the appropriate vehicle for the drug being examined, and statistical comparisons made with the corresponding vehicle-pretreated group. Doses of morphine were selected to produce an intermediate response which would enable either a decrease or an increase in effect to be observed. Vehicle effects were not examined systematically. However, following pretreatment with 50% ethanol/saline or 50% dimethylsulfoxide, higher doses of morphine were required to elicit the control response (cf. Figs. 1, 2 and 3 and Fig. 4 respectively).

Nociceptive Testing

Antinociception was quantitated using the radiant heat tail-flick test. A focused beam of light was applied to the dorsal surface of a section of the tail which excluded the upper and lower third, and the latency to a tail flick or removal from the beam of light determined. Baseline latencies were 2–4 s and an 8-s cutoff was imposed if no response was observed by then.

Experimental Design

On the day of testing, rats were randomly assigned to experimental groups. Following a baseline tail-flick determination, forskolin, Ro 20-1724 or the appropriate vehicle was injected intrathecally. This was followed after a 15-min interval by an IT injection of morphine. Subsequent tail-flick determinations were made at 15-min intervals for 60 min following the injection of morphine. In some experiments (rolipram and IBMX pretreatments), an additional baseline determination was included prior to the injection of morphine.

Data were converted to an Area Under the Curve (AUC) score which represents a cumulative increase in latency during the time course examined. This is the sum of differences in tail-flick latency at 15, 30, 45 and 60 min and the baseline value. The IT injection of saline or vehicle does not significantly alter tail-flick latencies from baseline values at these time intervals, indicating that this method is an appropriate approximation of the area under the time-response curve. Time course data was analysed using analysis of variance followed by the Student-Newman-Keuls test. AUC data were analysed using this test if multiple groups were being analysed, or by the Student's *t*-test if a single comparison was being made.

Drugs

Drugs were obtained from the following sources: morphine sulphate (British Drug Houses, Toronto, Ontario, Canada), forskolin (Calbiochem, La Jolla, CA), Ro 20-1724 [rac-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] (Hoffmann-La Roche, Basel, Switzerland), rolipram [4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone] (Schering, Berlin, Germany), 3-isobutyl-1-methylxanthine, L-noradrenaline bitartrate (Sigma, St. Louis, MO).

RESULTS

The IT injection of morphine 2.5–5 μg produces antinociception in the tail-flick test which develops within 15 min of injection and persists over the 60-min time interval examined (Figs. 1–4). The effect of IT pretreatment with forskolin 10 μg on this action is shown in Fig. 1. The 10 μg dose of forskolin was chosen because it had previously been shown to decrease the antinociceptive action of analogs of adenosine in the hot plate test (36). Forskolin 10 μg inhibited the action of morphine both in the time course and AUC score (Fig. 1). This dose of forskolin alone had no intrinsic effect on tail-flick latency (Fig. 1). (AUC values for saline over this time course range from 0–3 s.) A higher dose of forskolin was without significant effect on morphine.

The effects of a range of doses of phosphodiesterase inhibitors on the action of morphine are shown in Figs. 2–4. Ro 20-1724 produced a biphasic effect on spinal antinociception by morphine.

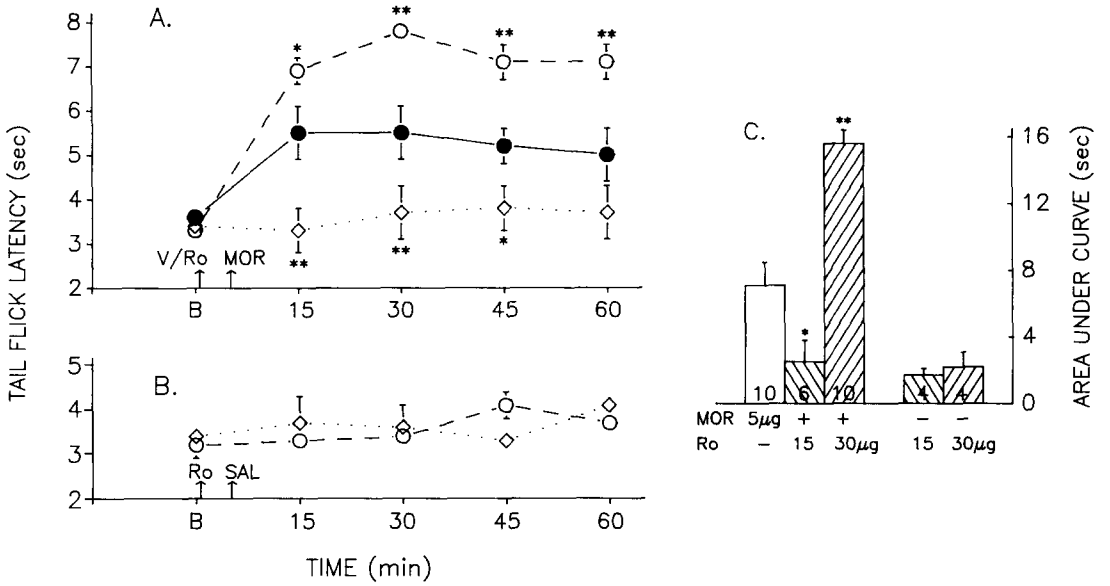

FIG. 2. Biphasic effects of Ro 20-1724 on spinal antinociception by morphine. (A) Time course of interactions. (B) Control responses to Ro 20-1724. The vehicle (V) 50% ethanol/saline (●), Ro 20-1724 (Ro) 15 μg (◇) or 30 μg (○) was injected intrathecally 15 min prior to the IT injection of morphine (MOR) (A) or saline (B). (C) Area under the curve summary of data. *$p<0.05$, **$p<0.01$ compared to vehicle-pretreated rats.

Thus the lower dose (15 μg) inhibited while the higher dose (30 μg) potentiated the action of morphine (Fig. 2). In this and other experiments where potentiation was observed, the degree of increase is underestimated because of the imposition of cutoff values (8 s). Following pretreatment with rolipram, only an increase in the effect of morphine was observed (10 and 30 μg), despite examining the widest dose range of any of the agents used (Fig. 3). With IBMX, inhibition of the action of morphine was observed at 10 μg, while a potentiation in the time course of action was observed at 30 μg (Fig. 4).

Phosphodiesterase inhibitors had no significant effect on basal tail-flick latencies. Ro 20-1724 was without effect on latencies throughout the entire time course (Fig. 2B). IBMX and rolipram (10 and 30 μg each) had no significant effect 15 min following injection (Figs. 3 and 4), or when examined over the entire time course 15–75 min following IT injection (AUC values 1.2 ± 0.7, 2.1 ± 0.9, 2.7 ± 1.6 and 1.9 ± 1.8 s respectively, compared to saline 1.6 ± 0.7 s, $n=3$ per group). The lack of effect of forskolin and phosphodiesterase inhibitors on basal tail-flick latencies may be due to there being multiple factors regulating the basal threshold in this test [e.g., tonic release of noradrenaline, 5-hydroxytryptamine and adenosine as respective receptor an-

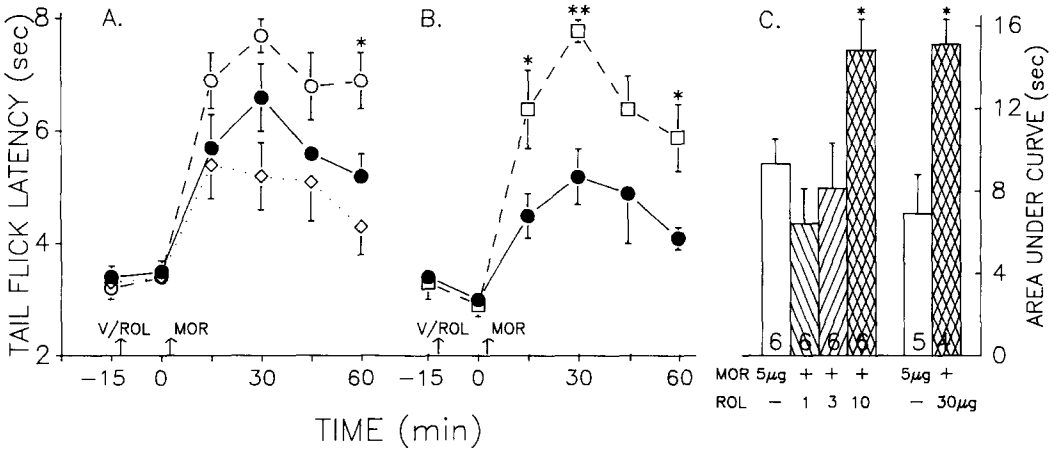

FIG. 3. Effect of rolipram on spinal antinociception by morphine. (A and B) Time course of action. The vehicle (V) 50% ethanol/saline (●), rolipram (ROL) 1 μg (◇), 10 μg (○) and 30 μg (□) was injected intrathecally 15 min prior to the IT injection of morphine (MOR). (C) Area under the curve summary of data. *$p<0.05$, **$p<0.01$ compared to vehicle-pretreated rats.

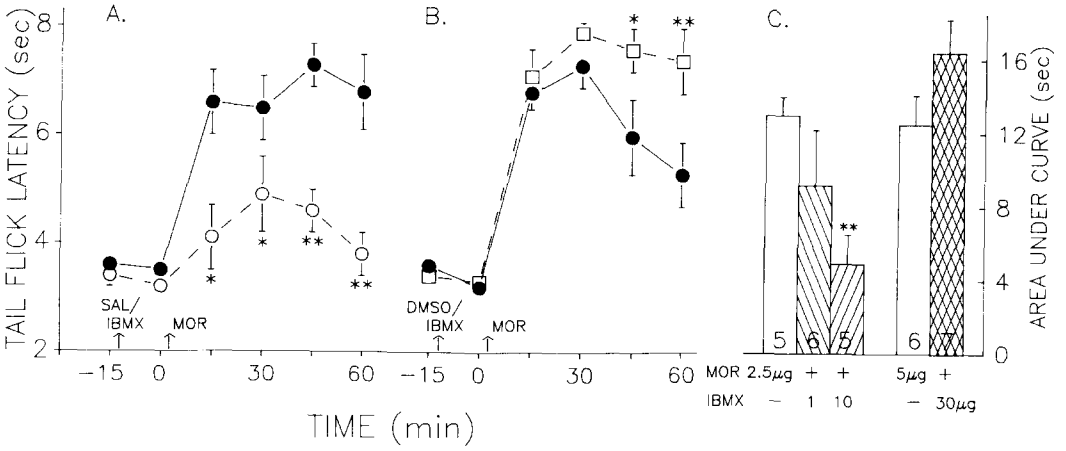

FIG. 4. Effects of 3-isobutyl-1-methylxanthine (IBMX) on antinociception by morphine. (A and B) Time course of effects. Saline (SAL) (left) or 50% dimethylsulfoxide/saline (DMSO) (right) (●) IBMX (10 μg) (○) or 30 μg (□) was injected intrathecally 15 min prior to the IT injection of morphine (MOR). (C) Area under the curve summary of data. *$p<0.05$, **$p<0.01$ compared to appropriate saline- or vehicle-pretreated rats.

tagonists produce hyperalgesia, (24, 32, 37)], and changes in adenylate cyclase may modify the contribution of only some of these factors.

DISCUSSION

The present study demonstrates that forskolin and the phosphodiesterase inhibitors Ro 20-1724 and IBMX can reduce the spinal antinociceptive action of morphine in the tail-flick test at some doses. In view of the hypothesis that if inhibition of adenylate cyclase was a major transduction mechanism for spinal antinociception by morphine, agents which increase cyclic AMP levels would reduce the action of morphine, this observation could be interpreted as supporting an opioid-mediated inhibition of adenylate cyclase in spinal antinociception. Other studies have demonstrated a similar reduction in inhibitory effects of morphine by forskolin and lipid soluble analogs of cyclic AMP in other preparations (1, 10, 53). However, in the present study, phosphodiesterase inhibitors uniformly potentiated spinal antinociception by morphine at higher doses. Although rolipram has a limited spectrum of activity in inhibiting phosphodiesterases (14), rolipram-sensitive phosphodiesterase plays an important role in regulating cyclic AMP levels in nervous tissue (46). IBMX has a wider spectrum of activity than rolipram, inhibiting multiple forms of phosphodiesterase (14,45), but it may be less useful for implicating changes in cyclic AMP production in the action of morphine because of opposing effects due to different pharmacological actions (see below). Nevertheless, the consistent increase in the action of morphine with phosphodiesterase inhibitors suggests that the hypothesis that opiate actions are mediated by inhibition of adenylate cyclase may be insufficient to account for opiate interactions with the adenylate cyclase system in the spinal cord.

The biochemical data on opiate and opioid effects on adenylate cyclase within the spinal cord supports complex interactions with this system. Thus opiates inhibit basal (3) and forskolin-stimulated adenylate cyclase activity (3,28) in explant and cultured spinal cord dorsal root ganglion preparations, and in membranes from adult spinal cord (3). Inhibition of adenylate cyclase was observed with δ and κ (28) of κ agonists only (3); selective μ agonists were ineffective in both studies. In other preparations, however, μ receptors have been linked to inhibition of adenylate cyclase (17,34). Spinal antinociception by morphine using a thermal test is normally mediated by activation of μ opioid receptors (19), although in the presence of μ receptor blockade, activation of δ opioid receptors also can occur (19). Opioids also have been reported to increase basal adenylate cyclase activity in cultured spinal cord preparations (28). The enhancement of antinociception by phosphodiesterase inhibitors is actually consistent with the possibility of stimulation of adenylate cyclase contributing to spinal actions of morphine. Opioids have been shown to exhibit dual excitatory and inhibitory electrophysiological effects in sensory neurons in spinal cord-ganglion cultures, both increasing and decreasing action potential duration at different doses (12). The increase in action potential duration was enhanced by forskolin and blocked by an inhibitor of cyclic AMP-dependent protein kinase, suggesting a role for stimulation of adenylate cyclase in this action of opioids [reviewed (11)].

Recently, it has been proposed that a significant component of the spinal antinociceptive action of morphine is due to release of adenosine and subsequent activation of adenosine receptors in the dorsal horn of the spinal cord [reviewed (38)]. The adenosine released by morphine originates from capsaicin-sensitive primary afferent neurons (48), while adenosine receptors are located predominantly on elements postsynaptic to primary afferent nerve terminals (4,18). The action of morphine could thus be considered a multistep process, with a potential for inhibition of adenylate cyclase at both pre- and postsynaptic sites of action. Inhibition of forskolin-stimulated adenylate cyclase activity has been demonstrated in both the spinal cord and ganglion components of cultured explants (28), suggesting this biochemical effect can occur at sites pre- and postsynaptic to afferent synapses. Analogs of adenosine inhibit (adenosine A1 agonists) or stimulate (adenosine A2 agonists) adenylate cyclase in the spinal cord (5), presumably reflecting a postsynaptic action. The antinociceptive effect of the adenosine analog cyclohexyladenosine (A1 agonist) is reduced by pertussis toxin, forskolin, Ro 20-1724 and rolipram, suggesting inhibition of adenylate cyclase may be involved in the action of the A1 agonist. [The effects of these agents on N-ethylcarboxamido adenosine, a mixed A1 and A2 agonist, are more complex, (36).] However, it is unlikely that the effects of forskolin and phosphodiesterase inhibitors on morphine simply reflect interactions with adenosine released endogenously by morphine because the doses of Ro 20-1724 and rolipram which reduce the action of

cylohexyladenosine (36) increased the action of morphine in this study. A potential role for cyclic AMP in the morphine-evoked release of adenosine from synaptosomes (presynaptic action) also has been examined directly (29). Forskolin and phosphodiesterase inhibitors increase basal release of adenosine but reduce the morphine-evoked release of adenosine (29). In view of the increase in the effect of morphine seen with phosphodiesterase inhibitors, the effects of these agents on release of adenosine also are insufficient to account for behavioural observations.

In addition to spinal actions of morphine which are mediated by release of adenosine, morphine produces effects which are independent of this action. For example, inhibition of the release of substance P from the spinal cord also may contribute to spinal antinociception (23,55), but adenosine does not inhibit substance P release from the spinal cord (51). A role for cyclic AMP in the effect of opioids on release of substance P has not been examined. However, cyclic AMP does not appear to be involved in the presynaptic inhibition of neurotransmitter release by opioids in the cortex (39). Substance P increases cyclic AMP levels in the spinal cord (30), but it is not known whether opioid inhibition of postsynaptic activation of neurons by substance P (22) involves cyclic AMP in any way.

So far, the effects of IBMX, Ro 20-1724, rolipram and forskolin have been considered in terms of interactions with the adenylate cyclase system. However, these agents have additional pharmacological actions which need to be considered in interpreting their effects. Thus IBMX also is an adenosine receptor antagonist (13,45). In view of the observation that methylxanthine adenosine receptor antagonists reduce spinal antinociception by morphine in the tail-flick test in rats (24,47) and mice (15), it is likely that inhibition of morphine by IBMX is due to this action. In contrast to IBMX, it is unlikely that inhibition of morphine by Ro 20-1724 is due to adenosine receptor antagonism because Ro 20-1724 does not bind to adenosine receptors (53). Ro 20-1724, rolipram and IBMX all have been shown to inhibit uptake of adenosine at higher concentrations (31). Thus the increase in the action of morphine by these agents might conceivably be due to blockade of reuptake of adenosine released endogenously by morphine. Finally, forskolin also has been reported to modulate K^+ channel activity directly rather than indirectly via activation of adenylate cyclase (21,26). In view of the observation that opioid-induced hyperpolarization of neurons mediated by an increase K^+ conductance is independent of cyclic AMP (2), the possibility that this action of forskolin contributes to its effect on morphine should be considered.

In summary, the effects of forskolin and phosphodiesterase inhibitors on spinal antinociception by morphine were examined to determine whether changes in cyclic AMP production was an important transduction mechanism in spinal antinociception. The inhibition of antinociception observed with forskolin and some doses of phosphodiesterase inhibitors provides evidence consistent with this hypothesis of action. However, phosphodiesterase inhibitors uniformly potentiated spinal antinociception by morphine at higher doses indicating that spinal interactions of opioids with the adenyl cyclase system are complex. An additional difficulty in interpreting behavioural data is that there are likely multiple components of action contributing to spinal antinociception (including pre- and postsynaptic interactions with purine and substance P systems), each of which with the potential to be differentially affected by the pretreatment. Finally, many of the agents used have multiple pharmacological actions which could contribute to their overall effects on spinal antinociception.

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