BRIEF COMMUNICATIONS

Adenosine 3',5'-Cyclic-Monophosphate-Dependent Regulation of α₁-Adrenergic Receptor Number in Rabbit Aortic Smooth Muscle Cells

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SUMMARY. The purpose of this study was to determine whether a cyclic adenosine 3',5'-monophosphate-dependent process can be involved in the regulation of vascular smooth muscle α₁-adrenergic receptor responsiveness. Experiments were performed in cultured rabbit aortic smooth muscle cells which were characterized previously according to α-adrenergic receptor-binding characteristics and receptor-coupled norepinephrine-stimulated ⁴⁵Ca⁺⁺ efflux. The addition of dibutyryl-cyclic adenosine monophosphate to the cell culture medium for 24 hours resulted in a concentration-related decrease in maximal [³H]prazosin-binding capacity (41 ± 4% decrease with 1 mM dibutyryl-cyclic adenosine monophosphate) without an effect on [³H]prazosin-binding affinity. Prostaglandin E₁ (10 nM) and forskolin (10 μM) caused similar decreases in maximal [³H]prazosin-binding capacity, whereas butyrate (1 mM) and dibutyryl-guanosine-3',5'-cyclic-monophosphate (1 mM) had no effect. Dibutyryl-cyclic adenosine monophosphate (1 mM) caused significant potentiation of the decrease in [³H]prazosin-binding caused by a submaximal (10 nM) but not a maximal (10 μM) concentration of norepinephrine, suggesting that cyclic adenosine monophosphate may act at a distal step in common with norepinephrine to reduce α-adrenergic receptor number. Despite the approximately 41% reduction in α-adrenergic receptor number following 24-hour incubation of cells with dibutyryl-cyclic adenosine monophosphate, maximal norepinephrine-stimulated ⁴⁵Ca⁺⁺ efflux was not reduced, consistent with the markedly nonlinear relationship between α-adrenergic receptor occupancy and maximal norepinephrine-stimulated ⁴⁵Ca⁺⁺ efflux in this cell system. These data provide evidence for a novel mechanism by which hormones or drugs which increase cyclic adenosine monophosphate levels can modulate α-adrenergic responsiveness in vascular smooth muscle. (Circ Res 58: 292-297, 1986)

THERE is considerable evidence that cyclic adenosine-3',5'-monophosphate (cAMP) can cause relaxation of vascular smooth muscle by reducing the availability of calcium to contractile proteins (Gerthoffer et al., 1984) and, possibly, by phosphorylation of myosin light chain kinase (Adelstein and Eisenberg, 1980). It has also been hypothesized that cAMP might affect vascular tone through an action on plasma membrane constituents such as receptor-operated and potential-dependent calcium channels, calcium adenosine triphosphatase (ATPase) or sodium/potassium ATPase (Jones et al., 1985); and recently it was demonstrated that agonist-induced β-adrenergic desensitization may be mediated, at least in part, by cAMP-dependent phosphorylation of the β-adrenergic receptor (Stadel et al., 1983).

Vascular smooth muscle cells derived from rabbit aorta and maintained in culture express α₁-adrenergic receptors that are coupled to calcium release from intracellular stores (Wikberg et al., 1983; Colucci et al., 1984a, 1985). We have characterized the relationship between α₁-adrenergic receptor occupancy and norepinephrine (NE)-stimulated ⁴⁵Ca⁺⁺ efflux in this system (Colucci et al., 1984a, 1985), and have observed that 24-hour exposure to NE results in a marked α-adrenergic receptor-mediated reduction in both α-adrenergic receptor number and maximum NE-stimulated ⁴⁵Ca⁺⁺ efflux (Colucci et al., 1984a, 1984b).

In this study, cultured rabbit aortic smooth muscle cells were used to test the hypothesis that a cAMP-dependent process can be involved in the regulation of vascular α-adrenergic receptor responsiveness, either through an effect on α-adrenergic receptor number or an alteration in the relationship between receptor occupancy and receptor-coupled calcium efflux.

Methods

Cell Culture

Rabbit aortic smooth muscle cells were cultured after enzymatic dissociation, as previously reported in detail
Experiments utilized cells grown for 7-14 days after plating at an initial density of $1 \times 10^6$ cells/cm². Cells were from several strains between passage levels 8 and 32, and were checked periodically to confirm that there was no change in basal [3H]prazosin-binding or NE-stimulated 45Ca²⁺ efflux.

[3H]Prazosin Binding

[3H]Prazosin-binding experiments were performed on a crude cellular homogenate, as previously described (Colucci et al., 1984a, 1985). Saturation-binding experiments utilized [3H]prazosin concentrations of 0.02-5 nM, and were analyzed by computerized nonlinear curve-fitting, using the LIGAND program as described by Munson and Rodbard (1980). The effect of experimental interventions on maximal [3H]prazosin-binding capacity (expressed as fmol/cm² of culture plate area) was determined at a single saturating concentration of [3H]prazosin (1.0-1.5 nM), and results were compared within each experiment to binding under control conditions which averaged 0.641 ± 0.089 fmol/cm² of culture plate area ($n = 10$). Addition of dibutyryl (db)-cAMP (1 mM) to cell culture medium for 24 hours had no effect on cell number (control, $2.19 ± 0.11 \times 10^5$ cells/cm²; db-cAMP-treated, $2.19 ± 0.13 \times 10^5$ cells/cm²; $n = 4$; $P = NS$).

45Ca²⁺ Efflux

45Ca²⁺ efflux was quantified as described previously in detail (Colucci et al., 1984a, 1985).

Adenylate Cyclase Assay

Adenylate cyclase activity was measured by the method of Salomon et al. (1974), as previously described (Mooney et al., 1982), using [γ-32P]ATP as substrate and directly measuring the [γ-32P]-labeled cAMP produced. Assay mixtures (final volume 50 μl) contained [γ-32P]ATP ($1.5 \times 10^6$ dpm), 1.0 mM ATP, 25 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM cAMP, 0.1% albumin, 10 mM theophylline, 1 mM ethylene glycol bis (β-aminoethyl ether)-N,N′-tetracetic acid (EGTA), and an ATP-regenerating system consisting of 20 mM creatine phosphate and creatine phosphokinase (1 mg/ml). Reactions were initiated by the addition of 10 μl of membrane suspension containing 20-50 μg of protein and incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 100 μl of 34 mM cAMP. Data were expressed as picomoles of cAMP formed per milligram of protein per minute.

Statistical Analysis

Statistical analysis was performed by analysis of variance and two-tailed nonpaired t-tests (Wallenstein et al., 1980). All data are presented as the mean ± se.

Chemicals

Forskolin was obtained from Calbiochem. All other chemicals were obtained from Sigma, or as previously described (Colucci et al., 1984a).

Results

Effects of db-cAMP, Forskolin, and PGE₁, on Maximal [3H]Prazosin-Binding Capacity

The addition of db-cAMP to the cell culture medium for 24 hours before the [3H]prazosin-binding assay resulted in a concentration-related decrease in maximum binding capacity, with a 41 ± 4% decrease at a db-cAMP concentration of 1 mM (Fig. 1). Saturation-binding experiments indicated that the binding affinity for [3H]prazosin was not affected by 24-hour incubation of cells with 1 mM db-cAMP ($k_d$ control, $0.06 ± 0.02$ nM; $k_d$ db-cAMP, $0.05 ± 0.03$ nM).
nm; n = 3; P = NS) (Fig. 2), and addition of db-cAMP (1 mM) directly to the [3H]prazosin-binding assay had no effect on maximal [3H]prazosin-binding capacity or affinity (data not shown). Butyrate alone (1 mM) and db-cGMP (1 mM) had no significant effects on [3H]prazosin binding, whereas forskolin (10 μM) and prostaglandin E1 (PGE1) (10 μM) added to the cell culture medium for 24 hours before assay resulted in substantial decreases in maximal [3H]prazosin-binding capacity of 36 ± 8% and 52 ± 8%, respectively (Fig. 1).

Effect of db-cAMP on NE-Induced α-Adrenergic Receptor Loss

Addition of NE to the incubation medium for 24 hours resulted in a concentration-related decrease in maximum [3H]prazosin-binding capacity: 10 nM and 10 μM concentrations of NE resulted in 28 ± 10% and 72 ± 8% decreases in maximal [3H]prazosin-binding capacity, respectively (Fig. 3, A and B, respectively). The addition of 1 mM db-cAMP plus 10 nM NE to the incubation medium for 24 hours before assay resulted in a significantly greater reduction in [3H]prazosin-binding capacity than did the addition of 10 nM NE alone (Fig. 3A). In contrast, addition of 1 mM db-cAMP, together with a maximally effective concentration of NE (10 μM), resulted in no greater receptor loss than the NE alone (Fig. 3B).

Effect of db-cAMP on 45Ca++ Efflux

Addition of db-cAMP (1 mM) to the 45Ca++ efflux assay at the initiation of efflux (t = 0) had no effect on the 6-minute basal or NE-stimulated 45Ca++ efflux, and likewise, incubation of cells with 1 mM db-cAMP for 24 hours had no effect on basal 45Ca++ content (data not shown). Despite a 42 ± 10% decrease in maximal [3H]prazosin-binding capacity in cells treated with db-cAMP for 24 hours (n = 4), there was no change in the magnitude of maximal NE-stimulated 45Ca++ efflux in response to 10 μM NE (Fig. 4A).

Basal and PGE1-Stimulated Adenylate Cyclase Activity

Basal adenylate cyclase activity was 11 ± 4 pmol/mg protein per min. 5'-guanylimido-diphosphate (GppNHp) (0.1 mM), NaF (10 mM), and forskolin (10 μM) resulted in 5 ± 2, 9 ± 4, and 11 ± 6-fold stimulation of basal adenylate cyclase activity, respectively (n = 3-5). PGE1 (10 μM) resulted in a 4 ± 1-fold increase in adenylate cyclase activity (n = 6).

Discussion

The major new finding of this study is a cAMP-dependent process which can regulate the density of α-adrenergic receptors in vascular smooth muscle cells. The nonhydrolyzable CAMP analog db-cAMP resulted in a concentration-related decrease in α-adrenergic receptor number without an alteration in binding affinity for the antagonist radioligand [3H]-prazosin, whereas butyrate alone in a comparable concentration had no significant effect on receptor...
Colucci/Regulation of α1-Adrenergic Receptors by cAMP

number or affinity. Direct stimulation of the adenylate cyclase catalytic unit by forskolin caused an effect similar to that of db-cAMP, as did PGE1, which was shown to be a potent stimulator of adenylate cyclase in a cell-free membrane preparation from these aortic smooth muscle cells. Finally, cGMP had no effect on α-adrenergic receptor number, indicating that cAMP is not acting through a cGMP-dependent protein kinase.

An approximately 40% reduction in α1-adrenergic receptors by db-cAMP resulted in no significant decrease in maximal NE-stimulated 45Ca++ efflux, a finding which is consistent with the markedly nonlinear relationship between α-adrenergic receptor occupancy and NE-stimulated calcium efflux in this vascular smooth muscle cell. We previously observed that occupancy of only approximately 7% of α-adrenergic receptors caused 50% of maximal NE-stimulated 45Ca++ efflux, and even a 69% reduction in α1-adrenergic receptor number by irreversible inactivation with phenoxybenzamine resulted in only a minimal decrease in maximal NE-stimulated 45Ca++ efflux (Colucci et al., 1985). As shown in Figure 4B, due to this nonlinearity of the α-adrenergic receptor occupancy-response relationship, a 40% decrease in α1-adrenergic receptor number would not be expected to result in a decrease in maximal NE-stimulated 45Ca++ efflux. The data also suggest that cAMP-mediated loss of α-adrenergic receptors is not accompanied by substantial uncoupling of the receptor from calcium efflux, since a decrease in NE-stimulated 45Ca++ efflux would have been anticipated. In contrast, although prolonged (24–48 hour) agonist exposure results in a decrease in α1-adrenergic receptor number (Colucci et al., 1981, 1984a), short-term agonist exposure of rabbit aortic cells (Colucci et al., 1984b; Colucci and Alexander, in press) or intact rabbit aorta (Lurie et al., 1985) causes a significant desensitization of norepinephrine-stimulated calcium efflux and contraction, respectively, prior to a decrease in receptor number. Therefore, it appears unlikely that cAMP plays a key role in rapid agonist-induced desensitization of α1-adrenergic responses in vascular smooth muscle, but, rather, may be important in the long-term tonic regulation of total α1-receptor number, possibly through an effect on receptor metabolism.

Since α-adrenergic receptor stimulation in this type of cell does not result in adenylate cyclase activation (unpublished data), it is unlikely that homologous regulation of α1-adrenergic receptor number by α1-adrenergic agonists is controlled by a cAMP-dependent process. However, these observations provide evidence for the heterologous regulation of α1-adrenergic receptors by adenylate cyclase-coupled hormones, or other agents affecting cAMP levels, such as phosphodiesterase inhibitors, and suggest that cAMP can modulate the regulatory effects of α1-adrenergic agonists on their receptor. In support of this thesis is the fact that db-cAMP significantly potentiated the ability of a submaximal concentration of NE (10 nM) to reduce α1-adrenergic receptor number. An analogous role for cAMP was suggested by the observation in BALB/c3T3 cells that cAMP potentiates the down-regulation of epidermal growth factor receptors by platelet-derived growth factor (Leof et al., 1982).

Whereas db-cAMP potentiated the effect of a submaximal concentration of NE to decrease α1-adrenergic receptor number, db-cAMP had no potentiating effect when a maximal concentration of NE was used, suggesting that the cAMP-dependent influence on α1-adrenergic receptor number is mediated at a step in common with the agonist-induced decrease in α1-adrenergic receptor number caused by NE. Since both PGE1, presumably acting through PGE1 receptors, and forskolin were able to decrease α-receptor number to a degree comparable to exog-
endogenous db-cAMP, it appears likely that endogenously produced cAMP is capable of significantly influencing α-adrenergic receptor number under appropriate conditions. However, since cAMP levels may be substantially lower than the near maximal levels stimulated by forskolin, the physiological importance of cAMP in the regulation of α1-adrenergic receptor cannot be determined from these data.

Although all known actions of cAMP appear to be mediated by activation of protein kinases, the present experiments do not allow conclusions regarding the substrate for the cAMP-dependent protein kinase in these cells, or its role in regulating cellular α-adrenergic receptor number. Agonist-induced desensitization of the turkey erythrocyte β-adrenergic receptor is associated with receptor phosphorylation, an action that can be mimicked partially by 8-bromo-cAMP (Stadel et al., 1983). By analogy, the present data raise the possibility that a cAMP-induced decrease in α-adrenergic receptor number may involve phosphorylation of the α-adrenergic receptor. Alternatively, cAMP-dependent protein kinases could regulate a variety of cellular mechanisms involved in the turnover of α-adrenergic receptors.

Considerable attention has been focused on the mechanism by which cAMP-elevating hormones and drugs cause vasodilation. The two major mechanisms that have been investigated are (1) an action of cAMP to reduce the availability of calcium to the contractile proteins (Adelstein and Eisenberg, 1980), and (2) an action of cAMP to phosphorylate myosin light chain kinase, thereby resulting in vascular smooth muscle relaxation (Ito et al., 1982; Miller et al., 1983; Saida and Van Breeman, 1984; Gerthoffer et al., 1984). Recently, it was suggested that cAMP could also influence vascular tone through an action on membrane components such as receptor-operated and potential-dependent calcium channels, calcium ATPase, or sodium/potassium ATPase (Jones et al., 1985). The data presented here provide direct evidence that a cAMP-dependent process can influence a membrane component, the α-adrenergic receptor, and thereby constitutes still a third possible mechanism by which cAMP can modulate vascular tone. As stressed by Hardman (1981), however, the observation that cAMP can affect a particular cellular process does not indicate that it must regulate that process under physiological conditions. It seems likely that the cAMP-dependent process identified in this study is only one of many mechanisms by which cAMP can influence vascular tone. These findings add to our understanding of the multiple factors involved in the regulation of vascular α-adrenergic responsiveness, and suggest a mechanism by which pharmacological interventions that cause an elevation in cAMP levels can influence α-adrenergic receptor-mediated responses in vascular smooth muscle.
Colucci/Regulation of α₁-Adrenergic Receptors by cAMP


INDEX TERMS: Vascular smooth muscle • α₁-Adrenergic receptor • [3H]Prazosin • Cyclic adenosine 3',5'-monophosphate • Calcium efflux
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