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Desensitization of β-Adrenergic Receptor-Mediated Vascular Smooth Muscle Relaxation

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SUMMARY

Responsiveness to catecholamines may be blunted after prolonged exposure to an agonist; this phenomenon, termed desensitization, is often mediated by receptor down-regulation. β-Adrenergic receptors mediate relaxation of vascular smooth muscle. We have examined the possibility that this response may be desensitized after prolonged exposure to increased concentrations of epinephrine. Rats were treated with epinephrine infusions (300 μg/kg/hr from a minipump) for 7 days and had levels of plasma epinephrine 70-fold greater than those of controls. The mesenteric artery rings from the epinephrine-treated rats contracted normally when exposed to serotonin; however, the extent of relaxation promoted by the β-adrenergic agonist isoproterenol was blunted (86 ± 4 vs. 43 ± 9%; $p < 0.05$). Acetylcholine and nitroglycerine, which may act through a cyclic GMP mechanism, caused virtually identical relaxation responses in both control and epinephrine-treated groups. To determine the mechanism for the loss in responsiveness to isoproterenol, we measured adrenergic receptors in individual mesenteric arteries using [^{125}I]cyanopindolol. Specific binding of [^{125}I]cyanopindolol was found to have the expected characteristics of interaction with β receptors. There was no difference in the number of β-adrenergic receptors between control and epinephrine-treated animals (24 ± 5 vs. 26 ± 6 fmol/mg of protein), although there was significantly marked down-regulation of β-adrenergic receptors in hearts (23 ± 2 vs. 10 ± 1 fmol/mg of protein; $p < 0.001$) and lungs (172 ± 29 vs. 76 ± 7 fmol/mg of protein; $p < 0.01$) in the same rats. The ability of isoproterenol to stimulate cyclic AMP production in the mesenteric arteries from the two groups was not significantly different (20.3 ± 3.5 vs. 23.8 ± 4.7 pmol of cAMP/mg of protein/2 min). Furthermore, mesenteric artery relaxation was found to be decreased in response to the cyclic AMP analogue dibutyryl cyclic AMP (45 ± 2.0 vs. 28 ± 2.0%; $p < 0.001$) in the epinephrine-infused rats. These data suggest that the desensitization of β-adrenergic receptor-mediated smooth muscle relaxation may be caused by a mechanism distal to cyclic AMP production.

INTRODUCTION

β-Adrenergic receptor-mediated responses may be attenuated by persistent exposure to catecholamines (1–4); this process has been referred to as desensitization. Desensitization has usually been correlated with decreased β-adrenergic receptor-mediated cyclic AMP production (1–4) and, in most circumstances, with β-adrenergic receptor loss from the cell's surface (2–4). While desensitization has been extensively studied in isolated cells treated *in vitro*, relatively little is known about the phenomenon as it occurs in intact organisms.

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In blood vessels, β-adrenergic receptors mediate catecholamine-induced relaxation of smooth muscle (5, 6). This vascular relaxing effect of β-adrenergic agents apparently involves stimulation of cAMP production with consequent activation of cAMP-dependent protein kinase (7). However, very little information is available concerning the regulation of β receptors in blood vessels, although an alteration in the sensitivity of vascular response to β adrenoceptor agonists has been observed in a number of physiological and disease states such as aging (8, 9) and hypertension (10).

We have investigated the possibility that prolonged exposure to catecholamines might modify subsequent sensitivity to β-adrenergic receptor-mediated smooth muscle relaxation. Rats were given prolonged infusions of epinephrine and the consequences for vascular smooth muscle relaxation were examined *in vitro* using the mes-

enteric artery as a model system. In an effort to characterize the mechanism for desensitization that was observed, β-adrenergic receptors and cAMP production were measured in individual mesenteric arteries. The results obtained suggest that desensitization of β receptor-mediated smooth muscle relaxation may be mediated by a potentially unusual mechanism distal to cAMP production.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled (±)-cyanopindolol was a generous gift from Dr. G. Engle of Sandoz (Basel, Switzerland). Carrier-free $Na^{125}I$ (Catalogue No. IMS30) was purchased from Amersham. Metoprolol was received as a gift from Ciba-Geigy Corp., Summit, NJ. All other reagents were from standard commercial sources.

Preparation of [^{125}I]cyanopindolol. (±)-CYP[1] was iodinated according to the method of Engel *et al.* (11). The reaction mixture contained 10 μl of 13.5 mM HCl containing 20 μg of CYP, 20 μl of potassium phosphate buffer (0.3 M, pH 7.6), 2 mCi of carrier-free $Na^{125}I$, and 20 μl of chloramine-T in aqueous solution (0.34 mg/ml). The mixture was incubated at room temperature for 5 min, after which the reaction was stopped with the addition of 300 μl of aqueous $Na_2S_2O_3$ (6.3 mM). Ten μl of 1 N NaOH was added to the tube and the reaction mixture was extracted with four 300-μl aliquots of ethyl acetate containing 0.01% phenol. The combined washes were subjected to descending paper chromatography for 6 hr at 4° on Whatman 3MM paper using 0.1 M ammonium formate (pH 8.5) as the mobile phase. The iodinated product was extracted from the appropriate strip of paper in methanol. [^{125}I]CYP was stable when stored at −20° in methanol for at least 8 weeks.

Treatment of rats with epinephrine. Male Sprague-Dawley rats weighing 150 to 200 g (5–6 weeks old) were treated with a continuous infusion of epinephrine at a rate of 300 μg/kg/hr from an Alzet minipump (model 2001). The minipump was filled with (−)-epinephrine HCl dissolved in acidified isotonic saline (0.001 N HCl), and was preincubated in isotonic saline at 37° for 3 hr before its implantation. The minipumps were implanted subcutaneously in the neck under light anesthesia with ether. The rate of infusion of fluid from the minipump was 1 μl/hr, delivering 300 μg/kg/hr of epinephrine for a period of up to 10 days.

The rats were killed 7 days after implantation of the minipump. The rats were anesthetized with pentobarbital (40 mg/kg intraperitoneally) and blood (2 ml) from the inferior vena cava below the level of the renal veins was rapidly collected through an abdominal incision and placed in ice-cold tubes containing 20 μl of 6% glutamine and 9% EGTA (pH 6.0 to 7.4) for each 1 ml of blood. Following centrifugation (350 × *g*), plasma was separated and stored at −70°. Plasma catecholamine concentrations were quantitated by high performance liquid chromatography with electrochemical detection (12). Mesenteric arteries, lungs, and hearts were rapidly removed from the rats and used as described below.

Isolation of rat mesenteric artery membranes. A particulate fraction of rat mesenteric artery smooth muscle was prepared by a modification of the method described by Wei *et al.* (13). Male Sprague-Dawley rats (150–200 g) were sacrificed as described above. The superior mesenteric vascular arcade was quickly removed, freed of adjacent tissues by blunt dissection, and placed in an ice-cold buffer (0.25 M sucrose, 5 mM Tris-HCl, and 1 mM $MgCl_2$, pH 7.4) in a Potter-Elvehjem homogenizer, and the fatty adventitial tissues were removed with two gentle strokes of a Teflon pestle. The artery cleaned in this fashion was then coarsely minced with scissors and homogenized with a Brinkmann Polytron (setting 7, 10 sec × 2) and then centrifuged at 1,500 × *g* for 10 min at 4°. The resulting supernatant was then recentrifuged at 100,000 × *g* for 30 min at 4°. The supernatant was then discarded, and the remaining membrane pellet was resuspended in buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM EDTA, pH 7.5) and used immediately in the β receptor-binding assays. Rat ventricular and pulmonary membranes were prepared by the method of Minneman *et al.* (14) with minimal modification. These preparations were used to determine possible changes in β receptors in the three tissues simultaneously from individual rats. Protein concentration was determined by the method of Lowry *et al.* (15) using bovine serum albumin as standard.

Radioligand binding assay. Binding assays were performed in duplicate by incubating 100 μl of membrane suspension with 25 μl of [^{125}I]CYP and 25 μl of various drugs in a final volume of 150 μl in polypropylene test tubes. Membranes were incubated with the radioligand for 55 min at 37° (heart), for 60 min at 25° (lung), and for 60 min at 25° (mesenteric artery), respectively. Preliminary experiments showed that equilibrium binding was obtained for each tissue under these conditions. The reaction was terminated with the addition of 5 ml of the incubation buffer at room temperature. The bound and free radioligands then were separated by rapid filtration over glass fiber filters (Whatman GF/C). The filters were washed with an additional 15 ml of incubation buffer at room temperature. The radioactivity retained on the filters was counted in a gamma counter at 70% efficiency. Nonspecific binding was defined as the amount of [^{125}I]CYP binding measured in the presence of 100 μM (−)-isoproterenol. The specific binding of [^{125}I]CYP generally represented 70–80% of the total radioligand bound.

Measurement of cAMP production. Mesenteric arteries were quickly removed and freed of adjacent tissues as described above and cut into six "rings" lengthwise. The rings were then incubated without tension at 37° for 2 hr in a modified Krebs solution of the following composition (in millimolar): NaCl, 118.2; KCl, 4.6; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 24.8; KH_2PO_4, 1.2; glucose, 10.0; and ascorbic acid, 1. The incubation was then bubbled with a gas mixture containing 95% O_2 and 5% CO_2 (pH 7.4). IBMX was then added to the bath at a final concentration of 10 μM, 5 min prior to the addition of other drugs as indicated. Drug effects on cAMP production were studied after a 2-min incubation with the indicated concentration of agonist, since preliminary experiments demonstrated that the effect of (−)-isoproterenol (10 μM) on the cAMP concentration in the vessels reached a plateau at that time. At the end of the incubation, the reaction was stopped by freezing the rings in liquid nitrogen. The tissue was stored at −70° until assayed. At the time of assay, the tissue was homogenized in 1 ml of 6% trichloroacetic acid at 4°. After centrifugation at 3000 × *g* for 45 min, cAMP was assayed in the supernatant by radioimmunoassay (16). cAMP concentrations are expressed as picomoles/mg of protein/2 min; the pellet was used for the determination of protein concentration.

In vitro mesenteric artery relaxation. Rings of superior mesenteric artery (3 mm wide), cut close to the abdominal aorta, were mounted in a 10-ml jacketed tissue bath kept at 37.5–38°. The composition of the modified Krebs solution was as described above. Tissue bath solutions were equilibrated with a 95% O_2 and 5% CO_2 gas mixture. An initial resting tension of 1.0 g was applied and the rings were allowed to equilibrate for 2 hr before exposure to drugs. Responses were recorded as changes in grams of tension on a physiological recorder with a strain gauge force displacement transducer (UC-2, Gould). Cumulative dose-response curves for drugs that cause relaxation were obtained by producing a stepwise increase in concentration of agonist as soon as a steady response was obtained from each preceding dose. Mesenteric rings were contracted with serotonin (5×10^{-6} M) before exposure to a relaxant drug. In preliminary studies, it was found that this concentration of serotonin causes approximately 80% of maximal tension (EC_{80}) in the vascular rings. Relaxant responses are expressed as percentage of the maximal possible relaxation, that is, relaxation of the contracted tissue back to the base line. Experiments were conducted in the presence of phentolamine (1×10^{-7} M) to avoid any α-adrenergic effects of catecholamines in these studies.

[1] The abbreviations used are: CYP, cyanopindolol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; IBMX, 3-isobutyl-1-methylxanthine.

When the contraction induced by serotonin reached a steady state plateau, relaxant agonist was then added. From the preliminary experiments, the serotonin response was observed to begin declining spontaneously after several minutes at plateau. For each concentration of isoproterenol, nitroglycerin, and acetylcholine, relaxation was prompt and the whole dose-response curves were completed before spontaneous relaxation began. However, relaxation to the dibutyryl-cAMP was found to be a more gradual process which continued beyond 10 min. Slow responses to dibutyryl-cAMP have also been observed in rat aorta; we have utilized a similar method of expressing the response to this analog (10). Therefore, since the serotonin-induced tone began to decline before relaxation to the dibutyryl-cAMP reached maximum, relaxation was measured 2 min after addition of each concentration of the analog. Therefore, relaxation to the dibutyryl-cAMP was considered as a relaxation rate.

Data analysis. The data from saturation curves were analyzed using nonlinear regression on a HP85 computer. These data were fit based on the law of mass action using a general program for the analysis of data in terms of models (17). The K_D values of competing drugs were calculated by the method of Cheng and Prusoff (18). The EC_{50} of each competitor was determined by a best fit of the four-parameter logistic equation (19). The experimental data given in text and figures are mean ± standard error of four to nine experiments as indicated. Student's unpaired t test was used to test for statistical significance.

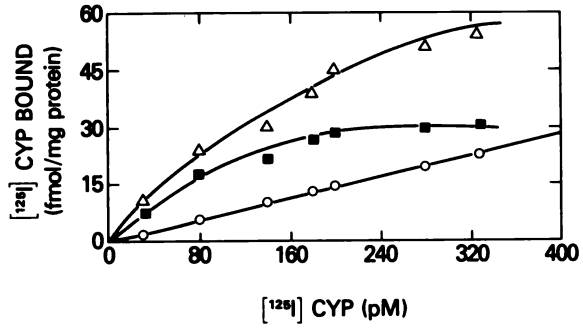

FIG. 1. *Saturation of [^{125}I]CYP-binding sites from rat mesenteric artery membranes*

The membranes for the experiment here derived from a single mesentery and the assay was performed as described in Materials and Methods. Specific binding (■) is the difference between total binding (△) and nonspecific binding (○) determined in the presence of 100 μM (−)-isoproterenol. The number of β receptors labeled by [^{125}I]CYP was 29.7 fmol/mg of protein. Each value shown is the mean of duplicate determinations. The experiment shown is representative of seven such experiments.

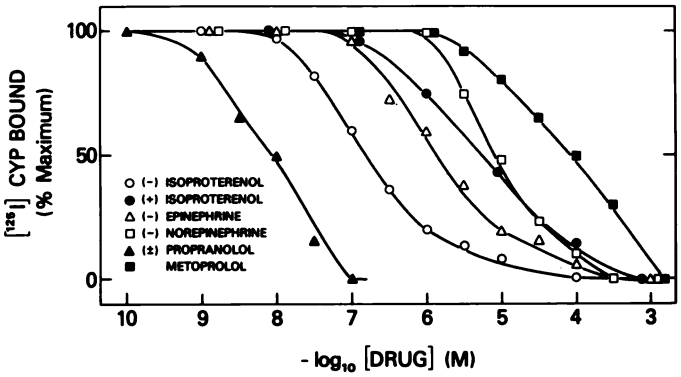

FIG. 2. *Competition curves of [^{125}I]CYP with β-adrenergic agonists and antagonists*

Each point represents the mean of duplicate determinations. The data are from a representative experiment.

RESULTS

Characterization of [^{125}]CYP binding in mesenteric artery. Specific binding of [^{125}I]CYP to the rat mesenteric artery particulate fraction was saturable (Fig. 1). Computer analysis of the saturation curves indicated that [^{125}I]CYP was binding to a single class of sites with dissociation constant of 82.6 ± 14.1 pM and a calculated total binding capacity of 29.7 ± 4.1 fmol/mg of protein (n = 7). Binding reached equilibrium in 50 min and remained stable for at least 30 more min (data not shown).

Catecholamines displaced [^{125}I]CYP with the expected β-adrenergic potency order of (−)-isoproterenol (EC_{50} = 0.26 μM) > (−)-epinephrine (EC_{50} = 0.8 μM) ≫ (−)-norepinephrine (EC_{50} = 15 μM) (Fig. 2; Table 1); and (−)-isoproterenol was approximately 20 times more potent than (+)-isoproterenol in competing for [^{125}I]CYP-binding sites (Fig. 2). The fact that (−)-epinephrine was much more potent than (−)-norepinephrine in competing for [^{125}I]CYP-binding sites suggests that the β-adrenergic receptors of the rat mesenteric artery are predominantly of the β_2 subtype. Also, the nonselective β-adrenergic antagonist (±)-propranolol (K_D = 7.4 nM) was much more potent in displacing [^{125}I]CYP than was the β_1-selective antagonist metoprolol (K_D = 39,000 nM), suggesting that β_2 receptors predominate in the mesenteric artery (Fig. 2). As has been observed with β-adrenergic receptors in other systems (for review, see Ref. 20), addition of 100 μM Gpp (NH)p decreased the affinity of agonists in competing for [^{125}I]CYP-binding sites (EC_{50} = 0.24 ± 0.01 vs. EC_{50} = 3.0 ± 0.2 μM; n = 4; $p < 0.05$) and increased the pseudo-Hill slope of the competition curve (0.4 ± 0.03 vs. 1.26 ± 0.17; n = 4; $p < 0.05$).

Effect of catecholamines on cAMP production in mesenteric artery. The cAMP contents of rat mesenteric artery after 2-min incubation are plotted in Fig. 3 as a function of concentrations of β-adrenergic agonists. The dose-response curves to (−)-isoproterenol, (−)-epinephrine, and (−)-norepinephrine showed the rank order of potency characteristic of the β_2-adrenergic system: (−)-isoproterenol > (−)-epinephrine ≫ (−)-norepinephrine. EC_{50} values were 0.7 ± 0.2 μM for (−)-isoproterenol, 12.4 ± 7.9 μM for (−)-epinephrine, and 220 ± 61 μM for (−)-norepinephrine, respectively. However, the maximum responses to these catecholamines were not the same. Isoproterenol and epinephrine increased the cAMP levels to about the same maximum 23.4 and 21.1 pmol of cAMP/mg of protein/2 min), whereas the maximal stimulation by norepinephrine (11.3 pmol of cAMP/mg of

TABLE 1

Affinity of adrenergic agonists for mesenteric artery β receptors

EC_{50} (mean ± SE) values were determined as described in Materials and Methods. n, number of experiments.

Compounds	EC_{50} (inhibition of [^{125}I]DYP binding)	EC_{50} (cAMP production)
(−)-Isoproterenol	$2.6 \pm 0.7 \times 10^{-7}$ (n = 6)	$7.3 \pm 1.5 \times 10^{-7}$ (n = 6)
(+)-Isoproterenol	$4.6 \pm 0.6 \times 10^{-6}$ (n = 4)	
(−)-Epinephrine	$8.0 \pm 1.0 \times 10^{-7}$ (n = 4)	$1.2 \pm 0.8 \times 10^{-5}$ (n = 6)
(−)-Norepinephrine	$1.5 \pm 0.2 \times 10^{-6}$ (n = 4)	$2.2 \pm 0.6 \times 10^{-4}$ (n = 6)

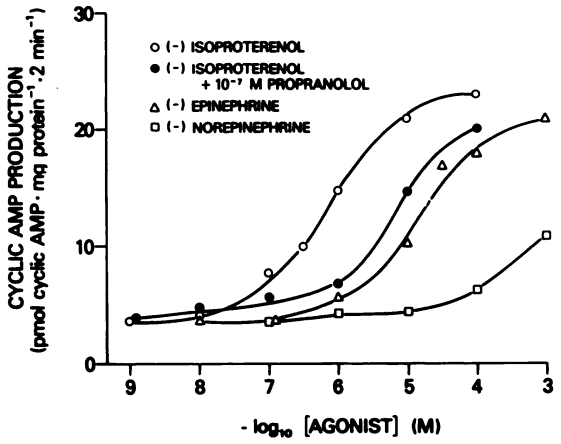

FIG. 3. *Cyclic AMP production in rat mesenteric artery*

Rat mesenteric artery rings were incubated at 37° for 2 min in the presence of 10 μM IBMX with various concentrations of agonists. For the experiments involving propranolol, the mesenteric rings were additionally preincubated with 0.1 μM propranolol for 5 min before various concentrations of (−)-isoproterenol were added. Each point represents the mean ± SE of six separate experiments.

protein/2 min) was approximately 50% of the other two agonists. An α-adrenergic blocking agent, phentolamine (10 μM), did not antagonize or potentiate the effect of these agonists on cAMP production. A β-blocking agent, propranolol, inhibited the isoproterenol-induced increase in cAMP content in mesenteric artery (Fig. 3). Thus, the results show that β-adrenergic receptors in mesenteric artery are linked to cAMP generation and can be characterized as predominantly β_2 as also shown by the radioligand-binding studies.

Relaxing response of rat mesenteric artery to isoproterenol. The addition of serotonin in a concentration of 5×10^{-6} M caused a sustained contraction in the mesenteric arterial strips. Mean values of the contractile tension developed by 5×10^{-6} M serotonin in mesenteric arteries from 150–200-g rats were 1516 ± 104 mg (n = 22). Preparations thus contracted relaxed well in response to vasodilating agents. The addition of (−)-isoproterenol in concentrations ranging from 1×10^{-9} to 1×10^{-6} M elicited a dose-dependent relaxation in the presence of phentolamine (10^{-7} M). Dose-response curves for the relaxing effect of (−)-isoproterenol in mesenteric arterial rings from this age rats showed the EC_{50} value was 8.6 ± 2.0 nM. Dose-response curves for the relaxing effect of (−)-isoproterenol in these rings were completely antagonized by 1×10^{-6} M propranolol, confirming that arterial relaxation by isoproterenol is due to its activation of β adrenoceptors. Also, isoproterenol is more potent in promoting relaxation than activating cAMP production.

Smooth muscle relaxation after chronic in vivo treatment with epinephrine. Analysis of plasma samples from control rats and rats treated by epinephrine demonstrated a marked increase in the levels of epinephrine in rats with minipump infusion. Plasma epinephrine concentration was much higher in rats with epinephrine infusion (14,300 ± 3,157 pg/ml; n = 18; $p < 0.0005$) compared to control rats (206 ± 91 pg/ml; n = 16), whereas plasma norepinephrine was not significantly different in the two groups: 756 ± 337 ng/ml (n = 16) in control and 825 ± 308 ng/ml (n = 18) in rats with epinephrine treatment.

Vascular smooth muscle relaxation. Mean values of the contractile tension developed by 5×10^{-6} M serotonin in mesenteric arteries from control groups and epinephrine-treated rats were 1440 ± 234 mg (control rats, n = 5) and 1630 ± 235 mg (epinephrine-treated rats, n = 5). The contractile response of the mesenteric artery to serotonin was not significantly different in the two groups. The addition of (−)-isoproterenol in concentrations ranging from 1×10^{-8} to 1×10^{-5} M elicited a dose-dependent relaxation in serotonin-contracted mesenteric arteries from both control and epinephrine-infused rats. The maximum relaxation from epinephrine-treated rats was significantly smaller than that from age-matched control rats (Fig. 4, 86 ± 4.3 vs. 43 ± 6.7%; $p < 0.05$). However, there was no difference in the relaxation response induced by acetylcholine (Fig. 5). Acetylcholine caused a 81.5 ± 1.6% relaxation in controls and 81.3 ± 2.6% relaxation in the epinephrine-treated group. Similarly, the nitroglycerine (1×10^{-6} M) caused greater than

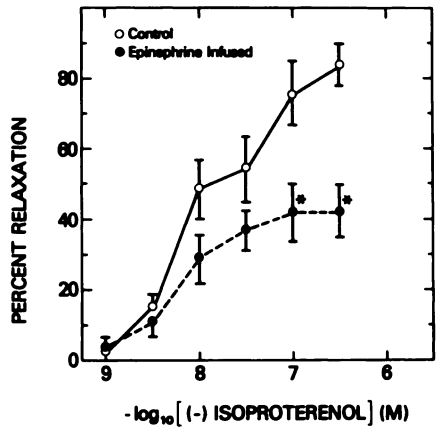

FIG. 4. *Dose-response for isoproterenol-induced relaxation of rat mesenteric artery rings from epinephrine-infused (●) and age-matched control rats (○)*

Results are the mean ± SE of five different animals in each group. *$p < 0.05$ difference from control group.

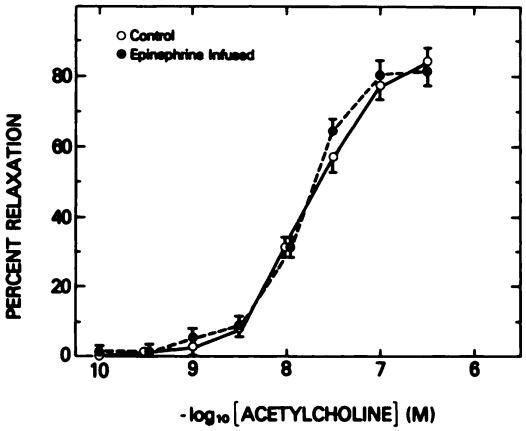

FIG. 5. *Dose-response for acetylcholine-induced relaxation of rat mesenteric artery rings from epinephrine-infused (●) and age-matched control rats (○)*

Results are the mean ± SE of six different animals in each group. Results do not differ at the $p < 0.05$ level of significance at any point.

95% relaxation in each of the two groups (data not shown).

Influence of chronic epinephrine administration on [^{125}I]CYP binding. In an effort to determine the mechanism for the desensitization of β receptor-mediated relaxation in the epinephrine-treated rats, the β receptors in mesenteric arteries were measured with [^{125}I]CYP. We found that there was no significant difference in the total number of β receptors in the arteries of control rats (24.4 ± 2.3 fmol/mg of protein; $n = 5$) compared with the vessels of epinephrine-treated rats (25.6 ± 2.7 fmol/mg of protein; $n = 5$) (Fig. 6). Nor was there any change in the affinity of [^{125}I]CYP for the β receptors in the control, K_D = 88.3 ± 11.3 pM, or epinephrine-treated groups, K_D = 107.7 ± 15.0 pM. However, a dramatic decrease was found in the number of β receptors in ventricular and pulmonary membranes prepared from tissues of the same animals (Fig. 6). The density of β receptors in the ventricles decreased 56% (22.6 ± 2.0 vs. 9.9 ± 0.9 fmol/mg of protein; $p < 0.001$), while the number of pulmonary β receptors was diminished 56% (172.0 ± 29.0 vs. 75.7 ± 6.7 fmol/mg of protein; $p < 0.01$). In both hearts and lungs, the affinities of the β receptors for the antagonist [^{125}I]CYP were not altered significantly by epinephrine infusion: ventricles, control, 118 ± 24.5 pM, and epinephrine-treated animals, 83 ± 20.5 pM; lung, control, 39.3 ± 7.3 pM, and epinephrine-treated animals 29.3 ± 5.1 pM.

Influence of epinephrine infusion on cAMP production in mesenteric artery. The concentration-response relationships of (−)-isoproterenol-induced accumulation of cAMP in rat mesenteric arteries from control and epinephrine-treated rats presented virtually identical characteristics: the threshold concentration was about 1×10^{-8} M; the EC_{50} values were 0.16 ± 0.07 μM (control, $n = 6$) and 0.10 ± 0.03 μM (epinephrine-treated, $n = 6$). No difference could be found in the maximal effect of (−)-isoproterenol between two groups (control, 20.3 ± 3.5; epinephrine-treated, 23.8 ± 4.7 pmol of cAMP/mg of protein/2 min) (Table 2).

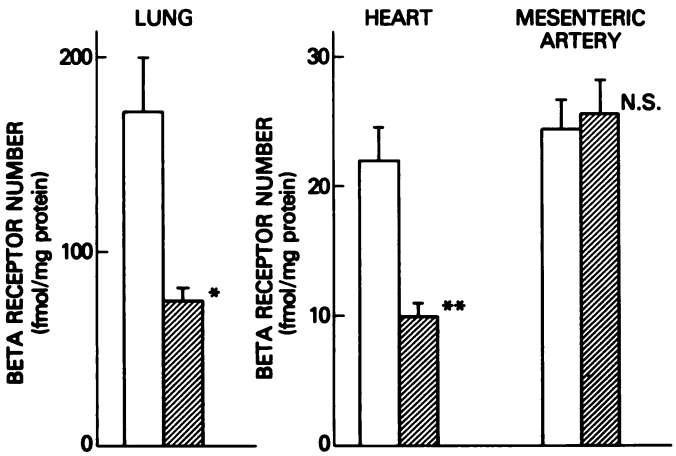

FIG. 6. *Changes in β-adrenergic receptors with epinephrine infusion*
β-Adrenergic receptors were determined as described in Materials and Methods. Open bars show the number of β-adrenergic receptors from control rats (±SE). Closed bars show the receptor numbers in membranes from epinephrine-infused rats. The affinities of β receptors for [^{125}I]CYP were not different between the two groups. *$p < 0.01$; **$p < 0.001$; NS, not significantly different.

TABLE 2

Effect of (−)-isoproterenol on cAMP production in rat mesenteric arteries of control and epinephrine-infused rats

Values are the mean ± SE of six different experiments. Results do not differ at the $p < 0.05$ level of significance between the two groups.

	Control rats	Epinephrine-infused rats
Basal (pmol cAMP/mg protein/2 min)	3.6 ± 1.4	4.5 ± 1.1
Isoproterenol (100 μM) (pmol cAMP/mg protein/2 min)	20.3 ± 3.5	23.8 ± 4.7
EC_{50} (μM)	0.16 ± 0.07	0.10 ± 0.03

TABLE 3

Relaxation in the presence of phosphodiesterase inhibitors

Mesenteric artery rings were prepared from control and epinephrine-infused rats as decribed in Materials and Methods. Isoproterenol-mediated relaxation of serotonin-contracted vessels was determined. Then, after extensive washing, either IBMX (10^{-5} M) or Ro-20-1724 (10^{-5} M) was added for a 5-min preincubation and the isoproterenol dose-response curve was redetermined in the serotonin-contracted vessels. The results are expressed as the percentage of the serotonin-induced contraction that could be relaxed with isoproterenol. As indicated in the text, the force of serotonin-induced contraction was less after both IBMX and Ro-20-1724. *n*, number of experiments.

Source of mesenteric arteries	Isoproterenol-mediated relaxation	
	EC_{50}	Maximal relaxation
	M	%
Control rats		
No preincubation	2.0×10^{-8} ($n = 4$)	85
Preincubated with IBMX (10^{-5} M)	1.0×10^{-9} ($n = 2$)	100
Preincubated with Ro-20-1724 (10^{-5} M)	2.5×10^{-9} ($n = 2$)	100
Epinephrine-treated rats		
No preincubation	3.5×10^{-8} ($n = 6$)	38[a]
Preincubated with IBMX (10^{-5} M)	3.0×10^{-8} ($n = 4$)	39
Preincubated with Ro-20-1724 (10^{-5} M)	2.5×10^{-8} ($n = 2$)	40

[a] $p < 0.01$ compared with control.

We utilized the phosphodiesterase inhibitor IBMX (10^{-5} M) in these experiments to magnify the isoproterenol-stimulated cAMP accumulation and facilitate its measurement. In an effort to make these results more comparable to the relaxation studies, additional experiments of isoproterenol-mediated relaxation in mesenteric artery rings were undertaken in the presence of either IBMX (10^{-5} M) or Ro-20-1724 (10^{-5} M) another more potent phosphodiesterase inhibitor. Mesenteric arteries from control and epinephrine-infused rats were incubated *in vitro* as described above. Dose-response curves of isoproterenol-mediated relaxation of serotonin-induced contraction were constructed and then, after extensive washing, either IBMX or Ro-20-1724 was added. After a 5-min preincubation, serotonin was added and the isoproterenol dose-response curve was recalculated. As indicated in Table 3, isoproterenol was less effective in promoting maximal relaxation in the epinephrine-infused rats as we had found previously. Because both IBMX and Ro-20-1724 are phosphodiesterase inhibitors and possibly nonspecific vasodilators, serotonin-induced contraction in the control vessels was sub-

stantially less after the addition of IBMX (38%) or Ro-20-1724 (69%). Isoproterenol, with increased potency, relaxed the remaining contraction (Table 3). In the mesenteric arteries from the epinephrine-infused rats, the serotonin-induced contractions after IBMX (70%) or Ro-20-1724 (90%) were more greatly maintained than in the control vessels. The isoproterenol dose-response curves in the vessels from the epinephrine-treated rats were essentially unaffected by the addition of either IBMX or Ro-20-1724 (Table 3). These results demonstrate that, even in the presence of phosphodiesterase inhibitors, relaxation is still impaired in vessels from epinephrine-infused rats. However, it is unclear why the sensitivity to isoproterenol did not increase in these vessels in the presence of IBMX or Ro-20-1724.

Cyclic nucleotide-induced relaxation of mesenteric artery rings from epinephrine-treated rats. Since the cAMP response to isoproterenol appeared to be intact, we next examined the responsiveness of desensitized mesenteric arteries to the analogue dibutyryl cAMP. The rate of relaxation after dibutyryl cAMP was less (45.5 ± 1.8 vs. 28.1 ± 1.7%; $n = 6$; $p < 0.05$) for mesenteric artery rings from epinephrine-infused rats as compared to the relaxation rate of mesenteric artery rings from age-matched rats (Fig. 7).

DISCUSSION

Utilizing a minipump infusion as a maneuver to obtain *in vivo* the chronic exposure to high concentrations of epinephrine, we detected a significant reduction in β-adrenergic receptor-mediated vascular relaxation. There was no loss in responsiveness to acetylcholine which acts by a cAMP-independent mechanism. The desensitization in responsiveness of the mesenteric arteries was not associated with down-regulation of β receptors or a blunting of the cAMP response in the arteries. However, the response of the epinephrine-treated vessels to the relaxing effect of dibutyryl cAMP was diminished. These data suggest that desensitization induced by epinephrine in the mesenteric artery is mediated by a change at the level of protein kinase or more distally rather than by alteration in the β-adrenergic receptor-adenylate cyclase system.

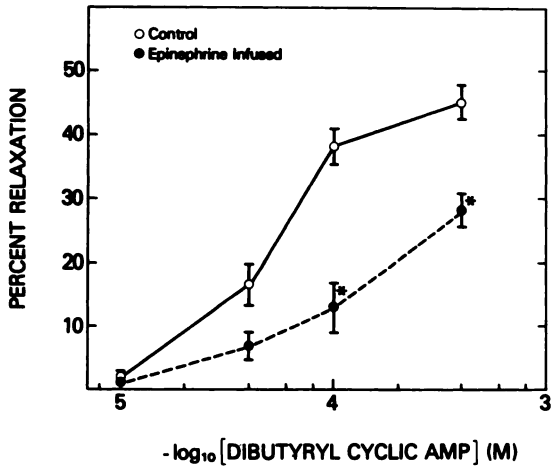

FIG. 7. *Dose-response curves for the relaxant effect of dibutyryl cAMP in mesenteric artery rings from epinephrine-infused (●) and age-matched control rats (○)*

Each point represents the mean value ± SE of rings from six animals. *Indicates those points differing significantly at $p < 0.05$.

In recent years, there has been intense interest in the mechanism by which a target cell or tissue may modulate its response to a neuroeffector or hormone. In particular, considerable attention has been devoted to studies of the effects of β-adrenergic agonists on adenylate cyclase activity and substantial advances have been made in elucidating such mechanisms in a number of *in vitro* and *in vivo* systems (4). From these studies, at least two general mechanisms of densensitization have been postulated, namely "uncoupled state" and "down-regulation" of receptors (3, 4). Whereas regulation at the level of the receptor/adenylate cyclase may represent one group of adaptive responses directed at blunting excess stimulation, these mechanisms do not exclude the role of modification of other cellular mechanisms in desensitization. Our study clearly supports the possibility that the *in vivo* epinephrine-induced reduction in sensitivity of the rat mesenteric artery smooth muscle to the relaxing action of isoproterenol seems to be accomplished by a mechanism distal to cAMP production. The absence of down-regulation of β receptors in the mesenteric artery is particularly interesting in view of the marked reduction in β receptors found in the hearts and lungs of the same animals. The mesenteric artery is a highly muscular vessel but contains cells other than smooth muscle cells such as endothelial cells and fibroblasts. Consequently, our measurements of β-adrenergic receptors and cAMP accumulation reflect a mixture of cell populations. However, the absence of changes in these parameters in the vessels from epinephrine-treated rats strongly suggests that there were actually no changes in the smooth muscle cells.

Relatively little is known regarding properties of β-adrenergic binding sites and cAMP production of the blood vessels, even though these are important for the regulation of vascular reactivity to catecholamines. We have chosen to characterize β receptor responses in the mesenteric artery rather than the more typically used rat aorta because the mesenteric artery is much more muscular, highly reactive, and richly innervated than a fibroelastic artery such as the aorta; thus, it is a desirable model to study mechanisms controlling vascular reactivity directly in muscular arteries that contribute to vascular resistance. We find that [^{125}I]CYP is a useful antagonist radioligand for characterization and quantitation of vascular β-adrenergic receptors. The limited previous reports on radioligand binding to vascular β-adrenergic receptors have yielded much less complete information about these receptors (21, 22). [^{125}I]CYP has a number of advantages for such studies. Compared to tritiated compounds ([^{3}H]dihydroalprenolol), it has a very high specific activity (≃2000 vs. 20 ~ 60 Ci/mmol) and is more selective than [^{125}I]hydroxybenzylpindolol, which has been reported to have significant affinity for α adrenoceptors (23) and serotonin receptors (24). The high specific activity of [^{125}I]CYP permitted the measurement of β receptors in individual arteries without requiring the use of pooled vessels from several animals.

The [^{125}I]CYP binding satisfied common criteria applied to receptor identification for the particulate fraction from individual rat mesenteric arteries: it was saturable, of high affinity, rapid, stereospecific, and of the pharmacological specificity of β_2 receptors. Consequently, [^{125}I]CYP should prove to be a useful tool for the measurement of vascular β-adrenergic receptors. Also, our results with [^{125}I]CYP are in good agreement with the experiments measuring cAMP production in the present studies and also classical pharmacological determinations of mesenteric artery relaxation (25).

An alteration in the sensitivity of blood vessels to β-adrenergic agonists has been observed in a number of physiological and disease states including estrogen treatment (26), thyroid treatment (27, 28), diabetes mellitus (29), aging (8, 9), and hypertension (10). The mechanisms for the altered vascular relaxation in these situations are uncertain. Cohen and Berkowitz have shown that isoproterenol and cyclic nucleotides exert diminished aortic relaxation with age (9) and hypertensive state (10). Their pharmacological findings that the decreased rat aortic relaxation which occurred with isoproterenol and cyclic nucleotides suggest possible analogies with our results involving prolonged catecholamine-stimulation of the vasculature. [^{125}I]CYP should be helpful in determining the possible role of β-adrenergic receptor regulation in these situations.

Our results suggest that the desensitization of vascular smooth muscle to β-adrenergic agonists after epinephrine treatment is not mediated by alteration in the β receptors or cAMP production. Consequently, acetylcholine and nitroglycerine were used as alternative vasodilators to determine if a nonspecific process was mediating the desensitization. It has been suggested that the vasodilators such as acetylcholine and nitroglycerine function by increasing the formation of cGMP in vascular smooth muscle (30, 31). Acetylcholine causes relaxation in an endothelial cell-dependent fashion rather than by a direct interaction with vascular smooth muscle cells. Neither of them modify cAMP concentrations (30). We found that the responses to both acetylcholine and nitroglycerine were intact in the mesenteric arteries from epinephrine-treated rats, indicating specificity of the desensitization for cAMP-dependent processes rather than those involving other intracellular nucleotides such as cGMP. This conclusion is also supported by our observation that responsiveness of the desensitized vessels to dibutyryl cAMP is reduced. While our data showed a decreased relaxation in response to dibutyryl cAMP in the mesenteric artery from epinephrine-infused rats, this finding should be interpreted with some caution principally because it is necessary to express the data in terms of relaxation rate rather than a straightforward maximal response. Also, isoproterenol and dibutyryl cAMP-induced vascular relaxation may not be identical since they are known to induce somewhat different protein phosphorylation profiles (32). Nevertheless, the data taken together support the hypothesis that the mechanism of reduced vascular relaxation ability subsequent to *in vivo* epinephrine infusion may be cAMP-specific and due to modification of a step distal to cAMP formation. Whether the lesion is at the level of cAMP-dependent protein kinase or is due to an alteration in its substrates requires further testing.

Our results indicate the complexity and diversity of mechanisms which may mediate desensitization to excessive β-adrenergic stimulation in various tissues. Not only may desensitization occur through β receptor down-regulation but also mechanisms distal to cAMP production may be involved in certain cells. Vascular smooth muscle may be a useful model system to pursue further these less well understood possibilities.

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