0026-895X/90/050725-12$03.00/0

MOLECULAR PHARMACOLOGY, 38:725-736

Two Pharmacologically Distinct α_1-Adrenoceptor Subtypes in the Contraction of Rabbit Aorta: Each Subtype Couples with a Different Ca^{2+} Signalling Mechanism and Plays a Different Physiological Role

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Received April 10, 1990; Accepted August 24, 1990

SUMMARY

Using the α_1-adrenoceptor subtype-selective antagonists chlorethylclonidine (CEC), WB4101, and 5-methyl-urapidil, we have examined the possible heterogeneity in the α_1-adrenoceptor populations in rabbit aorta. The α_{1b}-adrenoceptor alkylating agent CEC selectively inhibited the phasic component of the norepinephrine-induced contractile response, with little effect on the tonic component. The α_1-adrenoceptor occupancy-response relationship defined by the phenoxybenzamine inactivation method was rectangular hyperbolic for the tonic response, whereas that for the phasic response was linear, indicating the different degree of receptor reserve for the two responses. Radioligand binding studies with the nonselective α_1-adrenoceptor antagonist radioligand ^{125}I-BE2254 showed that 73–87% of the binding sites in rabbit aorta are CEC sensitive and they are predominantly low affinity sites both for WB4101 (pK_d = 8.1) and for 5-methyl-urapidil (pK_d = 7.1). Moreover, α_1-adrenoceptor-mediated phosphatidylinositol (PI) hydrolysis was CEC sensitive, and fractional inactivation of α_1 receptors with CEC showed equivalent increments in the reduction of PI hydrolysis and phasic contractile response, suggesting that both responses are linearly related to the CEC-sensitive receptor sites. The Schild plots for the competitive antagonists WB4101 and 5-methyl-urapidil against α_{1a}-adrenoceptor-selective agonist methoxamine-induced contraction were linear and had slopes not significantly different from unity, with a pA_2 of 9.07 ± 0.07 (n = 5) for WB4101 and 9.09 ± 0.05 (n = 3) for 5-methyl-urapidil. However, the Schild plots for these antagonists against norepinephrine were curvilinear. Computer-assisted analysis of these curvilinear Schild plots in a two-receptor system indicated that α_1-adrenoceptor populations responsible for the constrictive response are predominantly (~80–90%) low affinity sites for the two antagonists (pK_d ~8.1 for WB4101 and pK_d ~7.1 for 5-methyl-urapidil) and a small population (~10–20%) are high affinity sites (pK_d ~9.1 for both WB4101 and 5-methyl-urapidil), which was in good agreement with radioligand binding studies. The results indicate that rabbit aorta contains at least two pharmacologically distinct α_1-adrenoceptor subtypes, with approximately ~10–20% α_{1a} and ~80–90% α_{1b} receptors; however, a minor population of α_{1a} receptors rather than α_{1b} receptors predominantly mediate catecholamine-induced contraction because of their large receptor reserve. Each receptor subtype has a distinct role in the α_1-adrenoceptor-mediated vasoconstrictive response through different biochemical mechanisms for increasing intracellular Ca^{2+}; α_{1a} receptors cause a tonic response predominantly dependent on the influx of extracellular Ca^{2+}, whereas α_{1b} receptors stimulate PI hydrolysis/intracellular Ca^{2+} mobilization and cause a phasic response mainly independent of extracellular Ca^{2+}.

The sympathetic nervous system plays an important role in regulating the tone of the peripheral circulation. Catecholamines cause vascular smooth muscle contraction by activating α-adrenoceptors. It has now been clearly demonstrated that postsynaptic α-adrenoceptors in the peripheral circulation represent a mixed population of α_1- and α_2-adrenoceptors, with both receptors mediating vasoconstriction (1–3). Recent evidence, however, increasingly suggests that α_1-adrenoceptors do not have the same properties in all tissues (4–14).

The existence of distinct subtypes of α_1-adrenoceptors has been supported by a variety of pharmacological approaches (8–14). Recent evidence from radioligand binding experiments suggests that at least two subtypes of α_1-adrenoceptors can be differentiated by the competitive antagonists WB4101 (8) and 5-methyl-urapidil (13) and the alkylating agent CEC (9, 11); one subtype (the "α_{1a}" subtype) has higher affinities for WB4101 (pK_d ~9) and 5-methyl-urapidil (pK_d ~9) and is not inactivated by CEC ("CEC-insensitive"), whereas the other one

ABBREVIATIONS: CEC, chlorethylclonidine; ^{125}I-BE, ^{125}I-BE2254, ^{125}I-(2-β-(4-hydroxyphenyl)ethylaminomethyl)-tetralone; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; PI, phosphoinositide; IP, inositol 1-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; WB4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

(the "α_{1b}" subtype) has lower affinities for WB4101 (pK_d ~8) and 5-methyl-urapidil (pK_d ~7) and is inactivated by CEC ("CEC-sensitive"). These two receptor subtypes also have different affinities for the competitive antagonist benoxathian (10). Furthermore, the hypothesis that the subtypes of α_1-adrenoceptors may be coupled to different biochemical mechanisms for increasing $[Ca^{2+}]_c$ has been recently put forth (10); thus, one subtype (CEC-sensitive α_1-adrenoceptors, or α_{1b} receptors) stimulates inositol phosphate formation/intracellular Ca^{2+} mobilization and causes a contractile response mainly independent of extracellular Ca^{2+}, whereas the other (CEC-insensitive α_1 adrenoceptors, or α_{1a}) receptors do not stimulate inositol formation and cause physiological responses predominantly dependent on the influx of extracellular Ca^{2+}. However, very little information is available regarding their physiological role and signal transduction mechanism(s) in blood vessels.

In blood vessels, the contractile response to α_1-adrenergic stimulation can be divided into two components, the initial fast phasic response and the later slow tonic response (15). Each phase of contraction has a different dependence on extracellular Ca^{2+}, although the relative sizes of these phasic and tonic components of contraction vary with the muscle studied and also with the contractile stimulus (16). In rabbit aorta, rapid phasic contractions caused by norepinephrine are not dependent on the presence of extracellular Ca^{2+} and are mainly caused by Ca^{2+} release from internal stores, whereas slow tonic contractions require Ca^{2+} influx from the extracellular fluid for prolonged signals (17, 18). Based on the hypothesis described above, the simplest explanation that may account for the different dependence on extracellular Ca^{2+} of tonic and phasic contractile responses to α_1-adrenoceptor activation would be that they are mediated by pharmacologically distinct receptor subtypes.

The present study was undertaken to determine whether heterogeneous populations of α_1-adrenoceptors exist and what kind of physiological role they play in the contractile responses of rabbit aorta, an experimental model system for investigating vascular α_1-adrenoceptors. The data reveal that at least two pharmacologically distinct α_1-adrenoceptor subtypes coexist in rabbit aorta and that each subtype plays a distinct physiological role in the α_1-receptor-mediated vasoconstrictive response through different Ca^{2+} signalling mechanisms.

Experimental Procedures

Materials. Chemicals were obtained from the following sources: $LaCl_3$, (−)-norepinephrine bitartrate, *l*-phenylephrine HCl, (±)-propranolol HCl, yohimbine chloride, serotonin maleate salt, and histamine dihydrochloride, Sigma Chemical Co. (St. Louis, MO); CEC dihydrochloride, WB4101, and benoxathian hydrochloride, Research Biochemicals (Natick, MA); 5-methyl-urapidil, BYK Gulden Pharmazeutika (Konstanz, West Germany); $PGF_{2\alpha}$, Wako Pure Chemical Industries Ltd. (Osaka, Japan); and ^{125}I-BE (specific activity, ~2200 Ci/mmol) and *myo*-[2-^{3}H]inositol (16.5 Ci/mmol), New England Nuclear (Boston, MA). Dowex AG1-X8, 200–400 mesh, formate form, was obtained from Bio-Rad (Richmond, CA). Methoxamine (Burroughs Wellcome, Research Triangle Park, NC), prazosin (Pfizer, Sandwich, UK), phentolamine (CIBA-Geigy, Summit, NJ), nicardipine hydrochloride (Yamanouchi Pharmaceutical Co., Tokyo, Japan), and phenoxybenzamine hydrochloride (Smith Kline & French Laboratories, Philadelphia, PA) were generously supplied by each company. All other chemicals and reagents used were from standard commercial sources. Phenoxybenzamine solution was prepared by dilution into saline of a 1 mM stock solution prepared in absolute ethanol immediately before use.

Rabbit aorta preparation. Japanese White rabbits weighing 2.5–3.0 kg were injected with 1000 units of heparin subcutaneously and were sacrificed 15 to 30 min later by a blow to the head. The thoracic aortas were rapidly excised and placed in 95% O_2/5% CO_2 gassed Krebs-Henseleit solution (pH 7.4). The composition of this buffer was (in mM): NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; $CaCl_2$, 2.5; $MgSO_4$, 1.0; KH_2PO_4, 1.2; and dextrose, 11.1. While being maintained at room temperature in this buffer, adherent fatty and connective tissues were removed. Individual vessels were cut into approximately 6-mm-long ring segments, and their intimal surfaces were gently rubbed with a wooden stick to remove the endothelium (19). Ring segments were then used for organ bath experiments, measurement of PI hydrolysis, and radioligand binding studies. In all cases, Krebs-Henseleit buffer contained cocaine (3 μM) and propranolol (1 μM) to inhibit neural uptake and block β-adrenoceptors, respectively. In experiments with catecholamines, *l*-ascorbic acid (1%) was also included to inhibit oxidation.

Contractile response. The aortic ring segments were mounted over metal triangular supports and attached to force displacement transducers (DRM-T200; DIA Medical Co.), which were connected to a recorder (RJG-4124; Nihon Kohden Kogyo). The tissues were allowed to equilibrate for at least 1 hr, during which time the solutions were replaced three times every 20 min under a resting tension of 2.0 g (19), which was found to be optimal for inducing the maximal contraction from preliminary experiments. After an equilibrium period, the contractile response to 0.1 μM norepinephrine was first obtained. This procedure was found to improve tissue responsiveness (20). The rings were then washed repeatedly with fresh medium and equilibrated. Dose-response curves were constructed by the method of stepwise cumulative addition of the agonist. The concentration of agonist in the muscle chamber was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. When the maximum contraction to the agonist was reached, the agonist was washed from the preparation by the overflow method every 15 min for the next 120 min. Consecutive dose-response curves were always spaced at least 1 hr apart to ensure maximum washout of agonists and to minimize receptor desensitization. After determination of the control dose-response curve to the agonist, rabbit aortic segments were washed with drug-free medium for 20 min. After this period, the competitive antagonist was added to the organ bath and allowed to equilibrate with the tissue for no less than 60 min. After equilibration with the antagonist, the dose-response curve for the agonist was again determined. This procedure was repeated in some tissues with a higher concentration of the antagonist.

For experiments with Ca^{2+}-free solution, ring segments were incubated in the Ca^{2+}-free Krebs-Henseleit solution including 0.1 mM EGTA for 20 min before norepinephrine addition (21). In preliminary experiments, we observed that the maximal developed tension for norepinephrine obtained in a cumulative method was significantly smaller than that obtained by administering increasing doses individually, possibly due to the depletion of Ca^{2+} stores. Therefore, norepinephrine dose-response curves in Ca^{2+}-free solution were constructed by administrating doses of norepinephrine individually at increasing concentrations. This procedure was repeated with a higher concentration of norepinephrine after washing with Ca^{2+}-containing solution five times and equilibration for 60 min in 2.5 mM Ca^{2+}-containing solution to replenish Ca^{2+} stores.

In all studies, at least one tissue was run in parallel with the experimental tissues, but received no antagonists, and was used to correct for time-dependent changes in agonist sensitivity (22). Agonist potency was expressed as an EC_{50} (concentration producing 50% of maximum contraction), and effects of competitive antagonists on the EC_{50} for each agonist were examined.

Measurement of [^{3}H]PI hydrolysis. For experiments involving PI hydrolysis, rabbit aortic ring segments were exposed to 37° Krebs-

Henseleit buffer supplemented with *myo*-[2-^{3}H]inositol (8 μCi/ml), which was continuously gassed with 95% O_2/5% CO_2. This prelabeling incubation was continued for 180 min, by which time the incorporation of ^{3}H into inositol lipids had reached a plateau (12). The tissues were subsequently washed three times to remove excess [^{3}H]inositol and then resuspended in the Krebs-Henseleit buffer described above but without added [^{3}H]inositol. After 10 min of preincubation, tissues were transferred to flasks containing fresh Krebs-Henseleit solution and exposed to norepinephrine in the presence of LiCl (10 mM) (23), an IP phosphatase inhibitor, for 1 hr to prevent IP degradation. Some tissues were treated with CEC (four times, 20 min each) or exposed to the Ca^{2+} channel antagonist nicardipine for 60 min before addition of LiCl and norepinephrine. Additional tissues were stimulated by norepinephrine and LiCl for 1 hr in either Ca^{2+}-free Krebs-Henseleit solution containing 0.1 mM EGTA or La (5 mM)-containing Ca^{2+}-free Krebs-Henseleit solution. In experiments with La^{3+} (5 mM), PO_4^{3-} and SO_4^{2-} were replaced with Cl^- to avoid precipitation of La^{3+} salt.

Tissues were then frozen between clamps precooled in liquid nitrogen and were homogenized in 2 ml of 10% trichloroacetic acid at 4°. After centrifugation (low speed, 30 min) the supernatant was extracted six times with 2-ml portions of diethyl ether to remove trichloroacetic acid. The neutralized extracts were then subjected to ion exchange chromatography using 1 ml of Dowex AG1-X8 (formate form) ion exchange resin. The columns were then washed with 60 ml of water and IP was eluted with 8 ml of 0.2 M ammonium formate in 0.1 M formic acid. Scintillation cocktail (10 ml) was added to the elute and the samples were counted. Recoveries of IP throughout the ether extraction and column chromatography procedures was 80–90%. Our preliminary experiments confirmed that norepinephrine-induced accumulation of IP increased linearly with incubation time (up to 1 hr) and that norepinephrine increased IP accumulation in a concentration-dependent manner. Also, norepinephrine (10 μM)-induced IP accumulation was selectively inhibited by prazosin (1 μM) but not by yohimbine (1 μM) in rabbit aorta (data not shown).

^{125}I-BE binding. For the studies of α_1-adrenoceptors, crude particulate fractions were prepared as described previously (24), with modifications, and were used immediately in the receptor binding assays. Briefly, cleaned vessels were placed in 10 volumes of ice-cold buffer (10 mM Tris·HCl, pH 8.0 at 4°), minced finely with scissors, and then homogenized with a Polytron (Brinkman Instruments, Westbury, NY) by means of one 10-sec burst at a setting of 7 and then two 5-sec bursts at full speed. An equal volume of ice-cold buffer containing 500 mM sucrose, 10 mM Tris·HCl, and 2 mM EGTA (pH 7.5) was added and the homogenate was centrifuged at 250 × g for 10 min. The pellet was discarded and the supernatant was filtered through four layers of cheesecloth and centrifuged again at 250 × g for 10 min. The supernatant was then centrifuged at 75,000 × g for 30 min and the pellet was resuspended in ice-cold 50 mM Tris·HCl, 10 mM $MgCl_2$ buffer (pH 7.5). A final resuspension was filtered through four layers of cheesecloth and diluted to give a protein concentration of approximately 2 mg/ml.

Binding assays were routinely performed in polypropylene tubes in a total volume of 250 μl. Each assay tube contained 100 μl of membrane preparation (≅200 μg of protein), 100 μl of ^{125}I-BE (40–60 pM for competition experiments and 15–900 pM for saturation experiments), and 50 μl of a competing drug at various concentrations. Specific ^{125}I-BE binding was experimentally determined from the difference between counts bound in the absence and presence of 10 μM phentolamine. The receptor binding studies were conducted at 37° for 15 min. The reaction was terminated by addition of 10 ml of incubation buffer (at room temperature), followed by rapid filtration under vacuum through Whatman GF/C glass fiber filters, with a further wash with 10 ml of buffer. The radioactivity of the wet filters was determined in a γ-counter at an efficiency of 70%. All assays were conducted in duplicate or triplicate. Preliminary experiments on the specific binding of ^{125}I-BE in this crude particulate fraction showed appropriate kinetics, stereoselectivity, and rank order of potency of agonists characteristic of ligand binding to α_1-adrenoceptors, which were essentially similar to those described previously (24). The specific binding of ^{125}I-BE was routinely 70–90% of the total binding at radioligand concentrations near the dissociation constant (K_d).

Protein concentration was determined by the method of Lowry *et al.* (25), using bovine serum albumin as standard.

Fractional inactivation of α_1 receptors by phenoxybenzamine and by CEC. In some experiments to delineate the interrelationships between α_1-adrenoceptor occupation, PI hydrolysis, and contractile response in rabbit aorta, we examined the effects of phenoxybenzamine and CEC. In phenoxybenzamine inactivation experiments, aortic rings were exposed for 90 min to phenoxybenzamine (at final concentrations ranging from 0.1 to 10 nM) (12, 26–28). In experiments with CEC, preparations were repetitively treated with CEC (at final concentrations ranging from 0.1 to 100 μM; at least four times, 20 min each), because previous reports (11) and our preliminary experiments (as described below) showed that the incomplete access of the highly water-soluble CEC could be overcome by the repetitive treatments. After phenoxybenzamine and/or CEC pretreatment, aortic ring segments were washed five times and used for the contractile experiments, inositol phosphate studies, and radioligand binding assays. Also, in some radioligand binding experiments CEC treatment was performed on membrane preparations in hypotonic medium to gain complete inactivation (11). In these experiments, aliquots (usually 10 ml) of the resuspended membrane preparations were incubated with CEC for 10 min at 37° in Na HEPES buffer (10 mM, pH 7.6). Reactions were stopped by dilution with 20 ml of cold Na HEPES buffer, centrifugation at 75,000 × g for 30 min, and resuspension in ice-cold 50 mM Tris·HCl, 10 mM $MgCl_2$ buffer (pH 7.5). For receptor occupation, data are expressed as B/B_{max}, where B is the amount of ^{125}I-BE specifically bound under the experimental conditions and B_{max} is the amount of specific ^{125}I-BE binding in the absence of any phenoxybenzamine or CEC; for IP accumulation, as percentage of maximum response to norepinephrine (10 μM) in the absence of extracellular Ca^{2+}; and, for contractile measurement, as percentage of the maximal response obtained with 3 μM norepinephrine alone.

Calculation of α-adrenoceptor occupancy-response relationships. Data obtained from receptor inactivation studies were analyzed using the mathematical treatment of Furchgott and Bursztyn (29). According to the law of mass action, the relationship between the agonist curves obtained before and after partial receptor inactivation is described by the equation

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{1-q}{qK_A}$$

where $[A]$ and $[A']$ are corresponding equieffective concentrations of agonist before and after partial irreversible receptor inactivation, respectively, K_A is the equilibrium dissociation constant for the agonist, and q represents the fraction of active receptors remaining (i.e., receptors not alkylated) after partial irreversible receptor inactivation treatment (for derivation of this equation and its application to receptor theory, see Refs. 29–31). Plots of the reciprocals of norepinephrine concentration before fractional receptor inactivation (i.e., $1/[A]$) against the reciprocals of the corresponding equieffective concentrations of norepinephrine after receptor inactivation (i.e., $1/[A']$) yielded straight lines, as predicted by receptor theory (30, 31). The norepinephrine K_A as well as q were then calculated from the slope and intercept of the resulting "double-reciprocal" plots by the following equations.

$$K_A = \frac{\text{slope} - 1}{\text{intercept}}$$

and

$$q = \frac{1}{\text{slope}}$$

Data analysis. All data for each drug were averaged and the resulting dose-response curves from each group were analyzed simultaneously,

using the four-parameter logistic equation, on an APPLE IIe system (32). The resulting EC_{50} values and maximal responses (E_{max} values) were analyzed for significant differences using the ALLFIT program. The ALLFIT program is a modification of the program of DeLean and co-workers by Martin H. Teicher and was obtained from the Biomedical Computing Technology Information Center (Nashville, TN). Data derived from radioligand studies were analyzed by LIGAND, a nonlinear, computer-assisted, iterative, weighed least squares, curve-fitting procedure (33).

The experimental data given in the text and figures are the mean ± standard error of n experiments, as indicated. Differences between means within each experiment were evaluated by analysis of variance. If analysis of variance demonstrated a significant difference among means, Student's t test for unpaired observations were then used to determine which pairs of means were significantly different. Statistical significance was taken as a p value of <0.05.

Results

Effects of CEC on norepinephrine-induced vasoconstrictive response. Following four repetitive treatments (20 min each) with various concentrations of CEC, the norepinephrine-induced contractile response was examined both in the presence and in the absence of extracellular Ca^{2+}. In Ca^{2+}-free solution (Fig. 1, *left*), the CEC treatment (above 1 μM) reduced not only the peak response but also the subsequent maintained phase of norepinephrine (1 μM)-induced contraction, in a dose-dependent fashion. On the other hand, in the presence of 2.5 mM extracellular Ca^{2+}, the CEC treatment (0.1 to 10 μM) had little inhibitory effect on the contraction to 1 μM norepinephrine; however, higher concentrations of CEC treatment (above 30 μM) decreased the slope of the upstroke of the contractile response and reduced the peak developed tension (Fig. 1, *right*). In the absence of extracellular Ca^{2+}, the effects of CEC were examined not only on the maximum developed tension but also on the developed tension during the subsequent sustained phase (15 min after addition of norepinephrine). A period of fifteen minutes was decided upon because at that time the sustained response usually reached an equilibrium phase. As summarized in Fig. 2, the inhibitory effect of CEC treatment (1 to 10 μM) on the maximum developed tension (Fig. 2A) was essentially similar to that on the subsequent maintained phase (Fig. 2B); thus, in either case, the inhibitory effect of CEC was more marked in Ca^{2+}-free solution than in Ca^{2+}-containing solution.

The effects of CEC on contractile responses to other vasoactive stimuli were also examined, because CEC may have other inhibitory effects than those relevant to α_1-receptor inactivation. As summarized in Fig. 3, CEC treatment at concentrations

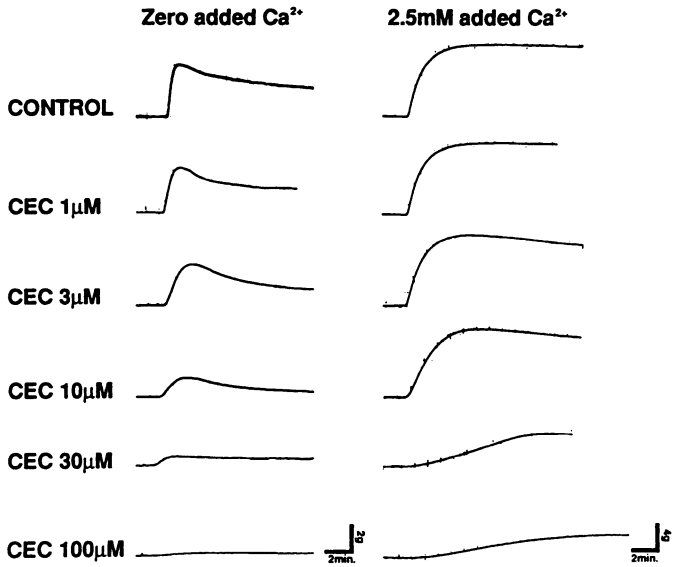

Fig. 1. Effects of CEC pretreatment (1 to 100 μM) on 1 μM norepinephrine-induced contractile responses of rabbit aorta in the presence (*right*) or the absence (*left*) of extracellular Ca^{2+}. CEC pretreatment consists of four successive 20-min exposures to the concentration indicated. The recording shown is a typical one from at least five independent experiments with similar results.

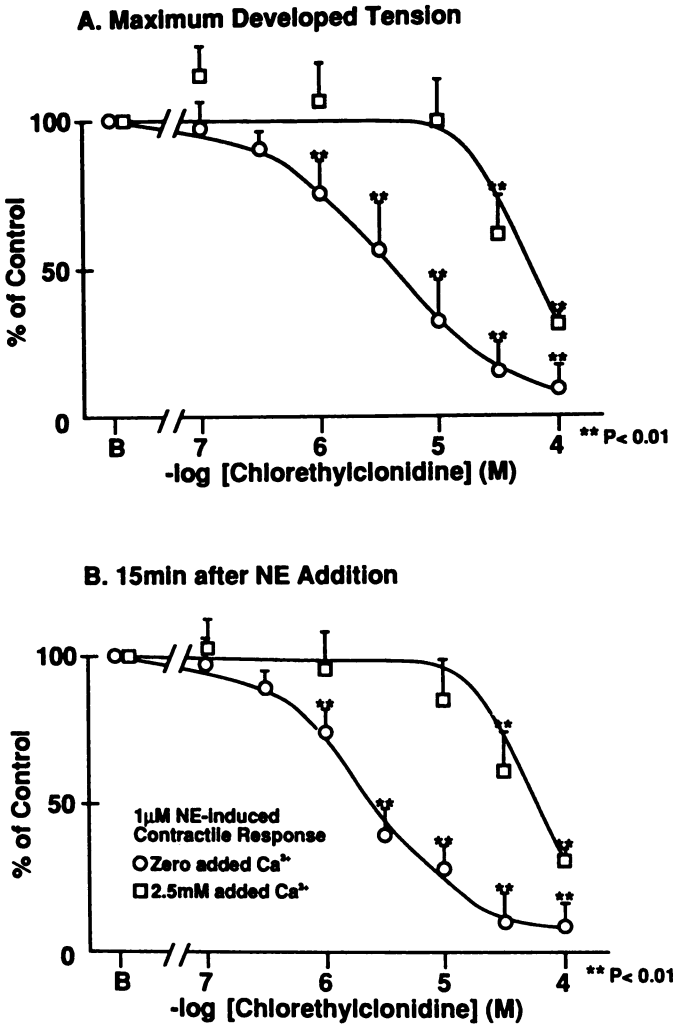

Fig. 2. A, Effects of CEC pretreatment (0.1 to 100 μM) on 1 μM norepinephrine-induced contractile responses of rabbit aorta, in the presence (□) or absence (○) of extracellular Ca^{2+}, at the peak of phasic contraction. Values are expressed as percentage of the maximal developed tension to 1 μM norepinephrine: 10.3 ± 0.45 g, $n = 5$, in the presence of extracellular Ca^{2+} and 3.6 ± 0.92 g. $n = 5$, in the absence of extracellular Ca^{2+}, respectively. *Points* and *bars*, mean ± standard error of at least five independent experiments. B, Effects of CEC pretreatment (0.1 to 100 μM) on 1 μM norepinephrine (*NE*)-induced contractile responses of rabbit aorta, in the presence (□) or absence (○) of extracellular Ca^{2+}, at 15 min after NE addition. This poststimulated time for measurement of developed tension was decided upon because at that time the phasic response usually reached the subsequent sustained phase. Values are expressed as percentage of the maximal developed tension to 1 μM norepinephrine: 10.3 ± 0.45 g, $n = 5$, in the presence of extracellular Ca^{2+} and 2.3 ± 0.58 g, $n = 5$, in the absence of extracellular Ca^{2+}, respectively. *Points* and *bars*, mean ± standard error of at least five independent experiments. ** $p < 0.01$, compared with control values of each group.

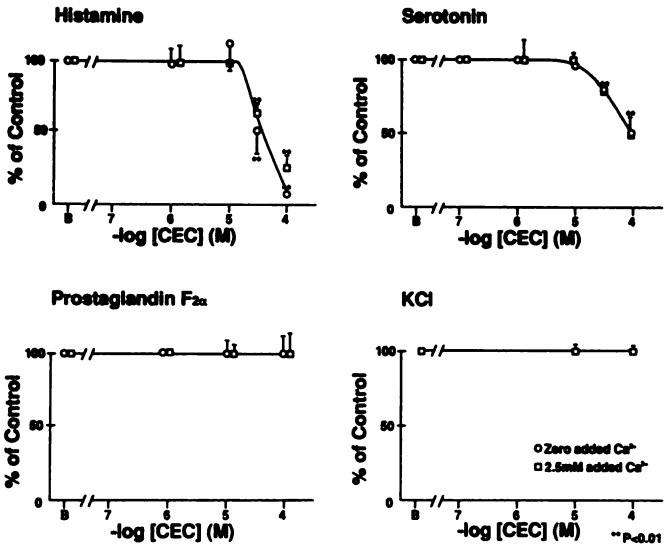

Fig. 3. Effects of CEC pretreatment (0.1 to 100 μM) on the contraction of rabbit aorta in response to histamine (10 μM), serotonin (100 μM), $PGF_{2\alpha}$ (10 μM), and KCl (40 mM), in the presence (□) or the absence (○) of extracellular Ca^{2+}. Values are expressed as percentage of the maximal developed tension for each agonist: 10 μM histamine, 5.0 ± 1.0 g and 2.0 ± 0.61 g, n = 5 each; 100 μM serotonin, 9.8 ± 0.6 g and 2.0 ± 0.2 g, n = 5 each; 10 μM $PGF_{2\alpha}$, 9.8 ± 0.6 g and 6.8 ± 0.6 g, n = 5 each, in the presence and absence of extracellular Ca^{2+}, respectively, and 40 mM KCl, 11.2 ± 0.86 g, n = 5, in the presence of extracellular Ca^{2+}. *Points* and *bars*, mean ± standard error of five independent experiments. **, $p < 0.01$, compared with control values of each group.

below 10 μM generally did not affect the contractions induced by serotonin (100 μM) or histamine (10 μM), in either the presence or the absence of extracellular Ca^{2+}. With higher concentrations of CEC treatment (above 30 μM), contractile responses to these vasoactive amines were significantly ($p <$ 0.05) attenuated in both the presence and the absence of extracellular Ca^{2+}. $PGF_{2\alpha}$ (10 μM)- and KCl (40 mM)-induced contractions were not affected at any concentration of CEC treatment examined (Fig. 3).

α_1-Adrenoceptor occupancy-response relationships defined by the phenoxybenzamine inactivation methods. The effects of progressive alkylation of α_1-adrenoceptors by phenoxybenzamine on the contractile responses elicited by norepinephrine were examined in either the presence or absence of extracellular Ca^{2+} (Fig. 4). Treatment with phenoxybenzamine produces the characteristic pattern of irreversible receptor alkylation in the response to norepinephrine under all conditions, with nonparallel rightward displacements of the dose-response curves and progressive reductions in the maximum developed tension (30, 31). Although the overall shapes of the dose-response curves to norepinephrine are similar after treatment with phenoxybenzamine in either the presence (Fig. 4A) or absence of extracellular Ca^{2+} (Fig. 4B), some marked and important distinctions may be discerned. It is clear from Fig. 4A that in the presence of extracellular Ca^{2+} the α_1-adrenoceptor-mediated dose-response curves for norepinephrine following pretreatment with lower doses of phenoxybenzamine (below 5 nM) are significantly displaced in a rightward direction without significant reductions in the maximum contractile response. Such an occurrence is characteristic of systems possessing large receptor reserves (30, 31). In contrast, in Ca^{2+}-free buffer blockade by phenoxybenzamine produces a significant reduction in maximum response to norepinephrine,

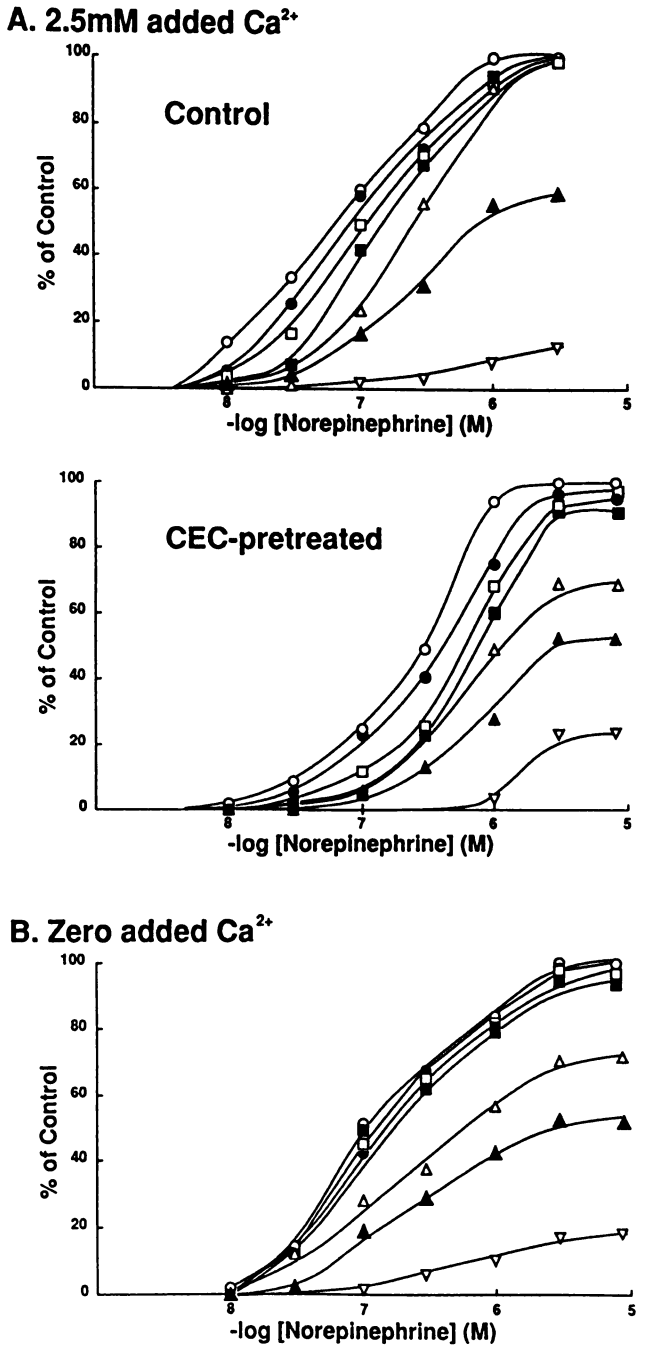

Fig. 4. Dose-response curves for the α_1-adrenoceptor-mediated contraction of norepinephrine, in either the presence (A) and absence (B) of extracellular Ca^{2+} following a 90-min incubation with various concentrations of the irreversible α-adrenoceptor antagonist phenoxybenzamine. The inactivation effects of phenoxybenzamine on contractile responses in the presence of extracellular Ca^{2+} were also examined following CEC (10 μM) pretreatment. Values are expressed as percentage of the control contractile response to 3 μM norepinephrine: 11.9 ± 0.5 g, n = 5 (control), and 12.2 ± 2.9 g, n = 5 (CEC-pretreated), in the presence of 2.5 mM extracellular Ca^{2+}, and 4.5 ± 1.2 g, n = 5, in Ca^{2+}-free solution. *Symbols* represent mean values (n = 5) of control (○) and those obtained following 0.1 nM (●), 1 nM (□), 2 nM (■), 5 nM (△), 10 nM (▲), and 30 nM (▽) phenoxybenzamine pretreatment, respectively.

without any initial rightward shift (Fig. 4B). This pattern is characteristic of systems lacking appreciable receptor reserves (30, 31). Furthermore, the inactivation effects of phenoxybenzamine on norepinephrine dose-response curves were examined

following the repetitive CEC (10 μM) pretreatment. In Ca^{2+}-containing buffer, the doses of phenoxybenzamine required to reduce the maximum response to norepinephrine became smaller (Fig. 4A), but still lower doses of phenoxybenzamine (less than 2 nM) are significantly displaced in a rightward direction without significant reductions in the maximum contractile response.

Based on the theoretical arguments and the graphical analysis described in Experimental Procedures, it is possible to determine the fraction of receptors remaining intact (i.e., q, the fraction of receptors not alkylated) following a given pretreatment with phenoxybenzamine. The analysis was performed on all the dose-response curves in the three groups of Fig. 4. The double-reciprocal plots of equieffective doses of norepinephrine obtained before and after phenoxybenzamine treatment are all linear (data not shown), in accord with receptor theory. From the slope and intercept of the double-reciprocal plots, the dissociation constants (K_A) of norepinephrine obtained in the presence of Ca^{2+} were 0.42 ± 0.17 μM (n = 5) and 1.45 ± 0.42 μM (n = 5) without and with CEC pretreatment, respectively. In the absence of extracellular Ca^{2+}, the value was 0.32 ± 0.10 μM (n = 5). Using the dissociation constant of norepinephrine obtained in each group, it is possible to calculate receptor occupancy at each bath concentration used in construction of the respective dose-response curves in Fig. 4. The respective occupancy-response relationships constructed for the α_1-adrenoceptor-mediated effects of norepinephrine are presented in Fig. 5. Correlation of fractional inhibition of maximal contraction responses (E_{max}) with the fraction of the initial receptor concentration remaining (q) approximates a nonlinear relationship in the presence of extracellular Ca^{2+}, whereas the relationship approaches linearity in Ca^{2+}-free buffer. The CEC pretreatment suppressed E_{max} in a range of lower q values (below 0.4), compared with suppression obtained without CEC treatment (Fig. 5A).

The abscissa in Fig. 5A has been transformed to a logarithmic scale (Fig. 5B), following conversion to the percentage of receptors not alkylated by phenoxybenzamine ($q \times 100$). This transformation has been shown to make hyperbolic and linear occupancy-response relationships parallel for the sake of comparison (31, 34). From the resulting curves presented in Fig. 5B, it is apparent that the difference in the relationships between maximum contractile response and the percentage of receptors remaining intact after phenoxybenzamine treatment (receptors that are free to interact with agonists) is approximately 50-fold more favorable for the CEC-insensitive α_1-adrenceptor-mediated response than for the CEC-sensitive α_1-adrenoceptor-mediated response in this model.

^{125}I-BE binding sites of rabbit aorta. We first examined the inactivation effect of CEC on the α_1-adrenoceptor binding sites. Following four times of repetitive treatment of rabbit aortic rings with 10 μM CEC (20 min each) in normal Krebs' solution, a condition which was the same as we used in the contractile experiments, we observed that this treatment caused a 73.2 ± 1.5% loss (n = 3) of ^{125}I-BE binding sites in saturation experiments (B_{max} = 14.0 ± 0.90 fmol/mg of protein, n = 3, in rabbit aorta unexposed to CEC versus 3.75 ± 0.45 fmol/mg of protein, n = 3, after repetitive CEC treatment, $p < 0.01$). Similarly, 10 μM CEC treatment of the membrane preparation under hypotonic conditions (10 mM Na HEPES, pH 7.6) (11) caused a 87.0 ± 9.2% loss (n = 3) of ^{125}I-BE binding sites (B_{max} = 16.9 ± 4.1 fmol/mg of protein, n = 3, in rabbit aorta membrane preparations unexposed to CEC versus 2.2 ± 1.1 fmol/mg of protein, n = 3, after CEC treatment, $p < 0.01$). The K_d values were 238 ± 77 pM (n = 3) in rabbit aorta membrane preparations unexposed to CEC and 200 ± 29 pM (n = 3) after treatment under hypotonic conditions.

Next we examined displacements of specific ^{125}I-BE binding by the competitive antagonists WB4101, benoxathian, and 5-methyl-urapidil; these agents had been reported to have different affinities for α_1-adrenoceptor subtypes (4, 8, 10, 13, 14). The Hill coefficients for these drugs were 1.00 ± 0.11 (n = 5) for WB4101, 1.08 ± 0.10 (n = 3) for benoxathian, and 0.75 ± 0.07 (n = 3) for 5-methyl-urapidil, and LIGAND analysis of the competition curves for these drugs showed no significant difference between a one-site fit and a two-site fit. K_I values were calculated as 7.76 ± 1.60 nM (n = 5) for WB4101, 35.5 ± 19.3 nM (n = 3) for benoxathian, and 83.3 ± 5.40 nM (n = 3) for 5-methyl-urapidil (Fig. 6). Additionally, the affinities of prazosin and phentolamine for ^{125}I-BE binding sites were examined, because the ratio of phentolamine versus prazosin was also useful in differentiating α_1-adrenoceptor subtype (8). LIGAND analysis of both prazosin and phentolamine competition curves showed no significant difference between a one-site fit

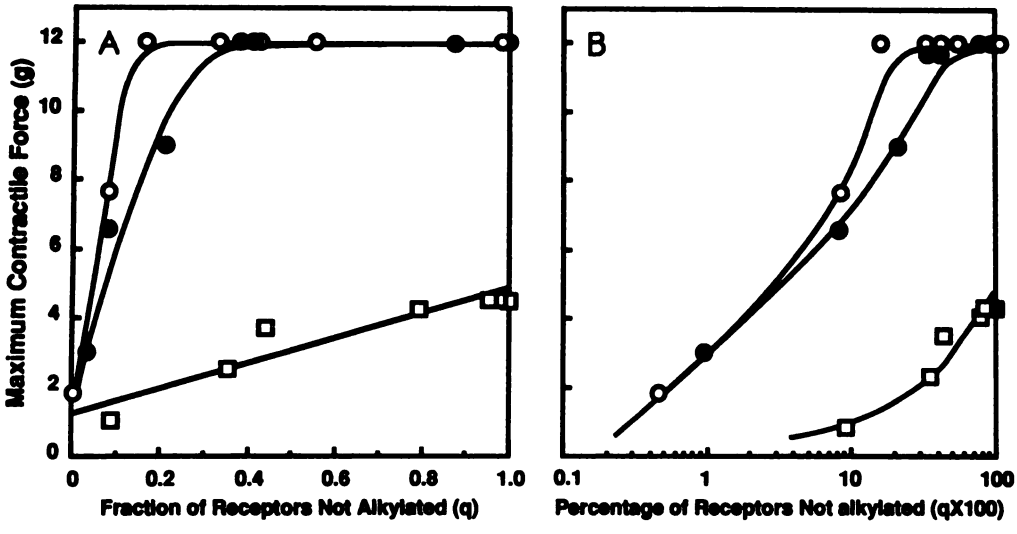

Fig. 5. A, Relationship between maximum contractile response and the fraction of α_1-adrenoceptors not alkylated by the irreversible α-adrenoceptor antagonist phenoxybenzamine. The results were determined using the mathematical treatment of Furchgott and Bursztyn (29), as described in Experimental Procedures, from the dose-response curves of Fig. 4. *Symbols* represent values obtained in controls (○) and after CEC pretreatment in the presence of Ca^{2+} (●) (Fig. 4A) and in the absence of Ca^{2+} (□) (Fig. 4B). B, The results replotted from A as a function of the logarithm of the percentage of α_1-adrenoceptors remaining intact [log ($q \times 100$)] after treatment with phenoxybenzamine.

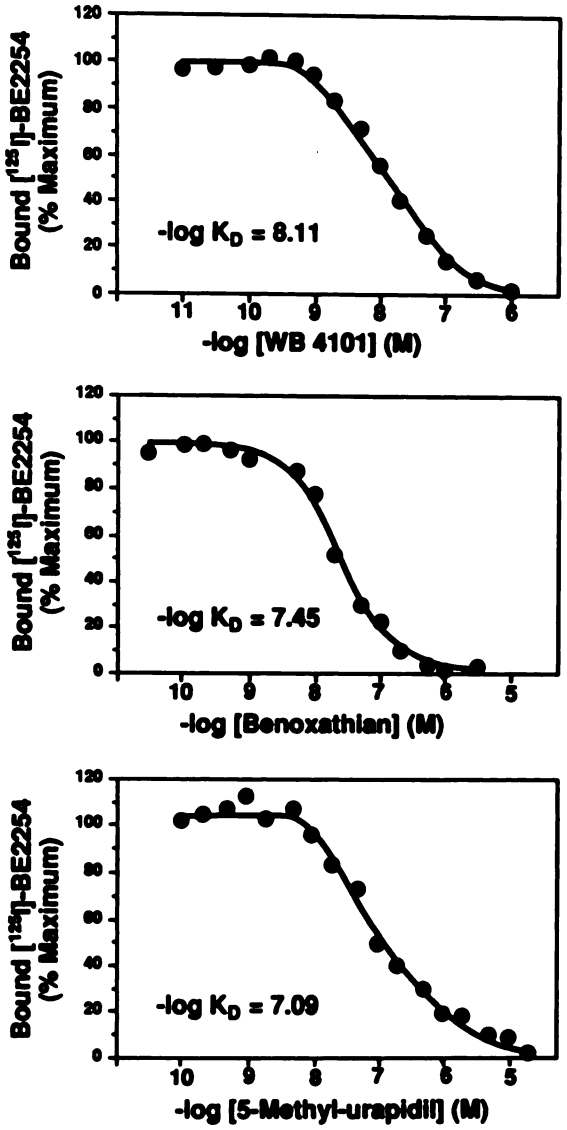

Fig. 6. Inhibition of specific ^{125}I-BE binding by the competitive antagonists WB4101 (*upper*), benoxathian (*middle*), and 5-methyl-urapidil (*lower*) in rabbit aorta membrane preparations. Specific receptor binding was defined as binding displaced by 10 μM phentolamine. Data are plotted as percentage of specific binding remaining in the presence of the indicated concentrations (M) of antagonist. Each value is the mean of data from five WB4101, three benoxathian, and three 5-methyl-urapidil experiments, performed in duplicate.

and a two-site fit. The K_I values were 1.2 ± 0.2 nM (n = 4) for prazosin and 26 ± 5.3 nM (n = 4) for phentolamine.

Effect of α_1-adrenoceptor inactivation with CEC on ^{125}I-BE binding, norepinephrine-stimulated IP formation, and phasic contraction. Because the CEC inactivation condition we used in previous experiments (12) was found to be incomplete, we reexamined the relationships between each α_1-adrenoceptor subtype and PI hydrolysis in rabbit aorta, using the four-time repetitive treatment (20 min each) with CEC. As summarized in Table 1, norepinephrine (10 μM) in the presence of 2.5 mM extracellular Ca^{2+} markedly stimulated IP production (1860 ± 155 cpm/mg of protein). Following the repetitive treatment with CEC (10 μM), the basal level of IP accumulation significantly ($p < 0.05$) increased; however, the CEC treatment suppressed norepinephrine-stimulated IP accumulation (69% decrease) (Table 1). In the absence of extracellular Ca^{2+}, norepinephrine-induced IP formation was markedly reduced (76% decrease), and CEC (10 μM) treatment further reduced the remaining IP accumulation response (Table 1). The Ca^{2+} entry required for IP production in rabbit aorta was further characterized with organic (nicardipine) and inorganic (La) Ca^{2+} entry blockers (Table 1). Treatment with nicardipine (1 μM) had little effect on norepinephrine-induced IP accumulation. With 5 mM La present in the buffer, on the other hand, norepinephrine-induced IP formation showed a marked reduction (about 77%), which was similar to the reduction obtained in the absence of Ca^{2+}. In addition, membrane depolarization by KCl (60 mM), which greatly stimulates Ca^{2+} influx-dependent contraction in rabbit aorta, produced no significant generation of IP (data not shown).

We next examined the effect of CEC inactivation on both ^{125}I-BE binding and norepinephrine-stimulated IP formation and compared them with the developed tension to norepinephrine in Ca^{2+}-free buffer (Fig. 7). Maximum ^{125}I-BE binding capacity was progressively decreased by exposure to increasing concentrations of CEC, reaching a minimal level of 1.8 ± 2.5% at a CEC concentration of 100 μM (n = 3). The CEC concentration necessary to block half of the ^{125}I-BE sites is 3.6 ± 1.0 μM (n = 3). To determine the effect of partial α_1 receptor inactivation on norepinephrine-stimulated IP formation, experiments were conducted both in the presence and in the absence of extracellular Ca^{2+}. Although norepinephrine-stimulated IP production was greatly enhanced in the Ca^{2+}-containing buffer, compared with that in the Ca^{2+}-free buffer (Table 1), data normalized to percentage of maximum response showed similar inhibitory responses of CEC both in the presence and in the absence of extracellular Ca^{2+}, with the IC_{50} values for norepinephrine-stimulated IP production being 3.0 ± 1.2 μM CEC (n = 5) and 3.7 ± 1.2 μM CEC (n = 5) in the presence and absence of extracellular Ca^{2+}, respectively. Thus, in Fig. 7, only the inhibitory effects of CEC obtained in the Ca^{2+}-free

TABLE 1

Effects of CEC (10 μM) pretreatment, nicardipine (1 μM)- and $LaCl_3$ (5 mM)-containing Krebs-Henseleit solution, and removal of extracellular Ca^{2+} on norepinephrine (10 μM)-induced accumulation of IP in rabbit aortas

Results are presented as the mean ± standard error of at least five different experiments.

Treatment	IP production
	cpm/mg of protein
Normal Krebs-Henseleit solution	
None	126 ± 17
Norepinephrine	1860 ± 155
CEC pretreatment	302 ± 32[a]
Norepinephrine + CEC pretreatment	579 ± 71[b]
Nicardipine	141 ± 28
Norepinephrine + nicardipine	1797 ± 208
La^{3+}-containing buffer	108 ± 18
Norepinephrine + La^{3+}-containing buffer	420 ± 57[b]
Ca^{2+}-free Krebs-Henseleit solution (containing 0.1 mM EGTA)	
None	81 ± 8[a]
Norepinephrine	455 ± 65[b]
CEC pretreatment	85 ± 10
Norepinephrine + CEC pretreatment	85 ± 17[c]

[a] $p < 0.05$, compared with corresponding basal IP production of control in the presence of extracellular Ca^{2+}.
[b] $p < 0.01$, compared with corresponding norepinephrine (10 μM)-induced IP production of control in the presence of extracellular Ca^{2+}.
[c] $p < 0.05$, compared with corresponding norepinephrine (10 μM)-induced IP production of control in the absence of extracellular Ca^{2+}.

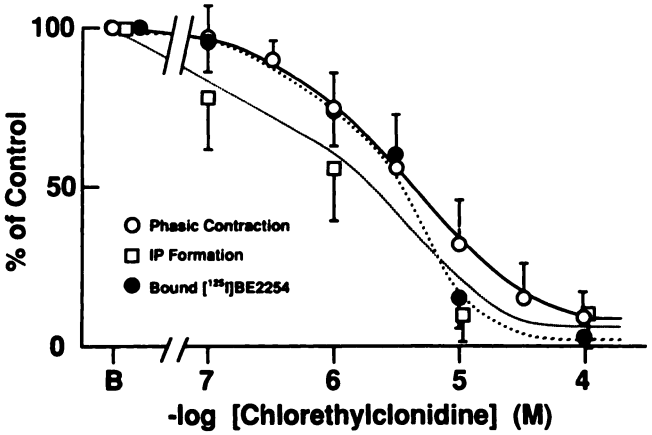

Fig. 7. Effects of treatment of rabbit aorta with several concentrations of CEC on maximal ^{125}I-BE binding capacity (●) and maximal norepinephrine-stimulated IP formation (□) and developed tension (○) in Ca^{2+}-free solution. Data are mean ± standard error of three ^{125}I-BE binding, five IP, or five contraction experiments.

buffer are shown. Moreover, fractional inactivation of α_1 receptors with CEC showed equivalent increments in the reduction of PI hydrolysis and phasic contractile response (Fig. 7), suggesting a close correlation between occupancy of "CEC-sensitive" α_1 receptor sites and the fractional responses of PI hydrolysis and phasic contractile response in rabbit aorta.

Schild regressions for WB4101 and 5-methyl-urapidil. Radioligand binding studies showed that the majority of ^{125}I-BE binding sites in rabbit aorta are CEC sensitive and have a low affinity for WB4101 and 5-methyl-urapidil; however, their receptor reserves were found to be markedly smaller than that of a small population of CEC-insensitive receptors. In an effort to assess the relative contribution of these parameters (receptor number and receptor reserve for each receptor site) in determining the pharmacological potencies of the competitive antagonists WB4101 and 5-methyl urapidil in final physiological responsiveness, we have examined the Schild regressions of the two antagonists.

The Schild regressions for WB4101 and 5-methyl-urapidil against methoxamine had slopes not significantly different from unity (0.95 ± 0.01, n = 5, for WB4101 and 0.93 ± 0.07, n = 3, for 5-methyl-urapidil) (Fig. 8A). The pA_2 values determined were 9.07 ± 0.07 (n = 5) for WB4101 and 9.09±0.05 (n = 5) for 5-methyl-urapidil. However, the Schild plots for these antagonists against norepinephrine had slopes significantly ($p < 0.05$) different from unity (0.90 ± 0.04, n = 5 for WB4101 and 0.53 ± 0.12, n = 3, for 5-methyl-urapidil) (Fig. 8A), suggesting that there is not a simple competitive antagonism of norepinephrine by WB4101 or by 5-methyl-urapidil at a single site (35). We further analyzed the Schild plots for norepinephrine-WB4101 and norepinephrine-5-methyl-urapidil competitions by the computer-assisted simulation. The theoretical Schild plots for two receptor subtypes are shown in Fig. 8B. They were computer simulated by assuming the specific parameters explained in Appendix. Varying the proportion of "low affinity" receptors, we found that the Schild plots experimentally obtained for norepinephrine-WB4101 or norepinephrine-5-methyl-urapidil competitions were best fitted to the curves simulated as a combination of approximately 10–20% high affinity sites and 80–90% low affinity sites.

Discussion

Using pharmacological tools, we have examined the possible heterogeneity in the α_1-adrenoceptor populations in rabbit aorta. Treatment with the α_{1b} receptor-selective alkylating agent CEC attenuated the phasic component of norepinephrine-induced contractile response more selectively than the tonic one. Radioligand studies indicated that rabbit aorta contains predominantly (73–87%) CEC-sensitive, low affinity sites for WB4101 and 5-methyl-urapidil (α_{1b} receptors) and a small population of CEC-insensitive sites (α_{1a} receptors); however, a minor population of α_{1a} receptors rather than α_{1b} receptors are functionally important because of their large receptor reserve. Also, α_1-adrenoceptor-mediated PI hydrolysis was CEC sensitive, and fractional inactivation of α_1 receptors with CEC shows that both PI hydrolysis and phasic contractile responses are linearly related to the CEC-sensitive α_1 receptor sites. The data suggest that two pharmacologically distinct α_1-adrenoceptor subtypes coexist in rabbit aorta and that each subtype has a distinctive physiological role in producing contractile responses through different biochemical mechanisms for increasing $[Ca^{2+}]_c$.

α_1-Adrenoceptors mediating phasic and tonic contractile responses are different not only in their pharmacological properties but also in their receptor reserves. Using the phenoxybenzamine inactivation methods in combination with CEC treatment, we found that the α_1 receptor occupancy-response relationship was markedly different for the α_{1b} receptor-mediated phasic response and the α_{1a} receptor-mediated tonic response. The relationship was hyperbolic for the α_{1a} receptor-mediated tonic response, whereas it was linear for the α_{1b} receptor-mediated phasic response. The markedly nonlinear relationship between these parameters indicates a sizable pool of "spare" receptors, whereas the linear relationship suggests an absence of a receptor reserve in activating the cellular response (30, 31). Although comparison of hyperbolic and linear occupancy-response curves is difficult, a common transformation used to make such relationships parallel for comparative purposes is to plot response against fractional (or percentage) receptor occupancy on a logarithmic scale (27, 31, 36). From this transformation (Fig. 5B), it is apparent that in rabbit aorta the occupancy-response relationship for the α_{1a}-adrenoceptor-mediated tonic contractile effect of norepinephrine is at least 50-fold more favorable than that for the α_{1b}-adrenoceptor-mediated phasic contractile effect of norepinephrine. Similar results have been previously obtained in rat aorta using $^{45}Ca^{2+}$ (37), although the experimental evidence indicates that the efficacy of coupling between α_1-adrenoceptor activation and the influx of extracellular Ca^{2+} is only 3–5-fold greater than that for the release of intracellular Ca^{2+} in this tissue.

Previously, Morrow and Creese and colleagues (4, 8) had reported that α_1-adrenergic receptor binding sites in rat brain could be subdivided into two classes based on their affinities for the competitive antagonist WB4101, as well as the ratio for pharmacological potency of phentolamine versus prazosin; the ratio at α_{1a} binding sites is approximately 4, whereas the same ratio at α_{1b} binding sites is approximately 80. Previous work reported that the ratio of phentolamine versus prazosin for contraction of rabbit aorta was 6.5 (38), and the present data showed that the pA_2 value of 9.07 for WB4101 in inhibiting methoxamine-induced contraction in rabbit aorta was close to that previously reported for high affinity binding sites of

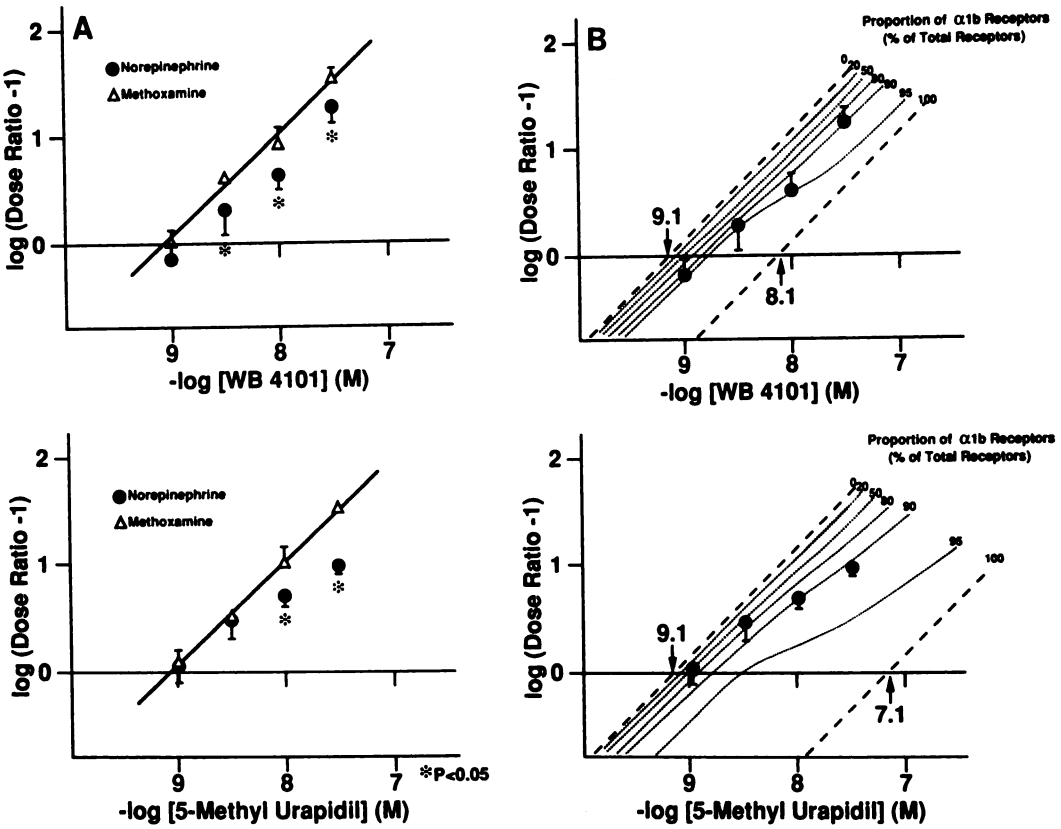

Fig. 8. A, Schild plots for the antagonists WB4101 (*upper*) and 5-methyl-urapidil (*lower*) against methoxamine (△) and norepinephrine (●) in rabbit aorta. *Points* and *bars*, mean ± standard error of five WB4101 independent experiments and three 5-methyl-urapidil independent experiments. *, $p < 0.05$, compared with corresponding values obtained using methoxamine as an agonist. B, Computer-simulated theoretical Schild plots for WB4101 (*upper*) and 5-methyl-urapidil (*lower*) against norepinephrine at various proportions of α_{1b}-adrenoceptors. Using the receptor occupancy-response relationship obtained for α_{1a} and α_{1b} receptors (Fig. 5), theoretical Schild plots were constructed as described in Appendix. Data experimentally obtained (●) are also shown.

WB4101 (10), indicating that α_{1a} receptors are present in rabbit aorta. In contrast to these functional results, radioligand binding studies showed that the displacement curve for WB4101 was best fitted by a single-site model with an affinity of 7.76 nM, which value corresponded well to that previously reported for low affinity sites (2.3–8.3 nM) (10). Also, our previous (24) and present characterization of α_1 receptor binding sites with ^{125}I-BE in rabbit aorta provides a ratio for pharmacological potency of phentolamine versus prazosin of 22–39, suggesting that α_{1b} receptors are preponderant in membrane preparations of rabbit aorta. Hence, there was an apparent disparity in pharmacological potency of WB4101 between functional and binding experiments. Such a discrepancy, however, can be explained in part when the receptor reserves for each receptor subtype are taken into account. Because the receptor reserves of α_{1a} receptors are markedly greater than those of α_{1b} receptors, the pharmacological potencies of antagonists in final functional responsiveness can be mainly determined by α_{1a} receptors, although their population is small in radioligand binding experiments that detect only recognition properties. We, thus, examined the effects of these parameters (receptor number and receptor reserve) for each receptor site on the pharmacological potencies of the competitive antagonist WB4101 in a functional study, by computer-assisted simulation of the Schild regression.

Computer-assisted analysis of the experimentally obtained nonlinear Schild plots for WB4101 and 5-methyl-urapidil against norepinephrine was found to be best fitted when the α_1-adrenoceptor populations responsible for the contraction of rabbit aorta are predominantly lower affinity sites for WB4101 and for 5-methyl-urapidil and a small population are high affinity sites for the two agents. The observation was apparently in good agreement with binding results. However, the results from theoretical Schild plots cannot be simply compared with the binding results. Effects of several important variables that determine theoretical Schild regressions should be critically assessed to evaluate the computer simulation. For example, the receptor occupancy-response relationship for the α_{1a} receptor subtype was experimentally determined by assuming that 10 μM CEC-pretreated rabbit aorta was behaving as a pure α_{1a}-adrenoceptor system. This assumption, however, clearly contradicts the experimental finding that the sum of E_{max} for tonic and phasic response is greater than the total response actually obtained (Fig. 5). In order to be consistent with the assumption that the total effects of the two receptor types on response are simply additive, we defined the E_{max} value for α_{1a} receptor-mediated tonic response as the value that subtracted the E_{max} of the phasic response from the total E_{max}. Obviously, it is possible that the total response is a more complex function of the two receptor-mediated processes rather than a simple addition as we assumed, and this type of assumption would have had an effect that underestimates the proportion of α_{1b} receptors.

Two pharmacologically distinct α_1-adrenoceptor subtypes in rabbit aorta appear to utilize different signal transduction mechanisms. As indicated in the Introduction, Han *et al.* (10) have recently extended the subclassification by incorporating functional responses at the second messenger level; the α_{1b} subtype stimulates PI hydrolysis and causes physiological responses that are independent of extracellular Ca^{2+}, whereas α_{1a} receptors cause physiological responses by stimulating the influx of extracellular Ca^{2+}. In good agreement with their proposal, we found that each α_1 adrenoceptor subtype in rabbit aorta was linked to different mechanisms for increasing $[Ca^{2+}]_c$ in developing contractile responses (Fig. 9). α_{1b}-Adrenoceptor

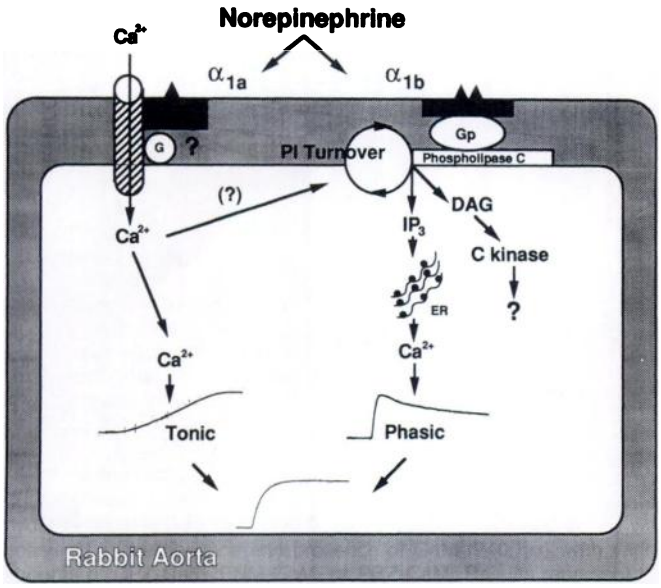

Fig. 9. Schematic diagram to illustrate two different Ca^{2+} signal transduction pathways linked to α_1-adrenoceptor subtypes in norepinephrine-stimulated contraction of rabbit aorta. See text for details. *DAG*, 1,2-diacylglycerol; *IP*$_3$, inositol 1,4,5-trisphosphate; *ER*, endoplasmic reticulum; *G*$_P$ and *G*, guanine nucleotide-binding protein.

activation causes PI hydrolysis and subsequently releases Ca^{2+} from intracellular stores, which is responsible for phasic contraction. α_{1a}-Adrenoceptor activation, on the other hand, causes Ca^{2+} influx, which is responsible for tonic contraction. Moreover, it is notable that α_{1b}-adrenoceptor-mediated PI hydrolysis in rabbit aorta can be facilitated by this α_{1a} receptor-mediated Ca^{2+} entry. The existence of such a link between the phospholipase C pathway and Ca^{2+} channels was inferred by Blakeley *et al.* (39) in the guinea pig ileum. The inhibitory effects of removal of extracellular Ca^{2+} on PI turnover in rabbit aorta may be a result of phospholipase C activation being dependent on Ca^{2+} (40, 41). The facilitation of PI hydrolysis by Ca^{2+} entry may work as a signal amplification mechanism and has a potential role in interrelating the Ca^{2+} signalling pathway coupled to each receptor subtype. Therefore, in rabbit aorta, which contains both receptor subtypes, the final physiological response of α_1 receptor-mediated contraction is caused by the $[Ca^{2+}]_c$ increase due to both α_{1a} receptor-mediated Ca^{2+} entry from extracellular fluid and α_{1b} receptor-mediated Ca^{2+} release from intracellular stores, with intracellular communication between the two pathways at the second messenger level. Also, a potentially important role for diacylglycerol, another putative second messenger generated in PI turnover that activates the ubiquitous protein kinase C (42), in each α_1-adrenoceptor subtype-mediated vasoconstrictive response is of great interest. Further studies to clarify these important questions are presently under investigation.

In conclusion, the present studies have shown that rabbit aorta contains two pharmacologically distinct α_1-adrenoceptor subtypes. Each α_1-adrenoceptor subtype couples with different signal transduction mechanisms of Ca^{2+} utilization, with a markedly different receptor reserve, and plays a differential role in the contractile response to norepinephrine. Our studies illustrate an approach of general applicability to resolving some of the complexities in characterizing the individual receptor subtypes with the relevant physiological response in tissues containing heterologous populations of receptor subtypes. Further work on regulation and interactions of these α_1-adrenoceptor subtypes in a variety of physiological and pathophysiological conditions should provide valuable insights concerning the α_1-adrenoceptor-mediated physiological consequences in smooth muscle.

Acknowledgments

Miss M. Yamada expertly prepared the manuscript. We particularly thank Dr. O.-E. Brodde and Dr. Gross (University of Essen, Germany) and Dr. Brian B. Hoffman (Department of Medicine, Stanford University Medical School, Palo Alto, CA) for valuable comments and continuing encouragement.

Appendix: Analysis of Schild Regressions in a Two-Receptor System

For any given agonist concentration $[A]$ in the presence of the reversible antagonist concentration $[I]$, the receptor occupancy y is derived from the mass-action relationship (22, 43, 44):

$$y = \frac{[A]}{[A] + K_A(1 + [I]/K_I)} \quad (1)$$

where K_A is the dissociation constant of the agonist-receptor complex and K_I represents that of the antagonist-receptor complex. The pharmacological response produced to the biological stimulus, S, generated by the agonist-receptor interaction, is proportional to receptor occupancy and is defined by:

$$S = \epsilon[R_t]y \quad (2)$$

where ϵ is the intrinsic efficacy of the agonist (30) and $[R_t]$ is the total receptor concentration. The final response, E, is often a logistic function of the biological stimulus, as shown by Kenakin (45):

$$E = E_{max}f(S) \quad (3)$$

where f is the function of the biological stimulus-response relationship and E_{max} is the maximum response (or capacity) of the tissue (or process). Eq. 3 defines the response mediated by any single process.

In a one-receptor system, experimental determination of the agonist concentration $[A']$ that produces a selected response in the absence of antagonist and the concentration $[A]$ that produces the same degree of response in the presence of antagonist concentration $[I]$, therefore, establishes, by way of Eqs. 1, 2, and 3, the identity:

$$E_{max}f\left(\frac{\epsilon R_T[A']}{[A'] + K_A}\right) = E_{max}f\left(\frac{\epsilon R_T[A]}{[A] + K_A(1 + [I]/K_I)}\right) \quad (4)$$

Rearrangement of Eq. 4 and conversion into logarithms, using r to denote $[A]/[A']$, yields the following expression (22, 30):

$$\log(r - 1) = \log [I] - \log K_I \quad (5)$$

By performing such an experiment with various concentrations of antagonist, data can be obtained for a plot of log $[I]$ as abscissa and log $(r - 1)$ as ordinate, the "Schild plot" (46). Inspection of Eq. 5 shows the result is a straight line with a slope of 1 and a y intercept of $-\log K_I$ (pA_2 value).

In analyzing the Schild plot in a two-receptor system, we have assumed that the total response E_T in a two-receptor system is the sum of the responses for the two independent receptor subtype-mediated processes (47), such that:

$$E_T = E_1 + E_2 \quad (6)$$

Subscripts 1 and 2 have been added to distinguish the two receptor types. In this equation E_1 is that component of the response mediated by the receptor subtype 1 and E_2 is that component dependent upon the other subtype, subtype 2. Then, E_T is defined by:

$$E_T = E_{\max_1} f_1 \left(\frac{\epsilon_1 [R_{t_1}][A]}{[A] + K_{A_1}(1 + [I]/K_{I_1})} \right) + E_{\max_2} f_2 \left(\frac{\epsilon_2 [R_{t_2}][A]}{[A] + K_{A_2}(1 + [I]/K_{I_2})} \right) \quad (7)$$

The theoretical Schild plots for two receptor subtypes were computer-simulated by assuming the specific parameters for Eq. 7 as follows.

1) In this equation, we assume that $\epsilon_1 = \epsilon_2 = 1.0$, and the combined total receptor concentration was arbitrarily defined as unity $(R_{t_1} + R_{t_2}) = 1.0$.

2) The dissociation constants used in the calculations (K_{A_1}, K_{A_2}) and each f was determined from the phenoxybenzamine and CEC inactivation experiments (Figs. 4 and 5). The K_A values for two receptor subtypes were 1.45 μM and 0.32 μM, respectively. In order to define f mathematically, we arbitrarily adopted a function f such that $f(q) = 100(1 - e^{aq+b})$. This equation has no theoretical implications but was selected because it simulates the occupancy-response curves of α_1 receptors in blood vessels (27, 34, 48). The "logistic function" used by Furchgott (30) and Kenakin (45) could be substituted with qualitatively similar results. Nonlinear regression analyses defined $f(q) = 100(1 - e^{0.13-5.75q})$ after CEC pretreatment in the presence of extracellular Ca^{2+}, which may reflect the function of the "CEC-insensitive" α_{1a} subtype, and the correlation coefficient of the curve is 0.83 The correlation in the absence of Ca^{2+}, which may reflect the function of the α_{1b} subtype, approximated a unitary relationship. The correlation has a slope of 1.02[$f(q) = 102q + 21.4$] and a correlation coefficient of 0.93, where q reflects the receptor occupancy and $f(q)$ the contractile response.

3) In order to the consistent with the assumption that the total effects of the two receptor types on response are simply additive, we defined the E_{max} value for the α_{1a} receptor-mediated tonic response as the value that subtracted the developed tension obtained during the sustained phase of the phasic response from the total E_{max}.

4) The pK_I values of high affinity sites and low affinity sites for WB4101 and for 5-methyl-urapidil were assumed to be 9.1 and 8.1 (for WB4101) and 9.1 and 7.1 (for 5-methyl-urapidil), respectively. These values approximate data obtained experimentally; the values of high affinity sites were obtained from Schild plots for methoxamine-WB4101 competition and for methoxamine-5-methyl-urapidil competition, and those of low affinity sites were obtained from displacement of specific ^{125}I-BE binding sites.

Substituting these parameters in Eq. 7, we performed the computer simulation of dose-response curves in the presence of various concentrations of WB4101 and 5-methyl-urapidil and determined each EC_{50} value to obtain the relationship between the agonist concentration [A'] that produces a selected response in the absence of antagonist and the concentration [A] that produces the same degree of response in the presence of antagonist. Using r to denote [A]/[A'], the relation between log ($r - 1$) and [I] was plotted. Then the effects on Schild plots of varying the ratio of $R_{t_2}/(R_{t_1} + R_{t_2})$ were investigated. Details and limitations of this analysis were previously described by Milnor (48).

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