Cross-tolerance between spinal neostigmine and morphine in the rat

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Background. Direct or indirect acting cholinergic muscarinic agonists such as neostigmine, are potent antinociceptives when administered intrathecally (i.t.). This study examines whether spinal neostigmine tolerance and cross-tolerance to spinal morphine occurs.

Methods. Rats (32/group) were implanted with miniosmotic pumps delivering either i.t. saline 1 μl h⁻¹ (S), morphine 10 nmol μl⁻¹ h⁻¹ (M), or neostigmine 3 nmol μl⁻¹ h⁻¹ (N). Latencies (infrared thermal withdrawal rear paw) were measured daily for 6 days after which four animals from each group were given one i.t. challenge dose of morphine (m) 0.1, 1, 10, or 100 nmol, or neostigmine (n) 0.3, 3, 10, or 30 nmol.

Results. Neostigmine and morphine-infused animals both developed tolerance to spinal neostigmine, but neostigmine-infused animals showed no significant cross-tolerance to spinal morphine; mean ED₅₀ nmol (CI 95%) dose–response values were Sn 2.6 (1.9–3.5), Mn 15.6 (9.9–24.6)*, Nn 18.7 (11.7–29.8)*, Sm 0.7 (0.4–1.1), Nm 1.2 (0.8–2.0), Mm 152 (50–461)* (*significance vs saline infused control group).

Conclusion. Thus, unidirectional cross-tolerance from morphine to neostigmine was evident. Previous studies suggest morphine has a cholinergic mechanism of action partially accounting for its antinociceptive effect, which may explain this observed unidirectional cross-tolerance.

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Neostigmine is a potent antinociceptive agent with a proposed action at spinal muscarinic receptors. Tolerance to systemic effects of cholinergic agents has been described; however, as yet there is just one spinal neostigmine study which failed to show tolerance in the rat treated for 4 days. Only two systemic studies have examined cross-tolerance between muscarinic and opioid agonists. One found no significant unidirectional cross-tolerance to morphine in animals treated with cholinergic agents. The second found no evidence of either unidirectional or bidirectional cross-tolerance. A lack of cross-tolerance between spinal morphine and spinal muscarinic agents could potentially be exploited for chronic pain management. Morphine is the gold standard for chronic spinal analgesic therapy. Substitution with, or the addition of other agents, is common in clinical practice to circumvent side effects that occur with higher concentrations of opioids. This study examines tolerance to the antinociceptive effect of neostigmine in the rat and whether cross-tolerance to spinal morphine occurs.

Methods
Institutional approval for the study was obtained. Male Sprague–Dawley rats (350–400 g) were implanted with an intrathecal (i.t.) catheter connected to a saline or drug filled pump (model 2001 delivering 1 μl h⁻¹; Alza, Palo Alto, CA). A proximal loop of catheter was passed to exit percutaneously. Rats (32/group) were randomly assigned to infusion groups; saline 1 μl h⁻¹ (S), morphine 10 nmol
µl⁻¹ h⁻¹ (M), and neostigmine 3 nmol µl⁻¹ h⁻¹ (N). A rear paw infrared thermal stimulator system (University of California, San Diego, CA) (cut off latency 20 s) was used to assess paw withdrawal latencies before implantation and at 08:00 hours each day for 6 days. On the sixth day, after testing, the external catheter was flushed (10 µl saline), and 24 h later, baselines were measured again. Four animals per group were then given one dose of i.t. neostigmine (n) (0.3, 3, 10, or 30 nmol), or i.t. morphine (m) (0.1, 1, 10, or 100 nmol) in 10 µl after which the catheter was again flushed (10 µl saline). Latencies were then measured at 15, 30, 60, 90, and 120 min. Drugs used: morphine (morphine sulfate; MW=334; Merck, Sharpe and Dohme, West Point, PA), naloxone hydrochloride (MW=363.8; Sigma Chemical Co., St Louis, MO), and neostigmine methylsulfate (MW=334.4; Gensia Laboratories, Ltd, Irvine, CA).

Analysis
Daily withdrawal latencies and dose–response data were converted to per cent maximum possible effect (mpe) and the mean values compared by ANOVA, repeated measures, significance P<0.05. The conversion of data to %mpe was by the following formula; (withdrawal latency – baseline latency) x 100 divided by (cut off – baseline latency) where 20 s= cut off value. Double reciprocal (Lineweaver–Burke) plots were made from dose–response data by plotting the reciprocal of log dose vs the reciprocal of %mpe from 20–80%. Analysis of the dose–response curves was obtained by a pharmacological software program⁸ to calculate the ED₅₀ (%mpe) values and the slope by using linear regression (95% confidence intervals). The tolerance ratio (the ratio of ED₅₀ in drug-infused animals to ED₅₀ of saline-infused animals) and 95% CI were also calculated.

Results
Daily latencies: implantation, daily testing, or the vehicle saline had no significant effect on latencies in the saline group (Fig. 1A). Latencies in both morphine and neostigmine groups rose maximally on day 1, and progressively decreased to values not significantly different from saline controls by day 6 (Fig. 1A). Dose–response studies: the morphine-infused group showed a significant right shift in dose–response to spinal morphine when compared with saline-infused controls (Fig. 1B). The neostigmine-infused group also showed a significant right shift in dose–response to spinal neostigmine when compared with saline-infused controls (Fig. 1C). The morphine-infused group showed a significant right shift in dose–response to spinal neostigmine when compared with saline-infused controls (Fig. 1C). In control neostigmine infused animals showed no significant shift in dose–response to spinal morphine when compared with saline-infused controls (Fig. 1B). Mean ED₅₀ nmol (CI 95%) dose–response values were Sn 2.6 (1.9–3.5), Mn 15.6 (9.9–24.6)*, Nn 18.7 (11.7–29.8)*, Sm 0.7 (0.4–1.1), Nm

![Fig 1](A) Daily paw withdrawal latencies in saline (S), morphine (M), and neostigmine (N) treated animals indicating that all groups are not different by day 6. Morphine and neostigmine dose–response curves in S, M, and N treated animals are shown in (ii) and (c), respectively. *Significance vs saline group.
infusion (SEM) were Sn 1, Mn 0.15 (0.05), Nn 0.14 (0.04), opioids. Thus, activation of this second messenger pathway may be responsible for spinal opioid tolerance. Cholinergic agonists act through spinal muscarinic (probably m4) receptors. This leads to activation of the spinal nitric oxide cascade system and alterations in cerebrospinal acetylcholine levels; however, tolerance to neostigmine cannot be explained by a loss of inhibition of acetylcholinesterase. Recent studies suggest NMDA receptor activation may be responsible for spinal opioid tolerance. Cholinergic agonists act through spinal muscarinic (probably m4) receptors. This leads to activation of the spinal nitric oxide cascade system and alterations in potassium channel conductance. Nitric oxide synthase is involved in the spinal nitric oxide cascade system and alterations in potassium channel conductance. 

Comment

These results show significant tolerance development after chronic i.t. neostigmine administration and unidirectional cross-tolerance from morphine to neostigmine as evidenced by (i) the significant right shift in dose–response to spinal neostigmine in morphine-infused animals but (ii) the preservation of effect of spinal morphine in neostigmine-infused animals (Fig. 1B,C). Tolerance remains a poorly understood phenomenon. I.T. neostigmine produces a rise in cerebrospinal acetylcholine levels; however, tolerance to neostigmine cannot be explained by a loss of inhibition of acetylcholinesterase. Recent studies suggest NMDA receptor activation may be responsible for spinal opioid tolerance. Cholinergic agonists act through spinal muscarinic (probably m4) receptors. This leads to activation of the spinal nitric oxide cascade system and alterations in potassium channel conductance. Nitric oxide synthase inhibitors have been reported to prevent tolerance to opioids. Thus, activation of this second messenger pathway may be common to tolerance development with both opioids and cholinergic agonists. This could explain cross-tolerance between neostigmine and morphine in morphine tolerant animals, but not the lack of cross-tolerance to morphine in neostigmine tolerant animals. Alternatively, a direct cholinergic effect of opioids could explain such unidirectional cross-tolerance. Recent studies have shown that acetylcholine release occurs after spinal morphine administration suggesting that opioids have an indirect spinal cholinergic mechanism of antinociception. Atropine also partially blocks the antinociceptive effect of spinal morphine. This indirect cholinergic effect of morphine would explain cross-tolerance to spinal neostigmine in morphine-tolerant animals, whereas neostigmine-tolerant animals would be expected to show little, or no cross-tolerance to the mu receptor effects of morphine, but only to the partial indirect and weaker cholinergic effects. Morphine-tolerant rats on the other hand would be expected to develop tolerance to both mu and cholinergic receptor agonists via activity through both receptors. Further studies using direct acting cholinergic agents may further evaluate such a hypothesis.

In summary, these results show that tolerance to the antinociceptive effect of spinal neostigmine occurs after chronic administration in the rat, and that unidirectional cross-tolerance to spinal neostigmine occurs where morphine tolerance is present. This unidirectional cross-tolerance may be explained by previous observations that morphine appears to have a partial cholinergic mechanism of antinociceptive action.

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