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Selective inhibition by organic mercurials of binding to the beta$_1$ population of rat renal cortical beta-adrenergic receptors*

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Binding of β-adrenergic agents to their receptors in various tissues is inhibited by thiol (SH) chelating agents [1–4]. It has been proposed that this effect may result, at least in part, from trapping of agonists within the receptor binding site [5]. A question that has not been answered, though, is whether the two different adrenergic receptor subtypes (β_1 and β_2) are equally sensitive to SH reagents. We reasoned that rat renal cortical membranes would be an appropriate tissue for addressing this question because we have found that such membranes are composed of 70% β_1 and 30% β_2 receptors [6]. We show here that the organic mercurials parahydroxymercuribenzoate (PHMB) and mersalyl—but not certain other SH-reactive agents (e.g. *N*-ethylmaleimide [NEM] and 5,5′-dithio-bis-(2-nitrobenzoic acid) [DTNB])—inhibit binding to renal cortical β-adrenergic receptors and that this inhibition is selective for the β_1 receptors.

Materials and methods

Materials. The following chemicals were received as gifts from the sources indicated: (-)propranolol HCl (Ayerst Research Laboratories); cyanopindolol HCl (Sandoz); practolol (ICI); and zinterol HCl (Mead Johnson). Carrier-free Na^{125}I (>350 mCi/ml) was purchased from New England Nuclear, and all other reagents were from standard sources. [^{125}I]Iodocyanopindolol (ICYP) was prepared as was described previously [6].

Preparation of membranes from renal cortex. Membranes were prepared as described previously [6]. Membrane suspensions (8–9 mg/ml protein [7]) were snap-frozen in liquid nitrogen and stored under the same conditions. No significant loss of specific binding of [^{125}I]iodocyanopindolol (ICYP) to β-adrenergic receptors was observed for periods up to 3 months. Radioenzymatic assays of catecholamine content (kindly performed by Dr. Michael Zeigler [8]) in freshly prepared membranes yielded values of 0.87 ± 0.09 ng norepinephrine/mg protein and 0.04 ± 0.01 ng epinephrine/mg protein (mean ± S.D.)

Radioligand binding assay. The reaction mixture (total volume 250 μl) contained Tris (50 mM, pH 7.5), $MgCl_2$ (10 mM) and ICYP (routinely about 250 pM unless stated otherwise). Assays were initiated by adding the membrane preparation (about 300 μg protein), and incubation was at 37° for 60 min. The reaction was terminated with the addition of 10 ml of the incubation buffer at 37°, and the bound and free radioligand were immediately separated by rapid (<10 sec) filtration through glass fiber filters (Whatman GF/C). The filters were washed with 10 ml of incubation buffer at 37°. The radioactivity retained on the filters was counted in a gamma counter at 86% efficiency. Nonspecific binding was defined as the amount of ICYP binding measured in the presence of 1 μM (-)propranolol. This assay has been validated previously in renal cortical membranes [6] Routinely, SH reagents were added directly to radioligand binding assays although preincubation of membranes with these reagents and subsequent wash out prior to binding gave similar results. Thin-layer chromatography of radioligand extracted from reaction mixtures with or without SH chelating agents showed that no breakdown of ICYP occurred during the course of incubation.

Data analysis. Values for K_D (equilibrium dissociation constant) and B_{max} (number of receptors) were calculated from Scatchard analysis of saturation isotherms. Competition curves using practolol (β_1) and zinterol (β_2) were used to determine β_1 and β_2 populations in renal cortical membranes [6]. As indicated previously [6], addition of guanine nucleotides failed to alter affinities of either practolol or zinterol and, thus, such addition was not included in the assays. Results for these competitive binding studies were analyzed either by Eadie–Hofstee plots or by a computer program (LIGAND) that performs iterative non-linear regression [9]. The number of β_1- and β_2-receptor subtypes present in renal cortical membranes was calculated by multiplying the relative percentage of each subtype (determined by practolol or zinterol competition for ICYP binding) by the B_{max} value determined from Scatchard analysis.

Results and discussion

Several different SH-reactive compounds were tested for their abilities to inhibit binding to rat renal cortical β-adrenergic receptors (Fig. 1). Two organic mercurials, PHMB and mersalyl, inhibited specific ICYP binding over a similar concentration range (IC_{50} ~100 μM), whereas NEM and DTNB, two SH reagents that are not organic mercurials, showed no inhibition of binding at concentrations up to 1 mM. NEM yielded less than 20% inhibition of ICYP binding at concentrations <10 mM (data not shown). The inhibition of ICYP binding produced by PHMB and mersalyl was noncompetitive and attributable to a decrease in number of receptors with no effect on K_D (Fig. 2). Values for K_D of ICYP in control membranes or membranes incubated with PHMB (0.2 mM) or mersalyl (0.4 mM) were 0.14 ± 0.02, 0.15 ± 0.02, and 0.17 ± 0.02 nM and for B_{max} were 45.3 ± 3.7, 24.9 ± 0.4 and 23.6 ± 0.3 fmoles/mg protein respectively ($\bar{x}$ ± S.E.M., N = 3). Attempts to reverse the inhibition of ICYP binding to renal cortical β-adrenergic receptors with dithiothreitol were complicated by the inhibition in binding produced by dithiothreitol alone (data not shown), an effect that has been observed for β receptors in other systems [10, 11].

Our recently published data [6] indicate that renal cortical membranes possess both β_1 and β_2 receptors, a conclusion which is at variance with another recent report [12]. Results of effects of PHMB and mersalyl on β_1- and β_2-adrenergic receptors in renal cortex provide additional data demonstrating that the renal cortex possesses both types of receptors (Figs. 3 and 4). Data analyzed by Eadie–Hofstee plots or by iterative fitting using the LIGAND program yielded similar results: the percentage of β_1 receptors decreased from 68 ± 4% in controls (a value virtually identical to that obtained previously [6]) to 43 ± 4 and 47 ± 9 in the presence of PHMB or mersalyl respectively (N = 3). When these percentages were applied to the change in β receptor number produced by these agents, the entire inhibition in ICYP binding was attributable to a decrease in β_1 receptors (Fig. 4).

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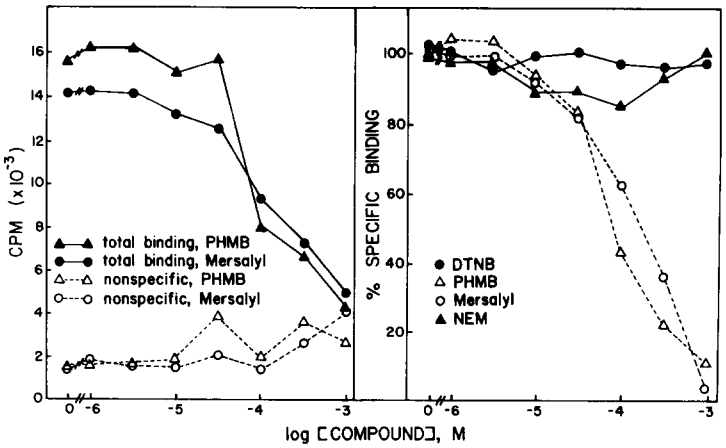

Fig. 1. Effects of SH-reactive reagents on ICYP binding to rat renal cortical membranes. Membranes were incubated with 100 pM ICYP and increasing concentrations of various SH reagents. Left panel: Inhibition of ICYP binding by PHMB (△, ▲) and mersalyl (○, ●) is shown for total (closed symbols) and nonspecific (open symbols) binding; nonspecific binding was determined in the presence of 1 μM (−)propranolol. Right panel: Effect on specific ICYP binding (total-nonspecific binding) of PHMB (△), mersalyl (○), NEM (▲) and DTNB (●). Similar data were obtained in three experiments.

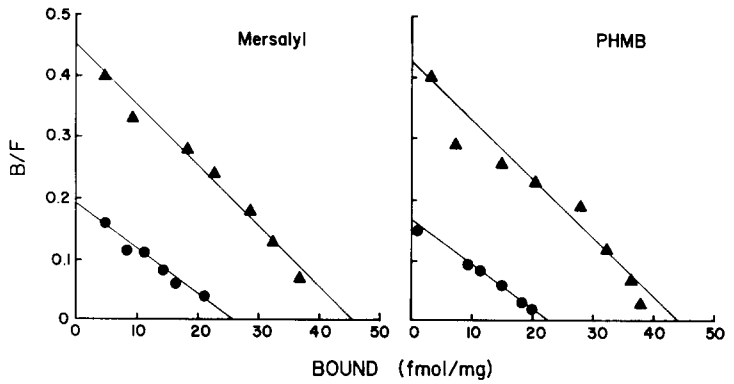

Fig. 2. Scatchard plots of inhibition of ICYP binding to renal cortical membranes by PHMB and mersalyl. Membranes were incubated with increasing concentrations of ICYP and either PHMB (0.2 mM) or mersalyl (0.4 mM). Right panel: Scatchard plot of saturation isotherm of specific ICYP binding in the presence (●) or absence (▲) of PHMB. Left panel: Scatchard plot of saturation isotherm of specific ICYP binding in the presence (●) or absence (▲) of mersalyl. In the figures, B is specific binding in femtomoles per milligram protein and B/F is the ratio of bound to free radioligand. Mean data obtained in three similar experiments are in the text.

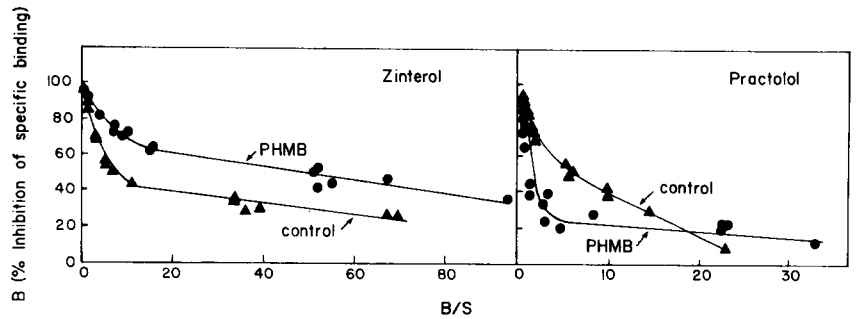

Fig. 3. Competition of practolol and zinterol for ICYP binding sites in PHMB-treated and control renal cortical membranes. Membranes were incubated with 0.2 mM PHMB, 220 pM ICYP and various concentrations of the β_1 antagonist, practolol, or the β_2 antagonist, zinterol. Control studies [6] demonstrated that both practolol and zinterol were full antagonists in binding assays. The data are shown as Eadie–Hofstee plots in which percent inhibition of specific ICYP binding is plotted on the ordinate and percent inhibition of ICYP binding divided by the concentration of competitor on the abscissa. Similar data were obtained in four separate experiments.

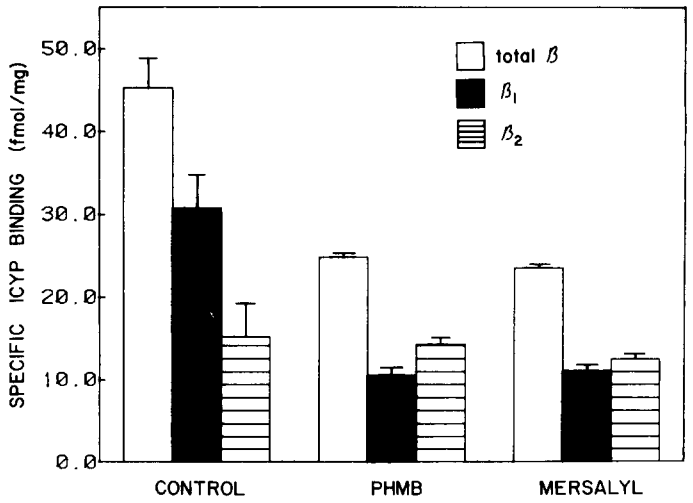

Fig. 4. Effects of PHMB and mersalyl on β-adrenergic receptor subtypes in rat renal cortical membranes. The data shown are from PHMB- (0.2 mM) treated, mersalyl- (0.4 mM) treated, or control membranes and are derived from estimates of B_{max} (from Scatchard plots) multiplied by estimates of the relative precentage of β_1 and β_2 receptors determined as described in Materials and Methods. Data shown are mean ± S.E.M. from three experiments.

These results indicate that rat renal cortical β_1 receptors are selectively decreased by treatment of membranes with organic mercurials. The possibility that catecholamine retained in our membrane preparation was preferentially trapped in β_1 receptors [5] and thereby decreased radioligand binding to these receptors seems unlikely because the affinity of epinephrine and norepinephrine for renal β-adrenergic receptors (K_D values ~1 μM) is much lower than the concentration of catecholamines that we assayed in our preparations (~6 nM and 0.3 nM for norepinephrine and epinephrine, respectively, in typical assay samples). The selective sensitivity of renal β_1 receptors to SH-reactive compounds is similar to the preferential sensitivity to such reagents demonstrated by solubilized β_1- (from heart) compared to β_2- (from lung) adrenergic receptors [13]. We are unaware of work conducted on the two subtypes of β-adrenergic receptors present in the same organ. The current and previous findings imply that β_1- and β_2-adrenergic receptors are either different structural entities or are located in distinct membrane domains with differential sensitivity to exogenous SH reagents. Although some data suggest that β_1 and β_2 receptors are distinct molecular entities [13, 14], other recent work suggests similarities in the subunit molecular weights of the two β-adrenergic receptor subtypes [15]. Thus, further studies will be needed to prove that differences in sensitivity to organic mercurials result from differences in primary structure of β_1 and β_2 receptors.

It is of interest that organic mercurials are much more effective in alternating renal β-receptors than are other types of SH agents. Studies with soluble protein preparations indicate that organic mercurials can recognize more SH groups than NEM [16] and that enzymatic activity can be altered to a much greater extent by organic mercurials than by non-mercurial SH-reactive reagents, such as NEM and DTNB [17]. The reasons for the difference in reactivity between organic mercurials and other sulfhydryl reagents in membranes as well as in soluble preparations are unclear but may have to do with differences in the hydrophobicity of the compounds and/or the ability of organic mercurials to denature protein and thus lead to expression of previously hidden sulfhydryl groups [18, 19].

It is attractive to imagine that alteration in renal cortical β_1 receptors could be related to the well-known effects of mercurial diuretics on mammalian kidney. Since beta receptors can regulate renal sodium and water transport [20, 21], such notions seem plausible and should be testable. Although definitive evidence has not been presented proving that β-adrenergic receptors on the renal tubule are of the β_1 subtype, some data have been presented that support this idea [22].

In summary, treatment of rat renal cortical membranes with the organic mercurials PHMB and mersalyl selectively inhibited binding to β_1-adrenergic receptors. The selective inhibition of β_1 receptors by organic mercurials extends our previous findings [6] regarding differences in the two β-receptor subtypes in rat renal cortex and provides additional evidence that the claim [12] that renal β-adrenergic receptors are of a homogenous class is incorrect. The differing sensitivity of β_1 and β_2 receptors to inhibition by organic mercurials suggests that SH groups are more critical for binding to β_1 than to β_2 receptors and that the primary structure of these two receptor subtypes is different.

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Adverse drug interactions with cimetidine: competitive inhibition of monooxygenase-dependent *N*-demethylation of morphine

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Inhibition by cimetidine of the monooxygenase-dependent metabolism of a number of other drugs is considered to be the source of the adverse side effects when subjects, receiving these drugs, also receive cimetidine [1]. However, studies of cimetidine as a monooxygenase inhibitor *in vitro* have shown extremely high concentrations of the drug to be required for demonstrable inhibition; and no clear mechanism of inhibition has emerged [2, 3]. In this communication we show unequivocally that cimetidine is a competitive inhibitor of the *N*-demethylation of morphine by a rat liver microsomal monooxygenase preparation. Furthermore, the demonstration of inhibitory action by low cimetidine concentrations provides tangible support for the hypothesis that the adverse drug interactions observed between this drug and morphine [4, 5], a matter of dispute [6], may be due to monooxygenase inhibition.

Materials and methods

Aliquots of a stock microsomal suspension prepared by the Netter method [7] from the livers of adult male, random outbred Wistar rats were stored at −75° prior to use. The microsomal cytochrome P-450 concentration, determined by the Estabrook procedure [8] with an Aminco DW-2a spectrophotometer in the dual wavelength mode, was 0.84 nmoles/mg protein. This monooxygenase preparation was diluted to a final pigment concentration of 1 μM for kinetic studies of the production of formaldehyde resulting from *N*-demethylation of morphine. Reaction mixtures (3 ml, pH 7.5) contained, in addition to microsomes, 0.125 M Tris–HCl, 0.025 M tetrasodium pyrophosphate, 2.5 mM glucose-6-phosphate, 1 mM NADP, 1 mM semicarbazide, D-glucose-6-phosphate dehydrogenase (1 μl, 0.7 U), morphine hydrochloride (8.0 μM to 1.64 mM), and cimetidine (0, 12, 37 or 95 μM): reaction mixtures were incubated at 26° for 25 min. Preliminary experiments had established the linear time-dependence, under these conditions, of formaldehyde production over the lower range of morphine concentrations (17–155 μM), where substrate depletion may have introduced error in the use of initial concentrations for the calculation of kinetic parameters. Preliminary experiments also showed that formaldehyde was not produced from cimetidine under these incubation conditions. Monooxygenase catalysis was initiated by the rapid addition of an aliquot of the stock microsomal suspension to the above reaction mixtures, and terminated by the addition of undiluted perchloric acid. Formaldehyde was measured by adding an equal volume of Nash reagent [9] to weighed aliquots (1.3–1.5 ml) of supernatant obtained by centrifuging the terminated reaction mixtures in an Eppendorf Zentrifuge (model 5412); and allowing colour development to proceed for 1 hr at 37° [10]. Absorbances at 410 nm were recorded on an Aminco DW-2a spectrophotometer operated in the split-beam mode; and converted to concentrations on the basis of a standard curve obtained with similarly treated formaldehyde solutions of predetermined concentration. Cimetidine was a gift from Smith Kline and French Laboratories (Australia) Limited, and morphine was a product of Macfarlan Smith (Batch 12666): NADP was a Sigma product. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer-Mannheim Corporation, and other chemicals were of reagent grade.

Results and discussion

Results of the kinetic experiments are summarized in Fig. 1, about which the following points are noted. First, *N*-demethylation of morphine by the rat liver monooxygenase preparation is adequately described by Michaelis–Menten kinetics with values of 300 ± 24 μM and 1.34 ± 0.05 min^{-1} for the Michaelis constant, K_m, and k_{cat}, respectively, the latter value being based on the pigment content of the assay mixtures. These estimates (±2 SEM) were obtained with a