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Perturbation of Ion Channel Conductance Alters the Hypnotic Response to the α_2-Adrenergic Agonist Dexmedetomidine in the Locus Coeruleus of the Rat

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Background: **The α_2-adrenergic agonists are members of a novel class of hypnotic–anesthetic agents that selectively bind to α_2 adrenoceptors in the locus coeruleus (LC) to initiate their pharmacologic action. The postreceptor molecular mechanism of the hypnotic action of α_2-adrenergic agonists remains unknown. In this study we addressed the role of conductance through a variety of calcium and potassium ion channels in the hypnotic action of dexmedetomidine in the LC of the rat.**

Methods: **Cannulas were inserted stereotactically into the LC of halothane-anesthetized rats (n = 318). After at least 48 h, rats were tested for loss of righting reflex in response to administration of the α_2-adrenergic agonist dexmedetomidine at a hypnotic (7.0 μg LC) or subhypnotic (3.5 μg LC) dose. To establish the mediating role of various species of calcium and potassium ion channels in the hypnotic response, rats were pretreated with the following drugs before the administration of dexmedetomidine LC: S(+)202791 (L-type calcium-channel activator), nifedipine and R(−)202791 (L-type calcium-channel blocker), SNX 111 (N-type calcium-channel blocker), SNX 230 (P-type calcium-channel blocker), quinine (calcium-activated and voltage-gated potassium-channel blocker), charybdotoxin (calcium-activated potassium-channel blocker), dendrotoxin (voltage-gated potassium-channel blocker), or glybenclamide (adenosine triphosphate–sensitive potassium-channel blocker). The drugs were used in doses not causing behavioral effects that could have confounded the interpretation of loss of righting reflex.**

Results: **SNX 230 and the dihydropyridines nifedipine and R(−)202791 produced loss of righting reflex in the presence of a subhypnotic dose of dexmedetomidine. The hypnotic-enhancing effects of the dihydropyridines could be blocked with S(+)202791, which also diminished loss of righting reflex in response to dexmedetomidine 7.0 μg LC. Quinine, dendrotoxin, and charybdotoxin each attenuated the hypnotic response to dexmedetomidine 7.0 μg LC. The hypnotic response to dexmedetomidine was not significantly altered by SNX 111 or glybenclamide.**

Conclusions: **Inhibition of ion conductance through L- or P-type calcium channels and facilitation of conductance through voltage-gated or calcium-activated potassium channels may be involved in the mechanism of hypnotic action of α_2-adrenergic agonists. These changes in ion conductance were capable of producing membrane hyperpolarization and decreasing neuronal excitability. There was no evidence for the involvement of adenosine triphosphate–sensitive potassium channels or N-type calcium channels in the hypnotic response to dexmedetomidine. (Key words: Brain: locus coeruleus. Hypnotic response. Ion channels: calcium; potassium. Receptors, adrenergic: α_2. Sympathetic nervous system, α_2-adrenergic agonists: dexmedetomidine.)**

THE MOLECULAR mechanism for anesthetic action remains unknown. The α_2-adrenergic agonists are a novel class of hypnotic–anesthetic agent with selectivity for specific binding sites through which these compounds exert their pharmacologic response.[1,2] This selectivity facilitated a systematic characterization of the molecular components involved in its hypnotic–anesthetic action. We showed that the locus coeruleus (LC) is a site that mediates the hypnotic response to the highly selective α_2-adrenergic agonist dexmedetomidine in the rat.[3] The precise localization allowed us to discretely probe the postreceptor molecular mechanism for the hypnotic action of α_2-adrenergic agonists. We demonstrated that the transduction pathway involves a pertussis toxin–sensitive G protein[4] and inhibition of adenylate cyclase.[5] Both of these molecular components can be considered as intermediate steps that do not, alone, alter neuronal excitability or produce central nervous system depression. The final effector mechanism for the hypnotic action of α_2-adrenergic agonists may be an alteration in transmembrane ion conductance resulting in hyperpolarization.[6]

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The role of transmembrane ion conductance in the hypnotic action of α_2-adrenergic agonists is considered in this study. Outwardly directed potassium conductance has been shown to mediate a decrease in neuronal firing rate after activation of α_2 adrenoceptors in an *in vitro* LC preparation.[7] The species of potassium channel involved in the transmembrane conductance was not identified. Activation of α_2-adrenoceptors can also inhibit calcium entry into neurons in the LC.[8] Again, the species of calcium channel involved was not characterized. To examine the role of the various species of calcium and potassium channels involved in the hypnotic action of α_2-adrenergic agonists, we administered a series of selective calcium and potassium channel blockers and activators directly into the LC and examined their effects on the hypnotic response to the highly selective α_2-adrenergic agonist dexmedetomidine. We thus were able to identify the channels that are involved by the way in which these ion channel blockers and activators altered the hypnotic response to the highly selective α_2-adrenergic agonist dexmedetomidine.

Materials and Methods

Animals

The experimental protocol was approved by the Animal Care and Use Committee at the Palo Alto Department of Veterans Affairs Medical Center. Male Sprague-Dawley rats (n = 318), weighing 250–300 g were used. The rats from the same litter were stratified into control and treatment groups and were matched for weight.

The left LC was stereotaxically cannulated with a 24-G stainless steel cannula according to the following coordinates: with the bregma as the reference, 1.2 mm lateral, 9.7 mm posterior, and at a depth of 6 mm from the skull.[3] At these ordinates the cannula was placed just above the LC (fig. 1). The surgical procedure was performed with the rat under halothane anesthesia, and the cannula was fixed in position with methylmethacrylate resin.

After a recovery period of 2–4 days, a 30-G stainless steel needle, connected to a polyethylene tubing, was inserted through the cannula and positioned 1 mm beyond its tip. For treatments in which the effect was reversible (*i.e.*, treatment with the nontoxins) the same rat was used with and without pretreatment and the order was randomly assigned and a minimum of 1 week separated the two dexmedetomidine drug administra-

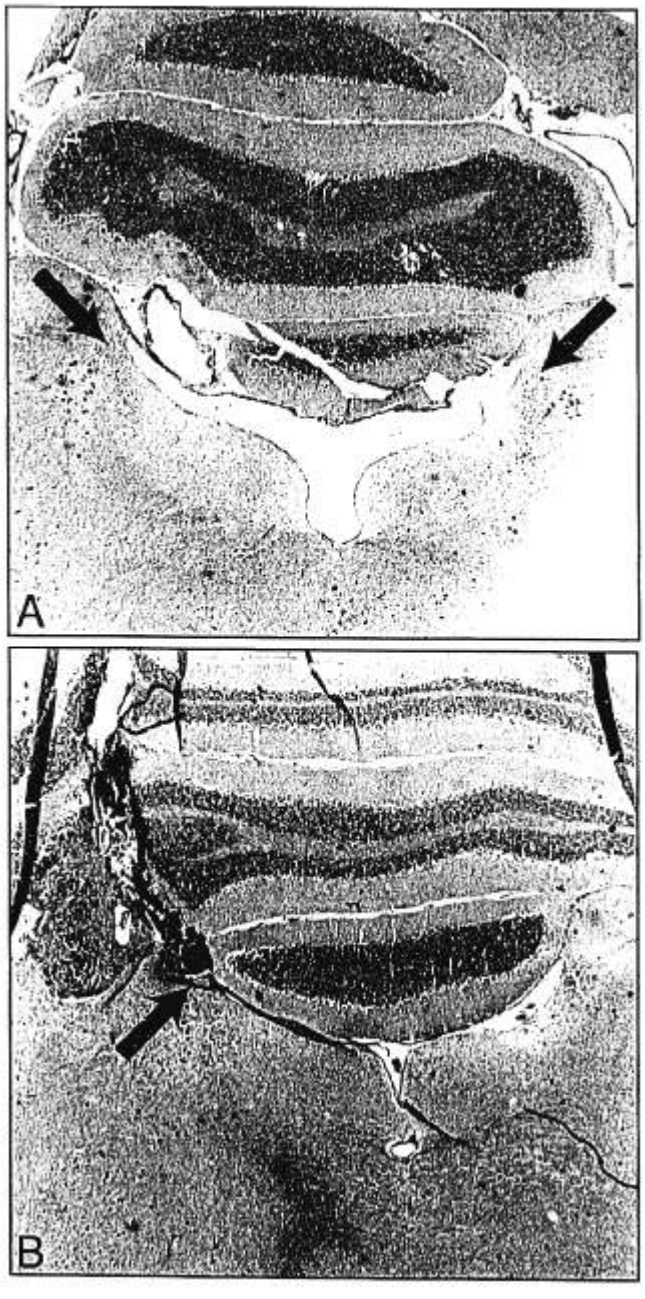

Fig. 1. Photomicrograph of the locus coeruleus in the rat. (*A*) Coronal section at the level of the fourth ventricle of the brainstem of the rat. Arrows indicate the cluster of noradrenergic neurons in the locus coeruleus. (*B*) After green dye (Davidson Marking System), 0.4 μl, was injected through the cannula, the rat was killed and the brain fixed for histologic section. The dye ends just at the upper level of the locus coeruleus and exemplifies a correctly placed cannula.

Table 1.

Drug	Dose	Route	Target	Action	Reference
Nifedipine	0.9 μg	LC	L-type Ca^{2+} channel	Blocker	11
S(+)202791	0.4 μg	LC	L-type Ca^{2+} channel	Activator	12
R(−)202791	0.4 μg	LC	L-type Ca^{2+} channel	Blocker	12
SNX 111	10–1,000 ng	LC	N-type Ca^{2+} channel	Blocker	13
SNX 230	80–10,000 ng	LC	P-type Ca^{2+} channel	Blocker	14
Quinine	0.7–7.0 μg	LC	Ca^{2+} activated K^+ channel	Blocker	15, 16
	0.5 g/kg	IP			
Dendrotoxin	4.5–4,500 pg	LC	Voltage-gated K^+ channel	Blocker	17
Charybdotoxin	4.4–880 pg	LC	Ca^{2+} activated K^+ channel	Blocker	18
Glybenclamide	0.8–8 μg	LC	ATP-sensitive K^+ channel	Blocker	19

tions. Pilot studies had indicated that repeated drug injections, 1 week apart, produced an equivalent hypnotic response. All drugs were injected by a pump (CMA/100 microinjection pump, Bioanalytical Systems, West Lafayette, IN) at a rate of 0.2 μl over a 30-s period and in a volume of 0.2 μl sterile water except as otherwise noted. Correct placement of the cannula at the superior border of the LC was confirmed histologically at the conclusion of the experiments. Green dye (Davidson Marking System, Bradley Products, Bloomington, MN) was injected through the cannula. Histologic sections were made in the standard fashion and if the dye or needle track were 2 mm or more from the LC, the data from that study was not included. This resulted in different group sizes, which are specified in the legends to the figures.

Behavioral Testing

All behavioral tests were performed between 10:00 AM and 4:00 PM. The hypnotic response was established if the rat lost its righting reflex. The rat was placed on its back as soon as it displayed evidence of sedation (stopped walking, remained quiet, or lay down flat on its abdomen). The rat was judged to have lost its righting reflex if it failed to right itself within 1 min of being placed on its back. If the rat still retained its righting reflex it was tested at 3-min intervals for up to 30 min after dexmedetomidine administration. A dose of dexmedetomidine 7 μg LC[3] or dexmedetomidine 50 $\mu g \cdot kg^{-1}$ intraperitoneally[9] was used because it produced loss of righting reflex in more than 95% of rats. In the experiments in which enhancement of the hypnotic response was tested, a subhypnotic dose of dexmedetomidine (3.5 μg LC) was administered. The observer was not blinded to the various treatments because the observations (loss and restoration of the rat's righting reflex) are unequivocal endpoints and are not subject to observer misinterpretation. To rule out a possible nonspecific hypnotic-reversing effect of the ion channel blockers, additional studies were performed with systemically administered pentobarbital. The γ-aminobutyric acid receptor chloride ionophore macromolecular complex is responsible for the hypnotic action of barbiturates with no involvement of calcium or potassium channels.[10] The timing of drug administration coincided with peak behavioral effects established by pilot experiments. For perturbing agents that do not cause behavioral effects when given alone (see below), an interval of 15 min was selected.

To investigate the role of calcium ion channels in the hypnotic response, rats were pretreated in the following manner (table 1):

1. nifedipine (L-type calcium blocker)[11] 0.9 μg LC, 15 min before dexmedetomidine 3.5 μg LC
2. S(+)202791 (L-type calcium activator)[12] 0.4 μg LC, 15 min before nifedipine 0.9 μg LC, 15 min before dexmedetomidine 3.5 μg LC
3. S(+)202791 0.4 μg LC, 15 min before R(−)202791 (L-type calcium blocker)[12] 0.4 μg LC, 15 min before dexmedetomidine 3.5 μg LC
4. ω conotoxin MVIIA (SNX 111; N-type calcium blocker)[13] 10–1,000 ng LC, 5 min before dexmedetomidine 3.5 μg LC
5. ω conotoxin MVIIC (SNX 230; P-type calcium blocker)[14] 80–10,000 ng LC, 5 min before dexmedetomidine 3.5 μg LC.

To investigate the role of potassium channels in the hypnotic response, rats were pretreated in the following manner:

1. quinine (calcium-activated potassium-channel blocker)[15] 0.1 and 0.5 $g \cdot kg^{-1}$ intraperitoneally,[16]

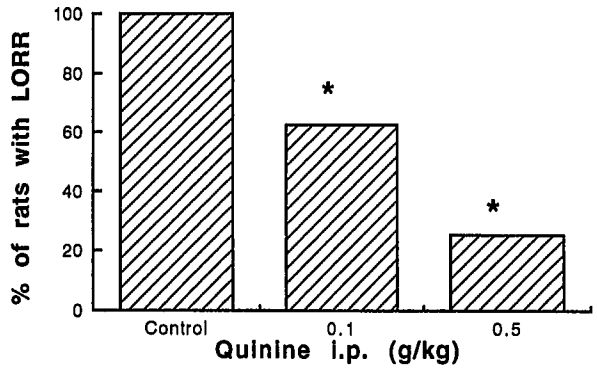

Fig. 2. Effect of intraperitoneal quinine on the hypnotic response to intraperitoneal dexmedetomidine. Quinine was administered 15 min before dexmedetomidine 50 $\mu g \cdot kg^{-1}$, and the loss of righting reflex was assessed. Data are presented as the percentage of rats exhibiting loss of righting reflex (LORR); n = 5 rats per group. *Significantly different from control.

15 min before dexmedetomidine 50 $\mu g \cdot kg^{-1}$ intraperitoneally

2. quinine 0.7–7.0 μg LC, 15 min before dexmedetomidine 7.0 μg LC
3. quinine 0.5 g/kg intraperitoneally, 15 min before pentobarbital 40 $mg \cdot kg^{-1}$ intraperitoneally
4. dendrotoxin (voltage-gated potassium-channel blocker)[17] 4.5–4,500 pg LC, 15 min before dexmedetomidine 7 μg LC
5. charybdotoxin (calcium-activated potassium-channel blocker)[18] 4.4–880 pg LC, 5 min before dexmedetomidine 7.0 μg LC
6. glybenclamide (adenosine triphosphate–sensitive potassium-channel blocker)[19] 0.8 and 8 μg LC, 15 min before dexmedetomidine 7.0 μg LC.

Because quinine (cardiorespiratory depression, seizures), conotoxin ("shakes" and motor deficits), charybdotoxin (sedation), and dendrotoxin (sedation) are capable of causing behavioral and toxic effects, the doses were selected in pilot studies to be below the behavioral and toxic thresholds. The drugs were dissolved in the following solvents; sterile water (dexmedetomidine, quinine, SNX 111, SNX 230 and pentobarbital), cremophor 5% in saline (nifedipine, R(−)202791, S(+)202791), saline (charybdotoxin), Tween 80 5% in sterile water (glybenclamide), and sodium phosphate buffer *p*H 7.6 (dendrotoxin). None of the solvents alone produced any behavioral effects.

For experiments involving a putative irreversible probe (*i.e.*, a peptide toxin), we tested the animals on two occasions; first, to confirm their uniform response to the α_2 agonist in the unperturbed state. Thereafter, the animals were stratified (according to weight) to be treated with the toxin or the vehicle before receiving the α_2 agonist. This response was then compared by contingency table analysis for proportional data. For experiments in which the drug effect is known to be reversible (*e.g.*, experiments with glybenclamide and quinine) each animal was tested with or without pretreatment with the order being assigned by random numbers.

Data were analyzed by the contingency table analysis for proportional data and by the unpaired Student's *t* test for parametric data. Data were considered to be statistically significantly different when the *P* value was <0.05.

Results

The hypnotic response to dexmedetomidine administered into the LC was blocked by quinine (the calcium-activated potassium-channel blocker) whether these drugs were delivered systemically (fig. 2) or directly into the LC (fig. 3). Conversely the hypnotic response to the barbiturate pentobarbital was uninfluenced by quinine (data not shown). Charybdotoxin (calcium-activated potassium-channel blocker) blocked the hypnotic response to dexmedetomidine LC when it was delivered directly into the LC (fig. 4). Similarly, dendrotoxin (voltage-gated potassium-channel blocker) blocked the hypnotic response to dexmedetomidine LC at low doses; this hypnotic-atten-

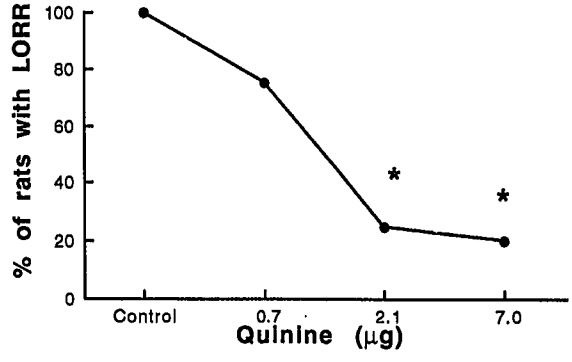

Fig. 3. Effect of quinine administered into the locus coeruleus (LC) on the hypnotic response to dexmedetomidine LC. Quinine or vehicle (control) LC was administered 15 min before the administration of dexmedetomidine 7.0 μg into the LC. Data are presented as the percentage of rats that exhibited loss of righting reflex (LORR); n = 4–7 rats per group. *Significantly different from the control (vehicle) group.

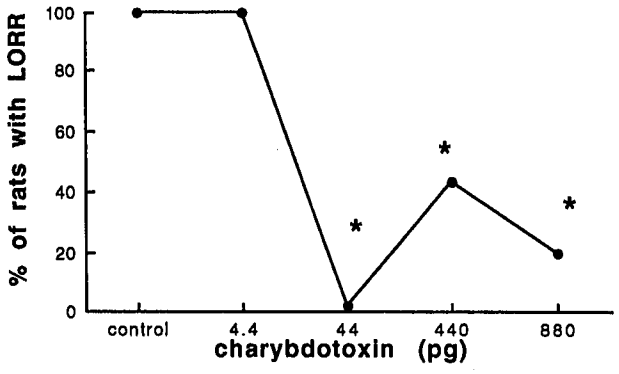

Fig. 4. Effect of charybdotoxin administered into the locus coeruleus (LC) on the hypnotic response to dexmedetomidine LC. Charybdotoxin or vehicle (control) LC was administered 5 min before dexmedetomidine 7.0 μg LC. Data are presented as the percentage of rats exhibiting loss of righting reflex (LORR); n = 4–7 rats per group. *Significantly different from control.

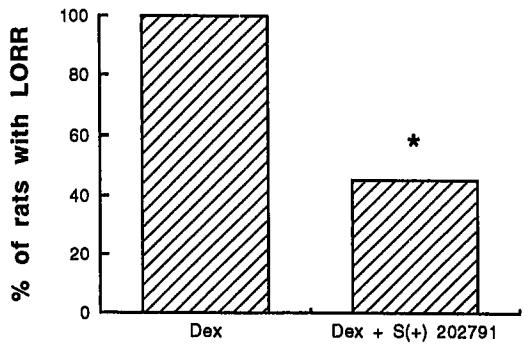

Fig. 6. Effect of S(+)202791 administered into the locus coeruleus (LC) on the hypnotic response to dexmedetomidine LC. S(+)202791, 0.4 μg LC, was administered 15 min before dexmedetomidine 7.0 μg LC. Data are presented as the percentage of rats exhibiting loss of righting reflex (LORR); n = 7 rats per group. *Significantly different from the control group.

uating effect disappeared at higher doses of dendrotoxin (fig. 5). S(+)202791 (L-type calcium activator) attenuated the hypnotic response to dexmedetomidine LC (fig. 6). The hypnotic response to a subhypnotic dose of dexmedetomidine administered into the LC, was enhanced by R(−)202791 (L-type calcium blocker) (fig. 7), administered LC. These hypnotic-enhancing properties were blocked by S(+)202791 (fig. 7). Qualitatively similar data were obtained with nifedipine, the less specific L-type calcium blocker (data not shown). SNX 230 enhanced the hypnotic response of dexmedetomidine (fig. 8). Neither glybenclamide nor SNX 111 significantly affected the hypnotic response to dexmedetomidine (data not shown).

Discussion

The LC in the rat is a collection of approximately 1,500 norepinephrine-containing neurons symmetrically located on each side in the floor of the fourth ventricle in the pontine brainstem.[20] Ascending efferent pathways leave the nucleus as a large dorsal bundle,

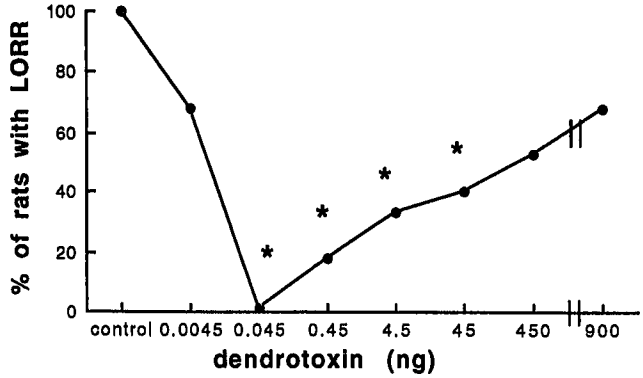

Fig. 5. Effect of dendrotoxin administered into the locus coeruleus (LC) on the hypnotic response to dexmedetomidine LC. Dendrotoxin or vehicle (control) LC was administered 15 min before dexmedetomidine 7.0 μg LC. Data are presented as the percentage of rats exhibiting loss of righting reflex (LORR); n = 7–10 rats per group. *Significantly different from control.

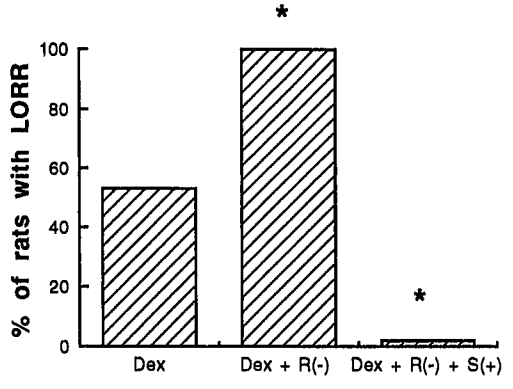

Fig. 7. Effect of R(−)202791 ± S(+)202791 administered into the locus coeruleus (LC) on the hypnotic response to dexmedetomidine LC. S(+)202791 0.4 μg or vehicle was administered 15 min before R(−)202791 0.4 μg, which was administered 15 min before dexmedetomidine 3.5 μg LC. Data are presented as the percentage of rats exhibiting loss of righting reflex (LORR); n = 8–13 rats per group. *Significantly different from dexmedetomidine (dex) alone.

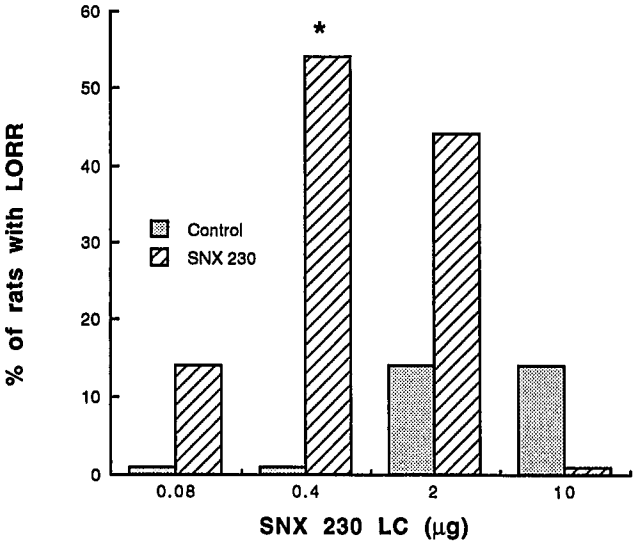

Fig. 8. Effect of ω conotoxin MVIIC (SNX 230) administered into the locus coeruleus (LC) on the subhypnotic response to dexmedetomidine LC. SNX 230 was administered 5 min before dexmedetomidine 3.5 μg LC. For each treatment dose cohort there was a corresponding control group that received the vehicle alone. Data are presented as the percentage of rats exhibiting loss of righting reflex (LORR); n = 7–9 rats per group. *Significantly different from the control group.

which splits in the forebrain and arborizes to innervate telencephalic terminal fields, and a smaller ventral bundle terminating in the basal forebrain. Descending efferent tracts convey fibers throughout the spinal cord. Afferent γ-aminobutyric acid–ergic inputs to the LC come from rostral medullary nuclei; other afferent fibers arise from the surrounding gray matter, hypothalamus and spinal cord. Destruction of the LC can enhance normal sleep[21] and decrease anesthetic requirements,[22] suggesting that the spontaneous discharge of noradrenergic pathways relaying from the LC are excitatory. This "firing rate" of the LC is suppressed by activation of the α_2 adrenoceptors[6] by altering transmembrane ion conductance.[7]

Each of the species of ion channels tested in this study are present in noradrenergic neurons in the LC[23] including the recently characterized P-type calcium channels.[24] Previously we demonstrated that LC-administered α_2 agonists induce loss of righting reflex.[3] Using the loss of righting reflex as a reflection of the alteration in the LC firing rate, we sought to determine the ion channels involved in the alteration of transmembrane conductance induced by α_2 agonists. The use of an *in vivo* model to address molecular mechanisms of drug action has certain limitations. The validity of such an approach depends on the specificity of the probes that are used to disrupt the molecular components. Previously, we demonstrated that systemically administered D-aminopyridine, dose-dependently, reversed the hypnotic response to dexmedetomidine.[9] D-Aminopyridine is relatively nonselective because it is capable of activating a variety of calcium channels and blocking multiple species of potassium channels. In the current study we used a series of compounds delivered, quite precisely, into a discrete site. When possible we studied multiple compounds to test for internal consistency in our data; also, the effects of the perturbing probe have largely been confined to an hypnotic site of action thereby minimizing the likelihood of a nonspecific response. Although we used various compounds and toxins for their selective action on a particular species of ion channel, these probes may exhibit other, as yet undefined nonspecific effects. We did not functionally assess whether the toxins produce cell death or injury. Of note, a biphasic response could be seen with dendrotoxin and SNX 230 that is not likely to be due to a progressive cell injury culminating in death. The multimodal dose response curves that were observed with dendrotoxin and charybdotoxin may be indicative of their action at more than one species of ion channel. Alternatively, the complex kinetics involved in the opening and closing of voltage-dependent ion channels[25] may give rise to the appearance of multiple sites of action. With these caveats in mind we demonstrated that the channels involved are sensitive to dendrotoxin, charybdotoxin, quinine, dihydropyridines, and one variety of ω conotoxin (SNX 230) and but not to another (SNX 111). These data suggest the participation of a voltage-gated potassium channel (sensitive to dendrotoxin and quinine), a calcium-activated potassium channel (sensitive to charybdotoxin and quinine), and both an L-type and a P-type calcium channel in the hypnotic response to α_2-adrenergic agonists. Our *in vivo* methodologic approach cannot distinguish between putative mediatory *versus* modulatory roles exerted by these ion channels.

Our findings regarding the potassium channel are largely consistent with previous *in vitro* studies. Activation of a transient outward potassium current is prominently involved in the inhibitory neuronal actions of α_2 agonists.[26] Also, α_2 adrenoceptor–mediated hyperpolarization of the plasma membrane secondary to increased potassium conductance appears to be calcium dependent in many systems, including cholinergic neurons in the myenteric plexus. While the lack

of participation of an adenosine triphosphate–sensitive potassium channel (glybenclamide-sensitive) agrees with other studies of central nervous system depression,[27] morphine analgesia was blocked with glybenclamide in doses similar to those used in our study.[28]

Decreased calcium conductance mediates the inhibitory effects of α_2-adrenoceptor activation on neurotransmitter release.[29] This effect is thought to involve direct regulation of calcium entry through N-type calcium channels.[29] Because SNX 111 did not significantly potentiate the subhypnotic effects of dexmedetomidine, we interpret this finding to indicate that a presynaptic site of action on conotoxin-sensitive N-type calcium channels may not be involved in the hypnotic action of α_2 agonists. This finding is consistent with our previous studies suggesting that presynaptic α_2-adrenoceptor activation is not pivotally involved in this hypnotic action of dexmedetomidine.[1] Conversely, there is convincing evidence for the involvement of an L-type calcium channel in the hypnotic response to dexmedetomidine because an L-type activator reversed the hypnotic response, whereas nifedipine (L-type blocker) was able to enhance the hypnotic response. The specificity of this action of nifedipine was demonstrate by the ability of the L-type calcium-channel activator to reverse the hypnotic-enhancing effect of nifedipine. Therefore we suggest that activation of the α_2 adrenoceptor in the LC decrease conductance through the L-type channel resulting in a hyperpolarized neuron that is less capable of achieving its firing threshold. A role for an L-type calcium channel in the anesthetic action of α_2 agonists has also been suggested by others.[30] Our data also suggest that the P-type calcium channel is involved in the hypnotic response to α_2 agonists. Defining the functional significance of this result must await the further characterization of the physiologic role of this channel in the mammalian nervous system.

Recently, we suggested that the phosphorylation state of key regulatory proteins may be pivotal in mediating the central nervous system effects of α_2 agonists.[5] In brief, drugs, such as dibutyryl cyclic adenosine monophosphate (cAMP) and rolipram, which functionally sustain intracellular cAMP content, reversed the hypnotic response to dexmedetomidine, an α_2 agonist.[5] This reversal of the hypnotic response to an α_2 agonist could be mitigated by blocking cAMP-dependent protein kinase activation with Rp-cAMPS. These findings suggest that the adenylate cyclase–, cAMP-dependent protein kinase pathway plays a pivotal role in the hypnotic response to an α_2 agonist in the LC. From these and our current findings, it appears that dephosphorylation of calcium-activated and voltage-gated potassium channels and voltage-gated P- and L-type calcium channels may be the final pathway in the development of α_2 mediated hyperpolarization. Earlier, others[31,32] had shown that a certain species of the large conductance, calcium-activated potassium channel (maxi-channel) from rat brain could be activated by phosphatase 2A and conversely could be inhibited by cAMP-dependent protein kinase. Similarly the conductance through the L-type calcium channel is also critically dependent on its state of phosphorylation.[33] In fact, the α_1 subunit of the purified L-type channel is completely incapable of conducting calcium in the dephosphorylated state.[34] Finally, conductance through voltage-gated potassium channels is also regulated by the state of phosphorylation[35] being significantly impaired by cAMP-dependent protein kinase.[36] Therefore, our findings are consistent with the hypothesis that stimulation of α_2 adrenoceptors shifts these key ion channels into the dephosphorylated state thereby enhancing outward flow of potassium ions *via* the voltage-gated and calcium-activated channels, and decreasing inward flow of calcium ions *via* the L- and P-type calcium channels.

Our data suggest that several, seemingly separate, channels may play a role in the hypnotic action of α_2 agonists. With our *in vivo* approach, we are able neither to characterize their stoichiometry nor to prioritize the importance of conductance through one channel species with respect to another. There is evidence that receptor-activated G proteins can couple to more than one effector mechanism[37]; in this manner the transduction of the hypnotic response to α_2 agonists may involve two or more primary effectors. Alternatively, one species of channel may be the principal effector mechanism with its properties modulated by alteration in ion conductance through other channels.

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