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Dissociation of Hypnotic-Anesthetic Actions of α_2 Agonists From Cyclic AMP in the Rat

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RABIN, B. C., T.-Z. GUO AND M. MAZE. *Dissociation of hypnotic-anesthetic actions of α_2 agonists from cyclic AMP in the rat.* PHARMACOL BIOCHEM BEHAV **57**(1/2) 23–29, 1997.—α_2 adrenergic agonists are used clinically for their anesthetic, analgesic, and sympatholytic actions in surgical patients. All α_2 adrenergic receptors, when activated by α_2-adrenergic agonists, are able to inhibit adenylate cyclase. We have examined the α_2-adrenoceptor-mediated anesthetic actions of dexmedetomidine, a highly selective α_2-adrenergic agonist, after pretreatment of the animals with rolipram, a cyclic AMP (cAMP)–specific phosphodiesterase inhibitor. cAMP accumulation and monoamine turnover were measured in the locus coeruleus (LC) and hippocampus (HC) following administration of rolipram [275 mg/kg, intraperitoneally (IP)] and dexmedetomidine (100–500 mg/kg, IP). The hypnotic response to dexmedetomidine was also measured in these animals. In other experiments, rats were stereotactically cannulated in the LC with an indwelling catheter, and after the second day, the tail-flick analgesic response to dexmedetomidine (3.5 mg/0.2 ml LC), following rolipram (275 mg/kg, IP) pretreatment, was assessed. In the presence of elevated cAMP levels, the hypnotic, analgesic, and sympatholytic effects of dexmedetomidine persisted. These data suggest that adenylate cyclase activity does not mediate the cellular responses to α_2-adrenergic agonists but instead may act in concert with other α_2-adrenoceptor–coupled effector mechanisms to transduce the anesthetic actions of these agents. © 1997 Elsevier Science Inc.

Sympathetic nervous system α_2 agonists   Dexmedetomidine   Brain   Locus coeruleus   Hippocampus   Cyclic AMP   Phosphodiesterase inhibitor   Rolipram

THE α_2-adrenergic agonists are being used perioperatively for their hypnotic, analgesic, and sympatholytic effects. To more precisely define the receptor-effector mechanism for these behavioral responses, we have sought a discrete site in the CNS at which these actions are transduced. Recently we have shown that the locus coeruleus (LC) is a crucial site for hypnotic (9), analgesic (15), and sympatholytic (33) effects of dexmedetomidine, a highly selective α_2-adrenergic agonists (35).

All α_2-adrenergic receptors, when activated, are able to inhibit adenylate cyclase (25). The resulting decrease in the accumulation of cyclic AMP (cAMP) reduces the stimulation of cAMP-dependent protein kinase and hence the phosphorylation of target regulatory proteins (40). Given the consistent temporal link between inhibition of adenylate cyclase and occupation of α_2 adrenoceptors, cAMP became the focus of initial studies of the ionic and second-messenger mechanisms underlying biologic responses elicited by α_2 agonists. In some paradigms inhibition of adenylate cyclase is pivotal to the cellular responses of α_2 agonists (2,7). In rat brain slices administration of dibutryl cAMP, a cAMP analog, reversed the hyperpolarization induced by clonidine (2).

In many cases, however, a decrease in cAMP production is insufficient to mediate α_2-adrenoceptor–induced physiologic effects. In both nonneuronal (28,44) and neuronal systems there is ample evidence of dissociation between the α_2-adrenoceptor responses and inhibition of adenylate cyclase. In neuronal systems, Holz et al. (19) reported that the ability of epinephrine to suppress voltage-dependent calcium conductance in chick dorsal root ganglion cells persisted even when cAMP levels were sustained at or above normal levels. Uhlen et al. (42) reported that the antinociceptive response to intratracheal injections of an α_2 agonist was unaffected when adenylate cyclase activity was maintained by forskolin stimulation. Previous studies have also demonstrated alternative pathways, independent of second messengers such as cAMP, through which α_2 adrenoceptors might signal their actions. These include membrane-delimited guanine nucleotide-binding proteins (G proteins) coupled directly to calcium (19,26) and potassium (4) channels.

Our expanding knowledge of signal transduction components and the potential for their selective activation via synthetic ligands or other pharmacologic agents, has made it increasingly important to characterize the functional significance of such components. The role of cAMP production in biologic

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responses elicited by α_2-adrenergic agonists can be tested by artificially maintaining this cyclic nucleotide at basal levels with rolipram, a selective inhibitor of the cAMP-specific phosphodiesterase (36). In previous studies, elevation of cAMP levels altered the hypnotic response to low doses of dexmedetomidine (8). The present study aimed to further delineate the role of cAMP levels by distinguishing between causal and coincidental relationships to the neurochemical, hypnotic, and analgesic effects of dexmedetomidine.

METHODS

Male Sprague–Dawley rats (250–300 g) were chosen as the experimental model following approval of the experimental protocol by the Animal Care and Use Committee at the Palo Alto Veterans Affairs Medical Center. Rat littermates were stratified to match weight distribution in the control and treated groups as closely as possible. All behavioral tests and animal sacrifices were performed between 0900 and 1300 h.

Animal Preparation

In subjects receiving LC injections, the LC was stereotactically cannulated with a 24-ga stainless-steel cannula according to the following coordinates: the bregma as the reference, 1.2 mm lateral, 9.7 mm posterior, and a depth of 6 mm from the skull (32). The surgical procedure was performed with the rat under halothane anesthesia, and the cannula was fixed in position with methylmethacrylate resin. Correct placement of the cannula at the superior border of the LC was confirmed histologically at the conclusion of the experiments. After a recovery period of 2–3 days, a 30-ga stainless-steel needle connected to polyethylene tubing was inserted through the cannula and positioned 1 mm beyond the tip. This served as the conduit for drug delivery.

Behavioral Measurement

In this study sleep was defined as the loss of righting reflex (LORR). LORR occurs when the animal is unable to return itself to a prone position when placed in a supine position. The following outcome measures related to LORR were assessed: latency to LORR and percentage of animals with LORR.

The analgesic response was measured by the tail-flick latency response as previously described (16). A high-intensity light was focused on the rat's tail and the time required for the rat to move its tail out of the light beam was automatically recorded (tail-flick aparatus; Columbus Instruments, Columbus, OH) and referred to as tail-flick latency. A different patch of the tail was exposed to the beam on each trial to minimize the risk of tissue damage. The animals were placed on the heating blanket to maintain the body and tail temperature during the experiment. A cutoff time of 10 s was chosen as the point at which the trial was terminated if no response occurred. data are expressed as maximum possible effect (MPE) according to the following formula:

$$\text{MPE (\%)} = \frac{\text{(Postdrug latency)} - \text{(Basal latency)}}{\text{(Cutoff latency)} - \text{(Basal latency)}} \times 100$$

Dexmedetomidine was used in the analgesic paradigm at a dose of 3.5 μg/0.2 ml (a 50% MPE dose) LC, and the tail-flick test was performed 5 min after dexmedetomidine.

cAMP Measurement

Animals were decapitated following 30 s exposure to CO_2, 30 min after an acute IP injection of dexmedetomidine (100–500 μg/kg) or saline. The tissues were prepared according to Gilman (14). The LC was removed from each side of the freshly harvested brain using the punch technique. Punches from 2-mm brain slices at the dorsal-ventral location of the LC were obtained. Brains were sliced fresh and the LCs removed over an ice-cold glass plate using an 0.8-mm-bore glass pipette. Two LC punches per sample (i.e., from both sides of one or two rats) were sonicated in 0.3 ml of ice-cold 5% trichloracetic acid. The disrupted tissue was centrifuged at $12{,}000 \times g$ at 4°C × 20 min. The supernatant was transferred to tubes containing 25 μl of 1 M HCl and extracted with ether, 0.75 μl × 3. The ether phase was discarded and the aqueous solution was evaporated under a stream of N_2 at 70°C. The extract was stored at 20°C overnight. On the following day, the extract was dissolved in assay buffer, provided by the cAMP Biotrak Enzyme Immunoassay System (Amersham, Buckinghamshire, UK), which contained 50 mM acetate buffer, pH 5.8, 0.02% bovine serum albumin, and 0.005% thimerosal. Briefly, the method is based on a competition between cAMP in the sample and a constant amount of peroxidase-labelled cAMP for a fixed number of binding sites on a cAMP-specific antibody. The bound antibody–peroxidase–cAMP complex is then firmly attached to a second antibody, which is precoated in the wells of a microtiter plate. All of the nonbound cAMP is washed away. The amount of peroxidase-labelled cAMP bound to the antibody is determined by adding a substrate, tetramethylbenzidine/hydrogen peroxide single-pot substrate (TMB), and stopping the substrate–peroxidase reaction by adding H_2SO_4. The resulting color change is read in a microtiterplate spectrophotometer at 450 nm. With increasing cAMP levels present in the unknown samples, there is less peroxidase-labelled cAMP bound to the antibody. The assay was linear between 100 and 3200 fmol cAMP/well. The cAMP levels were correlated to the amount of protein (pmol cAMP/mg per protein) using the Lowry method (27).

Monamine Measurement

To measure monoamine turnover, animals were injected with vehicle or dexmedetomidine and decapited following narcosis induced by a 30-s period of exposure to CO_2. The LC was removed from each side and the hippocampus (HC) from the left side of the freshly harvested brain. Samples were sonicated in an ice-cold 5% perchloric acid (PCA) solution and centrifuged to precipitate proteins and membranes. The supernatant was filtered to exclude molecules exceeding 5000 Da. These samples were stable for up to 3 mo when stored at −80°C.

Biogenic amines were measured by high-performance liquid chromatography and reverse-phase chromatography on a HR-80 column (70 mm) containing 3 mm spherical octadecylsilane beads. The electroactive biogenic amines and their metabolites were quantified by electrochemical detection (Coulochem II; ESA Chelmsford, MA). The limit of detection with this technique is 25 fmol.

The ratio of the concentration of the major metabolite of a brain monoamine neurotransmitter to brain monoamine concentration itself is used as an index of the overall turnover rate of the synaptic transmitter. Underlying this ratio is the understanding that high concentrations of the monoamine neurotransmitter such as norepinephrine (NE) will reduce neuronal activity by acting as an autoregulator at presynaptic α_2 adrenoceptors. Conversely, increased concentrations of a neuronal metabolite such as 3-methoxy-4-hydroxy phenylglycol (MHPG) are associated with increased neuronal firing.

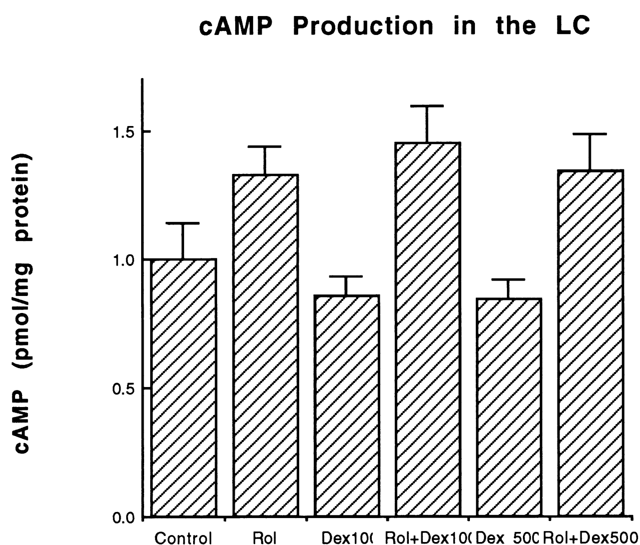

FIG. 1. Effect of rolipram and dexmedetomidine on cAMP production in the locus coeruleus. Rats were pretreated with 10% cremophor or rolipram (275 μg/kg), IP, 15 min before saline or dexmedetomidine (100 or 500 μg/kg). The locus coeruleus was harvested 30 min later. Data are expressed as a percentage of cAMP accumulation produced by 10% cremophor plus saline (control). Data are means ± SEM (n = 6–7/group).

This assumes that the formation and elimination of the monoamines and their metabolites are proportional to their concentrations (39).

Drug Administrations

Dexmedetomidine was injected into the LC by a pump (Harvard Apparatus no. 22; Cambridge, MA) at a rate of 0.4 μl/min and in a volume of 0.2 μl. In other animals, dexmedetomidine was administered IP in a volume of 1 ml/kg.

Rolipram was administered IP in a volume of 1 ml/kg.

Data Analysis

Data are expressed as mean ± SEM. The results of multiple groups were analyzed by analysis of variance followed by posthoc Scheffé's test. The comparison between two groups was performed by t-test for unpaired data. A p value < 0.05 was considered statistically significant.

RESULTS

Rolipram and cAMP Production

Rolipram (275 μg/kg), given as a pretreatment to dexmedetomidine (100 and 500 μg/kg), maintained cAMP production in the LC above basal levels (Fig. 1).

Rolipram and Dexmedetomidine Induced Decreases in Monoaminergic Neurotransmission

A significant decrease in NE turnover elicited by dexmedetomidine (100 and 500 μg/kg) in both the HC (Fig. 2A) and LC (Fig. 2B) is unaltered by rolipram (275 μg/kg). This dose of rolipram alone did not affect NE turnover.

A significant decrease in 5-hydroxytryptamine (5-HT) turnover elicited by dexmedetomidine (100 and 500 μg/kg) in the HC (Fig. 3) is unaltered by rolipram (275 μg/kg). This dose of rolipram alone did not affect 5-HT turnover.

Rolipram and the Hypnotic Response to Dexmedetomidine

Rolipram produces stereotypic behavior in rats as a result of inhibition of cAMP-specific phosphodiesterase, which results in an increase in the intracellular content of cAMP (45). This stereotypic behavior did not confound the assessment of LORR, since there was no associated sedation or hypnosis. The hypnotic response to dexmedetomidine (100 and 500 μg/kg) as measured by latency (Fig. 4) and percent LORR is not changed by rolipram (275 μg/kg). All rats treated with dexmedetomidine, including rats pretreated with rolipram, demonstrated LORR.

Rolipram and the Analgesic Response to Dexmedetomidine

The tail-flick analgesic response to dexmedetomidine (3.5 μg/2 ml) LC (Fig. 5) is not affected by rolipram (275 μg/kg). This dose of rolipram alone does not alter the basal analgesic response.

DISCUSSION

Rolipram, a cAMP-specific phosphodiesterase inhibitor, functionally sustains intracellular cAMP content above basal levels in the presence of dexmedetomidine. Despite the uncoupling of dexmedetomidine from its ability to reduce cAMP production, the drug is still able to elicit its neurochemical effects, decreases in monoaminergic neurotransmission, and concomitant behavioral responses—namely, hypnosis and tail-flick analgesia. These findings suggest that these α_2-induced neurochemical and behavioral effects are not mediated by adenylate cyclase inhibition in the LC.

Numerous previous findings show that central α_2-adrenergic receptors are coupled in an inhibitory fashion to adenylate cyclase. Uhlen et al. (42,43) showed that both UK-14,304 and guanfacine, selective α_2-adrenergic agonists, are capable of decreasing forskolin-stimulated cAMP production in rat and guinea pig spinal cords in vitro. In addition, α_2-adrenergic receptor-elicited decreases in cAMP production have been reported in isolated slices (12,24) and particulate membrane fractions (23,30) from the rat brain cortex.

Rolipram is known selectively to inhibit a cAMP-specific phosphodiesterase isozyme (3), resulting in increased brain cAMP levels (36). In animals treated with both rolipram and dexmedetomidine, cAMP production was clearly maintained above basal levels. Intracellular cAMP measurements in the LC following systemic administration of dexmedetomidine, however, failed to show a significant decrease in cAMP production. These data were clarified by a subsequent study showing dexmedetomidine's ability to reduce LC intracellular cAMP content in the presence of forskolin, an adenylate cyclase stimulator (34). Our data concur with past findings that UK-14,304, an α_2 agonist, dose dependently inhibited forskolin-stimulated cAMP accumulation but had no effect on basal cAMP accumulation (38). Moreover, these results parallel our understanding of signal transduction cascades: Nonstochiometric changes may be seen at downstream levels in the postreceptor signal transduction cascade, resulting in the amplification of signals of upstream messengers such as cAMP (40).

In addition to cAMP levels, monoamines were also measured in the LC, the central relay station for noradrenergic pathways in the mammalian CNS and a discrete area with high-density α_2 adrenoceptors, and the HC. The LC is a major

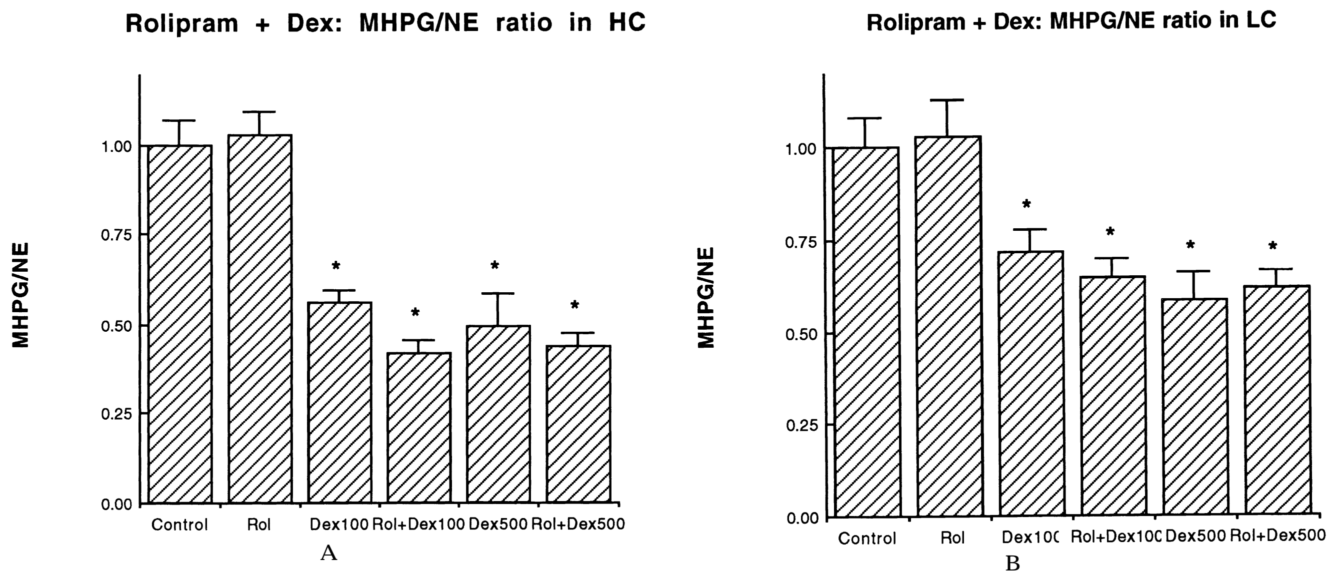

FIG. 2. Effect of rolipram on dexmedetomidine-elicited decrease in norepinephrine turnover in the hippocampus (A) and locus coeruleus (B). Rats were pretreated with 10% cremophor or rolipram (275 μg/kg), IP, 15 min before saline or dexmedetomidine (100 or 500 μg/kg). The hippocampus and locus coeruleus were harvested 30 min later. Catecholamines were measured by high-performance liquid chromatography with electrochemical detection. Data are expressed as a percentage of MHPG/NE produced by 10% cremophor plus saline (control). Data are means ± SEM (*n* = 6–7/group). *Significantly different from control by posthoc Scheffé's test.

site for the hypnotic (9,11) and antinociceptive (15) actions of dexmedetomidine. Both intraperitoneal administration of piperoxan, an α_2 antagonist, and direct electric stimulation to the LC increased MHPG concentrations in three areas whose noradrenaline (NA) innervation arises solely from the LC (i.e., cerebellum, HC, and cortex) (10,21,41). Thus, neurochemical analysis of NE turnover in one region innervated by the LC may be sufficient to accurately reflect the LC response at multiple terminal regions. Monoaminergic neurotransmission was also measured in the HC, which derives its norepinephrine input exclusively from the locus coeruleus (10,21), and serotonin input mainly from the dorsal raphe (18).

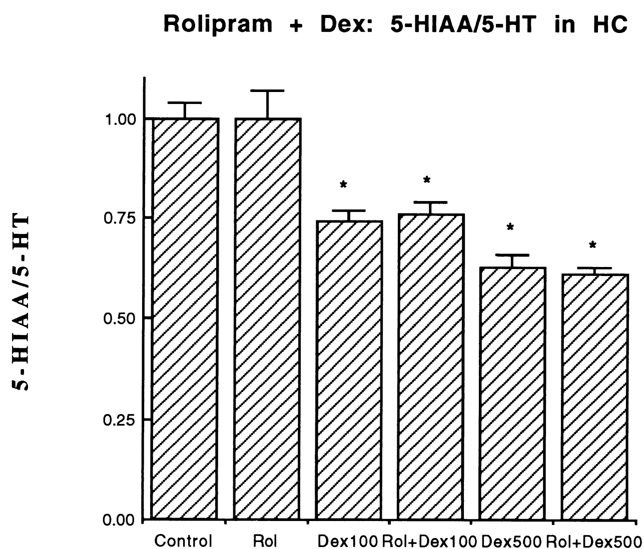

FIG. 3. Effect of rolipram on dexmedetomidine-elicited decrease in serotonin turnover in the hippocampus. Rats were pretreated with rolipram (275 μg/kg), IP, 15 min before dexmedetomidine (100 or 500 μg/kg). The hippocampus was harvested 30 min later. Indolamines were measured by high-performance liquid chromatography with electrochemical detection. Data are expressed as a percentage of 5-HIAA/5-HT produced by 10% cremophor plus saline (control). Data are means ± SEM (*n* = 7/group). *Significantly different from control by posthoc Scheffé's test.

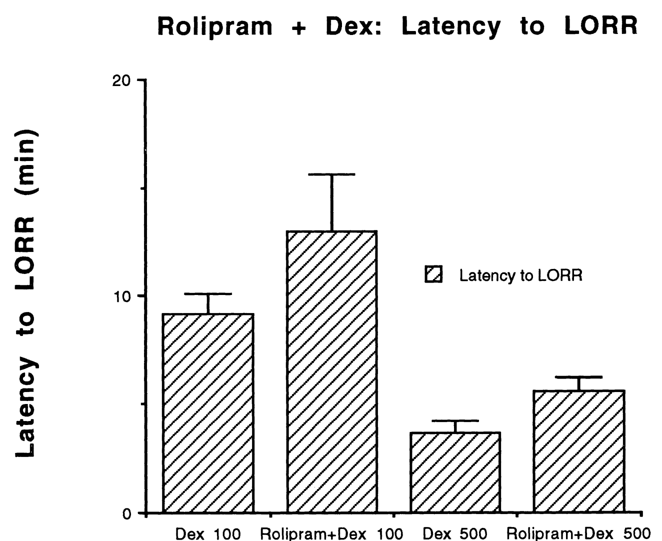

FIG. 4. Effect of rolipram on the hypnotic action of dexmedetomidine as measured by latency to sleep. Rats were treated with 10% cremophor or rolipram (275 μg/kg), IP, and 15 min later saline or dexmedetomidine (100 or 500 μg/kg). The latency to LORR (sleeptime) was determined and expressed as means ± SEM (*n* = 7/group).

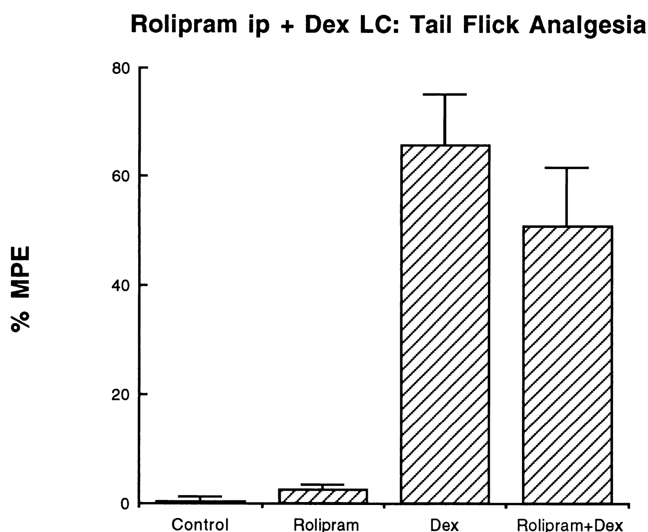

FIG. 5. Effect of rolipram on dexmedetomidine-induced tail-flick analgesia. Rats were treated with 10% cremophor or rolipram (275 μg/kg), IP, and 15 min later with saline or dexmedetomidine (3.5 μg/2 ml) LC. Data are means ± SEM (n = 8/group) for two groups receving saline and (n = 12/group) for two groups receiving dexmedetomidine.

Our method of assessing monoaminergic neurotransmission assumes that it is proportional to the turnover of the parent neurotransmitter (i.e., NE or 5-HT) and that the system is at steady-state conditions with no interference with either synthesis or reuptake of the parent neurotransmitters and their major metabolites [MHPG and 5-hydroxyindole acetic acid (5-HIAA)]. Previous studies have shown this to be the case (39).

While there is a clear link between α_2-adrenergic receptor stimulation and decreases in cAMP production, these biochemical changes are not sufficient to account for behavioral and neurochemical responses elicited by α_2 agonists. If attenuation of cAMP production is causally linked to biologic responses induced by α_2 adrenoceptors, then it follows that preventing a decrease in cAMP accumulation would blunt these responses. However, in the presence of elevated cAMP levels, following pretreatment with rolipram, dexmedetomidine's hypnotic, analgesic, and monoaminergic effects persisted. Also, the increases in cAMP levels induced by rolipram alone did not exert any independent nociceptive, hypnotic, or neurochemical effects. Rolipram is capable of elevating presynaptic norepinephrine content and release via stimulation tyrosine hydroxylase activity (22), but this effect is not apparent at the dose used in this study.

In previous experiments in this laboratory, rolipram altered the hypnotic response elicited by lower doses of dexmedetomidine (8). The present data elucidate these previous findings by delineating the dose range in which adenylate cyclase activity plays a modulatory role and demonstrating clearly that it is not the predominant effector mechanism for the transduction of certain biologic responses to α_2 agonists. It is possible that at the higher doses of dexmedetomidine used in this study, other non–cAMP-dependent transduction pathways are recruited.

These findings are well supported in the literature. Analogous to antinociceptive α_2-adrenoceptor pathways (42,43), the sympatholytic response to α_2-adrenoceptor stimulation appears to be unlinked to attenuations in cAMP accumulation. α_2-adrenergic receptors inhibit NA release in rat occipital cortex by a mechanism that does not involve decreasing cAMP levels (31). Similarly, in cultured rat superior cervical ganglion cells, UK-14,304 maintained its ability to reduce the release of tritiated norepinephrine in the presence of forskolin (38). Buchner et al. (6) showed that in vascular sympathetic nerve terminals adenylate cyclase inhibition played a minimal role in the negative feedback control by prejunctional α_2 adrenoceptors of NE release. B-Ht 933, an α_2-adrenoceptor agonist, elicited inhibition of NE release; was slightly reduced in the presence of 8-br-cAMP, a cAMP analogue; and was unaffected in the presence of forskolin. Finally, studies with chick sympathetic neurons suggest the postreceptor transduction mechanism of α_2-adrenoceptor–induced sympatholysis involves voltage-activated Ca^{2+} channels but not cAMP (5).

α_2-adrenergic agonists couple to a variety of signal transduction pathways not mediated by adenylate cyclase with diverse physiologic consequences. α_2-adrenoceptor activation can result in the alkalinization of the interior of certain cells by enhanced Na^+/H^+ exchange (20), which may in turn stimulate phospholipase A_2. Thus far, this antiporter mechanism appears to be physiologically relevant only in platelets. Enhanced flow through K^+ channels mediated by α_2 stimulation can elicit neuronal hyperpolarization, thereby suppressing cell firing (1,13) and/or secretion (37). In addition, α_2-adrenoceptor stimulation reduces Ca^{2+} entry into the nerve terminals (46). These electrophysiologic consequences of α_2-adrenoceptor activation may be membrane-delimited such that G proteins may couple directly to calcium (19,26) and potassium (4) channels.

Our laboratory has demonstrated the role of various signal transduction components and effector mechansms, including pertussis toxin–sensitive G proteins and K^+ and Ca^{2+} channels, in behavioral responses to α_2-adrenergic agonists (8,17,29). This study demonstrates that adenylate cyclase inhibition does not mediate signal transduction for the neurochemical and behavioral responses to α_2 agonists. Instead, it is likely that adenylate cyclase acts in a permissive role as a modulator of α_2-induced biologic responses by acting in concert with other effector mechanisms.

The principal intention of our study was to further characterize the role of adenylate cyclase in α_2-adrenergic receptor-elicited biologic responses, thereby enhancing our understanding of physiologically significant α_2-adrenergic receptor effector mechanisms. It is anticipated that this information will be essential in attaining a mechanistic understanding, at a molecular level, of pathologic conditions which stem from impaired functions of these pathways, and in developing rational, highly specific agents for therapeutic treatment. For example, the efficacy of α_2-adrenergic agonists has traditionally been determined by their ability to alter cAMP production. If, however, α_2-adrenergic receptors such as B receptors are coupled to multiple transduction pathways (47), then agents may have varying capacities to stimulate these pathways. More accurate characterization of drug efficacy and improvement in drug design will be predicated on a better understanding of the physiologic consequences of significant effector mechanisms.

ACKNOWLEDGEMENTS

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