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Antinociceptive Effects of Spinal Cholinesterase Inhibition and Isobolographic Analysis of the Interaction with μ and α_2 Receptor Systems

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Background: **Spinal cholinergic receptors have been shown to have a potent antinociceptive action, an effect that can be mimicked by spinal cholinesterase inhibitors. We (1) characterized the cholinergic receptor system through which intrathecally applied cholinesterase inhibitors produce their antinociceptive effect and (2) examined their interaction with spinal μ opioid and α_2-adrenergic receptors.**

Methods: **Rats were prepared with chronic intrathecal catheters and the nociceptive threshold was assessed by the use of the radiant heat-evoked hind paw withdrawal.**

Results: **Spinal administration of neostigmine, edrophonium, carbachol, clonidine, and morphine produced a dose-dependent increase on the thermally evoked hind paw withdrawal latency. The order of potency (dose producing a 50% effect, in nanomoles) was morphine (1.1) = neostigmine (1.2) > clonidine (4.4) > carbachol (15) >> edrophonium (112). Spinal pretreatment with atropine (35 nmol) attenuated the antinociceptive effect of intrathecal carbachol (55 nmol), neostigmine (15 nmol), and edrophonium (500 nmol) but did not affect the potency of intrathecal morphine (15 nmol) or clonidine (435 nmol). In addition, intrathecal pretreatment with naloxone (31 nmol) and yohimbine (28 nmol) attenuated the effects of intrathecally administered morphine and clonidine, respectively, but did not significantly affect the potency of carbachol, neostigmine, or edrophonium. The nicotinic receptor antagonist mecamylamine (60 nmol) did not affect thermal nociception. Isobolographic analysis revealed a synergistic interaction after the coadministration of neostigmine–clonidine ($P < 0.001$), edrophonium–clonidine ($P < 0.0001$), and edrophonium–morphine ($P < 0.01$) mixtures. Neostigmine–morphine exhibited simple additivity.**

Conclusions: **These data indicate that analgesia after spinal cholinesterase inhibition is mediated through muscarinic, but not nicotinic cholinergic, opioid, or α_2-adrenergic receptor systems, and that these spinal effects of cholinesterase inhibition interact synergistically with the antinociceptive effects of intrathecal μ and α_2 agonists. (Key words: Antagonists, acetylcholinesterase: edrophonium; neostigmine. Interaction (drug), analysis: isobologram. Receptors, spinal cord: α_2-adrenergic; muscarinic; nicotinic.)**

SPINAL injection of cholinergic agonists produces a dose-dependent antinociception in rats[1–4] and cats[1] mediated by a spinal muscarinic receptor. The mechanism of this muscarinically mediated antinociceptive effect is not known, but receptor autoradiography has identified significant densities of cholinergic binding found in the human and animal spinal cord[5–8] with significant muscarinic binding in the substantia gelatinosa (laminae II and III) of the dorsal horn.[9,10] Rhizotomies have been shown to significantly, but not completely, reduce the levels of muscarinic binding in the spinal dorsal horn.[11,12] Such observations are consistent with the possibility that spinal cholinergic systems may act to inhibit transmitter release from sensory neurons. In fact, such mechanisms have been postulated for a variety of spinal receptor classes, such as the μ and δ opioid and α_2 types, known to selectively alter pain behavior (see Hua *et al.*[13] for references).

These cholinergic receptors are acted upon by acetylcholine released from endogenous cholinergic terminals. Acetylcholine,[14] choline acetyltransferase[15,16] and acetylcholinesterase[17] have been identified in the spinal, dorsal, and ventral cords. Retrograde transport studies have failed to show brainstem cholinergic neurons projecting to the spinal cord.[18] This suggests that the spinal cholinergic terminals, particularly those within the dorsal horn, are associated with local interneuronal systems.

The potent analgesic actions of intrathecal muscarinic agonists and the presence of intrinsic cholinergic terminals raise the possibility that these endogenous systems play a role in modulating nociceptive transmission. Although the spinal delivery of atropine appears

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at best to result in only a modest hyperalgesia,[1,2,4] it is possible that a lack of evident tonic activity hinges on the rapid hydrolysis of acetylcholine resulting from local acetylcholinesterase. Consistent with this observation, it has been reported that the spinal delivery of physostigmine can augment the effects of exogenous acetylcholine[1] and alone will elevate nociceptive thresholds in rats[4,19] and sheep.[20] The nature of this interaction has not been completely characterized. Thus, some evidence indicates that cholinergic systems may be involved in the analgesic action of opioids.[21,22] Further, an interaction between the spinal α_2-adrenergic and cholinergic systems has been demonstrated in rats and humans.[19,23] Nevertheless, the characteristics and pharmacology of the interaction between the spinal cholinergic, α_2 and μ opioid receptor systems have not been defined. In the current study, we examined the antinociceptive properties of two cholinesterase inhibitors and sought to characterize their pharmacology. In addition, systematic studies were carried out to determine the characteristics of the interaction (linear or nonlinear) between the spinal cholinergic, α_2 and μ receptor systems.

Materials and Methods

The following investigations were carried out under a protocol approved by the Institutional Animal Care Committee, University of California, San Diego.

Animal Preparation

Male Sprague-Dawley rats (250–300 g; Harlan Industries, Indianapolis, IN) were housed in group cages with two or three rats and maintained on a 12 h light/12 h dark cycle. Animals had free access to food and water at all times. Chronic intrathecal catheters were implanted under halothane anesthesia according to a modification of the method described by Yaksh and Rudy.[24] Briefly, after the surgical area was shaved and blotted with povidone-iodine (Betadine), the atlantooccipital membrane was exposed and a polyethylene-10 catheter was advanced through an incision in the membrane to a position 9 cm caudal to the cisterna at the level of the lumbar enlargement. The catheter was externalized on the top of the skull and sealed with a piece of steel wire. The wound was closed with 3-0 silk sutures. Rats showing neurologic deficits postoperatively were killed immediately. After implantation of intrathecal catheters, rats were housed in individual stainless steel cages. Intrathecal injection studies were carried out 5–7 days after surgery and the animals were used two or three times in an experimental series. To eliminate the residual effects of the drug, an interval of 3–5 days was allowed to elapse between studies.

Intrathecal Drugs and Injection

The following drugs were used in the study: neostigmine methylsulfate (209.3 Da; International Medication Systems, South El Monte, CA), edrophonium chloride (201.7 Da; Sigma Chemical, St. Louis, MO), morphine sulfate (668.8 Da; Merk & Co., West Point, PA), clonidine hydrochloride (230.1 Da; Research Biochemicals, Natick, MA), carbamylcholine chloride (carbachol) (182.7 Da; Sigma), atropine sulfate (289.4 Da; Sigma), mecamylamine (167.29 Da; Sigma), naloxone hydrochloride (327.37 Da; Du Pont Pharmaceuticals, Garden City, NY), and yohimbine (354.43 Da; Sigma). Intrathecal administration of these drugs was accomplished using a hand-driven, gear-operated syringe pump. All drugs were injected in a total volume of 10 μl followed by 10 μl saline to flush the catheter. Yohimbine was prepared by dissolving it in 5% hydroxypropyl-β-cyclodextrin (Research Biochemicals, Natick, MA). Other drugs were routinely dissolved with physiological saline.

Nociceptive Threshold

The hind paw thermal nociceptive threshold was measured with a device similar to that previously reported.[25] The rats were placed in a clear plastic cage (10 cm/20 cm) on an elevated floor of clear glass (2 mm thick). A radiant heat source (halogen projector lamp CXL/CXP 50W 8V, Ushio, Tokyo, Japan) was contained in a movable holder placed beneath the glass floor. The radiant heat source's aperture was 4 mm in diameter, and bulb intensity was controlled by a constant voltage source. To reduce the variability, the interior of the box under the animal was prepared with a heat source such that the under-plate temperature was regulated to 30°C. The calibration of the thermal test system was such that the average response latency in normal untreated rats, measured before the initiation of an experimental series, was approximately 10 s. To initiate a test, the rat was placed in the box and allowed 5–10 min for adaptation. The under-floor heat source was then positioned such that it focused at the plantar surface of one hind paw where it was in contact with the glass. Care was taken not to focus the light source on the skin that was off of the glass plate. The light was then activated; activation of the light initiated a timing

circuit. The time interval between the application of the light beam and the brisk hind paw withdrawal response was measured to the nearest 0.1 s. Cut-off time in the absence of a response was 20 s. This value was then assigned as the response latency.

Study Paradigm

In all experiments, response latencies were determined twice for each rat before any drug injection and at 5, 10, 15, 30, 60 and 90 min after intrathecal injection.

Agonist Study. The first series of experiments defined the dose-dependency and time–response curves of intrathecally administered neostigmine (15, 4.8, 1.4 and 0.5 nmol), edrophonium (500, 185 and 50 nmol), carbachol (55, 16.4 and 5.5 nmol), morphine (15, 4.5, 1.5 and 0.4 nmol) and clonidine (435, 130, 26, 13 and 4.3 nmol).

Antagonist Study. The effect of the antagonists (atropine 35 nmol, mecamylamine 60 nmol, naloxone 31 nmol, and yohimbine 28 nmol) was determined for each agonist. Doses of antagonists were chosen based on previous studies with respective agents.[1,26,27,‡] In these experiments, an antagonist was injected intrathecally, followed 3 min later by the just maximally effective dose of each agonist, as defined in the immediately preceding study. Response latencies were determined 5 min after the agonist injection.

Isobolographic Analysis. To define the nature of the interaction between the drug classes, an equal dose ratio isobolographic analysis was undertaken. In this method, the dose producing a 50% effect (ED_{50}) is defined separately for each drug. The respective ED_{50} values of each drug are then administered concurrently. Subsequently, fractions (1/2, 1/4, 1/8, and 1/16) of this dose combination are delivered, and the fractional dose combination that produces a 50% effect is determined. In the current study, the isobolograms were used to characterize the effect of neostigmine–morphine (μ agonist), neostigmine–clonidine (α_2 agonist), edrophonium–morphine, and edrophonium–clonidine combinations.

Statistics

From the peak effect of the particular agent, dose–response curves, plotting percent maximal possible effect *versus* log dose, were obtained. In addition, the time course for the peak effect expressed as the area under the time–response curve was calculated by a trapezoidal rule. The percent maximal possible effect was calculated as ([postdrug latency − baseline latency]/[cutoff time (20 s) − baseline latency]) × 100.

Analyses of the dose–response curves and statistics were obtained using the pharmacologic software programs of Tallarida and Murray[28] and included calculation of the ED_{50} values and their 95% confidence intervals (CI). Other comparisons between groups were carried out with a one-way analysis of variance and a Newman–Keul multiple-range test. Differences yielding critical values corresponding to $P < 0.05$ were considered statistically significant.

Isobolographic analysis for drug–drug interaction was conducted according to the procedure of Tallarida *et al.*[29] This analysis has the advantage of being independent of the slopes of the dose–response curves; *i.e.*, parallelism does not have to be established. The isobolograms were constructed by plotting single-drug ED_{50} points on the dose coordinates of the isobologram, and a combined ED_{50} point in the dose field. A straight line joining the single-drug ED_{50} points is termed the "additive line." If the ED_{50} of a combination falls on the theoretical additive line, the effect of the drug mixture is additive. Points to the left of the theoretical additive line would be consistent with a synergistic interaction, whereas points to the right of the line would indicate a subadditive or antagonistic interaction. CIs for each point were calculated from the variances of each component alone. The CIs were evaluated for statistical significance with a Student's *t* test.

To define the type of interaction between the anticholinesterase agent/μ (or α_2) agonist, an algebraic (fractional) method of drug interaction (at ED_{50} level) was used.[30] The algebraic analysis was based on the expression of the component doses of the two agents given jointly as fractions of the doses that produce the same effect when given separately. The sum of the fractional doses, as expressed by the following equation, indicates the type of interaction:

$$da/Da + db/Db,$$

where Da and Db = the ED_{50} values of agents a and b given alone, and *d*a and *d*b = the doses of a and b that, when combined, are equipotent with Da or Db. Values near 1 indicate additive interaction, values greater than 1 imply an antagonistic interaction and values less than 1 indicate a synergistic interaction.

‡ Khan I, Taylor P, Yaksh T: Unpublished observations.

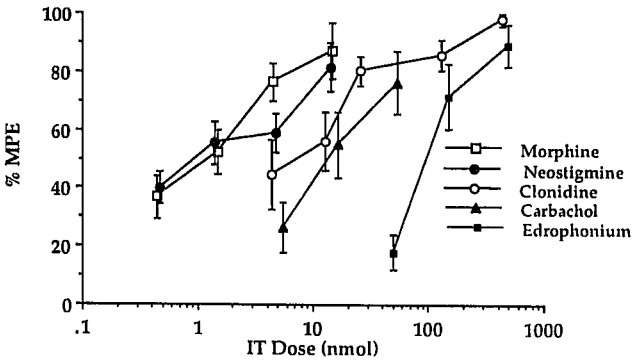

Fig. 1. Log dose–response curves for the effects of intrathecally administered morphine, neostigmine, clonidine, carbachol, and edrophonium on the thermal nociceptive threshold. The response is presented as maximal possible effect (%MPE) *versus* log dose in nanomoles. Each point on the graph represents the mean ± standard error of the mean for four to six rats.

Results

Dose–Response Analysis

Intrathecal administration of neostigmine, edrophonium, carbachol, clonidine and morphine resulted in a dose-dependent increase in the thermal response latency (fig. 1). The calculated intrathecal ED_{50} values and its 95% CIs are presented in table 1. The rank order of potency (and ED_{50}) was: morphine (1.1 nmol) = neostigmine (1.2 nmol) > clonidine (4.4 nmol) > carbachol (15 nmol) >> edrophonium (112 nmol) >> 0.

Time Course

Figure 2 displays the time course of the antinociceptive affects produced by each agent at the maximum dose examined in the absence of an antagonist. All drugs displayed the onset of peak effects within 5 (neostigmine, edrophonium, carbachol) to 15 (clonidine, morphine) min. As indicated, the duration of action of approximately equiactive concentrations was greater than 90 min for morphine and clonidine, 60 min for neostigmine and carbachol, and 45 min for edrophonium (fig. 2).

Intrathecal Antagonists

The antinociceptive effects of intrathecal neostigmine (15 nmol), edrophonium (500 nmol) and carbachol (55 nmol) were antagonized by pretreatment with

Table 1. ED_{50} Values and 95% CI for Intrathecally Administered Neostigmine, Edrophonium, Morphine, and Clonidine Alone and in Combination in a Fixed-Dose Ratio

Group	Neostigmine Component		Edrophonium Component		Morphine Component		Clonidine Component		Sum of ED_{50} Fractions
	Fraction of ED_{50}	Intrathecal Dose (nmol)	Fraction of ED_{50}	Intrathecal Dose (nmol)	Fraction of ED_{50}	Intrathecal Dose (nmol)	Fraction of ED_{50}	Intrathecal Dose (nmol)	
Single-drug study									
NEO	1.00	1.2 (0.5–3.0)	—	—	—	—	—	—	1.00
ED	—	—	1.00	112 (77–162)	—	—	—	—	1.00
MOR	—	—	—	—	1.00	1.1 (0.5–2.0)	—	—	1.00
CLON	—	—	—	—	—	—	1.00	4.4 (1.4–14)	1.00
Interaction studies									
NEO + MOR	0.39	0.46 (0.36–0.56)	—	—	0.40	0.44 (0.35–0.53)	—	—	0.79
NEO + CLON	0.29	0.34 (0.31–0.38)	—	—	—	—	0.29	1.3 (1.2–1.4)	0.58
ED + MOR	—	—	0.29	32 (26–40)	0.32	0.35 (0.27–0.42)	—	—	0.61
ED + CLON	—	—	0.08	9 (7.0–10)	—	—	0.08	0.34 (0.29–0.39)	0.16

NEO = neostigmine; ED = edrophonium; MOR = morphine; CLON = clonidine.

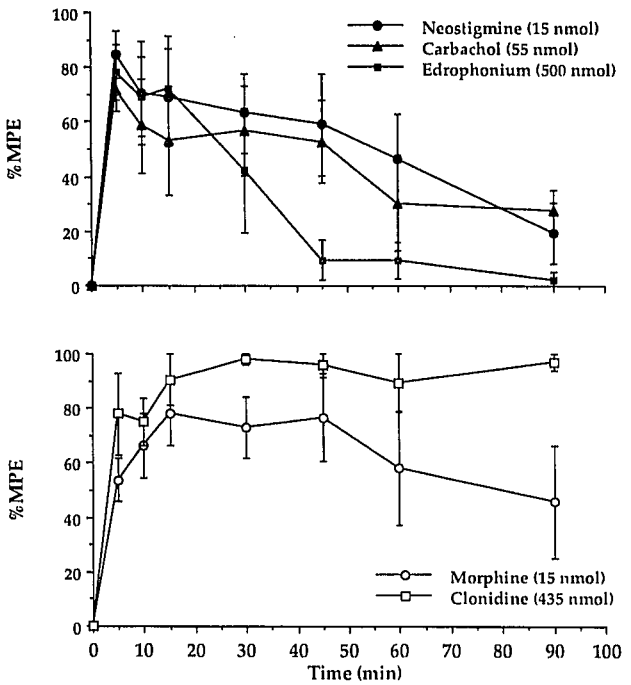

Fig. 2. The time course of the antinociceptive effects (percent maximal possible effect [%MPE]) of intrathecally administered neostigmine (15 nmol), carbachol (55 nmol), and edrophonium (500 nmol) (*top*) and morphine (15 nmol) and clonidine (435 nmol) (*bottom*) in rats. Each point represents the mean ± standard error of the mean for four to six rats.

atropine (35 nmol) (fig. 3). This dose of atropine had no effect when administered alone (data not shown). Pretreatment with the intrathecal nicotinic receptor antagonist mecamylamine (60 nmol), the μ-receptor antagonist naloxone (31 nmol) or the α_2-adrenergic receptor antagonist yohimbine (28 nmol) did not affect the thermal latency of neostigmine, edrophonium or carbachol. Naloxone and yohimbine were effective only in antagonizing the respective effects of morphine and clonidine, respectively (fig. 4).

Isobolographic Analysis

Figure 5 represents the neostigmine dose–response curves with and without the addition of morphine or clonidine in a fixed ratio of the individual ED_{50} dose, the neostigmine–morphine and the neostigmine–clonidine isobolograms. The experimentally determined mixture ED_{50} (and CI) was 0.46 (0.36–0.56) nmol for neostigmine and 0.44 (0.35–0.53) nmol for morphine. The theoretical additive ED_{50} (and CI) was calculated to be 0.6 (0.4–0.8) nmol for neostigmine and 0.5 (0.4–0.7) nmol for morphine. Though numerically less, the CIs of these points overlap, and the fractional analysis (0.79) does not differ significantly from "1" (table 1). On the other hand, isobolographic and fractional analyses demonstrated synergistic interaction between neostigmine and clonidine. The isobolographic calculations produced a theoretical additive

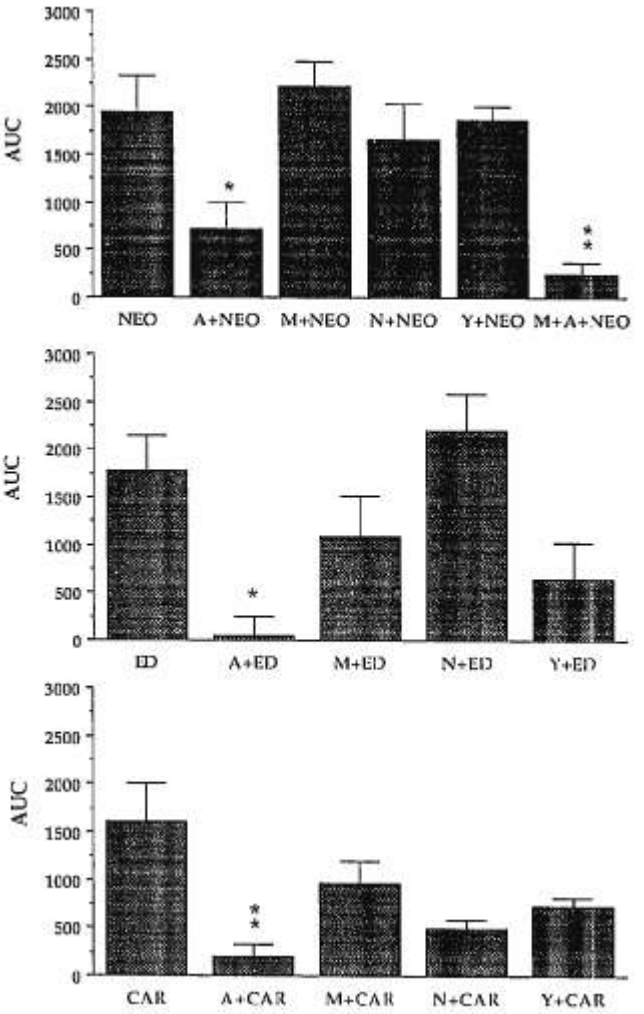

Fig. 3. To define the antagonist pharmacology for the agonists neostigmine ([NEO] 15 nmol; *top*), edrophonium ([ED] 500 nmol; *middle*), and carbachol ([CAR] 55 nmol; *bottom*), the intrathecal antagonists atropine ([A] 35 nmol), mecamylamine ([M] 60 nmol), naloxone ([N] 31 nmol), and yohimbine ([Y] 28 nmol) were administered 3 min before the administration of each agonist. An additional group of rats received both mecamylamine (60 nmol) and atropine (35 nmol) before the administration of neostigmine (*top*). Each bar represents the mean ± standard error of the mean for four to six rats. *$P < 0.05$; **$P < 0.01$.

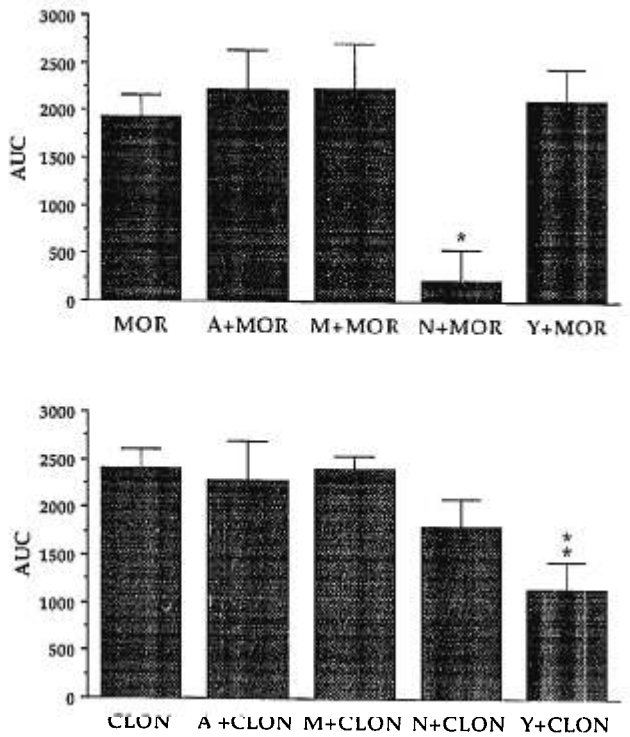

Fig. 4. To define the antagonist pharmacology for morphine ([MOR] 15 nmol; *top*) and clonidine ([CLON] 435 nmol; *bottom*), the intrathecal antagonists atropine ([A] 35 nmol), mecamylamine ([M] 60 nmol), naloxone ([N] 31 nmol), and yohimbine ([Y] 28 nmol) were administered 3 min before the administration of each agonist. Each bar represents the mean ± standard error of the mean for four to six rats. *$P < 0.05$; **$P < 0.01$.

ED_{50} (and CI) of 0.6 (0.5–0.7) nmol for neostigmine and 2.2 (1.7–2.6) nmol for clonidine. The experimentally determined ED_{50} was 0.34 (0.31–0.38) nmol for neostigmine and 1.3 (1.2–1.4) nmol for clonidine. The CIs of these points do not overlap. Furthermore, the fractional analysis of this interaction demonstrated synergism by virtue of the smaller fractional dose needed for the same effect with combined administration ($P < 0.05$, t test; 0.58 *vs.* 1; table 1).

The experimentally determined ED_{50} for the antinociceptive response of edrophonium–morphine mixture was 32 (26–40) nmol for edrophonium and 0.35 (0.27–0.42) nmol for morphine. The theoretical additive ED_{50} (and CI) was calculated to be 56 (40–72) nmol for edrophonium and 0.5 (0.4–0.7) nmol for morphine (fig. 6). The CIs of these points do not overlap, and the results of a Student's t test for potency ratio were significant ($P < 0.01$). Similarly, the isobolographic calculations for the antinociceptive effects of edrophonium–clonidine mixture produce a theoretical additive ED_{50} (and CI) of 56 (46–66) nmol for edrophonium and 2.2 (1.8–2.6) nmol for clonidine.

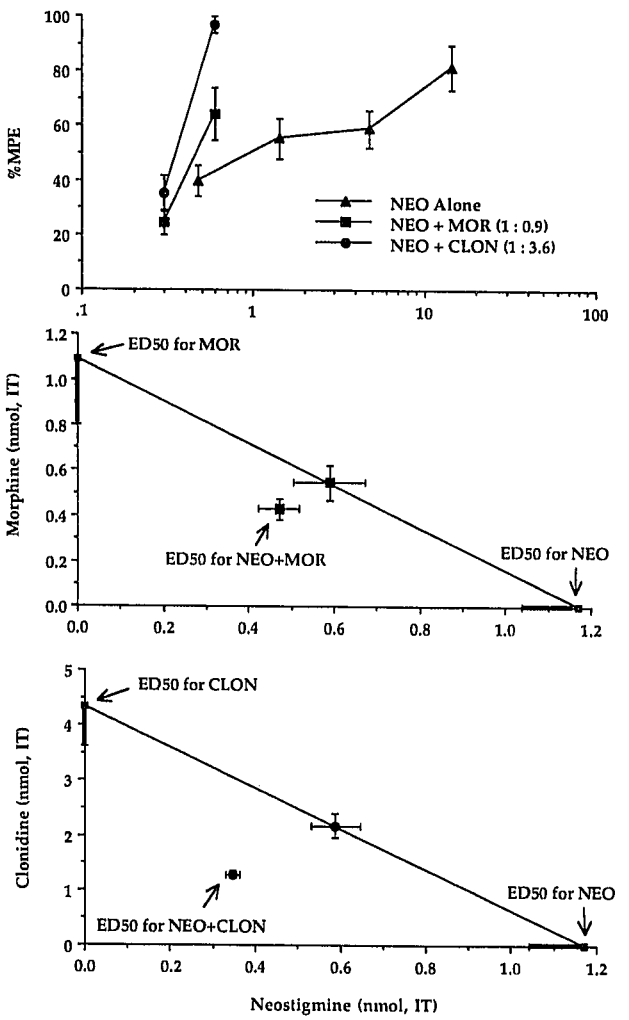

Fig. 5. Intrathecal neostigmine dose–response curves with and without the addition of morphine or clonidine in a fixed ratio of the individual dose producing a 50% effect (ED_{50}) (*top*). The ED_{50} isobologram for the interaction of the antinociceptive effect of intrathecal neostigmine–morphine (*middle*) and neostigmine–clonidine (*bottom*) mixtures when coadministered in a fixed dose ratio. The straight line connecting the single-drug ED_{50} points is the theoretical additive line, and the point on this line is the theoretical additive ED_{50} point (± standard error of the mean). The ED_{50} of the neostigmine–morphine mixture for the maximal possible effect, though numerically less, could not be statistically distinguished from a simple additive interaction. The experimental point for neostigmine–clonidine mixture was found to be significantly ($P < 0.001$) below the theoretical additive point, indicating synergistic interaction. Each point on the graph represents the mean ± standard error of the mean.

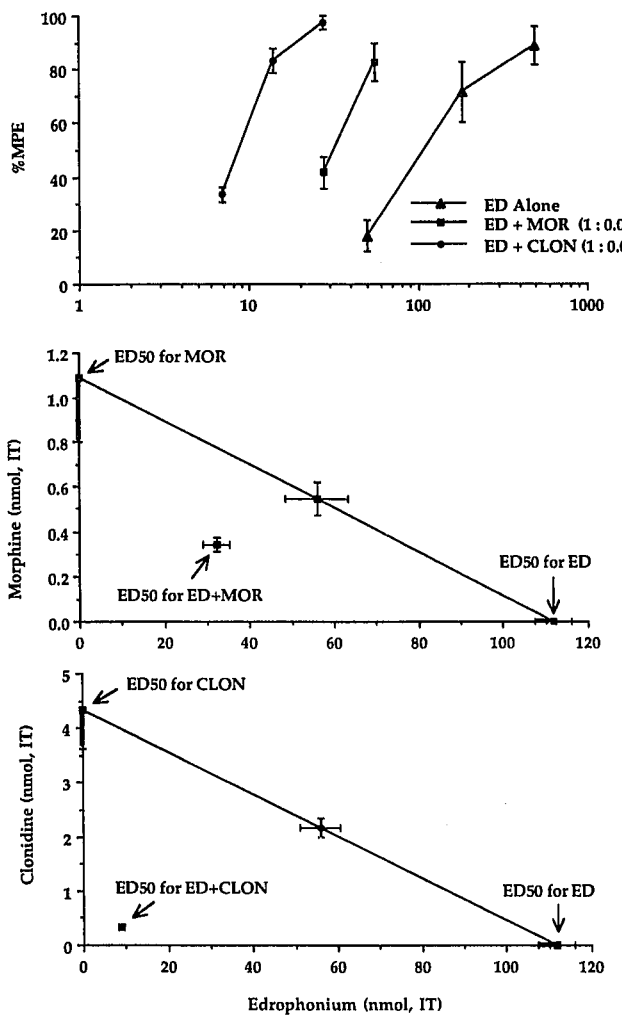

Fig. 6. Intrathecal edrophonium dose–response curves with and without the addition of morphine or clonidine in a fixed ratio of the individual dose producing a 50% effect (ED_{50}) (*top*). ED_{50} isobologram for the interaction of the antinociceptive effect of intrathecal edrophonium–morphine (*middle*) and edrophonium–clonidine (*bottom*) mixtures when coadministered in a fixed dose ratio. The straight line connecting the single-drug ED_{50} points is the theoretical additive line, and the point on this line is the theoretical additive ED_{50} point (± standard error of the mean). Both experimental points for edrophonium–morphine and edrophonium–clonidine mixture was found to be significantly ($P < 0.01$ and $P < 0.0001$, respectively) below the corresponding theoretical additive point, indicating synergistic interaction. Each point on the graph represents the mean ± standard error of the mean.

The experimentally determined ED_{50} (and CI) was 9 (7–10) nmol for edrophonium and 0.34 (0.29–0.39) nmol for clonidine. The CIs of these points do not overlap (fig. 6), and the results of a Student's *t* test for potency ratio were significant ($P < 0.0001$). Thus, the interaction between edrophonium–morphine and edrophonium–clonidine is uniformly synergistic.

General Observations

No motor impairment was observed in these animals after intrathecal drug administration. Tail grooming was observed within 5 min after intrathecal administration of carbachol, neostigmine, and edrophonium. This effect was noted in animals that had analgesia and lasted for approximately 15–20 min. It was blocked by atropine pretreatment, but not by mecamylamine, naloxone or yohimbine.

Rats that received intrathecal atropine pretreatment followed by neostigmine showed a behavior syndrome characterized by irritability, vocalization and truncal rigidity. This behavior was evoked either spontaneously or by a variety of nonpainful stimuli such as, for example, light touch or blowing of air to any part of the animal's body, or by noise. This effect was observed in about three fourths of the animals within 5 min after neostigmine injection, and the syndrome persisted for about 3–4 h. Pretreatment with mecamylamine (60 nmol) prevented the appearance of agitation otherwise evoked by atropine (35 nmol) in neostigmine (15 nmol)-treated rats. Approximately 75% of the rats that received intrathecal atropine pretreatment followed by edrophonium exhibited a milder form of the behavior syndrome that was observed with neostigmine. "Serpentine" movements of the tail were seen in the latter group as well.

Intrathecal administration of clonidine induced diuresis in all rats. Diuresis was dose-dependent and was not antagonized by yohimbine. It typically began within 5–10 min after intrathecal clonidine (435 nmol), peaked by 45 min and lasted for 2 h. However, with smaller doses of clonidine, the onset of diuresis was delayed and the duration was shorter.

Short-lived irritability (5 min) was noted in 50% of the rats that received intrathecal naloxone pretreatment followed by either intrathecal neostigmine, edrophonium or carbachol.

Discussion

Spinal Cholinergic System and Modulation of Nociceptive Processing

The results of this study emphasize that two different cholinesterase inhibitors are able to produce a dose-

dependent increase in the nociceptive threshold. These effects were uniformly antagonized by muscarinic and not by nicotinic antagonists. There are no data suggesting that these agents have direct agonistic activity at the cholinergic receptor. Previous studies with intrathecal neostigmine in the dose ranges used here have been shown to produce highly significant reductions in cholinesterase activity in the spinal cord, but not the medulla, of rats.[31] Moreover, pretreatment with intrathecal hemicholinium, leading to a reduction in the releasable pools of acetylcholine, significantly diminished the antinociceptive effects of intrathecal neostigmine.[2] These observations jointly suggest that these agents are acting through an alteration in the disposition of the hydrolysis of acetylcholine. Neostigmine contains a carbamate group that is transferred and chemically bonded to the esteratic subunit on the acetylcholinesterase. Edrophonium, on the other hand, binds electrostatically to the anionic subunit and by hydrogen binding to the esteratic site on the enzyme.[32] The equilibrium constant of such a reaction is small.[33] Therefore, it can be predicted that the *in vivo* activity of edrophonium should be rapid in onset but short in duration, which could explain the difference in the time course of the antinociceptive effects produced by neostigmine and edrophonium (fig. 2).

Several lines of evidence suggest that the effects of cholinesterase inhibitors reflect an action on specific sites. Both neostigmine and edrophonium inhibit acetylcholinesterase in a reversible fashion. The resultant increase in acetylcholine concentrations will stimulate both muscarinic and nicotinic recognition sites.

A finding of particular interest in this study was that cholinesterase inhibition in the presence of atropine would result in prominent signs of pain behavior. The fact that this effect was abolished by mecamylamine indicates that it was probably mediated via the cholinergic nicotinic receptors. Aversive effects such as gnawing, vocalization and hyperactivity have been reported after intrathecal administration of nicotinic receptor agonists (nicotine and cytisine) in the rat.[29] These effects are attenuated by mecamylamine[34] and suggest a stimulatory effect of nicotinic agonists at nicotinic receptors[9] on sensory elements within the dorsal horn.

Interaction of Spinal Cholinergic Receptor System with μ and α_2 Receptors

Considerable work has indicated that the spinal delivery of μ opioid (morphine)[35] and α_2-adrenoceptor agonists (clonidine)[1] will produce a powerful analgesia that is antagonized by naloxone and yohimbine, respectively. A previous study has suggested that depletion of spinal noradrenergic systems will attenuate the effects of cholinergic activation,[19] although competitive antagonists have been without effect on spinal cholinergic antinociception.[1,4] With regard to opioids, atropine fails to antagonize the effects of intrathecal morphine.[4] In the current study, neither the opioid receptor antagonist naloxone nor the α_2-adrenergic receptor antagonist yohimbine had any detectable effect upon the antinociceptive actions of neostigmine, edrophonium or carbachol. However, others have observed a partial attenuation of the effects of intrathecal clonidine by atropine.[19] Nevertheless, we interpret the body of data as suggesting that the antinociceptive effects of muscarinic activation at the spinal level are mediated by an action largely independent from those produced by either μ opioid or α_2 adrenoceptor activity.

Given the apparent independence of these receptor systems, it was of interest to define the nature of the interaction between these systems. The isobolographic analysis demonstrated a prominent dose sparing effect of the interaction of neostigmine and edrophonium with both clonidine and morphine. Based on the isobolographic analysis, the reduction in dose was statistically significant for all combinations with the exception of neostigmine and morphine. The magnitude of this interaction gave a dose fraction of 0.79, but the ratio failed to reach statistical significance. The reasons for this difference are not known. These results are in accord with the previous findings of Detweiler and Eisenach[20] who have shown a similar potentiating interaction between intrathecal clonidine and neostigmine in sheep.

The fact that this interaction was prominent for two structurally different cholinesterase inhibitors with two agonists interacting with two separate receptor systems suggests that these characteristics reflect upon a common property of the mechanisms of drug action. Recently, a synergy between μ, δ, and α_2 agents and nonsteroidal antiinflammatory drugs has been demonstrated.[36] Though speculative, the common factor in these interactions has been the observation that the agents with the ability to define a synergetic interaction are those that interact with receptors that diminish the evoked release of peptide transmitters from small primary afferent nerves (see Hua *et al.*[13] for references). It has been suggested that such an interaction might

serve to diminish the gain of the afferent input.[37] Reduced slopes of the stimulus response curves for single unit neurons have in fact been shown in physiological and pharmacological studies.[38-40] In this sense, an interaction would relate not to an additive change in baseline, but a synergistic function of the two slopes. Such an interaction would by its nature possess a significant nonadditive component.

Antinociception Produced by Inhibition of Transmitter Metabolism

The effects of spinal neostigmine and edrophonium emphasize the relevance of the release of endogenous acetylcholine from spinal systems. It is not clear whether this acetylcholine is released in response to high threshold afferent stimulation or whether there is a high tonic level of activity in unstimulated spinal dorsal horn. Eisenach[41] has shown that after spinal clonidine in humans, the levels of acetylcholine in lumbar cerebrospinal fluid are increased. The presence of a cholinesterase inhibitor would serve to augment cholinergic receptor tone. On the other hand, it should be stressed that this effect of cholinesterase inhibition is parallel to that found in studies examining the antinociceptive effects of tricyclic antidepressants that block amine reuptake[42-45] or enkephalinase inhibitors that prolong the action of endogenous enkephalins.[46-49] Both classes of drugs have been shown to have a spinal action that is presumed to reflect the presence of extracellular transmitters released by afferent input. The current work shows an antinociceptive effect of cholinesterase inhibition that equals or exceeds the effects observed with these other transmitter systems, suggesting that intrinsic spinal cholinergic terminals play a role in nociceptive processing that is at least as important as the better-defined modulatory substrates.

We note that intrathecal administration of clonidine induced diuresis in the rat. Although clonidine was shown to inhibit the release of antidiuretic hormone in anesthetized dogs,[50] this effect has not been demonstrated in the rat.[51] Evidence suggests that clonidine can block the renal tubular action of antidiuretic hormone[52] and affect the release of atrial natriuretic factor.[53] Whether these effects of intrathecal clonidine on diuresis reflect a central or peripheral action is not known.

Mechanisms of Spinal Cholinoceptive Modulation

As reviewed above, histochemical and autoradiographic studies have demonstrated the existence of cholinergic terminals and both muscarinic and nicotinic cholinergic binding sites in the spinal dorsal horn.[9,11,14-17] Lesion studies suggest that muscarinic receptors in the dorsal horn are located in the nerve terminals of the primary afferent, because a selective lesion of the primary afferent leads to a rapid loss of cholinergic receptors in this area.[11] Histochemical studies on the distribution of choline acetyltransferase positive cells in the dorsal horn have indicated that such immunoreactivity is found in dendrites and axons within the substantia gelatinosa. Immunopositive varicosities were found pre- and postsynaptic to the central varicosities typically associated with large and small axons. Such observations suggest that primary sensory fibers can excite cholinergic neurons and that in the dorsal horn, acetylcholine released by these local neurons may modulate by pre- and postsynaptic mechanisms, the input generated by small afferent nerves. The association of cholinergic terminals with large afferent systems raises the possibility that effects upon low threshold (large afferent) input might also be expected. Intracellular recording from dorsal horn neurons in spinal cord slices has indicated that muscarinic agonists will produce depolarization in approximately one third of the neurons examined and hyperpolarization in another one third.[54] The fact that muscarinic agonists may both excite and inhibit different dorsal horn cell systems raises the possibility that the antinociceptive actions of spinal muscarinic agonists (and the muscarinically mediated effects of cholinesterase inhibitors) reflect at least two modulatory mechanisms, one that excites inhibitory interneurons and one that hyperpolarizes dorsal horn projection neurons. These data, in combination with the behavioral data obtained with increasing intrinsic cholinergic tone (through the use of cholinesterase inhibitors), suggest that the physiologically dominant mechanism mediated by the muscarinic site in this spinal system may be the inhibition of high threshold afferent input. Importantly, monosynaptically driven ventral root reflexes are inhibited by conditioning input from adjacent dermatomes. This inhibition is mimicked by muscarinic agonists and facilitated by cholinesterase inhibitors (edrophonium).[55] Such observations indeed support the likelihood that afferent input can drive a reflexly evoked inhibition mediated by a local muscarinic site. Although this event occurs with a motor response, it offers parallels for dorsal horn connectivity in which output neurons may be reflexly regulated by an increased, afferent-driven cholinoceptive inhibition.

In conclusion, the current study demonstrates that spinal cholinergic muscarinic (but not nicotinic) receptors mediate the antinociceptive effect of intrathecally administered cholinomimetic and acetylcholinesterase-inhibiting agents. The synergistic effects observed after the coadministration of cholinesterase inhibitors and μ/α_2 agonists are suggestive of functional interactions between different spinal receptor systems involved in nociceptive processing. An important question remains to be answered regarding the identity of the subclasses of muscarinic sites that modulate spinal nociceptive activity.

References

1. Yaksh TL, Dirksen R, Harty GJ: Antinociceptive effects of intrathecally injected cholinomimetic drugs in the rat and cat. Eur J Pharmacol 117:81–88, 1985

2. Smith MD, Yang X, Nha J-Y, Buccafusco JJ: Antinociceptive effect of spinal cholinergic stimulation: Interaction with substance P. Life Sci 45:1255–1261, 1989

3. Gillberg PG, Gordh T Jr, Hartvig P, Jansson I, Pettersson J, Post C: Characterization of the antinociception induced by intrathecally administered carbachol. Pharmacol Toxicol 64:340–343, 1989

4. Zhuo M, Gebhart GF: Tonic cholinergic inhibition of spinal mechanical transmission. Pain 46:211–222, 1991

5. Scatton B, Dubois A, Javoy-Agid F, Camus A: Autoradiographic localization of muscarinic cholinergic receptors at various segmental levels of the human spinal cord. Neurosci Lett 49:239–245, 1984

6. Wamsley JK, Zarbin MA, Kuhar MJ: Distribution of muscarinic cholinergic high and low affinity against binding sites: A light microscope autoradiographic study. Brain Res Bull 12:233–243, 1984

7. Gillberg PG, Aquilonius S-M: Cholinergic, opioid and glycine receptor binding sites localized in human spinal cord by in vitro autoradiography. Acta Neurol Scand 72:299–306, 1985

8. Perry EK, Smith CJ, Perry RH, Whitford C, Johnson M, Birdsall NJ: Regional distribution of muscarinic and nicotinic cholinergic receptor binding activities in the human brain. J Chem Neuroanat 2: 189–199, 1989

9. Gillberg PG, d'Argy R, Aquilonius SM: Autoradiographic distribution of [3H]acetylcholine binding sites in the cervical spinal cord of man and some other species. Neurosci Lett 90:197–202, 1988

10. Bouchenafa O, Livingston A: The autoradiographic binding of [3H] quinuclidinyl benzilate to muscarinic: Receptors in the spinal cord of the sheep. J Vet Pharmacol Ther 14:345–350, 1991

11. Gillberg PG, Wiksten B: Effects of spinal cord lesions and rhizotomies on cholinergic and opiate receptor binding sites in rat spinal cord. Acta Physiol Scand 126:575–582, 1986

12. Gillberg PG, Askmark H: Changes in cholinergic and opioid receptors in the rat spinal cord, dorsal root and sciatic nerve after ventral and dorsal root lesion. J Neural Trans 85:31–39, 1991

13. Hua X-Y, Boublik JH, Spicer MA, Rivier JE, Brown MR, Yaksh TL: The antinociceptive effects of spinally administered neuropeptide Y in the rat: Systematic studies on structure-activity relationship. J Pharmacol Exp Ther 258:243–258, 1991

14. MacIntosh FC: The distribution of acetylcholine in the peripheral and the central nervous system. J Physiol 99:436–442, 1941

15. Feldberg W, Vogt M: Acetylcholine synthesis in different regions of the central nervous system. J Physiol 107:372–381, 1948

16. Kasa P, Mann SP, Hebb CO: Localization of choline acetyltransferase. Nature (Lond) 226:812–816, 1970

17. Roessmann U, Friede RL: The segmental distribution of acetylcholinesterase in the cat spinal cord. J Anat 101:27–32, 1967

18. Sherriff FE, Henderson Z, Morrison JF: Further evidence for the absence of a descending cholinergic projection from the brainstem to the spinal cord in the rat. Neurosci Lett 128:52–56, 1991

19. Gordh T Jr, Jansson I, Hartvig P, Gillberg PG, Post C: Interaction between noradrenergic and cholinergic mechanisms involved in spinal nociceptive processing. Acta Anaesthesiol Scand 33:39–47, 1989

20. Detweiler DJ, Eisenach JC: Intrathecal neostigmine potentiates the analgesic effects of intrathecal clonidine (abstract). ANESTHESIOLOGY 77:A840, 1992

21. Harris LS, Dewey WL, Howes JF, Kennedy JS, Pars H: Narcotic-antagonist analgesics: Interaction with cholinergic systems. J Pharmacol Exp Ther 169:17–22, 1969

22. Pleuvry BJ, Tobias MA: Comparison of the antinociceptive activities of physostigmine, oxotremorine and morphine in the mouse. Br J Pharmacol 43:706–714, 1971

23. Flodmark S, Wramner T: The analgesic action of morphine, eserine and prostigmine studied by a modified Hardy-Wolff Goodell method. Acta Physiol Scand 9:88–96, 1945

24. Yaksh TL, Rudy TA: Chronic catheterization of the spinal subarachnoid space. Physiol Behav 17:1031–1036, 1976

25. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77–88, 1988

26. Malmberg AB, Yaksh TL: Isobolographic and dose-response analyses of the interaction between intrathecal mu and delta agonists: Effects of naltrindole and its benzofuran analog (NTB). J Pharmacol Exp Ther 263:264–275, 1992

27. Takano Y, Yaksh TL: Characterization of the pharmacology of intrathecally administered alpha-2 agonists and antagonists in rats. J Pharmacol Exp Ther 261:764–772, 1992

28. Tallarida RJ, Murray RB: Manual of Pharmacologic Calculations with Computer Programs. 2nd edition. New York, Springer-Verlag, 1986

29. Tallarida RJ, Porreca F, Cowan A: Statistical analysis of drug-drug and site-site interactions with isobolograms. Life Sci 45:947–961, 1989

30. Berenbaum MC: Synergy, additivism and antagonism in immunosuppression. J Clin Exp Immunol 28:1–18, 1977

31. Takahashi H, Buccafusco JJ: The sympathoexcitatory response following selective activation of a spinal cholinergic system in anesthetized rats. J Auton Nerv Syst 34:59–67, 1991

32. Kitz RJ: The chemistry of acetylcholinesterase activity. Acta Anaesthesiol Scand 8:197–218, 1964

33. Wilson IB, Quan C: Acetylcholinesterase studies on molecular complementariness. Arch Biochem Biophys 73:131–143, 1958

34. Gillberg PG, Hartvig P, Gordh T, Sottile A, Jansson I, Archer T, Post C: Behavioral effects after intrathecal administration of cholinergic receptor agonists in the rat. Psychopharmacology 100:464–469, 1990

35. Yaksh TL: Spinal opiates: A review of their effect on spinal function with emphasis on pain processing. Acta Anaesthesiol Scand 31:25–37, 1987

36. Malmberg AB, Yaksh TL: Pharmacology of the spinal action of ketorolac, morphine, ST-91, U50488, and LPIA on the formalin test and an isobolographic analysis of the NSAID interaction. ANESTHESIOLOGY 79:270–281, 1993

37. Monasky MS, Zinsmeister AR, Stevens CW, Yaksh TL. Interaction of intrathecal morphine and ST-91 on antinociception in the rat: Dose-response analysis, antagonism and clearance. J Pharmacol Exp Ther 254:383–392, 1990

38. Yaksh TL: Inhibition by etorphine of the discharge of dorsal horn neurons: Effects upon the neuronal response to both high- and low-threshold sensory input in the decerebrate spinal cat. Exp Neurol 60:23–40, 1978

39. Gebhart GF, Sandkuhler J, Thalhammer JG, Zimmermann M: Quantitative comparison of inhibition in spinal cord of nociceptive information by stimulation in periaqueductal gray or nucleus raphe magnus of the cat. J Neurophysiol 50:1433–1444, 1983

40. Gebhart GF, Sandkuhler J, Thalhammer J, Zimmerman M: Inhibition in spinal cord of nociceptive information by electrical stimulation and morphine microinjections at identical sites in midbrain of the cat. J Neurophysiol 51:75–89, 1984

41. Eisenach JC. Cerebrospinal fluid (CSF) pharmacodynamics and a possible cholinergic mechanism of epidural clonidine in humans (abstract). ANESTHESIOLOGY 77:A870, 1992

42. Spiegel K, Kalb R, Pasternak W: Analgesic activity of tricyclic antidepressants. Ann Neurol 13:462–465, 1983

43. Bodnar RJ, Mann PE, Stone EA: Potentiation of cold-water swim analgesia by acute, but not chronic, desipramine administration. Pharmacol Biochem Behav 23:749–752, 1985

44. Ardid D, Eschalier A, Lavarenne J: Evidence for a central but not a peripheral analgesic effect of clomipramine in rats. Pain 45: 95–100, 1991

45. Ardid D, Guilbaud G: Antinociceptive effects of acute and 'chronic' injections of tricyclic antidepressant drugs in a new model of mononeuropathy in rats. Pain 49:279–287, 1992

46. Oshita S, Yaksh TL, Chipkin R: The antinociceptive effects of intrathecally administered SCH32615, an enkephalinase inhibitor in the rat. Brain Res 515:143–148, 1990

47. Al-Rodhan N, Chipkin R, Yaksh TL: The antinociceptive effects of SCH-32615, a neutral endopeptidase (enkephalinase) inhibitor, microinjected into the periaqueductal, ventral medulla and amygdala. Brain Res 520:123–130, 1990

48. Dickenson AH, Sullivan AF, Roques BP: Evidence that endogenous enkephalins and a delta opioid receptor agonist have a common site of action in spinal antinociception. Eur J Pharmacol 148:437–439, 1988

49. Suh HH, Tseng LL: Intrathecal administration of thiorphan and bestatin enhances the antinociception and release of met-enkephalin induced by beta-endorphin intraventricularly in anesthetized rats. Neuropeptides 16:91–96, 1990

50. Kimura T, Share L, Wang BC, Crofton JT: The role of central adrenoceptors in the control of vasopressin release and blood pressure. Endocrinology 108:1829–1836, 1981

51. Gellai M, Edwards RM: Mechanism of a_2-adrenoceptor agonist-induced diuresis. Am J Physiol 255:F317–F323, 1988

52. Smyth DD, Umemura S, Pettinger WA: a_2-Adrenoceptor antagonism of vasopressin-induced changes in sodium excretion. Am J Physiol 248:F767–F772, 1985

53. Chen M, Lee J, Huang BS, Grekin RJ, Malvin RL: Clonidine and morphine increase atrial natriuretic peptide secretion in anesthetized rats. Proc Soc Exp Biol Med 191:299–303, 1989

54. Urban L, Willetts J, Murase K, Randic M: Cholinergic effects on spinal dorsal horn neurons in vitro: An intracellular study. Brain Res 500:12–20, 1989

55. Yoshioka K, Sakuma M, Otsuka M: Cutaneous nerve-evoked cholinergic inhibition of monosynaptic reflex in the neonatal rat spinal cord: Involvement on M2 receptors and tachykininergic primary afferents. Neuroscience 38:195–203, 1990