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THE α-ADRENERGIC MEDIATED EFFECT IN RAT LIVER

CORRELATION BETWEEN [^{3}H]-DIHYDROERGOCRYPTINE BINDING TO PLASMA MEMBRANES AND GLYCOGEN PHOSPHORYLASE ACTIVATION IN ISOLATED HEPATOCYTES*

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Abstract—Alpha-adrenergic receptors of purified rat liver plasma membranes were characterized by the direct binding of [^{3}H]-dihydroergocryptine. Since glycogen phosphorylase (EC 2.4.1.1; 1,4-alpha-D-glucan: orthophosphate alpha-glucosyl transferase) activation in isolated rat hepatocytes is known to be mediated mainly through an alpha-adrenergic mechanism, we compared the abilities of various adrenergic agonists and antagonists in competing with [^{3}H]-dihydroergocryptine for the binding to alpha-sites in purified membranes and in activating phosphorylase or in inhibiting its activation in isolated hepatocytes. The activation of phosphorylase by agonists displayed an order of potencies typical of alpha-adrenergic mediators: (-)norepinephrine ($K_a = 0.31\ \mu M$) ≥ (−)epinephrine ($K_a = 0.76\ \mu M$) >> (−)isoproterenol ($K_a = 24\ \mu M$), and the stereospecificity expected: (−)epinephrine ($K_a = 0.76\ \mu M$) > (+)epinephrine ($K_a = 5.2\ \mu M$), (−)norepinephrine ($K_a = 0.31\ \mu M$) > (+)norepinephrine ($K_a = 3.3\ \mu M$). Alpha-adrenergic antagonists (phentolamine $K_{iapp} = 1.71\ \mu M$) were much more potent than beta-adrenergic antagonists [(−)propranolol $K_{iapp} = 113\ \mu M$] in preventing the activation of glycogen phosphorylase by epinephrine. In our system imidazoline derivatives such as clonidine appeared to be mainly antagonists for the alpha-adrenergic mediated events. Close correlations ($r = 0.92$, $P < 0.01$ and $r = 0.91$, $P < 0.001$) were observed, respectively, for adrenergic agonists and antagonists between either activation or prevention of the activation of the enzyme and competition with dihydroergocryptine for alpha-adrenoreceptors. These results validate the use of [^{3}H]-dihydroergocryptine as a marker of the physiological alpha-adrenergic receptor in rat liver.

In the past few years, [^{3}H]-dihydroergocryptine (DHEC)† has been used to characterize the alpha-adrenergic receptor in various systems (for a general review, see ref. 1). However, some reports have argued against this ligand as a good label for the alpha-adrenergic receptor. For uterine smooth muscle, Kunos *et al.* suggested that [^{3}H]-DHEC might label two classes of sites, one with low capacity and high affinity, corresponding to the true alpha-adrenoreceptors, and another class with higher capacity and lower affinity [2]. In rat liver plasma membranes, the number of alpha-adrenergic binding sites differed, depending upon the radioligand used, [^{3}H]-DHEC or [^{3}H]-catecholamines [3]. Furthermore, in rat brain, ergot alkaloids such as dihydroergocryptine were found to bind to serotonin [4] and dopamine receptors [5, 6] in addition to the alpha-adrenergic receptors. It was important, therefore, to correlate the binding to the alpha-adrenoreceptor of a ligand such as [^{3}H]-DHEC with a biological or enzymatic parameter as in the case of the beta-adrenergic receptor–adenylate cyclase system (for a general review, see ref. 1). Such a correlation has been obtained for platelets where the binding to the alpha-receptor was coupled to the inhibition of adenylate cyclase [7, 8] and to the aggregation of the platelets [8]. In rat parotid cells, the occupancy of the alpha-adrenoreceptor by catecholamines was related to the release of K^+ by the cells [9]. The integrity of the cell seems necessary for the alpha-adrenergic response, since no biological phenomenon in purified plasma membranes, except in platelets [10], has been connected, to date, with an alpha-adrenergic stimulus.

We characterized the alpha-adrenergic receptor in rat liver plasma membranes by the use of [^{3}H]-DHEC [11]. In order to verify that the binding sites identified with that ligand corresponded to the true alpha-adrenergic receptors, we correlated the binding data with a biological event. We chose the activation of glycogen phosphorylase in isolated rat hepatocytes. Indeed, the activation of phosphorylase through an alpha-adrenergic mechanism in rat liver has been described [12–15]. In this report, we present evidence that the binding sites defined with [^{3}H]-DHEC in rat liver plasma membranes correlate with the sites defined by the activation or prevention of activation of phosphorylase in rat hepatocytes. These results confirm the use of isolated rat liver cells as a tool for the study of alpha-adrenergic parameters and they validate [^{3}H]-DHEC as a label for the pharmacological alpha-adrenoreceptor in this system.

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† Abbreviations used: DHEC, dihydroergocryptine; hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

MATERIALS AND METHODS

Materials

Naphazoline, xylometazoline, phentolamine, (±)alprenolol (Ciba–Geigy), (+)epinephrine, (+)norepinephrine, (−)nordefrin (Sterling-Winthrop), phenoxybenzamine (Smith, Kline & French), yohimbine (Roussel Uclaf), (−)propranolol (Imperial Chemical Industries), dihydroergocryptine, hydroxybenzylpindolol, pindolol, 2-bromo-alpha-ergocryptine (CB 154) (Sandoz), azapetine (Hoffman–La Roche), labetalol, salbutamol (Allen & Hanbury), metaraminol (Merck Sharp & Dohme), clonidine (Boehringer Ingelheim), oxymetazoline (Farmex), timazoline (L.E.R.S.), prazosin and tetrahydrozoline (Pfizer) were obtained as gifts. (−)Epinephrine, (−)norepinephrine, (−)isoproterenol, (−)phenylephrine, (±)metanephrine, serotonin, glycogen type II from oyster, alpha-D glucose-l-phosphate grade 1, hepes, dopamine and glycylglycine were from Sigma Chem. Co. Crude collagenase, CLS type, was purchased from Worthington. Alpha-D-[^{14}C]-glucose-l-phosphate (0.3 Ci/mmole) was supplied from the Radiochemical Centre (Amersham, U.K.). (−) Propyl-2.3-[^{3}H] -dihydroergocryptine (24 Ci/mmole) was supplied from New England Nuclear Co. All other chemicals were from Merck (Darmstadt, F.R.G.).

Methods

Rat liver plasma membrane preparation. Plasma membranes were prepared from 100 g female, Wistar rats according to the procedure of Neville [16] up to step 11. The membrane preparations were stored in liquid nitrogen until use, without any loss of binding up to four months.

Rat hepatocyte isolation. Male Wistar rats (230–330 g body wt), fed laboratory chow *ad lib.*, were anaesthetized with nembutal, and liver parenchymal cells were isolated according to the procedure of Seglen [17] with the following modifications. (i) The Ca^{2+}-free perfusion buffer (pH 7.75) was used to prepare the collagenase buffer; 40 mg collagenase and 100 mg $CaCl_2$, $2H_2O$ were dissolved in 100 ml of the buffer and filtered through 0.45 μm Millipore filter before use. (ii) The 'washing buffer' was 1.3 mM $CaCl_2$ and was gassed with O_2:CO_2 (95%, 5%) before adjustment to pH 7.45. (iii) The suspension buffer, which was also used as incubation medium, was a Krebs–Ringer bicarbonate buffer (NaCl, 120 mM; KCl, 4.8 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 24 mM; $CaCl_2$, 1.3 mM) gassed with O_2:CO_2 (95%, 5%) and adjusted to pH 7.4. After dispersion, damaged and non-parenchymal cells were removed by three centrifugations at 90 *g* for 50 sec in the washing buffer and one centrifugation at the same speed in the suspension buffer. Following the last centrifugation, cells were resuspended in the suspension buffer under an O_2:CO_2 (95%, 5%) atmosphere. Cells were counted in a hemacytometer and the viability was expressed as the percentage of cells unstained by 0.45% trypan blue. Only preparations in which the viability exceeded 85 per cent were used. Hepatocytes were always prepared between 1:00 p.m. and 3:00 p.m. to minimize diurnal variations in metabolic processes.

Hepatocyte incubation. In a typical experiment, 50–100 × 10^6 cells in 5.8 ml of solution containing glucose, 4 g/l [18] were preincubated for 30 min at 37° in a 50 ml polypropylene Erlenmeyer undergoing rotational agitation, in order to reduce basal level of glycogen phosphorylase. For the study of agonists, the incubation of the hepatocytes, at 37° under an O_2:CO_2 (95%, 5%) atmosphere, was started by the addition of the preincubated cells (125 μl) to a 5-ml polypropylene tube containing the agonist (25 μl). The reaction was stopped 2 min later (unless otherwise stated) by freezing the tube in a dry ice–acetone mixture. For the study of antagonists, the reaction was started by the addition of the preincubated cells (125 μl) to a 5 ml polypropylene tube containing the antagonist (25 μl) and the incubation was performed at 37°. Six minutes later, the agonist (−)epinephrine (25 μl) was added at a final concentration of 5 μM and the reaction was stopped 1 min later by freezing the tube. The frozen tubes were kept at −80° until the phosphorylase a was assayed.

Phosphorylase a assay. The frozen hepatocytes were homogenized in 4 vol. of an ice-cold medium containing 100 mM NaF, 20 mM EDTA, 0.5% (w/v) glycogen and 50 mM glycylglycine, pH 7.4, according to Hue *et al.* [18]. The incubation was started by the addition of the homogenate (0.1 ml) to an equal volume of a solution containing 30 mM [^{14}C]-glucose-1-phosphate (0.01 μCi/μmole, 50,000 c.p.m.), 2% (w/v) glycogen, 0.3 M NaF, 1 mM caffeine [18], pH 6.1. After a 20–30 min incubation at 30°, the reaction was stopped by spotting 50 μl aliquots of incubation medium onto Whatman ET-31 filter papers (2.5 × 3 cm) which were dropped into ice-cold 66% (v/v) ethanol in order to precipitate glycogen. Each point was the mean of quadruplicate determinations which agreed within ± 5 per cent. The papers were washed and counted for radioactivity according to Gilboe *et al.* [19] with the following modifications: (i) the second ice-cold 66% ethanol washing solution was followed by a third 66% ethanol bath at room temperature; (ii) the three bath periods were at least 30 min each; (iii) the beakers contained 10 ml solution for each paper. Under these experimental conditions, blank values determined with boiled homogenate (70 ± 25 c.p.m.), represented 10–23 per cent of the radioactivity retained on the papers and 0.5 per cent of the radioactivity present in the incubation medium. The radioactivity was measured in vials containing 10 ml of Ready Solve EP (Beckman) scintillation mixture at an efficiency of 80 per cent. The basal level of the enzyme slightly varied from day to day experiment. The average of the basal level for 28 different batches of cells was 0.0094 ± 0.0004 (mean ± S.D.) μmoles of glucose-1-phosphate transformed per min per mg protein. However, for experiments performed with the same batch of hepatocytes, the basal level of the enzyme did not vary by more than 5 per cent. We verified that for the phosphorylase a assay the rate of the reaction was linear with respect to incubation time up to 60 min and linear with respect to protein concentration up to 9 mg/ml (in a typical experiment, the protein concentration was 2.5 mg/ml).

Binding assay of [^{3}H]-*DHEC to rat liver plasma membranes.* This assay was performed as previously described [20]. The specific binding of [^{3}H]-DHEC referred to the fraction of the radiolabeled ligand displaced by 0.01 or 0.1 mM phentolamine and represented 70–80 per cent of the total binding. Protein was measured using bovine serum albumin as standard according to the procedure of Lowry *et al.* [21].

Determination of K_a and $K_{i\ app}$ *values for phosphorylase a.* The K_a value for an agonist was taken as the concentration of the agent causing half the maximal phosphorylase a activation observed with that agent. The apparent K_i value for a competitive antagonist was calculated according to Cheng and Prusoff [22] by the equation

$$K_{i\,app} = EC_{50} \Big/ \left\{1 + \frac{[(-)\text{epinephrine}]}{K_a\ (-)\text{epinephrine}}\right\},$$

where EC_{50} is the concentration of antagonist causing a 50 per cent prevention of the activation obtained in the presence of 5 μM (−)epinephrine in the absence of any antagonist. [(−)Epinephrine] is the concentration of that agonist in the assay and K_a (−)epinephrine is as defined above. Since we measured a non-direct effect of antagonists upon the enzyme, but a prevention of activation through the receptor occupancy, we cannot calculate a true K_i by the method of Cheng and Prusoff, but an apparent K_i. K_a's and $K_{i\ app}$'s are the means of quadruplicate determinations, which do not vary by more that 10 per cent, each experiment being usually repeated twice.

Determination of the K_D *values for* [^{3}H]-*DHEC binding sites.* The K_D values for all compounds which inhibited [^{3}H]-DHEC binding were calculated according to Cheng and Prusoff [22], by the equation

$$K_D = EC_{50} \Big/ \left(1 + \frac{[\text{DHEC}]}{K_{D(\text{DHEC})}}\right),$$

where EC_{50} represents the concentration of compound causing half-maximal inhibition of [^{3}H]-DHEC binding, [DHEC] represents the concentration of the radiolabeled ligand present in the assay and $K_{D(\text{DHEC})}$ is the dissociation constant of [^{3}H]-DHEC [11]. K_D's are the mean of triplicate determinations, which do not vary by more than 10 per cent, each experiment being usually repeated twice.

RESULTS

The data reported in this paper compare the binding studies of [^{3}H]-DHEC to the alpha-adrenergic receptor of rat liver plasma membranes with the activation of glycogen phosphorylase in isolated rat hepatocytes. Preliminary studies on the activation of phosphorylase by catecholamines in rat hepatocytes indicated that the activation of the enzyme is very rapid (maximal activation obtained 30 sec to 2 min after addition of the hormone) and transient (the enzyme returned to its less active form 5–10 min after addition of the hormone) (Fig. 1). Preliminary results led us to think that the transient action of catecholamines might be due to a rapid degradation of the hormone in our experimental conditions. Under the experimental conditions used, no desensitization phenomenon could be observed, since readdition of fresh(−)norepinephrine restimulated the glycogen phosphorylase activity (Fig. 1).

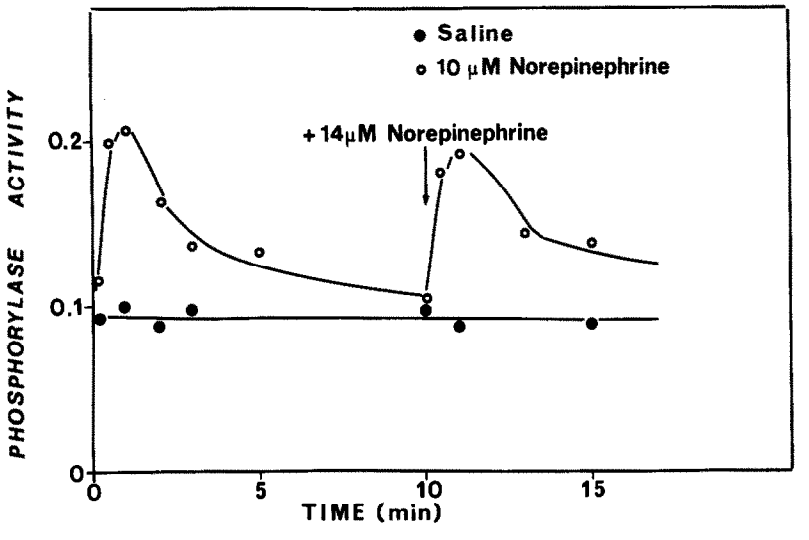

Fig. 1. Kinetics of activation of glycogen phosphorylase by (−)norepinephrine. Isolated rat hepatocytes (85×10^6 cells in 4.5 ml) were preincubated with glucose 4 g/l for 30 min at 37°. Portions (0.5 ml) of (−)norepinephrine 10 μM (final concentration) (○) or of buffer (●) were added and the reaction was stopped at various times studied by freezing 200 μl aliquots in a dry ice–acetone mixture. At time 10 min, 50 μl (−)norepinephrine 14 μM (final concentration) or buffer were added and the reaction stopped as described above. Phosphorylase a was assayed as described under Methods and its activity was expressed in μmoles of glucose-1-phosphate transformed in 20 min/mg protein. Each point is the mean of a quadruplicate determination. Basal and maximal activation values are 0.0047 and 0.011 μmoles/min/mg protein, respectively.

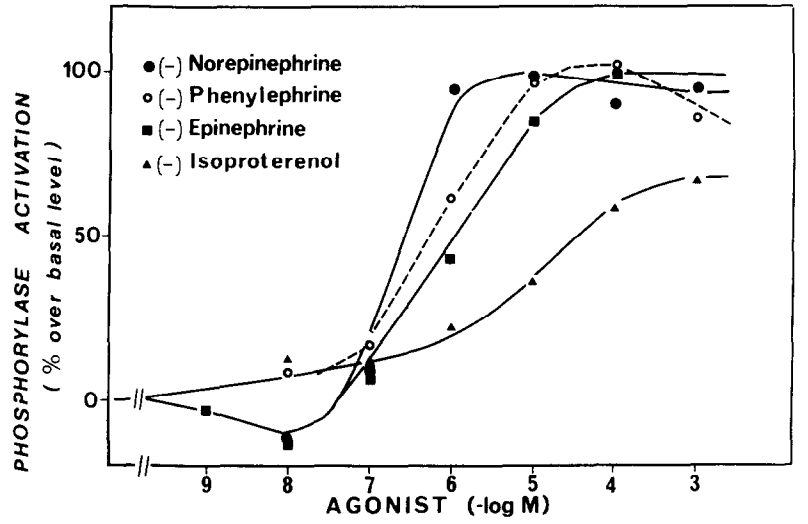

Fig. 2. Effect of various agonists on glycogen phosphorylase activity. Isolated rat hepatocytes (91×10^6 cells in 5.8 ml) were preincubated for 30 min at 37°, and then incubated with (−)epinephrine (■), (−)norepinephrine (●), (−)phenylephrine (○), (−)isoproterenol (▲) in various concentrations or buffer, as described under Methods. Basal level and maximal activity of the enzyme were, respectively, 0.025 and 0.05 μmoles of glucose-1-phosphate transformed per min/mg protein. These values are higher than the ones reported in Figs. 1 and 5, due to a shorter preincubation of hepatocytes in the presence of glucose. The enzyme activity is expressed as per cent of the activity above basal level. Each point is the mean of a quadruplicate determination.

Activation of rat liver glycogen phosphorylase is an alpha-adrenergic phenomenon. A number of alpha- and beta-adrenergic agonists were tested for their ability to activate the glycogen phosphorylase in isolated hepatocytes and for their ability to compete with [^{3}H]-DHEC for its binding sites in purified plasma membranes. Hepatocytes were incubated for 2 min with increasing concentrations (1 nM–1 mM final concentration) of four agonists. In Fig. 2, we show the effects of those agonists in stimulating the conversion of the less active phosphorylase b into the more active form of the enzyme, phosphorylase a. All those agonists activated the enzyme with the following order of potencies (Table 1): (−) norepinephrine ($K_a = 0.31$ μM) ≥ (−)epinephrine ($K_a = 0.76$ μM) ≥ (−)phenylephrine ($K_a = 1$ μM) >> (−)isoproterenol ($K_a = 24$ μM). This order was typical for an alpha-adrenergic mediated event [1].

The affinities of the same agents for the alpha-receptors identified by [^{3}H]-DHEC were also determined. In Fig. 3, we show the percentage of [^{3}H]-DHEC bound to the alpha-sites as a function of increasing concentrations of the agonists (10 nM–1 mM final concentration). The dissociation constants of those compounds for alpha-sites (Table 1) were: (−)norepinephrine ($K_D = 1.33$ μM) ≥ (−)

Table 1. Interaction of alpha and beta-agonists with [^{3}H]-DHEC binding sites and with glycogen phosphorylase*

Compound	[^{3}H]-DHEC binding sites K_D (μM)†	Phosphorylase	
		K_a (μM)	Maximal activation (% over basal level)
(−)Norepinephrine	1.33 (2)	0.31 (3)	100–120
(−)Epinephrine	1.68 (3)	0.76 (5)	100–160
(−)Phenylephrine	8.00 (2)	1.00 (2)	80
(−)Nordefrin	33.80 (3)	1.47 (2)	100
(+)Norepinephrine	46.00 (2)	3.30 (2)	85–95
(+)Epinephrine	54.00 (2)	5.20 (1)	160
Dopamine	72.00 (2)	12.50 (1)	55
(−)Isoproterenol	115.00 (2)	24.00 (3)	65
(±)Metanephrine	130.00 (2)	25.00 (1)	50
Metaraminol	100.00 (2)	27.50 (2)	25

* K_a and K_D values were determined as described under Methods. Maximal activation of glycogen phosphorylase is expressed as per cent of the activity over basal level. Numbers in parentheses represent the number of separate experiments performed for each compound tested.

† Some of the K_D values indicated here are slightly different from the ones given in ref. 11, due to the difference in the temperatures of incubation (30° here vs 37° in ref. 11).

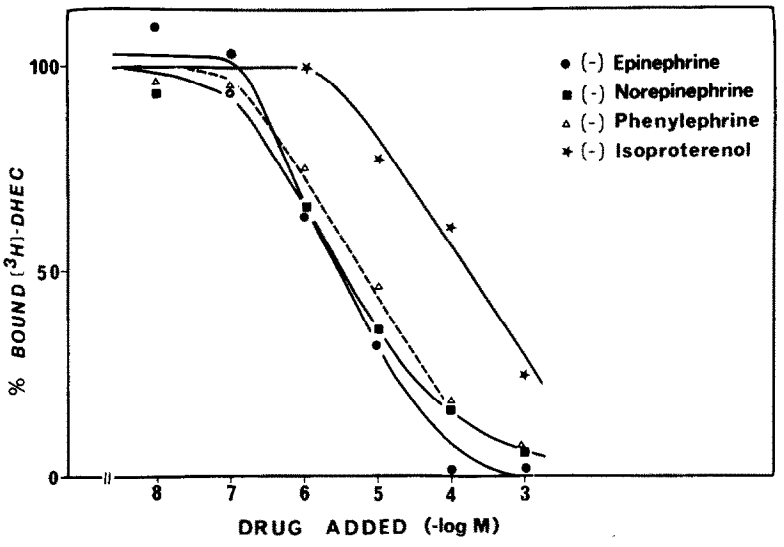

Fig. 3. Displacement of [^{3}H]-DHEC from alpha-receptors by various agonists. Purified plasma membranes (0.58 mg of protein/ml) were incubated at 30° for 10 min with 4.5 nM [^{3}H]-DHEC in the presence of increasing concentrations of (−)epinephrine (●), (−)norepinephrine (■), (−)phenylephrine (△) or (−)isoproterenol (★). Binding of [^{3}H]-DHEC was measured as described under Methods. Results are expressed as per cent of the amount of [^{3}H]-DHEC specifically bound in the absence of any agonist (820 fmoles of [^{3}H]-DHEC bound/mg of membrane protein). Each point is the mean of a triplicate determination.

epinephrine ($K_D = 1.68\ \mu M$) > (−)phenylephrine ($K_D = 8\ \mu M$) >> (−)isoproterenol ($K_D = 115\ \mu M$), as previously described [11]. The same order of potencies of those agonists was found either in activating the glycogen phosphorylase in isolated hepatocytes or in displacing [^{3}H]-DHEC from its binding sites in plasma membranes.

Stereospecificity. Both stereoisomers of epinephrine and norepinephrine were tested. The activation of the enzyme as well as the binding to alpha-adrenergic receptors was stereospecific: (+)epinephrine was 6-fold and 32 fold less potent, respectively, than (−)epinephrine either in activating the glycogen phosphorylase or in competing with [^{3}H]-DHEC for its binding sites. (+)Norepinephrine was 21-fold and 52-fold less potent, respectively, than the (−)isomer, in activating the enzyme and in competing for [^{3}H]-DHEC sites (Table 1).

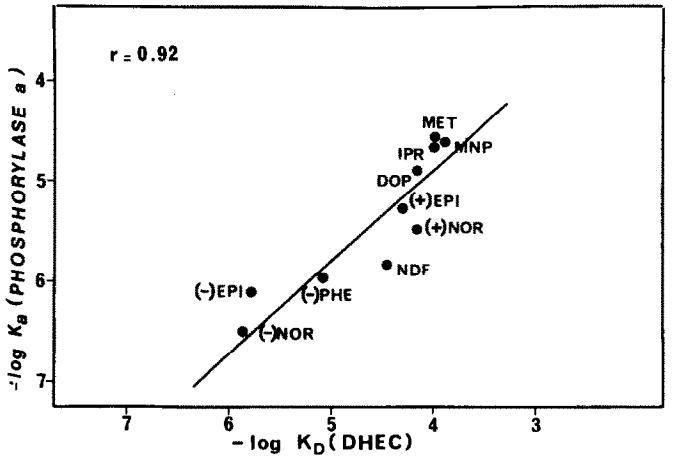

Fig. 4. Correlation between the activation of glycogen phosphorylase in isolated rat hepatocytes and displacement of [^{3}H]-DHEC from rat liver plasma membranes by adrenergic agonists. Along the abscissa are plotted the logarithms of K_D values of agonists for the alpha-adrenoreceptor defined by [^{3}H]-DHEC binding; along the ordinate are plotted the logarithms of K_a values of the agents in activating glycogen phosphorylase. (−)NOR, (−)norepinephrine; (−)EPI, (−)epinephrine; (−)PHE, (−)phenylephrine; NDF, (−)nordefrin; (+)EPI, (+)epinephrine; (+)NOR, (+)norepinephrine; DOP, dopamine; IPR, isoproterenol; MET, metaraminol; MNP, (±)metanephrine. Both K_D and K_a values are those reported in Table 1. The correlation coefficient ($r = 0.92$, $P < 0.01$) and the equation of the straight line were calculated by linear regression.

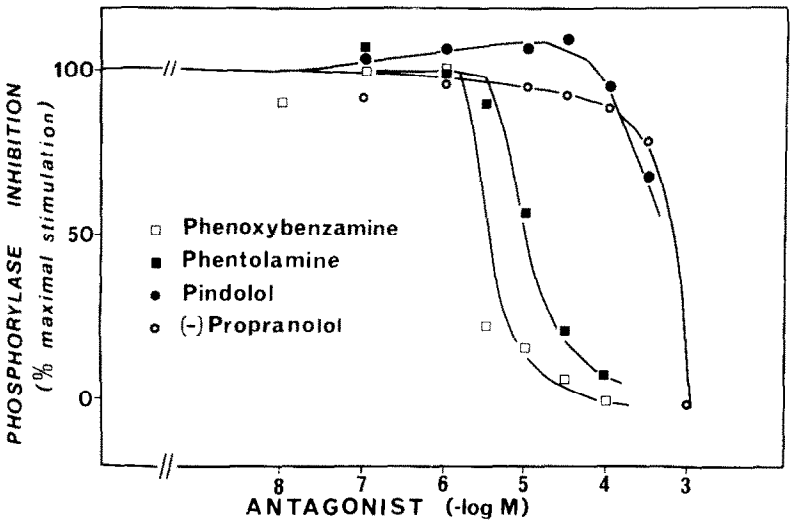

Fig. 5. Effect of various antagonists on the (−)epinephrine-stimulated glycogen phosphorylase activity. Isolated rat hepatocytes (77 × 10^6 cells in 5.8 ml) were preincubated at 37° for 30 min, and then incubated with phenoxybenzamine (□), phentolamine (■), (−)propranolol (○), pindolol (●) in various concentrations or buffer, as described under Methods. Basal level and maximal activity of the enzyme in the presence of (−)epinephrine alone were, respectively, 0.0125 and 0.03 μmoles of glucose-1-phosphate transformed per min/mg protein. The enzyme activity is expressed as per cent of the maximal response above basal level. Each point is the mean of a quadruplicate determination.

Other adrenergic agonists. Several other phenylethylamine derivatives which completely displaced [^{3}H]-DHEC from the alpha-adrenoreceptors in rat liver plasma membranes, acted as partial or total agonists for the glycogen phosphorylase in isolated rat hepatocytes (Table 1 and ref. 11). Salbutamol, a potent beta-adrenergic agonist, was a poor competitor for the alpha-adrenergic sites and did not activate phosphorylase up to 1 mM. Dopamine, the receptors of which are known to bind [^{3}H]-DHEC in brain [5–6], competed with this ligand in rat liver plasma membranes with a K_D of 72 μM, and was a partial agonist of phosphorylase in hepatocytes (K_a = 12.5 μM). Serotonin, the receptors of which also bind ergot alkaloids in brain [4], was a weak competitor of [^{3}H]-DHEC in plasma membranes (K_D = 104 μM) and was ineffective in activating the glycogen phosphorylase in rat hepatocytes up to 1 mM. From the data summarized in Table 1, we plotted the K_a values of agonists for the activation

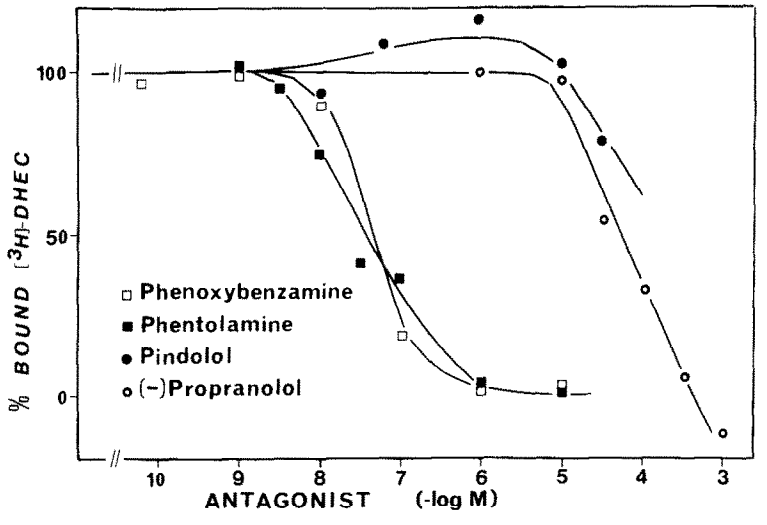

Fig. 6. Displacement of [^{3}H]-DHEC from alpha-receptors by various antagonists. Purified plasma membranes (1.05 mg protein/ml) were incubated at 30° for 10 min with 6 nM [^{3}H]-DHEC in the presence of increasing concentrations of phenoxybenzamine (□), phentolamine (■), (−)propranolol (○) or pindolol (●). Binding of [^{3}H]-DHEC was measured as described under Methods. Results are expressed as per cent of the amount of [^{3}H]-DHEC specifically bound in the absence of any antagonist (890 fmoles of [^{3}H]-DHEC bound/mg membrane protein). Each point is the mean of a triplicate determination.

Table 2. Interaction of alpha and beta-antagonists with [^{3}H]-DHEC binding sites and with glycogen phosphorylase*

Compound	[^{3}H]-DHEC binding sites K_D (μM)	Phosphorylase $K_{i\,app}$ (μM)
Prazosin	0.00047 (2)	0.00085 (2)
Dihydroergocryptine	0.003 (2)	0.25 (1)
Phenoxybenzamine	0.013 (1)	0.30 (2)
2-Bromo-alpha-ergocryptine	0.041 (2)	0.43 (1)
Phentolamine	0.014 (2)	1.71 (6)
Labetalol	0.170 (9)	3.30 (2)
Hydroxybenzylpindolol	0.200 (2)	6.20 (2)
(±)Alprenolol	1.800 (2)	8.00 (2)
Yohimbine	0.33 (1)	11.00 (1)
Azapetine	0.195 (2)	11.70 (2)
Pindolol	20.000 (1)†	75.00 (1)†
(−)Propranolol	18.000 (1)	113.00 (3)

* $K_{i\,app}$ and K_D values were determined as described under Methods. Numbers in parentheses represent the number of separate experiments performed for each compound tested.

† Extrapolated value, due to insolubility of the drug at high concentration.

of glycogen phosphorylase vs the K_D values of the same agents for the competition with tritiated DHEC for alpha-sites (Fig. 4). The two sets of data are directly correlated ($r = 0.92$, $P < 0.01$).

Studies with adrenergic antagonists. Various alpha- and beta-adrenergic antagonists were studied for their ability either to prevent the activation of phosphorylase by (−)epinephrine, or to compete with [^{3}H]-DHEC for alpha-sites. Hepatocytes were incubated for 6 min with increasing concentrations of antagonists (0.1 μM–1 mM final concentration). (−)Epinephrine was then added at a final concentration of 5 μM and the incubation was carried out for 1 min more. In Fig. 5, the percentage of enzyme activation by (−)epinephrine is plotted as a function of increasing concentrations of four antagonists. The alpha-adrenergic antagonists phenoxybenzamine ($K_{i\,app} = 0.3$ μM) and phentolamine ($K_{i\,app} = 1.71$ μM) were, respectively, 250- and 44-fold more potent than pindolol ($K_{i\,app} = 75$ μM) and 375- and 66-fold more potent than (−)propranolol ($K_{i\,app} = 113$ μM), two beta-antagonists, in preventing the activation of glycogen phosphorylase. The affinities of phenoxybenzamine, phentolamine, pindolol and (−)propranolol for the alpha-sites in rat liver plasma membranes were determined from competition curves with [^{3}H]-DHEC (Fig. 6). Phenoxybenzamine ($K_{D\,app} = 13$ nM) and phentolamine ($K_D = 14$ nM) were stronger competitors than (−)propranolol ($K_D = 18$ μM) and pindolol ($K_D = 20$ μM) for [^{3}H]-DHEC binding sites. Similarly, several other antagonists were studied (Table 2 and ref. 11). Among them, prazosin and yohimbine allowed the discrimination between alpha$_1$- and alpha$_2$-adrenoreceptor subtypes [23]. Prazosin appeared to be a very potent inhibitor of the activation of glycogen phosphorylase ($K_{i\,app} = 0.85$ nM) and a strong competitor of tritiated DHEC ($K_D = 0.47$ nM), while yohimbine was less effective either in preventing the activation of the enzyme ($K_{i\,app} = 13$ μM) or in competing with tritiated DHEC ($K_D = 0.33$ μM). Those results strongly suggest that the glycogen phosphorylase activation is mediated through an alpha$_1$-adrenergic receptor, and that the tritiated DHEC binding site possesses the characteristics of an alpha$_1$-site. Serotonin, tested as possible antagonist of the activation of glycogen phosphorylase by epinephrine, was ineffective in inhibiting the enzyme, up to 1 mM. It should be noted that none of the drugs tested influenced the basal level of the enzyme activity. From the data summarized in Table 2, the $K_{i\,app}$ values of antagonists for the prevention of the activation of the enzyme were plotted vs the K_D values of the same drugs for the competition with tritiated DHEC

Table 3. Interaction of imidazoline derivatives with [^{3}H]-DHEC binding sites and with glycogen phosphorylase*

Compound	[^{3}H]-DHEC binding sites K_D (μM)	Phosphorylase $K_{i\,app}$ (μM)
Naphazoline	0.25 (1)	6.9 (1)
Clonidine	0.30 (2)	10.0 (2)
Oxymetazoline	1.00 (1)	13.0 (2)
Tetrahydrozoline	1.54 (2)	5.3 (1)
Xylometazoline	1.86 (2)	7.5 (1)
Timazoline	7.66 (3)	20.0 (1)

* K_D and $K_{i\,app}$ values were determined as described under Methods. Numbers in parentheses represent the number of separate experiments performed for each compound tested.

for its binding sites (Fig. 7). The two parameters are linearly correlated ($r = 0.91$, $P < 0.001$).

Studies with imidazoline derivatives. Clonidine and related imidazoline derivatives previously have been reported to act as alpha-adrenergic agonists in peripheral tissues [24]. However, recent reports described these compounds as alpha-adrenergic antagonists in rat parotid cells [25] and in human platelets [26], while another report described imidazoline derivatives as partial agonists in human platelets [27]. Such discordant results led us to investigate how this class of compounds behaved in isolated rat liver cells.

Imidazoline derivatives were tested either for their ability to activate the glycogen phosphorylase or for their ability to prevent its activation by epinephrine and for their ability to displace tritiated DHEC from its binding site. All the compounds tested were able to prevent the enzyme activation. The $K_{i\ app}$'s are summarized in Table 3. When tested alone, the imidazoline derivatives acted as partial agonists for phosphorylase as compared with natural catecholamines. The maximal activations found were small (less than 50 per cent over basal level), required high concentrations of the drugs and were poorly reproducible. All those drugs behaved as competitors for the alpha-adrenergic sites defined with tritiated DHEC (Table 3).

DISCUSSION

The [^{3}H]-DHEC binding site in rat liver plasma membranes appeared to possess all the characteristics expected for an alpha-adrenergic receptor [11, 28]. However, some controversy has arisen recently about the true alpha-adrenergic nature of the sites identified with that ligand [2, 3]. On the other hand, a recent report described a direct correlation between [^{3}H]-DHEC binding and K^+ efflux in rat parotid cells [9], and preliminary results have shown [^{3}H]-DHEC binding to intact platelets in parallel with platelet aggregation [8]. This type of pharmacological correlation was obviously needed for the alpha-adrenergic receptor of rat liver. We decided, therefore, to compare the [^{3}H]-DHEC binding sites in rat liver plasma membranes with a physiological response, namely, the activation of glycogen phosphorylase in isolated cells. Ideally, it would have been more satisfactory to characterize [^{3}H]-DHEC binding sites in hepatocytes. However, our first attempts to identify alpha-adrenoreceptors in isolated hepatocytes with [^{3}H]-DHEC were unsuccessful. As stated by other groups, the difficulties in utilizing this ligand as a marker in intact cells might have been due to an important uptake of the drug by the cells or to a non-specific binding [8, 9].

The fact that the order of potencies of both agonists and antagonists is similar in the binding experiments and in the glycogen phosphorylase experiments (Figs. 4 and 7) strongly indicates that the DHEC binding site defined in plasma membranes is related to the glycogen phosphorylase in isolated rat liver cells.

However, in our system, the curves relating K_a or $K_{i\ app}$ to K_D values for agonists and antagonists do not fit on the same straight line. While there is less than

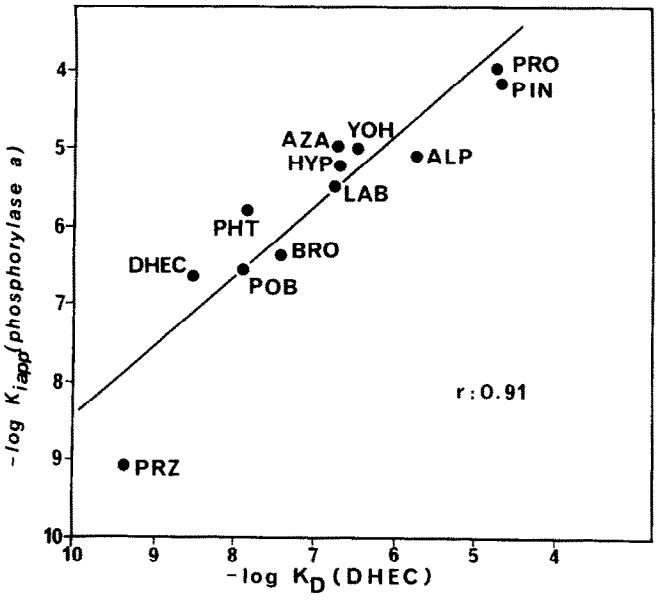

Fig. 7. Correlation between the inhibition of the (−)epinephrine-stimulated glycogen phosphorylase in isolated rat hepatocytes and displacement of [^{3}H]-DHEC from rat liver plasma membranes by adrenergic antagonists. The logarithms of K_D values of antagonists for the alpha-adrenoreceptor are plotted vs the logarithms of $K_{i\ app}$ values of the same drugs for the (−)epinephrine-stimulated phosphorylase. DHEC, alpha-dihydroergocryptine; POB, phenoxybenzamine; PHT, phentolamine; BRO, 2-bromo alpha-ergocryptine; LAB, labetalol; HYP, hydroxybenzylpindolol; AZA, azapetine; ALP, (±)alprenolol; PIN, pindolol; PRO, (−)propranolol; PRZ, prazosin; YOH, yohimbine. Both K_D and $K_{i\ app}$ values are those reported in Table 2. The correlation coefficient ($r = 0.91$, $P < 0.001$) and the equation of the straight line were calculated by linear regression.

one order of magnitude between K_D and K_a values for agonists, there are up to two orders of magnitude between K_D and $K_{i\ app}$ values for antagonists. Surprisingly, the antagonists appear to be 100-fold more potent in displacing tritiated DHEC than in inhibiting the enzyme. These results are not in agreement with the recent report by El Refai *et al.* using [^{3}H]-epinephrine as marker [3], in which agonists and antagonists fit on the same straight line with less than one order of magnitude between K_{50} (phosphorylase) and K_D (binding). A possible explanation is that the tritiated DHEC binding site and the site responsible for the glycogen phosphorylase activation belong to different alpha-subtypes, alpha$_1$ and alpha$_2$. However, this hypothesis is not valid; as shown in Table 2 and as we previously demonstrated [23], the two sites possess the same characteristics of an alpha$_1$-adrenoreceptor. Another explanation for the differences between agonists and antagonists may arise from differences in the experimental protocols: in binding experiments, the same protocol was used for agonists and antagonists, namely, a competition with tritiated DHEC, whereas in hepatocytes we used two different experimental procedures, a direct activation of the enzyme by agonists or a residual activation of the enzyme by an agonist after the prevention by an antagonist. We also may have underestimated the fraction of the receptors occupied by the antagonist when measuring the activity of phosphorylase, due to differences in the activation and binding kinetics of the various drugs tested. Nevertheless, if the discrepancy between K_D and $K_{i\,app}$ values may be surprising, such a phenomenon has been already observed in human platelets [7] and for phentolamine and oxymetazoline in guinea pig vas deferens [29]. Moreover, in liver, several reports indicate that large amounts of alpha-antagonists are necessary to antagonize the alpha-response, in contrast to other organs where the alpha-responses are inhibited by small concentrations of the drugs (for a general review, see ref. 30). The discrepancy we observed may be explained by the ability of hepatocytes to take up and inactivate drugs and hormones. If so, the real concentration in the incubation medium may be far less than the one supposed. Indeed, preliminary experiments performed in our laboratory suggest that there is a rapid and important uptake as well as a degradation of drugs by isolated hepatocytes. Those data contrast with the absence of degradation that we found with purified plasma membranes [11]. Since the experimental conditions are dissimilar (isolated membranes vs isolated cells, different buffer systems and incubation temperatures) and since preliminary results suggest that uptake and degradation of drugs reflecting the physiological liver function do exist with isolated hepatocytes, $K_{i\ app}$ and K_D values may not necessarily fit.

In conclusion, the major findings in the present report are the correlation between the orders of potencies of agonists or antagonists for both the binding and the enzyme experiments and the fact that both the tritiated DHEC binding site and the glycogen phosphorylase activation/inhibition belong to the alpha$_1$-adrenergic subtype. The recent work by El Refai *et al.* [3] reported that tritiated catecholamines could bind to two different types of binding sites in isolated rat liver plasma membranes, and that only the high affinity, low capacity type was related to phosphorylase activation. They suggest that the latter binding site was more representative of the actual alpha-adrenergic receptor than the DHEC binding-site. However, the suggestion that the DHEC binding site is also correlated to the actual physiological alpha-adrenoreceptor is based on the following lines of evidence: the exclusive alpha$_1$-nature of both the DHEC binding sites and the glycogen phosphorylase-linked adrenergic sites [23]; the monophasic appearance of all our displacement curves (Figs. 2–6); the same order of potencies of several adrenergic agonists and antagonists in competing for the DHEC binding sites and in activating/inhibiting the glycogen phosphorylase.

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