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SOLUBILIZATION OF RAT LIVER ALPHA$_1$-ADRENERGIC RECEPTORS

AGONIST SPECIFIC ALTERATION IN RECEPTOR BINDING AFFINITY

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Abstract—An improved method for the solubilization of the alpha$_1$-adrenergic receptors in rat liver, utilizing digitonin, glycerol and sonication, is described. The yield of solubilized receptors was approximately 20%. The soluble receptors showed characteristics similar to the membrane-bound alpha$_1$ receptors. However, upon solubilization, the affinity for the agonists (−)norepinephrine and (−)epinephrine increased 35- to 66-fold when compared to the affinity in the membranes. The affinity for antagonists remained unchanged. A number of synthetic partial agonists showed a less marked (5- to 10-fold) increase in affinity upon solubilization. These data are consistent with the notion that these receptors might be capable of existing in two distinct conformational states with the high affinity state for agonists being favored by solubilization.

Alpha-adrenergic receptors have been classified into alpha$_1$ and alpha$_2$ subtypes [1, 2]. These receptors appear to be membrane-bound integral proteins. The successful isolation of integral proteins requires their solubilization which generally is achieved by using a suitable detergent. In some previous studies, the alpha$_1$ receptor was solubilized with the non-ionic detergent Lubrol PX [3] or the ionic detergent deoxycholate [4]. The use of Lubrol PX has the disadvantage that the soluble receptor loses its binding activity for alpha-adrenergic ligands presumably due to alterations of the tertiary structure of the receptor. To identify the solubilized receptor, it had to be prelabeled in membranes by covalently incorporating [^{3}H]phenoxybenzamine [3]. Also, when deoxycholate was used, prelabeling was required [4]. However, it would be a great advantage if the receptor could be solubilized without prelabeling since this would enable the use of several important techniques, such as affinity chromatography. We and others have reported previously that the plant glycoside digitonin solubilized native alpha$_1$ receptors [5, 6]. In the present report, we describe an improvement of our previous solubilization procedures for alpha$_1$ receptors from rat liver, utilizing digitonin in conjunction with glycerol and sonication, which consistently solubilizes over 20% of the membrane-bound receptors. We also report that, upon solubilization, there was a marked increase in affinity of the alpha$_1$ receptors for agonists whereas that for antagonists was not altered.

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MATERIALS AND METHODS

Materials. Yohimbine, hydrochloride, (−)epinephrine bitartrate, (−)norepinephrine bitartrate, (−)phenylephrine hydrochloride and bovine serum albumin were from the Sigma Chemical Co. (+)Epinephrine bitartrate was from Sterling-Winthrop. HEAT (BE2254, 2-[3-(4-hydroxyphenyl)-ethylaminomethyl]-tetralon) was from Beiersdorf, Hamburg, prazosin from Pfizer Pharmaceuticals, rauwolscine hydrochloride from Roth, Karlsruhe, West Germany, phentolamine hydrochloride from Ciba-Geigy, digitonin from Analar and reagent grade glycerol from Mallinckrodt. Oxymetazoline was from Draco, Lund, Sweden, and (±)ethylnorphenylephrine from Boehringer Ingelheim. KABI 2023 [2 - methylammonio - 1 - (spiro[cyclopentane - 1,1' -indene]-3'-yl)ethanol] was a gift from Dr. B. Sparf, Kabivitrum AB, Stockholm, Sweden. [^{125}I]HEAT was prepared by iodination of cold HEAT as described [7] or obtained from the New England Nuclear Corp. [^{3}H]Prazosin (33 Ci/mmole) was from the Amersham Corp. Sephadex G-50, Sepharose 4B-CL and Blue Dextran were from Pharmacia, Uppsala, Sweden. Guanyl-5'-yl-imidodiphosphate (Gpp(NH)p) was from Boehringer Mannheim. Other chemicals were from commercial sources and generally of the highest available quality.

Membrane purification. Hepatic plasma membranes were prepared essentially as described previously [8] and stored in 50 mM Tris–HCl (pH 7.5) at −80° until used.

Solubilization procedure. The standard procedure for solubilization was as follows. Membranes (1–2 mg protein/ml) were suspended in ice-cold 10 mg/ml digitonin, 48% glycerol (by volume) and 50 mM Tris–HCl (pH 7.5) and sonicated for 90 sec

with a Sonic 300 dismembranator (Artek, Systems Corp., Framingdale, NY) using the smallest probe at 35% of maximal output. Cooling of the sample was assured by keeping it in an ice bath. The sample was thereafter diluted 2-fold with 10 mg/ml digitonin, 50 mM Tris–HCl (pH 7.5) to achieve a final glycerol concentration of 24% and spun in a Sorvall OTD-75B ultracentrifuge at 200,000 *g* for 60 min using a Sorvall T-875 fixed angle rotor. In some experiments, 24% glycerol was used instead during sonication, and this sample was not diluted before centrifugation. This preparation gave results identical to those obtained when 48% glycerol was used.

Membrane alpha$_1$ receptor assay. Membranes (10–20 μg protein) were incubated for 30 min at 25° or for 6–8 hr at 4° (which in both cases was sufficient time to achieve equilibrium) in a final volume of 100 μl of 25 mM Tris (pH 7.5) containing appropriate amounts of [^{125}I]HEAT and drugs. Termination of the incubation was by filtering and washing on Whatman GF/C glass fiber filters with 20 ml of ice-cold buffer. The activity retained on the filters was determined in a Packard Auto-gamma 800 gamma counter at a counting efficiency of 75%. In a few experiments [^{3}H]prazosin was used to assay alpha$_1$ receptors as described by Hoffman *et al.* [9].

Soluble alpha$_1$ receptor assay. Fifty microliters of the soluble preparation was added to 50 μl of [^{125}I]HEAT and drugs and incubated at 4° usually for 6–8 hr. The bound radioactivity was determined by "desalting" on Sephadex G-50 columns [10] using ice-cold 0.1% digitonin, 50 mM Tris–HCl (pH 7.5) as elution buffer. The assays of soluble preparations were determined either in duplicate or triplicate. Non-specific binding was estimated in the presence of 1×10^{-5} M prazosin. In a few experiments [^{3}H]prazosin was used to assay soluble alpha$_1$ receptors. Soluble preparation (450 μl) was incubated with 50 μl of [^{3}H]prazosin and drugs at 4° for 8 hr. Separation of bound activity from free was as described above. When [^{3}H]prazosin was used, non-specific binding was estimated in the presence of 1×10^{-4} M phentolamine. Protein was determined by the "Amidoschwarz" protein staining method described by Schaffner and Weissman [11] using bovine serum albumin as standard. Neither digitonin nor glycerol interfered with this assay.

Statistical analysis. Computer modelling of binding data was as described previously [12]. Briefly, the data were fitted to equations derived from the law of mass action by least squares regression utilizing Marquardt's algorithm. The data were first fitted to equations assuming that the ligands bound to one site and subsequently the same data were refitted under the assumption that the ligands bound to two (independent) sites and so forth. A model involving, for example, two sites was accepted only if it gave a significant improvement in the goodness of fit as compared to a one-site model whereas a three-site fit did not further improve the regression model.

RESULTS

Solubilization of alpha$_1$ receptors. Preliminary studies confirmed the observation [5] that 1% digitonin was able to solubilize active alpha$_1$ receptors. The degree of solubilization was low and variable, however, and generally ranged from 1 to 5% of the membrane bound receptors. To improve the solubilization, digitonin was used in combination with glycerol and sonication. As shown in Fig. 1A, sonication in 1% digitonin improved the solubilization slightly. Addition of 24% glycerol induced a further 2- to 3-fold improvement of the solubilization, when the sample was sonicated for up to 150 sec. Increasing the glycerol concentration to 48% induced a further increase in solubilization, the total improvement by glycerol and sonication being up to 15-fold over the use of digitonin alone. Whereas glycerol and sonication maximally solubilized slightly more than 20% of the alpha$_1$ receptors, the relative fraction of protein solubilized was much higher. Sonication slightly increased the solubilization of protein when 1% digitonin was used alone. Addition of 24% glycerol only marginally increased this solubilization whereas addition of 48% glycerol caused a substantial increase in protein solubilization (Fig. 1B). The specific activity of the solubilized preparation increased

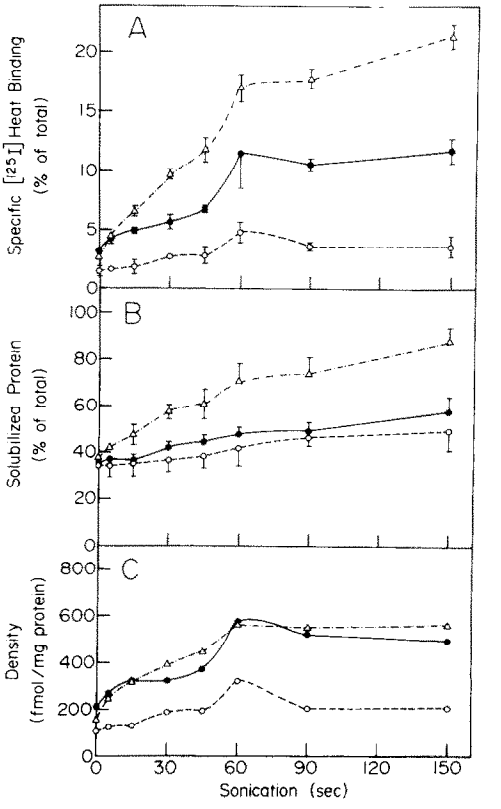

Fig. 1. Solubilization of alpha$_1$ receptors and protein from rat liver membranes. Membranes containing approximately 2 pmoles alpha$_1$ receptors/mg protein were suspended in 1% digitonin (○---○), 1% digitonin plus 24% glycerol (●—●) or 1% digitonin plus 48% glycerol (△-·-△) in 50 mM Tris–HCl buffer (pH 7.5) and sonicated for the time indicated. The activity in the soluble preparation was measured after ultracentrifugation at 200,000 *g* for 60 min. (A) The fraction of alpha$_1$ receptors solubilized was estimated at a [^{125}I]HEAT concentration of 1.5 nM. Non-specific binding was estimated in the presence of 1×10^{-5} M prazosin. (B) Fraction of protein solubilized. (C) Specific activity of alpha$_1$ receptors in the soluble preparation.

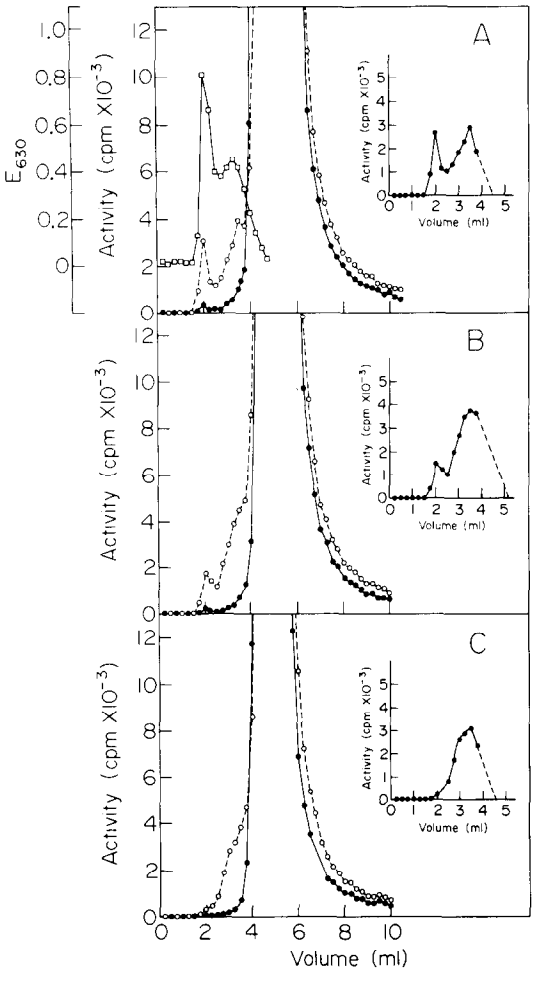

Fig. 2. Chromatography of solubilized liver plasma membrane on Sepharose 4B-CL. Membranes were solubilized as described in the legend of Fig. 1 using 24% glycerol and then spun as indicated below. The supernatant fraction was divided into two parts and incubated with 0.2 nM [^{125}I]HEAT in the presence or absence of 1×10^{-4} M phentolamine. Then 250 μl each of the samples was applied to identical 0.6 × 15 cm columns packed with Sepharose 4B-CL equilibrated with 50 mM Tris (pH 7.5), 12% glycerol and 0.1% digitonin. The radioactivity was then eluted by applying 250 μl aliquots of the buffer. In panels A–C (○---○) represents total binding and (●—●) non-specific binding (i.e. in the presence of 1×10^{-4} M phentolamine) of [^{125}I]HEAT. Inserts: Specific binding (i.e. total − non-specific binding). (A) Sample spun down at 50,000 g for 30 min. The squares (□—□) represent the elution profile of Blue Dextran. (B) Sample spun down at 100,000 g for 30 min. (C) Sample spun down at 200,000 g for 60 min. Observe that in panel C there is a total disappearance of the high molecular weight [^{125}I]HEAT binding activity which was eluted in the void volume in the experiments shown in panels A and B.

about 2.5-fold when glycerol and sonication were used (Fig. 1C). However, 48% glycerol was not more effective than 24% glycerol due to the fact that the former concentration not only improved the solubilization of alpha$_1$ receptors but also increased the amount of protein solubilized.

The alpha$_1$ receptor binding activity "solubilized" with digitonin, glycerol and sonication was not due to particulate receptors such as those present in, for example, membrane fragments, since it was shown by molecular sieving on Sepharose 4B-CL that the receptor was well included in the gel (Fig. 2C). However, it was necessary to spin down the unsolubilized membranes at 200,000 g for 60 min since lower g forces (50,000–100,000 g) and shorter times (30 min) gave rise to a heterogeneous preparation, with a substantial amount of high molecular weight material, that was excluded from the Sepharose gel (Fig. 2A–B).

A number of other conditions were also tested in an attempt to improve solubilization. Thus, for example, combinations of digitonin with mono- and divalent cations, chelators, sodium cholate or phospholipids did not give better solubilization than the use of digitonin alone. Several other detergents besides digitonin were also tested and found to be ineffective. Thus, Zwittergents 214, 308, 310 and 312 and dodecyl-β-D-maltoside proved to be ineffective. Brij 36T, 56 and 58 and Zwittergents 314 and 316 proved to be useless due to high partition of [^{125}I]HEAT into the detergent micelles. Previous data from this laboratory also indicated that Tritons X-100, X-305, and X-405, Lubrol PX, Tween 80 and octyl sodium sulfate were ineffective [5].

Stability of the soluble alpha$_1$ receptor. At 4° the receptor binding activity was found to be essentially stable, at least for up to 8 hr (Fig. 3). However, at higher temperatures the receptor was inactivated fairly rapidly—the half-life being approximately 15 and 60 min at 37° and 25° respectively (Fig. 3). Due to these findings, all subsequent experiments were performed in the cold.

Kinetics of [^{125}I]HEAT binding to the soluble preparation. The interpretation of the binding of [^{125}I]HEAT to the soluble preparation was complicated by the observation that it apparently bound to two different sites. The evidence for this assumption will be presented in detail below. However, it appeared that prazosin could displace the iodinated ligand from only one of these sites, which is the presumptive alpha$_1$ receptor (i.e. "specific binding site"), whereas cold HEAT besides inhibiting binding to the alpha$_1$ site also caused inhibition at the

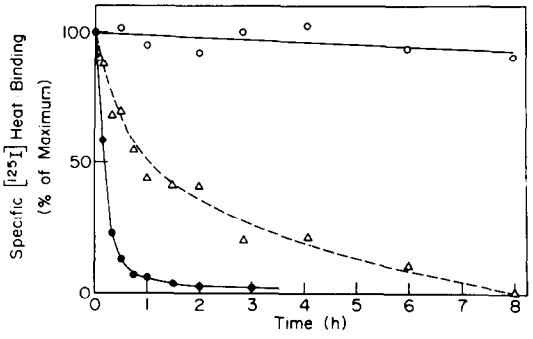

Fig. 3. Stability of the soluble preparation. The soluble preparation was incubated at 4° (○—○), 25° (△—△) and 37° (●—●), and the specific binding activity was estimated at timed intervals. Each point represents the mean from duplicate determinations from a single experiment.

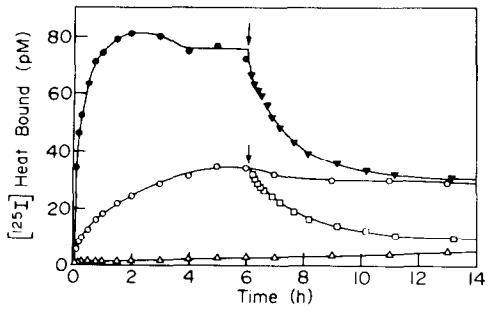

Fig. 4. Time course of binding of [^{125}I]HEAT to the soluble preparation. The soluble preparation was incubated with 0.4 nM [^{125}I]HEAT at 4° in the presence or absence of drugs, and the binding was estimated at timed intervals by "desalting" on G-50 columns. Shown is native sample (●—●), and sample pretreated with 1×10^{-5} M prazosin (○—○) and 1×10^{-4} M HEAT (△—△) respectively. Arrows indicate the addition of 1×10^{-5} M prazosin (▼—▼) and 1×10^{-4} M HEAT (□—□) respectively. Each point is the mean of duplicate or triplicate determinations of a single experiment.

other site. The latter site will be referred to below as the HEAT site.

Binding of [^{125}I]HEAT to the soluble preparation was rapid and reached equilibrium after approximately 2 hr (Fig. 4). After blocking the binding of [^{125}I]HEAT to the alpha$_1$ site with prazosin (1×10^{-5} M), it was revealed that a slower component of binding was present. The latter component did not reach equilibrium until after approximately 6 hr. After protecting both the alpha$_1$ and HEAT site with cold HEAT (1×10^{-4} M), the binding of radiolabeled HEAT was negligible (Fig. 4). To investigate whether binding of [^{125}I]HEAT to the alpha$_1$ and HEAT sites was reversible, the soluble preparation was incubated with the iodinated ligand until equilibrium was reached at respective sites whereafter either cold prazosin or HEAT was added. As can be seen in Fig. 4, prazosin caused complete dissociation of [^{125}I]HEAT binding down to the level of equilibrium binding observed when prazosin was present from the beginning of the incubation. HEAT reversed the binding almost completely so that it approached the level observed when HEAT was present from the start of the incubation. Thus, these data indicate that [^{125}I]HEAT binding to both the alpha$_1$ and HEAT site is a completely reversible reaction.

Equilibrium binding of [^{125}I]HEAT to the soluble preparation. To estimate the equilibrium binding constants of [^{125}I]HEAT to the soluble preparation, saturation experiments were performed. In Fig. 5A a typical experiment is shown. It was revealed that prazosin (1×10^{-5} M) partially inhibited the binding of [^{125}I]HEAT whereas cold HEAT (1×10^{-4} M) caused almost total inhibition. By simultaneously fitting these data into equations derived from the law of mass action by non-linear least squares regression ("computer modelling"; [12]), it was revealed that the assumption that [^{125}I]HEAT bound to two sites resulted in significantly better fits than when a one-site model was assumed ($P < 0.001$; $N = 3$). The regression estimates of the K_d values were for the

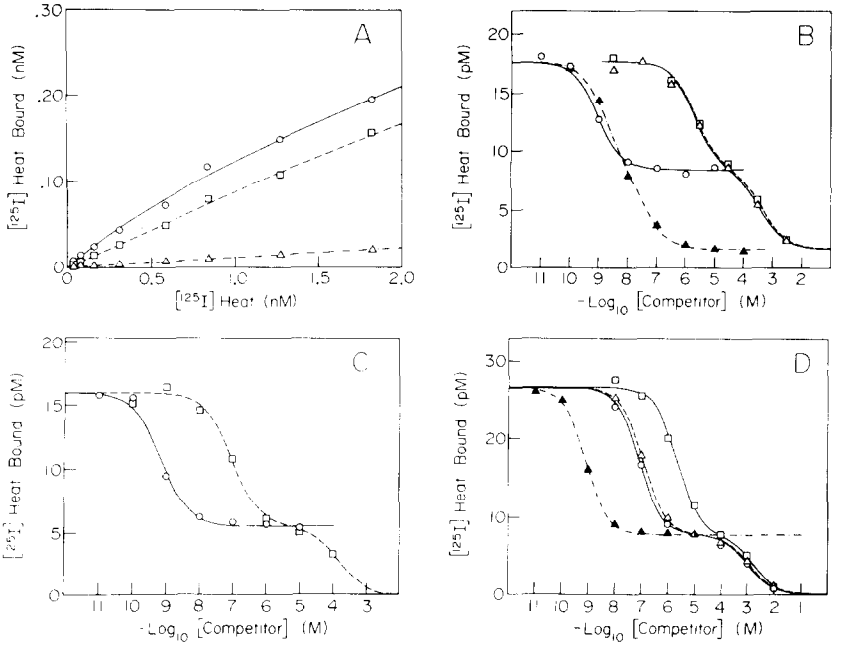

Fig. 5. Equilibrium binding of [^{125}I]HEAT to the soluble preparation at 4°. (A) Saturation curve of [^{125}I]HEAT. Shown is the total binding (○—○), and the binding in the presence of 1×10^{-5} M prazosin (□--□) and in the presence of 1×10^{-4} M HEAT (△--△). (B–D) Competition curves of antagonists and agonists. The concentration of [^{125}I]HEAT was 0.2 nM. (B) Prazosin (○—○), HEAT (▲--▲), rauwolscine (△—△) and yohimbine (□---□). (C) Prazosin (○—○) and phentolamine (□---□). (D) Prazosin (▲--▲), (−)epinephrine (○—○), (−)norepinephrine (△---△) and (+)epinephrine (□--□).

Table 1. Estimates of K_d values of alpha ligands competing for [^{125}I]HEAT in liver membranes and soluble preparation determined by computer modelling

Drug	K_d (nM)			
	Membranes		Soluble preparation*	
	25°	4°	Alpha$_1$ site	HEAT site
Prazosin	1.0†(3)	0.59 (2)	0.54	— (6)
HEAT	4.1 (3)	1.2 (2)	1.2	32 (2)
Phentolamine	88 (2)	68 (2)	66	140,000 (2)
Yohimbine	1,300 (4)	570 (2)	1,500	420,000 (2)
Rauwolscine	2,700 (4)	720 (2)	1,300	340,000 (2)
(−)Epinephrine	1,800‡(4)	2,600‡(2)	52	1,000,000 (2)
(−)Norepinephrine	4,100‡(4)	1,900‡(4)	62	1,400,000 (4)
(+)Epinephrine	33,000 (2)		1,500	1,600,000 (2)
Oxymetazoline	1,600 (4)	1,300 (4)	1,600	50,000 (2)
KABI 2023	5,600 (2)	4,200 (2)	1,500	19,000 (2)
(−)Phenylephrine	21,000 (2)	8,800 (2)	920	460,000 (2)
(±)Ethylnorphenylephrine	130,000 (2)	55,000 (2)	9,900	280,000 (2)

* The soluble preparation was assayed at 4°.
† Number of experiments.
‡ K_d values were not changed by the addition of 1 mM ascorbate and 0.1 mM catechol.

prazosin-sensitive site (presumed alpha$_1$ site) 0.48 ± 0.05 nM and for the HEAT-sensitive site 6.6 ± 3.2 nM (N = 3). The proportion of the alpha$_1$ and HEAT sites were, respectively, 22 and 78% of the total [^{125}I]HEAT binding capacity. The validity of using [^{125}I]HEAT to estimate alpha$_1$ receptors in the soluble preparation was confirmed by utilizing [^{3}H]prazosin. In one batch of soluble preparation, the specific binding of 4 nM [^{3}H]prazosin amounted to 199 fmoles/ml whereas the specific binding of 1.5 nM [^{125}I]HEAT was nearly identical—175 fmoles/ml. The low specific activity of [^{3}H]prazosin (33 Ci/mmole) as compared to that of [^{125}I]HEAT (2200 Ci/mmole) was a great disadvantage and precluded the routine use of the tritiated ligand.

Competition experiments gave clear support for the notion that [^{125}I]HEAT bound to two sites since on all occasions tested computer modelling to a two-site model gave significantly better fits than a one-site model (generally at $P < 0.001$) (Fig. 5B–D). On no occasion could the data be fitted into models assuming three sites. The estimates of K_d values for the antagonists and agonists tested are summarized in Table 1. The data fully support the notion that the high affinity site for [^{125}I]HEAT is the alpha$_1$ receptor site since the alpha$_1$ antagonist prazosin was more than 2000-fold more potent than both of the alpha$_2$ antagonists yohimbine and rauwolscine. The high affinity site also displayed stereoselectivity since (−)epinephrine was almost 20-fold more potent than (+)epinephrine. By contrast, the low affinity site (HEAT site) did not display stereoselectivity, and the affinities of the tested drugs for this site were, in general, very low and did not match any known pharmacological profile. Only HEAT showed comparatively high affinity for this site. Prazosin up to 1×10^{-5} M did not bind to the HEAT site. From the computer modelling in the different competition experiments it was revealed that the alpha$_1$ site constituted 17–22% of the total [^{125}I]HEAT binding capacity, whereas the HEAT site constituted 78–83%. This is in excellent agreement with the proportion of the two sites determined from the saturation experiments described above.

Comparison of [^{125}I]HEAT binding to soluble preparation and liver membranes. For comparative purposes the binding of [^{125}I]HEAT was tested in liver membranes. Time-association experiments revealed that, at 25°, [^{125}I]HEAT reached equilibrium binding within 20 min (data not shown). Computer modelling of saturation curves indicated that [^{125}I]HEAT bound to only one site with a K_d of 0.15 ± 0.03 nM (N = 3) (Fig. 6A). In comparison with the soluble preparation, the non-specific binding was much lower in the membranes. Identical results were obtained when either 1×10^{-4} M phentolamine or 1×10^{-5} M prazosin was used to estimate non-specific binding. On no occasion could the data be fitted into a two-site model. Also, in these tests an excellent agreement was found between the number of alpha$_1$ sites estimated by [^{125}I]HEAT to those estimated by [^{3}H]prazosin. Thus, in one batch of membrane preparation the amount of alpha$_1$ receptors determined by computer modelling of saturation curves for [^{125}I]HEAT was 8.2 pmoles/ml whereas that determined with [^{3}H]prazosin was 8.5 pmoles/ml.

Competition data of both agonists and antagonists also modelled to one-site fits, indicating that [^{125}I]HEAT bound only to one site in the liver membranes. The order of potencies for antagonists was that expected for an alpha$_1$ receptor (Fig. 6B), and the estimates of K_d values were very close to those obtained in the soluble preparation (Table 1). The catecholamine agonists also showed the expected order of potencies for alpha receptors, as well as stereoselectivity (Fig. 6C). However, the affinities of (−)norepinephrine and (−)epinephrine were 66- and 35-fold higher in the soluble preparation than in the membranes (Table 1). The lower affinities in membranes were not due to degradation of the catecholamines since the K_d values were the same when estimated in the presence of 1 mM ascorbate and 0.1 mM catechol. To further evaluate this effect,

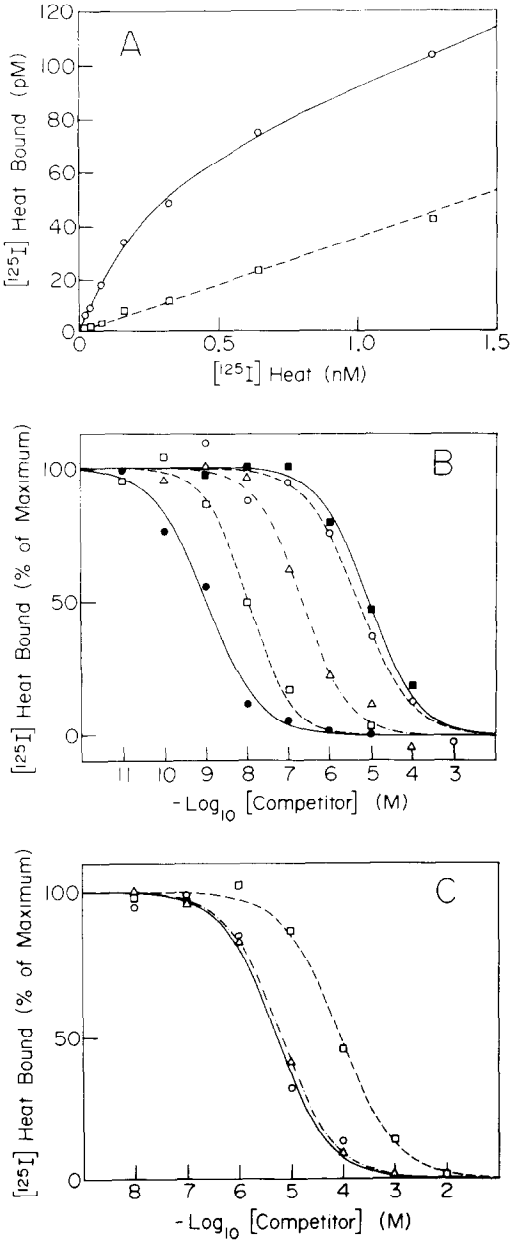

Fig. 6. Equilibrium binding of [^{125}I]HEAT to liver membranes at 25°. (A) Saturation curve from one typical experiment. Total binding (○—○) and binding in the presence of 1×10^{-4} M phentolamine (□—□). (B–C) Competition curves. The concentration of [^{125}I]HEAT was 0.2 nM. (B) Antagonists: prazosin (●—●), HEAT (□---□), phentolamine (△-·-△), yohimbine (○---○) and rauwolscine (■—■). Each point represents the mean of two to three different experiments, each performed with duplicates. (C) Agonists: (−)epinephrine (○—○), (−)norepinephrine (△-·-△) and (+)epinephrine (□---□). Each point represents the mean of two different experiments performed with duplicates.

a number of synthetic alpha-adrenergic agonists were also tested. The data are summarized in Table 1. In the membranes, tests were performed at 4° to make the data comparable to that in the soluble preparation since in the latter the binding was performed only in the cold. Lowering the temperature induced a slight but inconsistent increase in the affinities of both agonists and antagonists in the membranes. The change in affinity was small and never exceeded a 3- to 4-fold shift. Thus, when compared to the affinities in the membranes in the cold, (−)epinephrine and (−)norepinephrine were still 30- to 50-fold more potent in the soluble preparation. Also, some of the synthetic agonists were more potent in the soluble preparation, although the difference was less than for the natural catecholamines. Thus, the alpha$_1$ selective agonists (−)phenylephrine and KABI 2023 [13*] were, respectively, 10- and 5-fold more potent in the soluble preparation than in membranes at 4°. The alpha agonist (±)ethylnorphenylephrine was also 6-fold more potent in the soluble preparation. However, the preferential alpha$_2$ agonist oxymetazoline [13] was equipotent in the membranes and soluble preparation. For the antagonists the K_d values were in excellent agreement also when comparison was made between the soluble preparation and membranes assayed at 4°.

Effect of guanine nucleotides in the soluble preparation. It was suggested recently that the alpha$_1$ receptor in rat liver membranes existed in two distinct affinity states for agonists, and that the presence of guanine nucleotides abolished the high affinity state [14]. It was therefore of interest to investigate the effects of guanine nucleotides in the soluble preparation. The addition of 1×10^{-4} M guanyl-5'-yl imidodiphosphate (Gpp(NH)p) did not affect the affinity of (−)epinephrine for the alpha$_1$ site of the soluble preparation (data not shown). When the membranes were preincubated with 8×10^{-4} M Gpp(NH)p for 20 min at 25° and subsequently solubilized, the affinity of (−)epinephrine was also not altered (data not shown). In this context it may also be mentioned that, whereas some investigators have found evidence for high and low affinity states and effects of guanine nucleotides for alpha$_1$ receptors, others have failed to demonstrate such effects [15].

DISCUSSION

In the present study we have developed a method for solubilizing rat liver alpha$_1$ receptors utilizing digitonin, glycerol and sonication. The mechanism by which this procedure increased the solubilization is unknown although several possibilities exist. Sonication might disintegrate the membrane into smaller fragments which could be more easily attacked by the detergent micelles. The role of glycerol might be to stabilize the alpha$_1$ receptors but it seems likely that it was more directly involved in the solubilization process since the total amount of protein solubilized increased in its presence. The role of glycerol could also be to alter the structure of the digitonin micelles or it might even alter the structure of the liver membranes themselves. The polarity of the aqueous phase would also be decreased by glycerol which might aid in the solubilization process. In any case, once the alpha$_1$ receptors are solubilized it seems as if glycerol is not essential since upon removal of the glycerol

* J. E. S. Wikberg, Abstr. XVI, *Scand. Congr. Physiol. Pharmac.* p. 77 (1979).

by desalting on Sephadex G-50 the alpha$_1$ receptors can be quantitatively recovered (unpublished observations).

Our data clearly confirm the value of [^{125}I]HEAT for identification of alpha$_1$ receptors. There are already a number of studies which have shown that [^{125}I]HEAT selectivity labels alpha$_1$ receptors in membrane preparations derived from various tissues [7, 16, 17]. The K_d values of drugs obtained in competition with [^{125}I]HEAT agree well with those reported by others when [^{3}H]prazosin was used in the liver [9]. In our work [^{3}H]prazosin labels the same number of specific binding sites, both in the soluble preparations and the membranes as does [^{125}I]HEAT. This observation further strengthens the validity of using [^{125}I]HEAT to identify alpha$_1$ receptors. The advantages of using a radioiodinated ligand with high specific activity are obvious. Nonetheless there were some disadvantages in using [^{125}I]HEAT since it bound to a non-specific site (HEAT site) in addition to the alpha$_1$ receptor sites in the soluble preparation. The HEAT site does not appear to represent binding to the digitonin micelles since the blank values for binding to the solubilization medium were negligible. Thus, it is likely that the HEAT site represents a component solubilized from the liver membranes. At present the molecular nature of this component is completely unknown but it seems likely that it is a protein. Although the HEAT site made the interpretation of the data more difficult, the availability of computer modelling techniques still allowed an accurate analysis of the binding data. In the membranes we could not detect the HEAT site under the experimental conditions examined. This is in agreement with several previous studies utilizing [^{125}I]HEAT in membranes from various tissues [7, 16, 17]. In the membranes much lower non-specific binding was observed than in the soluble preparation. It is likely, therefore, that the HEAT site does not become exposed until it is solubilized from the membrane environment.

The binding data indicate that the soluble receptor is derived from the membrane bound alpha$_1$ receptors. However, there was a striking difference between the soluble preparation and the membranes in that agonists showed up to 60-fold higher affinities in the former, whereas the affinities of antagonists were the same. Several possible explanations for this phenomenon may be envisaged. One possibility is that the alpha$_1$ receptor in the membranes is heterogeneous and that the solubilization procedure solubilizes preferentially a species which has higher affinity for agonists. Although this possibility cannot be rigorously excluded due to the fact that the procedure employed in the present work solubilizes only approximately 20% of the membrane-bound receptors, we consider this explanation as highly unlikely. If such receptors were present in the membrane, they should have been detected by the computer modelling technique that was used.

Another possibility, which we favor, is that upon solubilization the alpha$_1$ receptor acquires the property of binding agonists more strongly than is apparent in the membranes. There are several possible mechanisms by which the alpha$_1$ receptor could acquire this property. It seems likely that this is related to a conformational change of the receptor (i.e. a change in its tertiary structure). Such a structural change could be due to release of a constraint of the membrane upon solubilization exerted by factors such as lipids or regulatory proteins. Although there exists some data in the literature suggesting that guanine nucleotides might regulate the alpha$_1$ receptors in the rat liver [14], our data show that these do not affect the affinity of agonists for the soluble alpha$_1$ receptors.

We also observed that the increase in affinity for agonists upon solubilization was less for the synthetic agonists. Thus, the magnitude of the affinity increase for agonists was: (−)epinephrine = (−)norepinephrine > (−)phenylephrine > (±)ethylnorphenylephrine > KABI 2023 > oxymetazoline. Although we did not perform any tests to evaluate the physiological actions of these substances in the rat liver, this order is quite consistent with their known intrinsic activities at the alpha$_1$ receptor. For example, with respect to activation of phosphorylase by the alpha receptor in rat hepatocytes, phenylephrine shows almost as great an intrinsic activity as the natural catecholamines whereas oxymetazoline actually appears to be an antagonist in this system [18]. Also, with respect to stimulation of phosphoinositol turnover in the rat hepatocytes, phenylephrine appears to have less intrinsic activity than epinephrine or norepinephrine [19]. The observation that oxymetazoline behaves as an antagonist at rat liver alpha$_1$ receptors [18] is very much in line with the hypothesis that the affinity shift upon solubilization is quantitatively related to the intrinsic activity, since for this agent we found no solubilization shift at all.

The fact that the effect of solubilization was observed only for agonists strongly suggests that it is in some way related to the mechanism by which the alpha$_1$ receptor is activated by these agents. The observation that the solubilization shift was less for partial agonists also seems to lend support to this hypothesis. The data presented here are consistent with a "two-state" receptor model in which an inactive form (R) and an active (R*) form of the receptor are in equilibrium with each other. Agonists show higher affinity for R* than for R and therefore stabilize R*. Antagonists, on the other hand, show equal affinity for R and R* and therefore do not differentiate between the two receptor states. Partial agonists also stabilize R* but presumably to a lesser extent than full agonists [20, 21]. As discussed above, solubilization appears to stabilize R* which may represent a high affinity state for agonists as well as the active conformation of the receptor. With such a model both agonist and antagonist binding competition curves are steep and uniphasic, although the dissociation constants calculated from such curves are only apparent and, in fact, reflect contributions from the affinity of the ligand for both the R and R* forms of the receptor when these exist in equilibrium. As noted above, in the present study both agonist and antagonist curves were steep and uniphasic. This behavior is in marked contrast to that observed in beta-adrenergic systems where a ternary association of the receptor with a coupling protein leads to shallow (i.e. the curves are resolved to two-site fits

in computer modelling) agonist competition curves [22]. It may be noted, however, that others [14] have been able to observe two affinity states for agonist binding to alpha receptors in the rat liver membranes. The reason for this discrepancy is at present not clear. One possibility is that it is due to differences in the experimental protocols used in the two studies (i.e. different incubation times or the presence or absence of divalent cations). Thus, for now the question should remain open as to whether the alpha receptors are capable of being involved in more complicated interactions than those described by a "two-state" model.

There exist in the literature other examples of receptor systems which show increases in affinities for agonists without affinity changes for antagonists on solubilization. Thus, such observations have been made for beta-adrenergic receptors from turkey erythrocytes [23] and nicotinic cholinergic receptors from *Torpedo* [24] and *Electrophorus* [24–26]. Although no definite explanation has been provided for the observed effects in these systems, it is possible that different mechanisms were responsible in each, given the very different biochemical mechanisms of action of these receptors.

In summary, we have described an improved method for solubilizing hepatic alpha$_1$ receptors utilizing digitonin, glycerol and sonication. The receptors "solubilized" appeared to be truly soluble and showed characteristics similar to those of membrane-bound receptors. However, one distinct difference was that the affinities for agonists increased upon solubilization, whereas those for antagonists remained unaltered. An hypothesis is proposed to explain this observation in which it is assumed that the alpha$_1$ receptor exists in distinct conformational states with differing affinities for agonists but not antagonists.

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