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Glycogen Phosphorylase Activation by Two Different α_1-Adrenergic Receptor Subtypes: Methoxamine Selectively Stimulates a Putative α_1-Adrenergic Receptor Subtype (α_{1a}) that Couples with Ca^{2+} Influx

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SUMMARY

We compared the effects of methoxamine on α_1-adrenergic receptor-mediated phosphorylase activation in rat hepatocytes and rabbit aorta. Although methoxamine is a potent agonist in activating phosphorylase of rabbit aorta, it had little effect in rat hepatocytes. Using the phenoxybenzamine inactivation method, we found that the quantitative relationship between ^{125}I-BE2254 (^{125}I-BE) binding capacity and maximal norepinephrine-stimulated phosphorylase activation was nonlinear in rabbit aorta, whereas it was linear in rat hepatocytes. The potency of methoxamine in inhibiting specific ^{125}I-BE binding is significantly ($p < 0.05$) higher in rabbit aorta (K_d, 96.4 ± 7.7 μM), compared with rat hepatocytes (K_d, 283 ± 16 μM). However, these quantitative differences could not fully explain the blunted $[Ca^{2+}]_c$ and phosphorylase responses to methoxamine in rat hepatocytes. Treatment with chlorethylclonidine dose dependently suppressed ^{125}I-BE binding sites and norepinephrine-induced phosphorylase activation in rat hepatocytes, whereas in rabbit aorta it resulted in only a 31% decrease in ^{125}I-BE binding sites, with little effect on phosphorylase activation. Furthermore, α_1-adrenergic receptor-mediated cellular events of phosphatidylinositol (PI) hydrolysis and phosphorylase activation were unaffected by the removal of extracellular Ca^{2+} in rat hepatocytes, whereas both responses were markedly attenuated in rabbit aorta. The results indicate that two different α_1-adrenergic receptor subtypes activate glycogen phosphorylase, through different mechanisms for increasing $[Ca^{2+}]_c$ in the two systems. In rat hepatocytes, α_1 receptors are closely linked to PI hydrolysis and Ca^{2+} release from intracellular stores and cause phosphorylase activation. In rabbit aorta, on the other hand, activation of α_1 receptors increases $[Ca^{2+}]_c$ by Ca^{2+} influx from the extracellular fluid as well as by Ca^{2+} release, and both PI hydrolysis and phosphorylase activation are caused mainly by the Ca^{2+} entry. Methoxamine interacts with both chlorethylclonidine-sensitive and -insensitive α_1 receptor subtypes but selectively stimulates the α_1 receptor subtype that closely couples with the Ca^{2+} influx.

α-Adrenergic receptors have been divided into two subclasses, i.e., α_1 and α_2, on the basis of their affinity for agonists and antagonists and their mechanisms of signal transduction (1–3). However, increasing evidence suggests that α_1-adrenergic receptors do not have the same pharmacological properties in all tissues. Differences in potency ratios of agonists and antagonists have been observed in functional experiments (e.g., the contraction of smooth muscle) (4, 5). Also, differences in the potencies of certain competitive and noncompetitive antagonists are found in radioligand binding studies on membrane preparations (6, 7); in particular, the irreversible alkylating agent CEC has recently been found to inactivate only a subpopulation of the α_1-adrenergic receptor binding sites in different tissues (8, 9). These results raise the possibility that there may be distinct subtypes of α_1-adrenergic receptors. Furthermore, the hypothesis that the subtypes of α_1-adrenergic receptors may couple with different signal transduction mechanisms has recently been put forth (10); thus, one subtype (CEC-sensitive α_1-adrenergic receptors or α_{1b} receptors) stimulates PI hydrolysis/intracellular Ca^{2+} mobilization and causes functional response mainly independent of extracellular Ca^{2+}, whereas the other (CEC-insensitive α_1-adrenergic receptors, α_{1a} receptors) does not stimulate inositol formation and causes physiological responses predominantly dependent on the influx of extracellular Ca^{2+}.

Methoxamine is well known as a potent α_1-adrenergic receptor agonist in a variety of cell systems. However, its exceptionally low efficacy in activating glycogen phosphorylase in rat

ABBREVIATIONS: CEC, chlorethylclonidine; ^{125}I-BE, ^{125}I-BE2254; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; PI, phosphatidylinositol; IP, inositol monophosphate; quin2/AM, quin2-tetraacetoxymethyl ester; PIPES, 1,4-piperazine-bis(ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

hepatocytes has been noted (11). The reason for this low efficacy of methoxamine in hepatic phosphorylase activation is not clear. Variables that could explain this observation involve the ability of liver cells to rapidly take up and metabolize methoxamine (12) and the different drug-receptor relationship for methoxamine in the liver cells. The differential effects of methoxamine in different tissues have never been examined particularly from the point of view of α_1-adrenergic receptor heterogeneity.

The present study was undertaken to investigate the mechanism(s) for the differential effects of methoxamine in different tissues. For the purpose, we characterized the phosphorylase activation in rabbit aorta, another model system for α_1-adrenergic receptors, and compared it with that in rat hepatocytes. We first determined the quantitative relationships between fractional occupation of α_1-adrenergic receptors and the phosphorylase activation in both systems. Also, we examined the effects of methoxamine on each step in the α_1-adrenergic receptor-mediated phosphorylase activation cascade, such as receptor binding, PI hydrolysis, and intracellular Ca^{2+} response. Furthermore, utilizing CEC, which is now thought to selectively inactivate one of the α_1-adrenergic receptor subtypes (α_{1b}) (8, 9), we examined the possibility that the differential effects of methoxamine in the two tissues reflect the functionally discrete subtypes of α_1-adrenergic receptors.

Experimental Procedures

Materials

Chemicals were obtained from the following sources: quin2/AM, Dojindo Laboratories (Kumamoto, Japan); collagenase (Type II), Worthington Biochemicals (Freehold, NJ); lanthanum chloride ($LaCl_3$), (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, *l*-phenylephrine HCl, (±)-propranolol HCl, and yohimbine HCl, Sigma Chemical Co. (St. Louis, MO); CEC and WB 4101, Research Biochemicals (Natick, MA); and ^{125}I-BE (specific activity, ~2200 Ci/mmol), myo-[2-^{3}H(*N*)]inositol (16.5 Ci/mmol), and α-D-[U-^{14}C]glucose-1-phosphate, New England Nuclear (Boston, MA). Aequorin was purchased from Dr. J. R. Blinks of The Mayo Foundation (Rochester, MN) as 1 mg of lyophilized protein in KCl and PIPES buffer. 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, U.K.), phentolamine (CIBA-GEIGY, Summit, NJ), nicardipine HCl (Yamanouchi Pharmaceutical Co., Tokyo, Japan), and phenoxybenzamine HCl (Smith Kline & French Laboratories, Philadelphia, PA) were generously supplied by each company. All other chemicals and reagents used were from standard commercial sources.

Rat Hepatocyte Isolation

Isolated liver cells were prepared from 250–300 g (body weight) male Sprague-Dawley rats by the collagenase perfusion method, as described previously (13). The yield of cells from one liver averaged 1.5×10^8, with 90–95% viability as estimated by trypan blue exclusion.

Rabbit Aorta Preparation

Thoracic aortic segments from adult male Japanese white rabbits (2–2.5 kg of body weight) were used. The thoracic aortas were rapidly excised and placed in Krebs-Ringer bicarbonate buffer that was bubbled with 95% O_2/5% CO_2 (pH 7.4). While being maintained at room temperature under an aerated buffer, the adherent adventitia and fatty tissue were removed. For use in inositol phosphate and glycogen phosphorylase studies, individual vessels were cut into 8 to 10 0.5–0.6-cm ring segments, and their intimal surfaces were rubbed with a scalpel to remove the endothelium.

Measurement of Glycogen Phosphorylase Activation

Freshly isolated hepatocytes (6 to 15 × 10^6 cells/ml) were preincubated for 30 min, before the stimulation by agonists, in Krebs-Ringer bicarbonate buffer supplemented with 40 mM glucose and 2% dialyzed bovine serum albumin, whereas antagonists were added as required after 20 min of preincubation. At the end of incubation, aliquots of the cell suspension were stimulated by the drugs, as indicated, for 2 min and were immediately immersed in the liquid nitrogen to terminate the reaction. The frozen hepatocytes were stored at −80° until assay.

Aortic rings were allowed to equilibrate, in Krebs-Ringer bicarbonate buffer that was supplemented with 40 mM glucose and 50 mM sucrose, for 2 hr before the addition of any drugs. Vessels were stimulated for 8 min, during which interval the phosphorylase activation was found to reach equilibrium, from preliminary time-course experiments, in 50 ml of aerated Krebs-Ringer bicarbonate buffer in the presence of agonists as indicated. Propranolol (1 μM) was included in all assays except for the data described in Table 1. At the end of 8 min, the vascular ring segments were quickly blotted and then frozen in liquid nitrogen-cooled clamps. The tissues were then stored at −80° until assayed. From preliminary experiments, we have confirmed the previous observation by Namm (14) that most of phosphorylase activity of the whole rabbit aortic preparations resided in the smooth muscle (media-intima layers), with little activity in the connective tissues of the adventitia.

For the assay, the frozen samples were first mixed with either 120 μl (hepatocytes) or 400 μl (aortic ring) of buffer containing (in mM): 30 MOPS, 150 NaF, 15 EDTA, and 3 dithiothreitol, pH 7.0, and were then homogenized. The homogenates were centrifuged at 12,000 × g_{max} for 5 min at 4°, and the supernatant was used for the assay of glycogen phosphorylase. Glycogen phosphorylase *a* activity was measured using a filter disc assay similar to that of Gilboe *et al.* (15), as modified (16). Data are expressed as micromol of [^{14}C]glucose from α-D-[U-^{14}C]glucose-1-phosphate incorporated into total assay glycogen/min/100 mg of protein or as the activity ratio representing the percentage of activity in the active form (phosphorylase *a* activity/phosphorylase *a* + *b* activity).

Measurement of $[Ca^{2+}]_c$ Movement in Rat Hepatocytes

Measurement of $[Ca^{2+}]_c$ in isolated hepatocytes was achieved by utilizing either the photoprotein aequorin or the fluorphore quin2, because these indicators have been reported to reflect different aspects of Ca^{2+} homeostasis; aequorin may be more sensitive to local and low levels of Ca^{2+} transients, whereas quin2 is more sensitive to the average or diffuse $[Ca^{2+}]_c$ (17).

Aequorin. Incorporation of aequorin into isolated hepatocytes was performed by the gravity loading method described by Borle *et al.* (18), with modification (19). The aequorin-loaded cells were resuspended in modified Hanks' buffer and bioluminescence was measured by a modified photomultiplier tube-aggregometer (Chronolog Lumiaggregometer, Havertown, PA) under constant stirring at 37°. Resting $[Ca^{2+}]_c$ was calculated from a calibration curve previously published by Borle and Snowdowne (20), assuming the even distribution of calcium in the cell. $[Ca^{2+}]_c$ in stimulated cells was not calibrated because no information was available as to the spatial distribution of calcium in the cells. None of the drugs used in the present study quenched luminescence of aequorin added to a cell-free suspension. Tracings presented in Results are the representatives of at least five experiments with similar results.

Quin2. Quin2 loading was achieved as described previously (19). After a 20-min incubation with quin2/AM (100 μM), when maximal accumulation of quin2 had occurred, the cells were washed twice and resuspended in HEPES buffer with the albumin concentration reduced to 0.2%. Quin2 fluorescence measurements were performed in a Hitachi Model F-3000 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) equipped with a thermostat-controlled cuvette holder and magnetic stirring. Excitation and emission wavelengths were 340 and 490

nm, respectively. Calibration and determination of $[Ca^{2+}]_c$ were done as described by Tsien *et al.* (21).

Measurement of [^{3}H]PI Hydrolysis

For experiments involving PI hydrolysis, hepatocytes (3 to 6 × 10^6 cells/ml) were suspended in Krebs-Ringer bicarbonate buffer that was supplemented with 2% dialyzed bovine serum albumin and myo-[2-^{3}H]inositol (15 μCi/ml) (22). This prelabeling incubation was continued for 90 min, by which time the incorporation of ^{3}H into inositol lipids had reached a plateau. The cells were subsequently washed three times to remove excess [^{3}H]inositol and then resuspended (1 to 3 × 10^6 cells/ml) in the bicarbonate buffer described above but without added [^{3}H]inositol. After 10 min of preincubation, 10 mM LiCl (23) was added and this pretreatment was continued for a further 10 min before addition of agents. Because we observed that norepinephrine-induced accumulation of IP increased linearly with longer periods of incubation in preliminary experiments, the reaction was terminated after incubations, with agents for 60 min by adding 0.2 ml of 60% (w/v) trichloroacetic acid kept on ice to the cells plus medium. After centrifugation (5000 × *g* for 10 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 on 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 (23).

Rabbit aortic ring segments were exposed for 3 hr to 8 μCi/ml [^{3}H]-inositol in 37° Krebs-Ringer bicarbonate solution, which was gassed with 95% O_2/5% CO_2. Tissues were then transferred to flasks containing fresh Krebs-Ringer bicarbonate solution and were exposed to agonist in the presence of LiCl (10 mM) for 1 hr. Tissues were then frozen between clamps precooled in liquid nitrogen and were homogenized in 10% trichloroacetic acid, extracted with ether, and assayed for inositol phosphates as previously described (24). Our preliminary experiments confirmed that norepinephrine-induced accumulation of IP increased linearly with longer periods of incubation (up to 1 hr) and that norepinephrine increased IP accumulation in a concentration-dependent manner.

In experiments with antagonists or the Ca^{2+} channel modulator nicardipine, tissues were exposed to the agent for 1 hr before addition of LiCl and norepinephrine. In some experiments with Ca^{2+}-free solution (Ca^{2+}-free Krebs-Ringer bicarbonate solution containing 0.5 mM EGTA) or La-containing solution, tissues were equilibrated in Ca^{2+}-containing solution after [^{3}H]inositol loading and were then transferred to the respective solution without preincubation. In experiments with La^{3+} (5 mM), PO_4^{3-} and SO_4^{2-} were replaced with Cl^- to avoid precipitation of La salt. All data on IP production were normalized with norepinephrine (10 μM)-induced IP production in the presence of 2 mM extracellular Ca^{2+} as the reference full agonist. In preliminary experiments, we confirmed that norepinephrine (10 μM)-induced IP accumulation was selectively inhibited by prazosin (1 μM) but not by yohimbine (1 μM) in both tissues (data not shown).

^{125}I-BE Binding

For the studies of α_1-adrenergic receptors, particulate fractions of rat hepatocytes and rabbit aortas were prepared by homogenization and centrifugation, as described previously (25, 26), and were used immediately in the receptor binding assays.

Measurement of specific ^{125}I-BE binding was usually performed (26) by incubating 100 μl of tissue preparation with ^{125}I-BE (40–60 pM for competition experiments and 15–900 pM for saturation experiments) in a total volume of 150 μl for 20 min at 37°, in the presence or absence of competing drugs. The reaction was terminated by the addition of 5 ml of room temperature incubation buffer. The bound and free radioligands were separated by rapid filtration over glass fiber filters (Whatman GF/C). The filters were washed with an additional 15 ml of incubation buffer and the radioactivity retained on the filters was counted. Nonspecific binding was defined as the amount of ^{125}I-BE binding measured in the presence of 10 μM phentolamine.

Protein concentration was determined by the method of Lowry *et al.* (27), using bovine serum albumin as standard.

Fractional Inactivation of α_1-Receptors by Phenoxybenzamine and CEC

In some experiments to delineate the linkage between α_1-adrenergic receptor occupation and phosphorylase response, we examined the effects of phenoxybenzamine (28–30) and CEC in both tissues. In these experiments, either phenoxybenzamine (in final concentrations ranging from 10^{-12} to 10^{-5} M) or CEC (in final concentrations ranging from 10^{-8} to 10^{-3} M) was added to the incubation buffer for hepatocytes and aortic ring preparations and was incubated for 20 min at 37°. Hepatocytes and aortic ring segments were then washed three times and used for radioligand binding assay and phosphorylase experiments. Data are expressed as percentage of control, thus, for receptor occupation 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, and for phosphorylase measurement as percentage of the phosphorylase response obtained with 10 μM norepinephrine alone.

Data Analysis

All data for each drug were averaged and the resulting concentration-response curves from each group were analyzed simultaneously using the four-parameter logistic equation (31). The resulting ED_{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 (32).

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 was then used to determine which pairs of means were significantly different. Criterion for statistical significance was a p value of less than 0.05.

Results

The agonists epinephrine, norepinephrine, and phenylephrine stimulated glycogen phosphorylase in a dose-dependent manner in both rat hepatocytes (Fig. 1A) and rabbit aorta (Fig. 1B). Methoxamine caused little stimulation of hepatic phosphorylase, whereas it worked as an effective agonist in the activation of phosphorylase in rabbit aorta. The activity of glycogen phosphorylase in adult male rat hepatocytes has previously been demonstrated to be mediated predominantly by α_1-adrenergic receptors (13), which was confirmed in the present study. As summarized in Table 1, a maximal increase of the enzyme activation induced by epinephrine (10 μM) was potently inhibited by the α_1-selective antagonist prazosin (1 μM) by 85% ($p < 0.01$), whereas both the α_2-selective antagonist yohimbine (1 μM) and the β-antagonist propranolol (1 μM) were without effect. Similarly, phosphorylase activation by epinephrine (10 μM) or methoxamine (100 μM) in rabbit aorta was potently and selectively inhibited by prazosin (Table 1).

Explanations for methoxamine being unable to stimulate hepatic α_1-adrenergic receptors would include the existence of a high capacity uptake system that effectively removes methoxamine from the medium (12). We examined this possibility in two ways. 1) Assuming that dilution of the cell suspension would minimize this complication, we examined the effects of

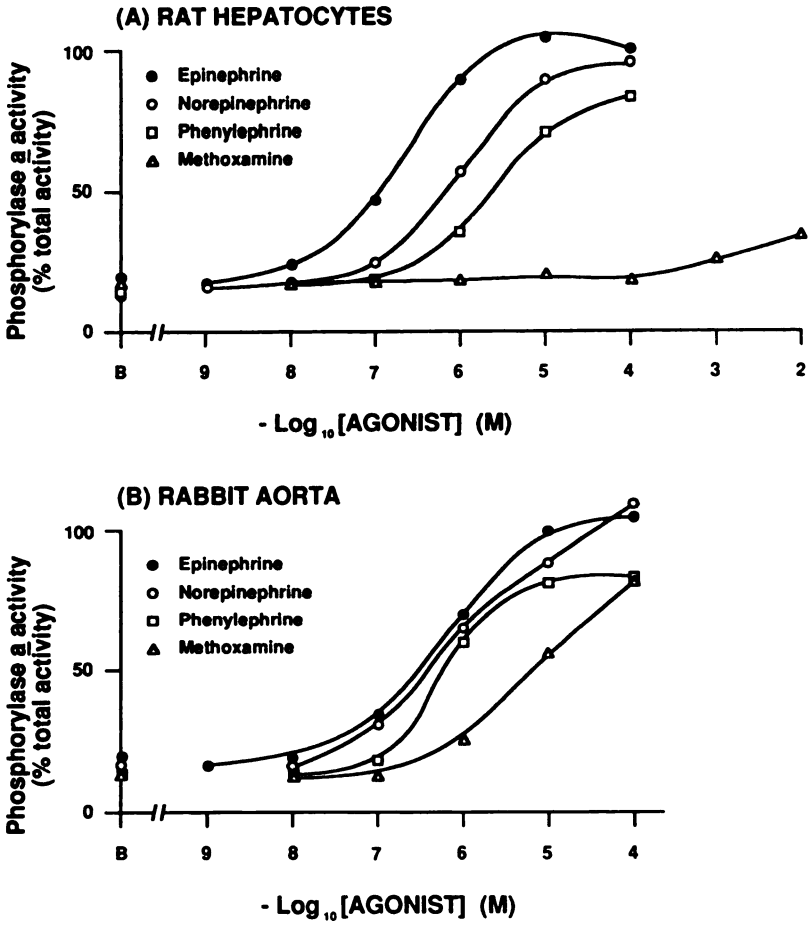

Fig. 1. Dose-response curves for activation of glycogen phosphorylase by epinephrine, norepinephrine, phenylephrine, and methoxamine in isolated rat hepatocytes (A) and rabbit aortic ring segments (B). Experimental conditions were as described in Experimental Procedures. The basal level of the glycogen phosphorylase *a* activities were 17 ± 2% total activity; 1.03 ± 0.18 units/100 mg of protein, n = 7 in rat hepatocytes, and 14 ± 1% total activity; 1.18 ± 0.11 units/100 mg protein, n = 7 in rabbit aorta, respectively. The results are the mean of four to seven different experiments.

TABLE 1

Effect of α- and β-adrenergic antagonists on epinephrine- and methoxamine-activated glycogen phosphorylase activity in rat hepatocytes and rabbit aortic ring segments

Experimental conditions were identical with those of Fig. 1 except that the hepatocytes or aortic segments were preincubated with each antagonist for 10 min at the time of activation by the indicated concentration of agonist. Basal phosphorylase activities were 18 ± 3% total activity; 1.16 ± 0.25 units/100 mg of protein, n = 6 in rat hepatocytes, and 13 ± 3% total activity; 1.04 ± 0.20 units/100 mg of protein, n = 6 in rabbit aorta, respectively. Values shown are the means ± standard errors of four to six different experiments.

	Glycogen phosphorylase activity ratio
	%
Hepatocytes	
Epinephrine (10 μM)	106 ± 8
Epinephrine (10 μM) + prazosin (1 μM)	17 ± 4*
Epinephrine (10 μM) + yohimbine (1 μM)	115 ± 10
Epinephrine (10 μM) + propranolol (1 μM)	109 ± 7
Rabbit aorta	
Epinephrine (10 μM)	99 ± 8
Epinephrine (10 μM) + prazosin (1 μM)	25 ± 3*
Epinephrine (10 μM) + yohimbine (1 μM)	86 ± 9
Epinephrine (10 μM) + propranolol (1 μM)	92 ± 7
Methoxamine (100 μM)	90 ± 0
Methoxamine (100 μM) + prazosin (1 μM)	22 ± 3*
Methoxamine (100 μM) + yohimbine (1 μM)	80 ± 10
Methoxamine (100 μM) + propranolol (1 μM)	72 ± 6

*$p < 0.01$ difference from agonist alone.

the concentration of rat liver cells on methoxamine-induced hepatic phosphorylase. Methoxamine (100 μM)-induced phosphorylase activations either in concentrated (~5 × 10^7 cells/ml) or diluted (~1 × 10^6 cells/ml) cell suspensions were compared. Methoxamine did not activate hepatic phosphorylase in either preparation (data not shown). 2) In addition, we examined the biological activity of methoxamine in cell suspensions. Hepatocytes (~1 × 10^7 cells/ml) were incubated with methoxamine (10^{-4} M) for 2 min and then centrifuged (1000 × g for 10 min). The biological activity of methoxamine in the resulting supernatant was compared with that in the freshly prepared one. In smooth muscle contraction (either in contraction of rabbit aorta or in the pressor response of pithed rats) and also in the phosphorylase activation of rabbit aorta, the dose-response relationships of methoxamine were comparable for both preparations (data not shown), suggesting that the biological activity of methoxamine was retained even in the concentrated cell suspension. These experiments, thus, provided no evidence to support the existence of a hepatic uptake mechanism that decreased the concentration of methoxamine in the incubation medium.

We next examined the effect of methoxamine on the $[Ca^{2+}]_c$ response in rat hepatocytes, because α_1-adrenergic agonists are thought to activate phosphorylase via an increase in $[Ca^{2+}]_c$. Estimated resting $[Ca^{2+}]_c$ values in the presence of 1 mM extracellular Ca^{2+} for isolated hepatocytes were 123 ± 22 nM (n = 7) and 145 ± 18 nM (n = 8) for aequorin-indicated and quin2-indicated $[Ca^{2+}]_c$, respectively. As shown in Fig. 2, meth-

oxamine caused little or no change in either aequorin- or quin2-indicated $[Ca^{2+}]_c$ signals. However, in the hepatocytes, where methoxamine induced no change in $[Ca^{2+}]_c$, norepinephrine (1 μM; Fig. 2) or phenylephrine (10 μM; data not shown) caused a rapid increase in $[Ca^{2+}]_c$ indicated by both aequorin and quin2, although the time course of the $[Ca^{2+}]_c$ signal differed between the two indicators (Fig. 2). The results suggest that the defect for methoxamine to activate hepatic phosphorylase lies in the initial step in α_1-receptor-mediated signal transduction, proximal to $[Ca^{2+}]_c$ increase.

We examined the potencies of several α-adrenergic agonists in binding to α_1 receptor sites in both tissues. As shown in Table 2, epinephrine, norepinephrine, and phenylephrine showed similar affinities in both tissues. However, methoxamine showed a slightly but significantly ($p < 0.05$) higher affinity in rabbit aorta when compared with hepatocytes. The Hill slopes for the agonists in inhibiting specific ^{125}I-BE binding were close to 1.0 in rat hepatocytes, whereas those values were significantly ($p < 0.05$) lower in rabbit aorta (Table 2).

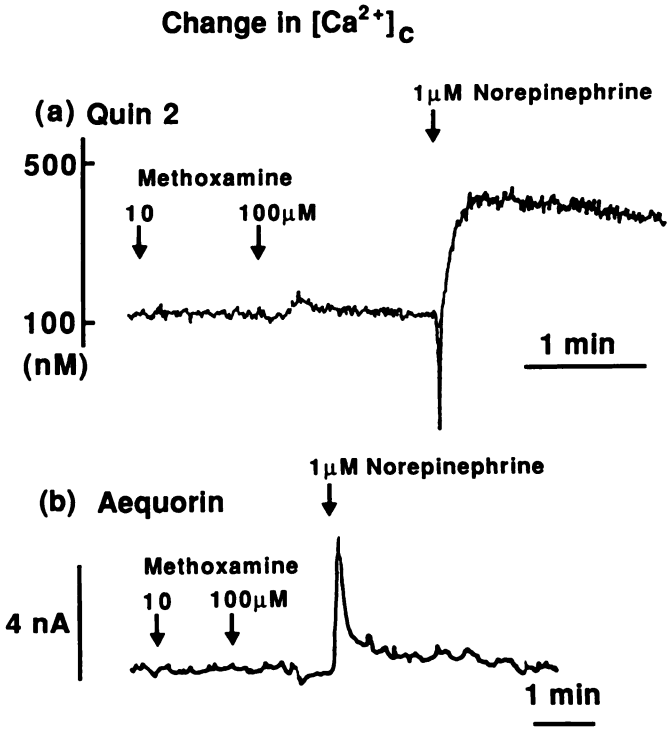

Fig. 2. Changes in $[Ca^{2+}]_c$ indicated by quin2 (a) and by aequorin (b) in response to methoxamine (10 and 100 μM) and norepinephrine (1 μM) in isolated rat hepatocytes. Quin2 and aequorin-loaded hepatocytes were prepared as described in Experimental Procedures. Quin2 fluorescence and aequorin bioluminescence measurements were collected every 0.5 sec and sequentially, respectively. The results presented are representative experiments of at least five experiments.

TABLE 2

Competition for specific ^{125}I-BE binding sites by adrenergic agonists in rabbit aorta and rat hepatocyte

Inhibition of specific ^{125}I-BE binding was compared in crude particulate fractions from rabbit aorta and rat hepatocytes. Competition curves were analyzed by computer as described in Experimental Procedures. Data shown are means ± standard errors of six or seven experiments.

Competing drug	K_d		Hill slopes	
	Rabbit aorta	Rat hepatocytes	Rabbit aorta	Rat hepatocytes
	μM			
(−)-Epinephrine	3.85 ± 1.1	3.95 ± 1.3	0.66 ± 0.06	0.97 ± 0.05*
(−)-Norepinephrine	6.22 ± 2.1	7.14 ± 1.6	0.72 ± 0.03	0.95 ± 0.02*
L-Phenylephrine	38.4 ± 4.4	29.1 ± 5.8	0.73 ± 0.05	0.92 ± 0.03*
Methoxamine	96.4 ± 7.7	283 ± 16*	0.88 ± 0.08	1.04 ± 0.03*

*$p < 0.05$ compared with rabbit aorta.

The next series of experiments was designed to examine important factors at receptor sites that could affect the efficacy of the agent (33). Because agonists with lower efficacy (or intrinsic activity) are associated with lower or no receptor reserve, one hypothesis is that there may exist a receptor reserve, or "spare receptors," for rabbit aorta but not for rat hepatocytes. If there exists a receptor reserve only for α_1 receptors in rabbit aorta, then one would anticipate that methoxamine would cause aortic phosphorylase activation to a greater degree there than in hepatocytes, even if the receptor occupancy by methoxamine were equal in both systems.

We examined the quantitative relationship between fractional occupation of α_1 receptor and phosphorylase responses. Fractional inactivation of α_1-adrenergic receptors were performed by the addition of the irreversible antagonist phenoxybenzamine to the incubation buffer for 20 min (37°) before the assays for ^{125}I-BE binding and phosphorylase activation. Maximal ^{125}I-BE binding capacity was progressively decreased by exposure to increasing concentrations of phenoxybenzamine in both rat hepatocytes and rabbit aorta, reaching a maximal reduction of 95 ± 6% ($n = 5$) and 87 ± 4% ($n = 5$) at a phenoxybenzamine concentration of 10 μM for hepatocytes and aorta, respectively (Fig. 3). The phenoxybenzamine concentrations necessary to block half of the ^{125}I-BE sites in 20 min are $3.8 \pm 1.6 \times 10^{-7}$ M ($n = 5$) and $2.3 \pm 1.3 \times 10^{-10}$ M ($n = 5$) for rat hepatocytes and rabbit aorta, respectively. The effect of partial α_1 receptor inactivation on maximal norepinephrine-stimulated phosphorylase activation in both tissues is also shown in Fig. 3. The half-maximal inhibitory concentrations (IC_{50}) for norepinephrine-stimulated phosphorylase activation are $3.9 \pm 0.8 \times 10^{-7}$ M ($n = 4$) and $3.0 \pm 0.4 \times 10^{-9}$ M ($n = 5$) phenoxybenzamine for rat hepatocytes and rabbit aorta, respectively. Correlation of fractional inhibition of norepinephrine-induced phosphorylase activation with fractional inhibition of ^{125}I-BE binding approximated a unitary relationship for liver cells, whereas the relationship was markedly nonlinear for rabbit aorta (Fig. 3, *inset*). These results indicate that methoxamine-induced phosphorylase activation is greater in rabbit aorta than in liver cells even when the receptor occupancy by methoxamine is equal in the two systems. However, the blunted $[Ca^{2+}]_c$ and phosphorylase responses to higher concentrations of methoxamine cannot be fully explained only by this quantitative relationship. In addition, in an effort to determine whether methoxamine works as an antagonist on the hepatic α_1 receptor system, its effect on the action of epinephrine was tested. Methoxamine (1 to 100 μM) was found to produce little shift of the dose-response curves for epinephrine-induced hepatic phosphorylase activation (data not shown), suggesting it has little antagonistic effect on hepatic α_1 receptors.

We next examined the possibility that there exist different subpopulations of α_1 receptors in rat hepatocytes and rabbit aorta and that methoxamine can selectively activate those in rabbit aorta. For this purpose, we first compared the effect of CEC on α_1 receptor binding sites in both tissues, because CEC has been recently shown to be useful in differentiating α_1 receptor subtypes (8, 9). A 20-min pretreatment with CEC

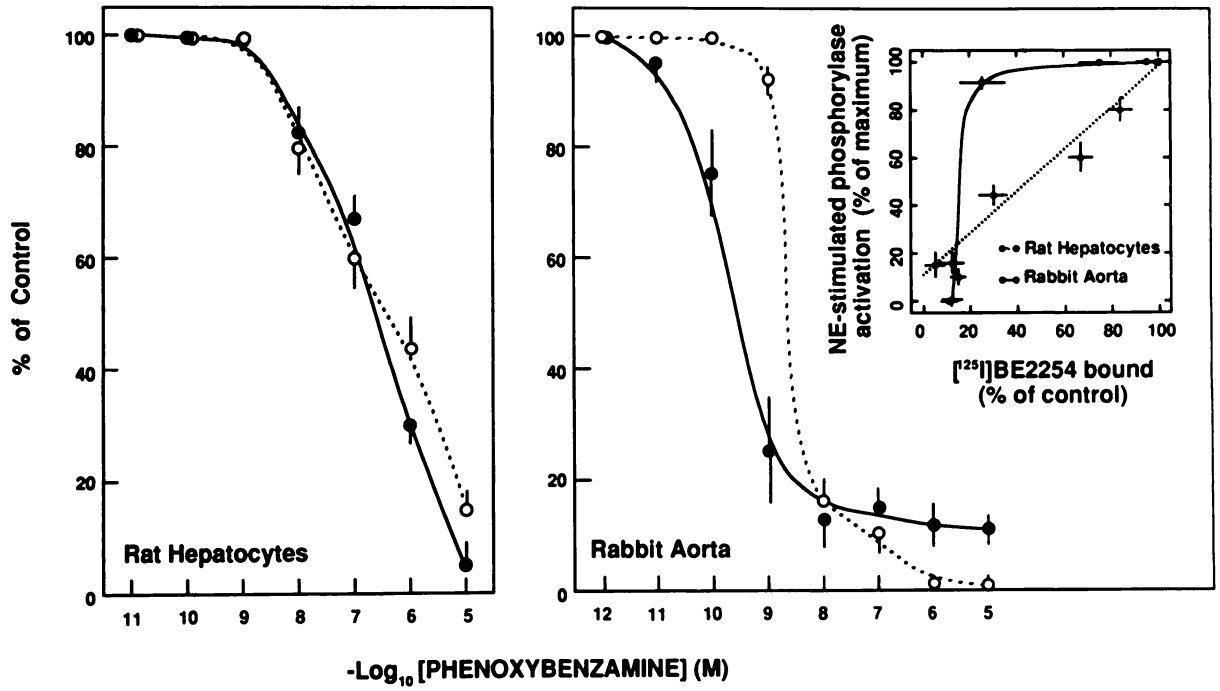

Fig. 3. Effect of a 20-min incubation of rat hepatocytes (*left*) and rabbit aortic ring segments (*right*) with several concentrations of phenoxybenzamine on maximal ^{125}I-BE binding capacity (●) and maximal norepinephrine (10 μM)-stimulated phosphorylase activation (○). Data are means ± standard errors of five ^{125}I-BE binding or four or five phosphorylase experiments. Maximal specific ^{125}I-BE capacities were 126.4 ± 11.6 fmol/mg of protein (n = 5, rat hepatocytes) and 17.4 ± 1.9 fmol/mg of protein (n = 5, rabbit aorta), respectively. *Inset*, relationship between maximal ^{125}I-BE binding capacity and maximal norepinephrine (10 μM)-stimulated phosphorylase activation in rat hepatocytes (●) and rabbit aortic ring segments (○) after incubation with phenoxybenzamine as described in Fig. 3. Data depicted are replotted from Fig. 3.

caused markedly different effects on the specific ^{125}I-BE binding sites of each system. Maximal ^{125}I-BE binding capacity was progressively decreased by exposure to increasing concentrations of CEC in rat hepatocytes, reaching a maximal reduction of 95 ± 2% (n = 5) at a CEC concentration of 100 μM, whereas the treatment with CEC (up to 1 mM) had no significant effect in rabbit aorta. There was little effect on the K_d for ^{125}I-BE in rabbit aorta, whereas a small but significant ($p < 0.05$) increase was observed in rat liver cells at higher concentration of CEC (K_d; 53 ± 9 pM, n = 6 in untreated cells, versus 154 ± 26 pM, n = 5 in 100 μM CEC-treated cells). Examining the effects of washing on the inactivation caused by CEC in hepatocytes, we confirmed the previous observation made by Han *et al.* (8) that there was no recovery of specific ^{125}I-BE binding sites after repeated washing of intact cells, which was consistent with an irreversible inactivation of binding sites (data not shown). As one of the possible reasons for our not being able to observe the inactivation effects of CEC in rabbit aorta, incomplete access of the highly water-soluble CEC to α_1-adrenergic receptor sites should be considered (9), especially because we treated intact tissue rather than isolated cells. Because this problem has been reported to be overcome by repetitive pretreatment with CEC (9), we also examined specific ^{125}I-BE binding sites after five repetitive treatments (5 min each) with 100 μM CEC. Although there was no significant loss of ^{125}I-BE binding sites after a single period of treatment (B_{max}, 18.5 ± 2.4 fmol/mg of protein, n = 6 in rabbit aorta unexposed to CEC, versus 17.4 ± 3.2 fmol/mg of protein, n = 4 after a single treatment of 100 μM CEC), five sequential treatments with 100 μM CEC resulted in a significant 31% loss of binding sites (12.8 ± 1.3 fmol/mg of protein, n = 6, $p < 0.05$). Additionally, we confirmed that CEC had similar potencies in both tissues as a competitive antagonist (the K_i values for CEC were 1.4 ± 0.40 μM, n = 3 in rat hepatocytes, versus 1.8 ± 0.36 μM in rabbit aorta, n = 4).

To determine whether the differential inactivation of ^{125}I-BE binding sites reflected effects on functional receptors, the effects of CEC pretreatment on norepinephrine-stimulated phosphorylase activation were examined in both tissues. Pretreatment with various concentrations of CEC for 20 min caused a progressive decrease in the maximal norepinephrine-induced phosphorylase activation in rat hepatocytes, reaching a total reduction at a CEC concentration of 100 μM (Fig. 4), whereas the 20-min incubation with CEC had no significant effect in rabbit aorta (data not shown). Furthermore, we found that even five sequential treatments of rabbit aorta with 100 μM CEC, which caused a 31% loss of binding sites, had little effect on the maximal norepinephrine-induced phosphorylase activation in the presence of extracellular Ca^{2+} (Fig. 5, *upper*). Examining the effect of a 20-min incubation of rat hepatocytes with several concentrations of CEC on maximal norepinephrine-stimulated phosphorylase activation, we observed that the relationship between maximal ^{125}I-BE binding capacity and maximal norepinephrine-stimulated phosphorylase activation, as defined by the CEC inactivation method, is linear for the liver cells (Fig. 4, *inset*) as was delineated by the phenoxybenzamine inactivation method. Additionally, the antagonist potency of WB 4101 was examined in the two systems; the K_b values for WB 4101 in inhibiting the phosphorylase responses to norepinephrine were 23 ± 5 nM (n = 4) and 1.6 ± 0.8 nM (n = 3) in rat hepatocytes and rabbit aorta, respectively.

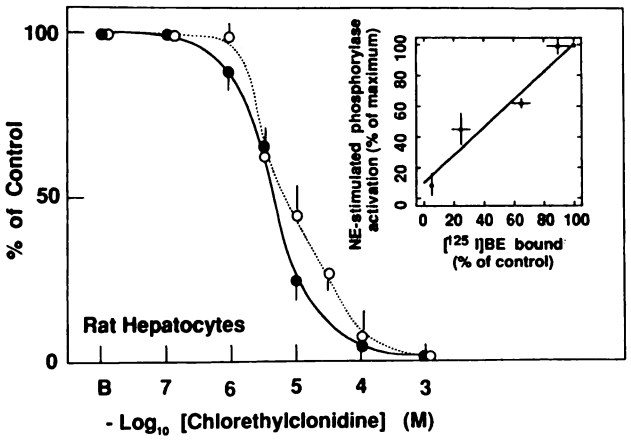

Fig. 4. Effect of a 20-min incubation of rat hepatocytes with several concentrations of CEC on maximal ^{125}I-BE binding capacity (●) and maximal norepinephrine (10 μM)-stimulated phosphorylase activation (○). Maximal specific ^{125}I-BE capacities were 131.7 ± 10.5 fmol/mg of protein (n = 5, rat hepatocytes) and 16.8 ± 1.7 fmol/mg of protein (n = 5, rabbit aorta), respectively. Data are means ± standard errors of five ^{125}I-BE binding or four phosphorylase experiments. *Inset*, relationship between maximal ^{125}I-BE2254 binding capacity and maximal norepinephrine (10 μM)-stimulated phosphorylase activation in rat hepatocytes after incubation with CEC as described in Fig. 4. Data depicted are replotted from Fig. 4.

Because each α_1-adrenergic receptor subtype may be linked to different signal transduction mechanisms for increasing intracellular Ca^{2+} (10), we next examined the phosphorylase activation by α agonists in both tissues in the presence and absence of extracellular Ca^{2+}. Norepinephrine- and phenylephrine-induced phosphorylase activations were not much influenced by the removal of Ca^{2+} from the extracellular fluid in rat hepatocytes (10 μM norepinephrine-induced phosphorylase *a* activities were 86 ± 9% of total activity, n = 6, and 78 ± 8% of total activity, n = 6, in the presence and absence of extracellular Ca^{2+}, respectively; 100 μM phenylephrine-induced phosphorylase *a* activities were 78 ± 8% of total activity, n = 6, and 65 ± 6% of total activity, n = 6, in the presence and absence of extracellular Ca^{2+}, respectively). Also, either with or without extracellular Ca^{2+}, phosphorylase activation induced by these agonists were completely suppressed by pretreatment with 10 μM CEC for 20 min (data not shown).

In contrast, even after five repetitive pretreatments with 10 μM CEC, phosphorylase activation to norepinephrine, phenylephrine, and methoxamine in the presence of extracellular Ca^{2+} was not inhibited at all in rabbit aorta (Fig. 5, *upper*). Treatment with nicardipine (1 μM) decreased phosphorylase activation to α-adrenergic agonists and KCl; the greatest reduction was in KCl-induced activation (55 ± 1.7% decrease in percentage of total activity, n = 6) (Fig. 5, *middle*). With the presence of La (5 mM) in the buffer the basal activity for phosphorylase was substantially ($p < 0.05$) increased (27 ± 3.4% in percentage of total activity, n = 5 in La-containing solution, versus 8 ± 2.9% in percentage of total activity in control, n = 6); however, the increase in phosphorylase activation over basal activity for all stimulants was markedly suppressed, compared with those in control (Fig. 5, *middle*). In the absence of extracellular Ca^{2+}, all of these agonist-induced responses were significantly ($p < 0.05$) decreased, compared with those in the presence of 2 mM extracellular Ca^{2+}; in particular, the decrease in phosphorylase activity was significantly more marked with methoxamine

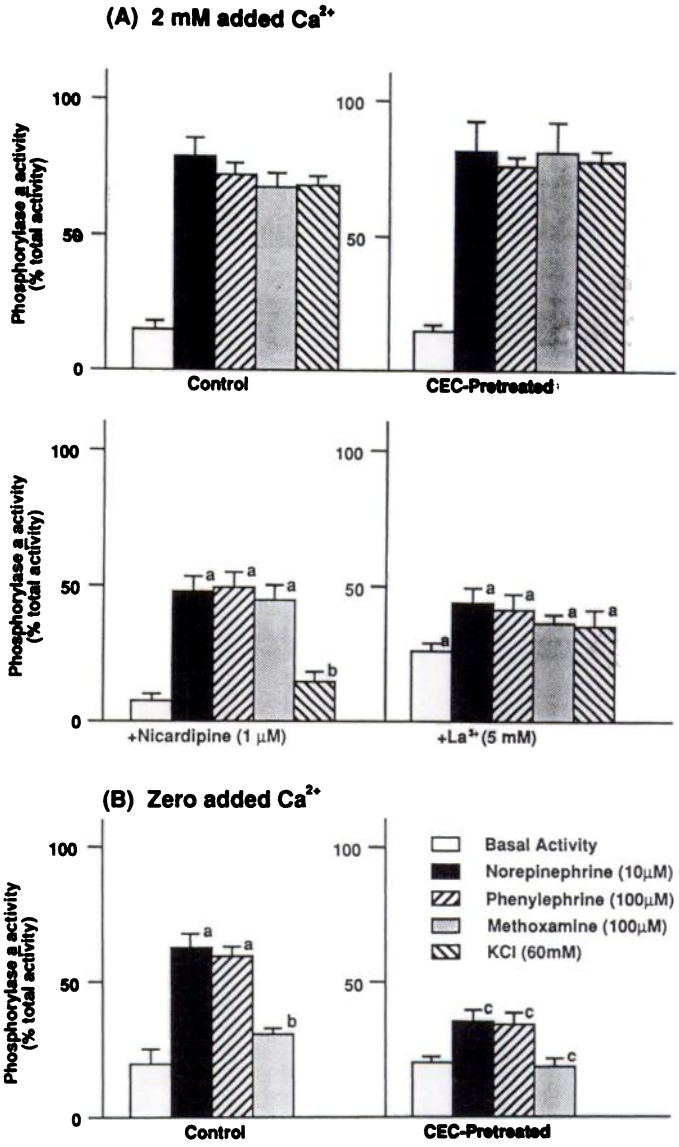

Fig. 5. Effects of CEC pretreatment (*upper*), nicardipine (1 μM)- and $LaCl_3$ (5 mM)-containing Krebs-Ringer bicarbonate solution (*middle*), and removal of extracellular Ca^{2+} (*lower*) on norepinephrine (10 μM)-, phenylephrine (100 μM)-, and methoxamine (100 μM)-induced activation of phosphorylase *a* activity in rabbit aorta. Because ^{125}I-BE binding experiments showed that a single treatment with CEC had no effect on aorta α_1 receptors, in these experiments aortic rings were given five repetitive treatment with CEC (10 μM) before stimulation by agonists. The basal level of the glycogen phosphorylase *a* activities in control in the presence of extracellular Ca^{2+} was 15 ± 2% total activity; 1.36 ± 0.21 units/100 mg of protein, n = 8. Results are the mean ± standard errors (*bar*) of five to nine experiments. $^a p < 0.05$ compared with corresponding phosphorylase *a* activity of control in the presence of extracellular Ca^{2+}. $^b p < 0.01$ compared with corresponding phosphorylase *a* activity of control in the presence of extracellular Ca^{2+}. $^c p < 0.05$ compared with corresponding phosphorylase *a* activity of control in the absence of extracellular Ca^{2+}.

treatment (decrease in percentage of total activity, 38 ± 2.1%, n = 9) than with the other α adrenergic agonists (17 ± 2.2%, n = 7 for norepinephrine, and 12 ± 1.8%, n = 7 for phenylephrine, respectively) (Fig. 5, *lower*). Furthermore, a combination of both removal of extracellular Ca^{2+} and repetitive CEC (10 μM) treatment completely abolished the phosphorylase response to methoxamine, whereas the phosphorylase response to either

norepinephrine or phenylephrine was not eliminated by the combination (Fig. 5, *lower*).

We further examined the relationships between each α_1 receptor subtype and PI hydrolysis. In rat hepatocytes, norepinephrine (10 μM) potently stimulated IP accumulation (764 ± 51 cpm/mg of protein, $n = 8$, and 711 ± 62 cpm/mg of protein, $n = 8$, in the presence and absence of extracellular Ca^{2+}, respectively), whereas methoxamine (up to 1 mM) was inactive in either the presence or absence of extracellular Ca^{2+} (1 mM methoxamine-induced IP accumulations were 468 ± 61 cpm/mg of protein, $n = 7$, and 446 ± 57 cpm/mg of protein, $n = 7$, in the presence and absence of extracellular Ca^{2+}, respectively; basal levels were 459 ± 48 cpm/mg of protein, $n = 8$, and 440 ± 68 cpm/mg of protein, $n = 8$, in the presence and absence of extracellular Ca^{2+}, respectively). A 20-min pretreatment of rat hepatocytes with CEC (10 μM) completely suppressed the norepinephrine-stimulated IP formation either with or without extracellular Ca^{2+} (data not shown).

In rabbit aorta, methoxamine (100 μM) activated IP formation as potently as norepinephrine (10 μM) in the presence of 2 mM extracellular Ca^{2+} (Fig. 6, *upper*). Although the repetitive treatment of rabbit aorta with CEC (10 μM) significantly ($p < 0.05$) increased the basal level of IP accumulation (8 ± 1.8%, $n = 6$, versus 17 ± 1.6%, $n = 6$), it potently suppressed norepinephrine-stimulated IP accumulation (6 ± 4.8% decrease in percentage of E_{max}, $n = 6$); however, the CEC treatment had a significantly ($p < 0.05$) less inhibitory effect on methoxamine-induced IP formation (29 ± 3.2% decrease in percentage of E_{max}, $n = 6$) (Fig. 6, *upper*). Membrane depolarization by KCl (60 mM), which potently stimulates phosphorylase activation in rabbit aorta, produced no significant generation of IP. In contrast to phosphorylase activations, treatment with nicardipine (1 μM) had little effect on the agonist-induced IP accumulations (Fig. 6, *middle*). With the presence of 5 mM La in the buffer, on the other hand, both norepinephrine- and methoxamine-induced IP formations were markedly reduced; the reduction is much greater in methoxamine-induced response (decrease in percentage of E_{max}, 71 ± 7.2%, $n = 6$ for norepinephrine, versus 90 ± 4.3%, $n = 6$ for methoxamine) (Fig. 6, *middle*). In the absence of extracellular Ca^{2+}, similar results as in experiments with La-containing solution were obtained (Fig. 6, *lower*). Both norepinephrine- and methoxamine-induced IP formations were markedly reduced; the reduction is much greater in methoxamine-induced response (decrease in percentage of E_{max}, 70 ± 3.3%, $n = 6$ for norepinephrine, versus 92 ± 2.6%, $n = 6$ for methoxamine). CEC (10 μM) treatment further reduced the remaining IP accumulation response (Fig. 6, *lower*).

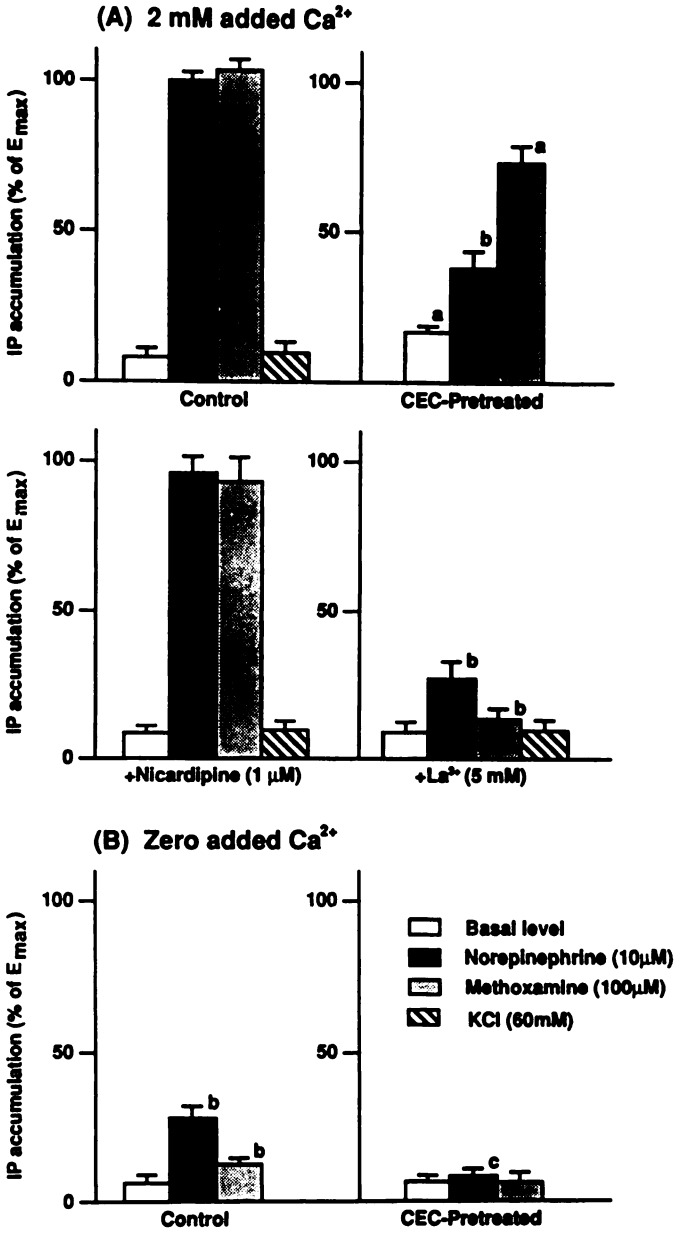

Fig. 6. Effects of CEC pretreatment (*upper*), nicardipine (1 μM)- and $LaCl_3$ (5 mM)-containing Krebs-Ringer bicarbonate solution (*middle*), and removal of extracellular Ca^{2+} (*lower*) on norepinephrine (10 μM)-, phenylephrine (100 μM)-, methoxamine (100 μM)-, and KCl (60 mM)-induced [^{3}H]IP accumulation in rabbit aorta. Because ^{125}I-BE binding experiments showed that a single treatment with CEC had no effect on aortic α_1 receptors, in these experiments aortic rings were given five repetitive treatment with CEC (10 μM) before stimulation by agonists. Results are presented as the means ± standard errors (*bars*) of at least five different experiments. Values for each treatment are expressed as percentage of the maximal response to norepinephrine (10 μM, E_{max} = 100%; 1589 ± 112 cpm/mg of protein). $^a p < 0.05$ compared with corresponding phosphorylase *a* activity of control in the presence of extracellular Ca^{2+}. $^b p < 0.01$ compared with corresponding phosphorylase *a* activity of control in the presence of extracellular Ca^{2+}. $^c p < 0.05$ compared with corresponding phosphorylase *a* activity of control in the absence of extracellular Ca^{2+}.

Discussion

By examining responses in rat hepatocytes and rabbit aorta, we found that α_1-adrenergic receptor-mediated phosphorylase activation in each system was markedly different quantitatively and also qualitatively. As defined by the phenoxybenzamine inactivation method, the α_1 receptor occupancy-phosphorylase activation relationship is linear in rat hepatocytes, whereas it is nonlinear in rabbit aorta. Also, α_1 receptor-mediated phosphorylase activation in rat hepatocytes is CEC sensitive, whereas that in rabbit aorta is CEC-insensitive. Furthermore, as summarized in Fig. 7, we found that these pharmacologically distinct α_1 receptors in the two systems were coupled with different Ca^{2+}-signalling mechanisms for phosphorylase activation. Methoxamine interacted with α_1 receptor sites in both systems but stimulated PI hydrolysis and phosphorylase activation only in rabbit aorta, mainly by increasing Ca^{2+} entry.

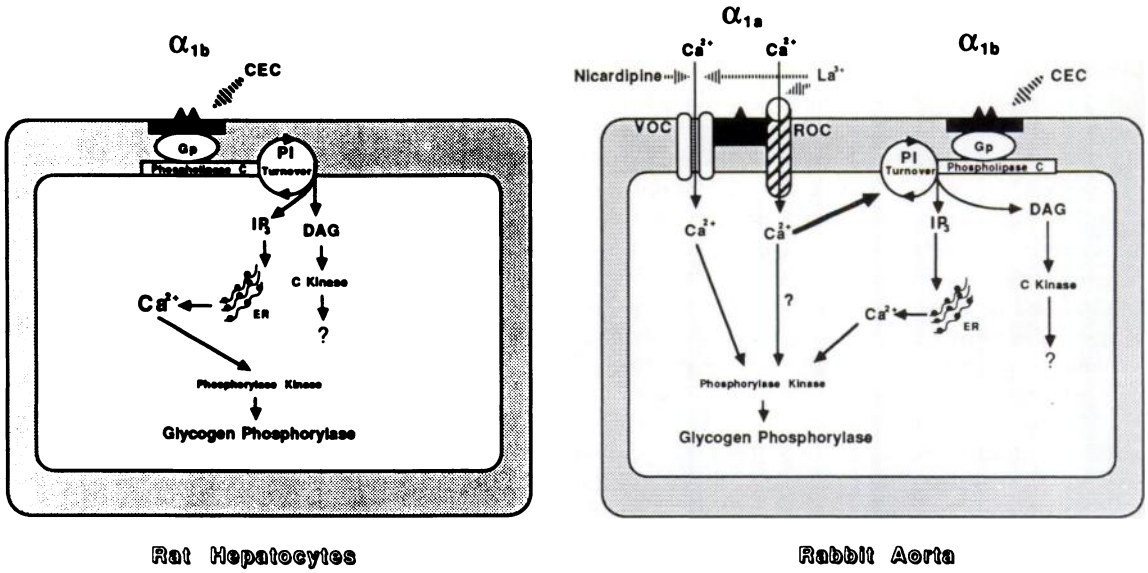

Fig. 7. The proposed mechanisms for Ca^{2+} signal transduction linked to α_1-adrenergic receptor subtypes in activating glycogen phosphorylase of rat hepatocytes and rabbit aorta. See text for details.

The results suggest that methoxamine is a selective agonist for a putative α_1 receptor subtype (CEC insensitive or α_{1a}), which predominantly couples with Ca^{2+} influx.

The α_1 receptor occupancy-phosphorylase response relationship defined by the phenoxybenzamine inactivation method is linear in rat hepatocytes. Recently we have also observed the linear relationship between occupancy of α_1 receptor binding sites and $[Ca^{2+}]_c$ response in the same system (19). The linear relationships between these parameters indicate an absence of a receptor reserve in activating these intracellular events in rat liver cells, which is in good agreement with previous observation by Lynch *et al.* (34) on the agonist concentration-response relationship in this tissue. In a sharp contrast to rat liver cells, we observed the marked nonlinearity between the same parameters in rabbit aorta, indicating the presence of a sizable pool of spare receptor. Alternatively, this nonlinear relationship may indicate the presence of a postreceptor amplification, as proposed by Sastre *et al.* (35). Because phosphorylase activation, a step distal to the increase in $[Ca^{2+}]_c$, was evaluated, the exact locus of the amplification step cannot be determined from the present experiments. Comparison with rat hepatocytes, however, suggests that the nonlinear relationship in the rabbit aorta occurs, at least in part, at a step proximal to the increase in $[Ca^{2+}]_c$.

α_1-Adrenergic receptors for phosphorylase activation in the two systems are different in their pharmacological properties as well. In rat liver cells, CEC completely inactivated the α_1 receptor binding sites responsible for phosphorylase activation, whereas in rabbit aorta even the repetitive CEC treatment had little effect on norepinephrine-mediated phosphorylase activation in the presence of extracellular Ca^{2+} with only a 31% inactivation of total α_1 receptor sites. The results suggest that α_1 receptors in rat liver are mostly the CEC-sensitive type, whereas rabbit aorta contains both CEC-sensitive and CEC-insensitive types, with the CEC-insensitive type predominating. Also, in rat liver cells we observed the similar unitary α_1 receptor occupancy-phosphorylase activation relationship with CEC as defined by the phenoxybenzamine inactivation method, suggesting that α_1 receptors functionally responsible for activating phosphorylase (CEC sensitive type) have little receptor reserve in this tissue. This finding confirmed and extended the previous observation by Minneman *et al.* (9) that CEC treatment could inactivate most $[Ca^{2+}]_c$ receptors in the slice preparation of this tissue.

Although CEC-sensitive α_1 adrenergic receptors are a minor population in rabbit aorta, they clearly have some contribution in activating phosphorylase. In the presence of extracellular Ca^{2+}, the CEC treatment was found to have no inhibitory effect on agonist-induced phosphorylase activation. On the other hand, in the absence of extracellular Ca^{2+}, the CEC treatment further reduced phosphorylase activation to α agonists. Thus, in rabbit aorta, CEC-sensitive α_1 receptors (α_{1b}) clearly play a role in activating phosphorylase but their effects appear to be masked in the presence of extracellular Ca^{2+}. The results imply that the simultaneous activation of CEC-insensitive α_1 receptor, which predominantly couple with the Ca^{2+} influx, can mask CEC-sensitive α_1 receptor-mediated phosphorylase activation in this tissue.

Previously, Morrow and Creese and colleagues (6, 7) 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 antagonists phentolamine and WB 4101; they termed the high and low affinity sites for WB 4101 α_{1a} and α_{1b}, respectively. In the present study, the competitive potencies of WB 4101 in inhibiting norepinephrine-induced phosphorylase activation in rabbit aorta and rat hepatocytes corresponded well to those previously reported for high and low affinity sites of WB 4101 (10). Morrow and Creese (7) also proposed that the ratio of phentolamine to prazosin at α_{1a} binding sites is approximately 4, whereas the same ratio at α_{1b} binding sites is approximately 80. According to their literature survey, for rabbit aorta (in contraction experiments), the ratio was 6.5 (36), indicating that α_1 receptors in rabbit aorta can be classified as the α_{1a} type, whereas the ratios in rat liver obtained from Ca^{2+}-dependent phosphorylase activation (37) and [^{3}H]-prazosin binding experiments (38) were 57 and 212, respec-

tively, suggestive of pure α_{1b}. Interestingly, however, our previous characterization of α_1 receptor binding sites by I-BE in rabbit aorta (26) provides a ratio of 39; the intermediate ratio indicates that the tissue contains both subtypes.

As indicated in the introduction, Han *et al.* (10) have recently extended the subclassification by incorporating functional responses at the second messenger level; one subtype (CEC sensitive or α_{1b} receptor) stimulates PI hydrolysis and causes a physiological response that is independent of extracellular Ca^{2+}, and the other (CEC insensitive or α_{1a} receptor) causes a physiological response by stimulating the influx of extracellular Ca^{2+}. In good agreement with their proposal, we found that α_1-adrenergic receptors in the two systems were linked to different mechanisms for increasing intracellular Ca^{2+} in activating phosphorylase (Fig. 7). α_1-Adrenergic receptor activation in rat hepatocytes causes PI hydrolysis and subsequently releases Ca^{2+} from intracellular stores, which is responsible for phosphorylase activation. In rabbit aorta, on the other hand, norepinephrine causes both PI hydrolysis and Ca^{2+} influx, and the Ca^{2+} introduced through the latter mechanism seems to facilitate PI hydrolysis. Therefore, in rabbit aorta the final physiological response of α_1 receptor-mediated phosphorylase activation is caused by the $[Ca^{2+}]_c$ increase due to both Ca^{2+} release from intracellular stores and Ca^{2+} entry from extracellular fluid, with Ca^{2+} influx predominantly working.

It is notable that α_1-adrenergic receptor-mediated PI hydrolysis in rabbit aorta can be facilitated by the CEC-insensitive (α_{1a}) receptor-mediated Ca^{2+} entry. The inhibitory effects of removal of extracellular Ca^{2+} on PI turnover in rabbit aorta may be due to the observations that phosphorylase C activation is Ca^{2+} dependent (39, 40). The facilitation of PI hydrolysis by the Ca^{2+} entry may work as a signal amplification mechanism and may possibly mask CEC-sensitive (α_{1b}) receptor-mediated responses. Another interesting point is that the entry of Ca^{2+} across the plasma membrane for PI hydrolysis activation occurred through a different pathway than that for phosphorylase activation. α_1 Receptor-mediated Ca^{2+} entry responsible for phosphorylase activation is through both a La-sensitive and a nicardipine-sensitive pathway, whereas that for PI hydrolysis is through a La-sensitive but nicardipine-insensitive pathway. Thus, a voltage-operated channel (dihydropyridine-sensitive channel) is involved in increasing $[Ca^{2+}]_c$ for phosphorylase activation, whereas the Ca^{2+} introduced through this pathway seems not to be working in facilitating PI hydrolysis. Additionally, the observation that K^+ depolarization potently activates phosphorylase but not PI hydrolysis in this tissue strongly supports the proposal that the Ca^{2+} entry through a voltage-operated channel is involved only in phosphorylase activation.

Based on the pharmacological differences between the two tissues, the observation that methoxamine potently stimulates α_1 receptors in rabbit aorta (predominantly CEC insensitive) but has little effect on those in rat hepatocytes (mostly CEC sensitive) suggests that the agent is a selective agonist for CEC-insensitive α_1 receptors. Because the greatest reduction in both IP production and phosphorylase activation after removal of extracellular Ca^{2+} was observed for methoxamine among the agonists examined in rabbit aorta, methoxamine appears to potently stimulate Ca^{2+} influx and, thereby, causes PI hydrolysis and phosphorylase activation in this tissue. It is, however, notable that methoxamine-induced phosphorylase activation in rabbit aorta can be further reduced by CEC treatment in the absence of extracellular Ca^{2+}, indicating that methoxamine may be able to interact with CEC-sensitive α_1 receptors as well as directly stimulate them. However, the extent to which methoxamine can stimulate IP accumulation and phosphorylase activation is more dependent on the CEC-insensitive, extracellular Ca^{2+}-influx pathway, compared with other α-adrenergic agonists. Taken together, our data suggests that methoxamine selectively activates α_1-adrenergic receptors, which predominantly couple with Ca^{2+} influx.

In summary, our results strongly support the existence of two pharmacologically distinct α_1-adrenergic receptor subtypes, each of which appears to be linked to different mechanisms for increasing $[Ca^{2+}]_c$ in activating phosphorylase. Regulation of these subtype receptors and interactions between Ca^{2+}-signalling mechanisms linked to each subtype may be of physiological importance. Also, development of selective agents for each subtype may be of pharmacotherapeutic significance. Rat hepatocytes and rabbit aorta should provide useful model systems to pursue these potentially important issues.

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