LABORATORY INVESTIGATIONS

Alpha-2 Adrenoceptor Agonists Decrease Cyclic Guanosine 3',5'-Monophosphate in the Mouse Brain

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**Background:** In the central nervous system neurotransmitters, drugs or conditions that excite cyclic guanosine 3',5'-monophosphate (cGMP), an effect mediated by the neuromodulator nitric oxide, whereas those that sedate decrease cGMP. Volatile anesthetics were shown to decrease cerebellar cGMP, an effect that correlates with their anesthetic and anti-inflammatory effect. Because alpha-2 adrenoceptor agonists have anesthetic properties, the role of the nitric oxide–cGMP pathway in the action of the alpha-2 adrenoceptor agonists clonidine and dexmedetomidine was investigated.

**Methods:** Groups of mice were given, intraperitoneally, one dose of either 30–600 μg/kg clonidine, or 3–300 μg/kg D-methylen (dexmedetomidine) or L-metedomidone. The alpha-2 adrenoceptor antagonists, 0.3–5 mg/kg yohimbine or 1 mg/kg atipamezole, 1 mg/kg of the alpha-1 antagonist prazosin, and 10–300 mg/kg of the nitric oxide synthase inhibitors, NO-nitro-L-arginine methylester and NO-nitro-L-arginine, were given 10–20 min before the agonist. The mice were killed by microwave radiation focused to the head. Cyclic GMP was measured by radioimmunoassay in deproteinized extracts from different brain areas.

**Results:** Clonidine and dexmedetomidine, at sedative doses, dose-dependently decreased cerebellar cGMP (ED₅₀: 100 and 50 μg/kg for clonidine and dexmedetomidine, respectively). This effect was inhibited by yohimbine and atipamezole, but not by prazosin, confirming the alpha-2 nature of the response to the agonists. L-metedomidone, which has no sedative/hypnotic effect, did not decrease cGMP. Pretreatment of the mice with a maximum dose of 100 mg/kg of a nitric oxide synthase antagonist abolished the cGMP response to the agonists. Similar results were obtained in the cerebral cortex, hippocampus and caudate nucleus.

**Conclusions:** The results suggest that the nitric oxide–cGMP pathway is an effectors system coupled to the alpha-2 adrenoceptor mediating sedation. (Key words: Animals: mouse; Brain: caudate nucleus; cerebellum; cerebral cortex; hippocampus. Pharmacology: Alpha-2 adrenoceptor agonists, clonidine; dexmedetomidine. Nitric oxide synthase antagonists: NO-nitro-L-arginine methylester; NO-nitro-L-arginine. Neuromodulator: nitric oxide. Intracellular mediators: cGMP.)

ALPHA-2 adrenoceptor agonists have potent analgesic, sedative/hypnotic, anxiolytic properties in humans and in experimental animals. They also were shown to decrease anesthetic requirements and produce perioperative hemodynamic stability.1 In recent studies, it was suggested that they may protect from cerebral ischemic injury.2 Because of these properties, these compounds may prove to be clinically useful. However, the cellular mechanism(s) by which they produce their effects remains to be elucidated.

Alpha-2 adrenoceptors are found ubiquitously in the central nervous system and have been identified pre- and post-synaptically.3–6 Presynaptically, alpha-2 adrenoceptor agonists are known to suppress the release of norepinephrine and other neurotransmitters.7 The sympatholytic activity accounts, in part, for the hemodynamic stability and the decrease in anesthetic requirement.6,8 The function of the postsynaptic alpha-2 adrenoceptor is not well understood. The alpha-2 adrenoceptor has been negatively coupled to adenylate cyclase, to inhibit conductance of voltage-dependent calcium channels, and to activate potassium channels and the Na⁺/H⁺ antiporter, all functions associated with depressed neuronal excitability.9 In our earlier studies, we showed that the alpha-2 adrenoceptor is also linked to the cGMP pathway in the heart.10

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It is well documented that in the central nervous system, cGMP concentrations are increased by neurotransmitters, drugs, or conditions that excite and are decreased by those that sedate. We reported that volatile anesthetics, at anesthetic concentrations, decrease cerebellar cGMP, a change that correlates with the loss of righting reflex and their aniconvulsant effect. In addition, glutamate, the major central nervous system excitatory transmitter, increases cGMP, an effect recently shown to be mediated by nitric oxide, an important neuromodulator widely distributed throughout the brain. There is also accumulating evidence that anesthetic agents interact with the nitric oxide–cGMP pathway, and it was reported that nitric oxide synthase antagonists prevent the alpha-2 adrenoceptor-mediated analgesic effect of clonidine. Therefore, in the present study, we tested the hypothesis that the alpha-2 adrenoceptor pathway is associated with the nitric oxide–cGMP system in the brain. Clonidine and dexmedetomidine were used as representative alpha-2 adrenoceptor agonists. Dexmedetomidine is a new, potent, highly selective agonist being tested in clinical trials for use in anesthesia.

Materials and Methods

The protocol for the experiments was approved by the Animal Care and Use Committee at this institution (167). Male Swiss-Webster mice, weighing 20–25 g, were allowed free access to Purina chow and water until 2 h before the experiment. The mice were housed in cages kept at 22°C and maintained at a 12 h light–dark cycle. All experiments were performed between 9 am and 5 pm. On the day of the experiment, mice were divided into groups of 4 to 6. All the experimental drugs or saline in control mice were given intraperitoneally in a volume of 0.1 ml/10 g body weight. To determine the dose-dependency and stereospecificity of the effect of alpha-2 adrenergic agonists on cGMP concentration, each mouse received one dose of 10, 30, 100, or 300 μg/kg dexmedetomidine; 30, 100, or 600 μg/kg clonidine; or 100 or 300 μg/kg l-norepinephrine. Pilot experiments determined that the optimal time of exposure to the drugs was 15 min for clonidine and 25 min for dexmedetomidine. The mice were killed by microwave radiation focused to the head for 2.5 s, a treatment that stops all enzymatic activity. The cerebellum and, in selected experiments, the cerebral cortex, hippocampus and caudate nucleus, were immediately dissected out and stored at −60°C until processed for cGMP determination. To establish the involvement of alpha-2 adrenoceptors in the effect of dexmedetomidine and clonidine on cGMP, mice were pretreated with either of two selective alpha-2 adrenergic antagonists—0.3, 1, 2, or 5 mg/kg yohimbine; or 1 mg/kg atipamezole—10–20 min before 100 μg/kg dexmedetomidine or 150 μg/kg clonidine. To determine whether the effect of dexmedetomidine on cGMP concentration is associated with the nitric oxide pathway, mice were pretreated with a nitric oxide synthase antagonist, 10–30 mg/kg Nω-nitro-l-arginine methyl ester (l-NAME) or Nω-nitro-l-arginine (l-NA), 20 min before 100 μg/kg dexmedetomidine or 150 μg/kg clonidine. The control groups received vehicle and/or the antagonist.

Cyclic GMP Determination

The tissue samples were homogenized in 1- to 2-ml bidistilled water. The homogenates were heated in boiling water for 3 min and centrifuged. Cyclic GMP was measured in aliquots of the supernatant by radioimmunoassay after acetylation of the nucleotide. The pellet was used for protein determination with the method of Bradford. Reagent blanks and appropriate standards were run in parallel with each set of samples. Determinations were made in triplicate, and the results are expressed in pmol/mg protein.

The data are reported as mean ± SEM. Statistical analysis was performed on absolute values. One way analysis of variance was used to compare the responses to different doses or treatments. Significant differences between groups were determined by Student's t test for unpaired samples. Bonferroni correction was used to control for multiple comparisons. Statistical significance was set at P < 0.05.

Materials

Dexmedetomidine HCl and atipamezole HCl were a gift from Farmos-Group, Ltd. (Turku, Finland). Clonidine HCl, yohimbine HCl, prazosin HCl, l-NAME, and l-NA were purchased from Sigma Chemicals (St. Louis, MO), and the cGMP radioimmunoassay kit from Biomedical Technologies (Stoughton, MA). All other compounds were reagent grade.

Results

Both clonidine and dexmedetomidine produced a dose-dependent decrease in cerebellar cGMP content, dexmedetomidine being approximately twice as po-
tent as clonidine (ED$_{50}$, 50 and 100 µg/kg for dexamethasomidine and clonidine, or 211 and 577 µmol/kg, respectively) (fig. 1).

The decrease in cerebellar cGMP induced by clonidine and dexamethasomidine was dose-dependently reversed by the selective alpha-2 adrenergic antagonists yohimbine and atipamezole, but was not affected by the alpha-1 antagonist prazosin (figs. 2A and B). Conversely, the levoisomer of metedetomidine, which also binds to alpha-2 adrenoceptors, though with lower affinity and less selectively than dexamethasomidine, but has no hypnotic effect, increased cGMP content at a dose of 100 or 300 µg/kg, doses at which dexamethasomidine produced more than a 70% decrease in cGMP (fig. 3).

Dexamethasomidine and clonidine also decreased cGMP content in the cerebral cortex, the hippocampus and the caudate nucleus. In agreement with published data, the cGMP content in these brain areas is lower than in the cerebellum. Figure 4 shows that, in these tissues, 100 µg/kg dexamethasomidine decreased cGMP levels by 90%, 58%, and 45%, respectively.

L-NAME and L-NA (10-500 mg/kg) decreased cGMP in a dose-dependent manner, to a maximum of approximately 10% of control (data at submaximum doses not shown). Pretreatment of the mice with a maximum dose of 100 mg/kg L-NAME prevented the effect of dexamethasomidine and clonidine (fig 5). Similar results were obtained with L-NA (not shown).

Discussion

Cyclic GMP is found throughout the brain, but the content of cGMP in cerebellar tissue is 5-10 times greater than in other brain areas. Cerebellum contains alpha-2 adrenoceptors and has high nitric oxide synthase and guanylate cyclase activity, and not a site associated with the cerebellum in control of muscle coordination and local activity has been performed in laboratory animals. A model of choice test has been developed based on the requirement for the cerebellum and cerebellar cGMP production and adrenoceptors activated by the levoisomer of metedetomidine, demonstrating a disproportionately twofold increase in cGMP. This suggests that the adrenoceptor affinity of dexamethasomidine is more than twice that of clonidine.

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guanylate cyclase activity.\textsuperscript{3,15} Although the cerebellum is not a site associated with the anesthetic state per se, anesthetics have a significant effect on the cerebellar control of muscle coordination and motor activity.\textsuperscript{12,28} Motor activity has been used as an index of anesthetic action in laboratory animals. Therefore, the cerebellum was a model of choice to test our hypothesis.

Based on the results of this study, dexmedetomidine and clonidine produce a dose-dependent decrease in cerebellar cGMP, an effect mediated by alpha-2 adrenoceptors activated by the physiologically active dextroisomer of medetomidine.\textsuperscript{6,28} Dexmedetomidine is approximately twice as effective as clonidine at decreasing cGMP. This reflects, at least in part, the higher affinity of dexmedetomidine for alpha-2 adrenoceptors. Binding studies have shown that dexmedetomidine has more than twice the affinity of clonidine for the alpha-2 adrenoceptor.\textsuperscript{21} In addition, clonidine and dexmedetomidine are also agonists at the alpha-1 adrenoceptor, dexmedetomidine showing a much greater selectivity than clonidine for alpha-2 versus alpha-1 adrenoceptors. In binding studies, the relative alpha-2/alpha-1 activity ratio was 1.620 for dexmedetomidine and 220 for clonidine.\textsuperscript{21} Because, in neuronal systems, activation of alpha-1 adrenoceptors is associated with an increase in cGMP,\textsuperscript{30} the lower efficacy of clonidine to decrease cGMP could also be related to the stronger opposing alpha-1 action. This does not appear to be the case at the lower concentrations, because the alpha-1 adrenergic antagonist prazosin did not enhance the effect of 100 \textmu M clonidine. Another factor accounting for the lower efficacy of clonidine may relate to its partial agonist properties.\textsuperscript{31} In contrast, within the same range of concentrations, the levo derivative of medetomidine...
increased cGMP. The levo derivative, like dexmedetomidine, has affinity, though lower, for alpha-2 and alpha-1 adrenoceptors. It has been shown, however, to be functionally ineffective at the alpha-2 adrenoceptor, but active at the alpha-1 adrenoceptor.²⁷⁻²⁸ This suggests that the increase in cGMP may reflect its alpha-1 agonistic affinity unopposed by the more potent alpha-2 activity. Other factors may be involved, because the concentrations that increase cGMP are at the lower end of the alpha-1 selective range (Ki ~ 3 μM). Studies were not further pursued, because l-metodetomidine has no sedative/hypnotic properties.⁵⁻²⁸

A major pathway of cGMP formation is activation of guanylate cyclase by nitric oxide.⁴⁻³伍 When the mice were pretreated with nitric oxide synthase inhibitors, the cerebellar cGMP concentration decreased to approximately 10% of its basal value, indicating that, in this tissue, cGMP is mainly regulated by nitric oxide. Because the cGMP response to dexmedetomidine is eliminated when nitric oxide synthase is inhibited, these data suggest that the alpha-2 adrenoceptors are linked to the nitric oxide-cGMP pathway. In the central nervous system, nitric oxide acts as an intracellular messenger of hormone action and as an excitatory neurotransmitter released from nitricergic nerve endings.¹⁴ Therefore, there are different ways by which activation of alpha-2 adrenoceptors may modulate the nitric oxide-cGMP pathway: inhibition of guanylate cyclase or of nitric oxide synthase activity, inhibition of the release of neurotransmitters that stimulate nitric oxide synthase or of the release of nitric oxide itself. It is well documented that alpha-2 adrenergic agonists inhibit autonomic neurotransmission by preventing the release of neurotransmitter.⁵ Recent evidence indicates that they also inhibit the release of excitatory neurotransmitter from nonadrenergic noncholinergic nerves (e.g., glutamate).³⁶ Whether the nitricergic nerve endings have alpha-2 adrenoceptors that modulate the release of nitric oxide is not known at this time. Inhibition of glutamate release is an attractive proposition, because the basal level of cGMP in the brain is regulated, in great part, by activation of NMDA receptors by glutamate released by spontaneous synaptic activity.³⁶ Inhibition of guanylate cyclase is unlikely, because, except for the particulate guanylate cyclase that is an integral domain of the atrial natriuretic peptide receptor, it has never been possible to demonstrate direct regulation of the enzyme by a hormone or neurotransmitter.⁴³

Correa-Sales and colleagues⁶⁵ and Nacler-Cloelho et al.⁶⁶ identified several putative effector pathways in the transduction of the hypnotic effect of alpha-2 agonists: inhibition of adenylate cyclase, hyperpolarization through an increase in potassium conductance, and a decreased calcium conductance of L-type calcium channel. In our earlier studies, a good correlation was found between the loss of righting reflex and the decrease in cerebellar cGMP induced by volatile anesthetics.¹²⁻¹³ Even though no attempt was made in the current study to correlate the changes in cGMP with the sedative effects of dexmedetomidine and clonidine, the data suggest that the nitric oxide-cGMP pathway is another effector coupled to the alpha-2 adrenoceptor that may mediate their action. The behavioral responses to dexmedetomidine and clonidine that were observed during the course of the study corroborate those reported in the literature.⁵⁷⁻⁵⁸ The decrease in cerebellar cGMP induced by dexmedetomidine and clonidine occurs within the range of concentrations that produce sedation and hypnosis and reduce the minimum alveolar concentration of volatile anesthetics. At these concentrations (<1 mg/kg, intraperitoneally), the behavioral and biochemical responses are antagonized by an alpha-2 adrenergic antagonist, but are not influenced by an alpha-1 antagonist. L-metodetomidine has no hypnotic action and does not decrease cGMP levels.²⁸⁻⁵⁷

Clonidine and dexmedetomidine, within the range of concentrations that decrease cGMP in the cerebellum, also decrease cGMP in the cerebral cortex, hippocampus and caudate nucleus, areas of the brain endowed
with alpha-2 adrenoceptors and elements of the nitric oxide-cGMP system. Because anesthetics agents have been shown in vitro and in vitro to alter cellular functions in these brain areas, these results give further support to the proposal that the nitric oxide-cGMP pathway is involved in the sedative/hypnotic action mediated by alpha-2 adrenoceptors. Correa-Sales and collaborators provided evidence that identified the locus coeruleus as a site for the hypnotic action of dexmedetomidine. They demonstrated that direct activation of alpha-2 adrenoceptors in the locus coeruleus, which suppresses the spontaneous firing of the locus coeruleus noradrenergic efferent neurons, is associated with a loss of righting reflex. The locus coeruleus is a small, distinct cluster of neurons, situated in the upper brain stem under the floor of the fourth ventricle, that plays a role in the regulation of a variety of physiologic functions, including sleep and wakefulness. The locus coeruleus is an extremely complex structure that contains a wide variety of neurochemicals. The majority of the neurons are noradrenergic, and were shown to express mRNA for α-2 adrenoceptors. Neurotransmitters or elements of pathways known to regulate cGMP (e.g., glutamate, nitric oxide) were identified, suggesting that the alpha-2 adrenoceptor also may be associated with the nitric oxide-cGMP pathway in the locus coeruleus. Based on the results of another study, injection of a cGMP derivative or of a nitric oxide donor directly into the locus coeruleus causes excitation of locus coeruleus neurons. Whether alpha-2 agonists also decrease cGMP in the locus coeruleus remains to be shown.

The locus coeruleus sends noradrenergic projections throughout the brain and exerts a modulatory role on neuronal responsiveness to afferent synaptic inputs. Therefore, it is possible that, in addition to a direct activation of alpha-2 adrenoceptors in the cerebellum, cerebral cortex, hippocampus or caudate nucleus, the alpha-2 adrenoceptor-mediated inhibition of the firing of locus coeruleus excitatory noradrenergic efferents is responsible, in part, for the decrease in cGMP observed in other brain areas. It is well documented that the cerebellar cortex receives signals from the periphery and from other brain areas, including the locus coeruleus, and that the activity of the cerebellar Purkinje cells, which provide the major output to the motor pathways, is determined by the intensity of excitatory and inhibitory input converging on these cells. The cerebellar cortex cGMP content, which represents mainly the cGMP of the Purkinje cells, changes with the Purkinje cell activity: an increase in Purkinje cell activity is accompanied by an increase in cGMP level and a decrease in Purkinje cell activity with a decrease. Therefore, inhibition of the noradrenergic input from the locus coeruleus to the cerebellum may modulate cerebellar cGMP concentrations, Purkinje cell activity, and motor activity.

In summary, the results of this study provide evidence that alpha-2 adrenoceptors are coupled to the nitric oxide-cGMP effector pathway in the mouse brain.

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