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# Classification of Phenoxybenzamine/Prazosin-Resistant Contractions of Rat Spleen to Norepinephrine by Schild Analysis: Similarities and Differences to Postsynaptic *Alpha*-2 Adrenoceptors

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## ABSTRACT

The striking resistance of norepinephrine contractions of rat splenic strips to antagonism by the selective *alpha*-1 adrenoceptor antagonist prazosin was examined by Schild analysis. Prazosin was a simple competitive antagonist of contractions to phenylephrine indicating that this tissue possesses *alpha*-1 adrenoceptors. In contrast, the Schild regression for prazosin, with norepinephrine as the agonist, was nonlinear and had an overall slope of 0.24. These data indicated that norepinephrine activated a prazosin-resistant adrenoceptor in this tissue. As a working hypothesis, it was assumed that the prazosin-resistant receptor was an *alpha*-2 adrenoceptor; the concomitant addition of yohimbine, in concentrations below those required to block *alpha*-1 adrenoceptors, converted the atypical Schild regression for prazosin (norepinephrine as agonist) to a linear regression identical with that found for antagonism of phenylephrine responses. Selective alkylation of *alpha*-1 adrenoceptors with phenoxybenzamine (POB) eliminated responses to phenylephrine but not those to norepinephrine. After POB-alkylation and in the presence of a concentration of prazosin that was sufficient to produce a profound blockade of *alpha*-1 adrenoceptors, a response to norepinephrine remained. It was determined that the POB/prazosin-resistant response most likely was mediated by a homogeneous population of receptors by the finding that the Schild regressions for both yohimbine and idazoxan were identical with respect to slope and elevation when either norepinephrine or cobefrin were utilized as agonists, *i.e.*, a difference in the regressions for these antagonists would be expected if the two agonists activated a heterogeneous receptor population. The working hypothesis was not supported by the fact that the Schild regressions for both yohimbine and idazoxan in POB/prazosin-treated rat spleen were significantly different from those found in rat vas deferens (inhibition of twitch response assumed to be a classical *alpha*-2 adrenoceptor-mediated response). It should be noted that receptor classification data with antagonists for *alpha* adrenoceptors are remarkably variable and that the differences observed between the rat vas deferens and POB/prazosin-resistant rat spleen may simply reflect this variability. Therefore, although the Schild analysis described in this paper specifically indicates that the receptors mediating norepinephrine responses are not homogeneous with *alpha*-2 adrenoceptors as defined by antagonism in the rat vas deferens, the noted heterogeneity of such classifications within the larger group currently defined as *alpha*-2 adrenoceptors makes it not possible to classify these receptors with existing data. Although all obvious precautions were taken, it is not clear whether these data support other literature proposing the further subclassification of postsynaptic *alpha* adrenoceptors or whether these differences are due to experimental or technical factors. Assuming that equilibrium conditions were attained in these experiments, the data are provocative in terms of further exploring *alpha* adrenoceptor-mediated mechanisms.

The quantitative use of Schild regressions (Arunlakshana and Schild, 1959) is perhaps the most powerful single tool available to pharmacologists to detect and characterize receptor heterogeneity. This technique has the advantage of classifying receptors that respond to agonists and thus are known to be coupled to stimulus-response mechanisms; this often is not the case for multiple curve-fitting procedures in ligand binding studies.

Experimentally, it was observed that prazosin clearly did not produce simple competitive antagonism of contractions of rat splenic strips to norepinephrine; this raised the possibility that the contractions produced by norepinephrine in this tissue were mediated by a heterogeneous population of receptors for this agonist. Several models have been described to predict the behavior of Schild regressions in heterogeneous receptor systems (Furchgott, 1981; Lemoine and Kaumann, 1983; Kenakin, 1984a, 1985, 1987). This present paper describes attempts to characterize the *alpha* adrenoceptors mediating isometric contractions of rat spleen using Schild analysis.

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**ABBREVIATION:** POB, phenoxybenzamine.

## Methods

**Splenic strips.** Rats (male Sprague-Dawley, 300–350 g) were sacrificed by cervical dislocation; the spleens were removed rapidly, placed in oxygenated (95% $O_2$-5% $CO_2$) Krebs-Henseleit solution and cut lengthwise into strips of width approximately 3 to 5 mm. The composition of the Krebs-Henseleit solution was (in millimoles/liter); $Na^+$, 151; $K^+$, 3.4; $Ca^{++}$, 2.15; $Mg^{++}$, 1.2; $Cl^-$, 128.4; $HCO^-$, 30; $SO_4^=$, 1.2; $H_2PO^-$, 1.0; and D-glucose, 5.5. One end of the strip was tied (5-0 silk thread) to a perspex tissue holder and the other to a Grass FT .03 isometric transducer. Tissues were placed in 27-ml organ baths heated to 37°C filled with oxygenated Krebs-Henseleit solution and tied under a resting tension of 1.0 g. This resting tension was readjusted frequently over an equilibration period of 2 hr; isometric tension was recorded on a Sensormedics R-611 dynograph recorder. Desmethylimipramine (0.3 μmol/l), corticosterone (30 μmol/l) and propranolol (0.1 μmol/) were added to the bathing medium to block the neuronal and extraneuronal uptake of catecholamines and *beta* adrenoceptors, respectively.

After an equilibration period of 60 min, tissues were exposed to norepinephrine (10 μmol/l) and washed to base line for a further 1 hr. Some tissues were treated with the alkylating agent POB (1.0 μmol/l for 10 min) and then washed for 30 min with medium containing thiosulfate ion (100 μmol/l) as a scavenger for residual aziridinium ion (alkylating species) and then a further 60 min with thiosulfate-free Krebs-Henseleit solution. Before determination of initial dose-response curves, tissues were equilibrated in the organ baths for a total period not less than 2.5 hr with frequent adjustment of resting tension to 1.0 g. Dose-response curves to agonists were obtained cumulatively (van Rossum, 1963) for norepinephrine and cobefrin by addition of the agonist in volumes of less than 0.2 ml to the organ baths. Dose-response curves to phenylephrine (and in one experiment, cobefrin) were obtained by addition of one concentration, observation of steady-state response and washing with agonist-free solution.

After determination of the control dose-response curve to the agonist, splenic strips were washed with drug-free medium for 20 min. After this period, the antagonist was added to the organ bath and allowed to equilibrate with the tissue for no less than 60 min. After equilibration with the antagonist, the dose-response curve to the agonist again was determined. This procedure was repeated in some tissues with a higher concentration of antagonist; no more than two concentrations of antagonist were tested in any one tissue.

**Rat vas deferens.** Rats (male, Sprague-Dawley, 300–350 g) were sacrificed by cervical dislocation, the vasa deferentia were dissected quickly and placed in oxygenated (95% $O_2$-5% $CO_2$) Krebs-Henseleit solution. Tissues were trimmed free of fat and connective tissue, tied to perspex tissue holders between bipolar platinum electrodes and placed in 27-ml heated (30°C) organ baths containing oxygenated Krebs-Henseleit solution. The other end of the tissue was tied to a Grass FT .03 isometric transducer under a resting tension of 0.5 g. Tissues were equilibrated in the organ baths for 30 min (with frequent changes in bath fluid) and then field stimulated *via* a Grass S88 electronic stimulator (square wave, 0.1 Hz, 3-msec duration, threshold voltage + 30%). Twitch contraction was recorded on a Sensormedics R-611 dynograph recorder. The bathing medium contained desmethylimipramine (10 nmol/l), corticosterone (40 μmol/l) and propranolol (1.0 μmol/l) to block neuronal and extraneuronal uptake of norepinephrine and *beta* adrenoceptors, respectively.

After an equilibration period of 30 min, cumulative dose-response curves to clonidine were obtained by addition of clonidine in volumes of less than 0.2 ml to the organ baths in 3-fold incremental increases in concentration. Higher concentrations were added only after attainment of steady-state responses to the previous concentration or 5 min in the absence of a response. Dose-response curves to clonidine were comprised of concentrations that did not produce greater than 80% inhibition of twitch height because it was found that if these concentrations were exceeded, the control twitch response (before clonidine) could not be regained by washing. The maximal response was assumed to be complete inhibition of twitch as separate experiments indicated that clonidine was a full agonist in this preparation with respect to inhibition of twitch height.

After determination of control responses to clonidine, electrical stimulation was stopped and the tissues were washed for 30 min. During this period, the antagonist was added to the medium. For Schild analysis, the antagonist was equilibrated with the tissue for no less than 60 min. The sensitivity to clonidine then was assessed by determination of a dose-response curve to clonidine in the presence of the antagonist. The effects of no more than two doses of antagonist were measured in any one tissue.

**Analysis of antagonism.** The potency and competitivity of antagonists was measured by Schild analysis. In this method, the ratios of equiactive concentrations of agonist, in the absence and presence of various concentrations of antagonist, were calculated (dose-ratios, *dr*) and a regression of these dose-ratios (as logarithms) upon the logarithms of the molar concentrations of antagonist that produced them was made (Schild regression). This regression was compared to the Schild equation (Arunlakshana and Schild, 1959):

$$\log(dr - 1) = n \log B - \log K_B \quad (1)$$

where $[B]$ is the molar concentration of antagonist and $K_B$ the equilibrium dissociation constant of the antagonist-receptor complex. Equation 1 is the equation for a straight line, thus, if the experimental regression was linear with a slope of unity ($n = 1$), then the intercept (log (dr-1) = 0) was taken to be an estimate of the $K_B$; this is a chemical term unique to every antagonist-receptor pair and thus of value in the classification of drug receptors (Arunlakshana and Schild, 1959).

If experimental regressions did not conform to the criteria for competitiveness, namely linearity with unit slope, then the intercept was considered to be a qualitative measure of antagonist potency referred to as the $pA_2$. This is the minus logarithm of the molar concentration on antagonist that produces a 2-fold shift to the right of the agonist dose-response curve ($dr = 2$, log (dr-1) = 0).

**Statistical analysis.** Schild regressions were analyzed by standard linear regressional techniques. Two Schild regressions were compared with an analysis of covariance of regression lines as described by Snedecor and Cochran (1967). This procedure calculates a statistic $F$ for comparison of the lines with respect to slope and another $F$ value for the relative elevation of the lines. This latter test can be used to determine whether or not the regression lines are coincident or separated along the $x$-axis.

**Drugs.** Drugs used in these studies were *l*-norepinephrine, phenylephrine HCl, propranolol HCl, corticosterone, desmethylimipramine HCl, yohimbine HCl (all from Sigma Chemical Co., St. Louis, MO), clonidine (Boehringer Ingelheim Ltd., Ridgefield, CT) and POB HCl (Research Biochemicals, Wayland, MA). Prasozin was a generous gift from Pfizer Laboratories (New York, NY), idazoxan from Reckitt and Coleman Pharmaceutical Div. (Hull, U.K.) and cobefrin (*alpha*-methylnorepinephrine) from Sterling-Winthrop Laboratories (Rensselaer, NY). All drugs were dissolved in ascorbate (10 μmol/l) except for corticosterone, yohimbine and prazosin, which were solubilized in concentrated form in dimethylsulfoxide and then diluted into ascorbate. POB was prepared in 0.1 N HCl. All drugs were kept on ice throughout the experimental procedures.

## Results

**Antagonism by prazosin and yohimbine.** Prazosin produced simple competitive antagonism of splenic responses to phenylephrine (fig. 1); the Schild regression for prazosin for antagonism of splenic contractions to phenylephrine was linear, had a slope of unity (1.0, 95% CL of 0.94–1.05) and indicated a $pK_B$ of 9.2 (95% CL of 8.9–9.5; fig. 1). In contrast, although prazosin produced a shift to the right of dose-response curves to norepinephrine in rat splenic strips, the resulting Schild regression was not linear and did not have a slope of unity (slope = 0.24, 95% CL of 0.22–0.27; fig. 1).

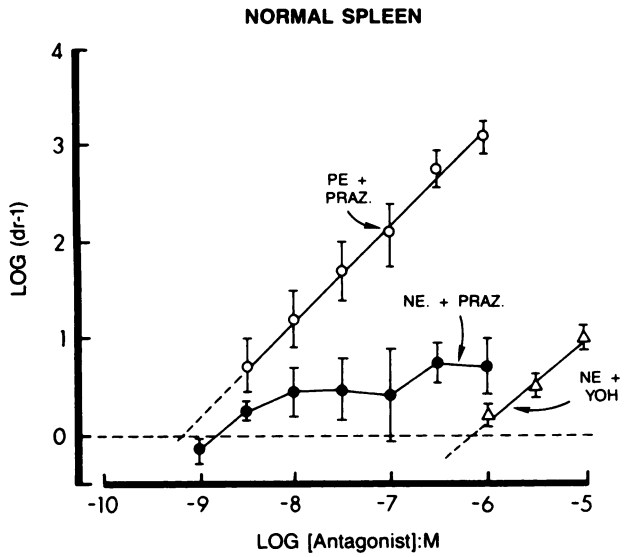


**Fig. 1.** Schild regressions for prazosin and yohimbine in normal rat splenic strips. Ordinates; logarithms of equiactive dose-ratios in the absence and presence of antagonist minus 1 (according to equation 1). Abscissae: logarithms of molar concentrations of antagonist. Schild regressions for prazosin (phenylephrine as the agonist) designated PE + PRAZ (○, $n$ = 24), prazosin (norepinephrine as the agonist) designated NE + PRAZ (●, $n$ = 36) and yohimbine (norepinephrine as the agonist) designated NE + YOH (△, $n$ = 8). Bars represent S.E.M.

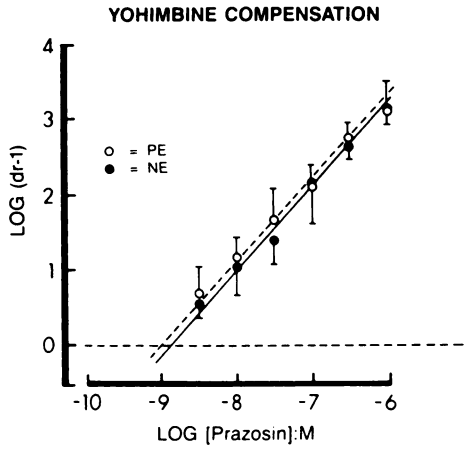


**Fig. 2.** Schild regressions (ordinates and abscissae as for fig. 1) for prazosin in normal rat splenic strips with phenylephrine (PE) (○, $n$ = 24) as the agonist and norepinephrine (NE) (●, $n$ = 38) as the agonist. For this latter regression, yohimbine was added concomitantly to make the following combinations of antagonist present in the receptor compartment: PRAZ 30 nmol/l + YOH 0.3 μmol/l; PRAZ 0.1 μmol/l + YOH 1.0 μmol/l; PRAZ 0.3 μmol/l + YOH 3.0 μmol/l; PRAZ 1 μmol/l + YOH 10 μmol/l. Bars represent S.E.M.

Responses to norepinephrine also were antagonized by yohimbine; the Schild regression was linear, had a slope of 0.78 (0.70–0.83) and a $pA_2$ of 6.2 (6.1–6.35; see fig. 1).

**Antagonism by prazosin with yohimbine compensation.** As a working hypothesis, it was assumed that the difference between the Schild regressions for prazosin when phenylephrine and norepinephrine were used as agonists was due to a mixed population of *alpha*-1 and *alpha*-2 adrenoceptors. The results were consistent with a selective activation of *alpha*-1 adrenoceptors by phenylephrine (therefore prazosin produced simple competitive antagonism) and activation of both *alpha*-1 and *alpha*-2 adrenoceptors by norepinephrine. In this latter instance, a prazosin-resistant component of contraction would be observed (*i.e.*, activation of *alpha*-2 adrenoceptors) and the potency of prazosin would be grossly underestimated.

To test this hypothesis, a Schild regression to prazosin was repeated in the presence of added concentrations of yohimbine. The concentrations of yohimbine were chosen carefully so as not to block *alpha*-1 adrenoceptors and complicate the antagonism by prazosin. It was assumed that the $pK_B$ of yohimbine for *alpha*-1 adrenoceptors was 6.0 (see fig. 1) and for *alpha*-2 adrenoceptors that the $pK_B$ was 8.1 (Doxey *et al.*, 1983). The "compensating" concentrations of yohimbine added to the prazosin-containing medium were chosen to block receptors for which yohimbine had a $pK_B$ of 8.1 (*i.e.*, *alpha*-2 adrenoceptors) but would produce no appreciable blockade of receptors for which yohimbine had a $pK_B$ of 6.0 (*i.e.*, *alpha*-1 adrenoceptors). For example, without yohimbine present, a concentration of 30 nmol/l of prazosin produced a dose-ratio for norepinephrine of 3.8 ± 4.2 (fig. 1). As seen in figure 2, this same concentration of prazosin produced a dose-ratio of 50 ± 42 when phenylephrine was the agonist (selective *alpha*-1 adrenoceptor activation). In the presence of 30 nmol/l of prazosin with 1.0 μmol/l of yohimbine present, the dose-ratio for norepinephrine increased to 30 ± 22, a value not significantly different from that found for prazosin-phenylephrine antagonism. This added concentration of yohimbine would produce an insignificant blockade of *alpha*-1 adrenoceptors (compared to that produced by prazosin) but a 100-fold shift to the right of a dose-response curve for *alpha*-2 adrenoceptor activation.

Figure 2 shows the effects of the addition of compensating concentrations of yohimbine on the Schild regression to prazosin with norepinephrine as the agonist; for comparison, the Schild regression for phenylephrine antagonism also is shown. The regression for antagonism of norepinephrine responses was linear with a slope of 1.1 (1.0–1.2) and a $pK_B$ of 8.9 (8.6–9.2). The regressions for antagonism of phenylephrine responses and norepinephrine responses under these circumstances were not significantly different with respect to slope ($F$ = 2.2, dF = 1, 59) or elevation ($F$ = 0.6, dF = 1, 60).

**Effects of alkylation of *alpha*-1 adrenoceptors by POB.** To test this hypothesis further, rat splenic strips were treated with the *alpha* adrenoceptor alkylating agent, POB, a drug reportedly selective as an irreversible antagonism of *alpha*-1 adrenoceptors (Constantine and Lebel, 1980; Minneman, 1983). After a 10-min exposure to POB (1.0 μmol/l) and washing with thiosulfate ion (100 μmol/l for 30 min) and drug-free Krebs-Henseleit solution for a further 60 min, the responses of rat splenic strips to phenylephrine were considerably depressed (fig. 3A). The addition of prazosin (0.3 μmol/l) to the medium depressed the remaining responses to phenylephrine to nearly insignificant levels (fig. 3A). In contrast, the same alkylation procedure produced a much smaller depression of the responses of rat splenic strips to norepinephrine (fig. 3B); the addition of prazosin had little further effect on the POB-resistant responses to norepinephrine (fig. 3B). This concentration of prazosin is sufficient to produce a 475-fold shift to the right of a dose-response curve for activation of *alpha*-1 adrenoceptors by phenylephrine (see broken line in fig. 3B).

These results indicated that a nearly complete blockade of the responses to phenylephrine could be produced by alkylation with POB and addition of prazosin in a concentration sufficient to produce a fractional occupancy of *alpha*-1 adrenoceptors of 99.8%. This same procedure produced a much smaller inhibi-

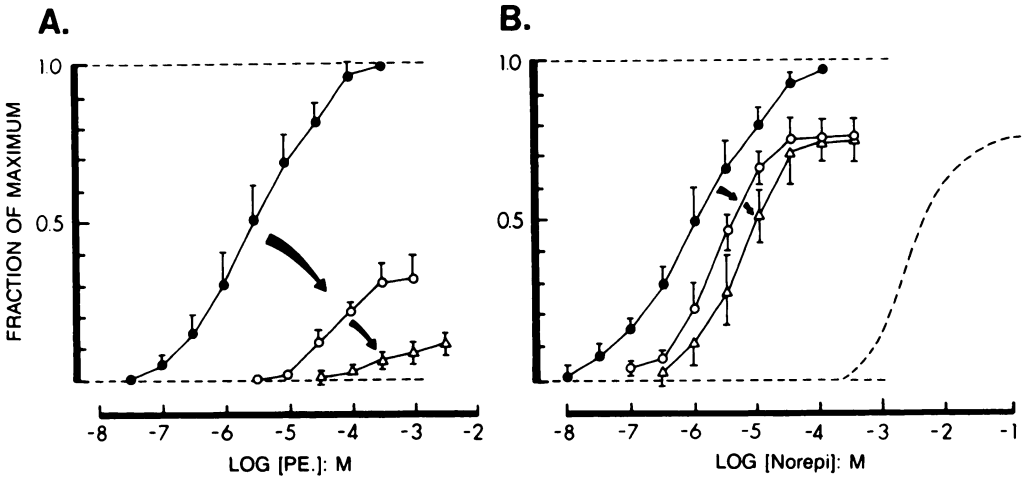


**Fig. 3.** The effects of controlled receptor alkylation by POB and prazosin on response of rat splenic strips to (A) phenylephrine and (B) norepinephrine. Ordinates: isometric contractions of rat splenic strips as fractions of the maximal contraction to either phenylephrine (A) or norepinephrine (B) before POB. Abscissae: logarithms of molar concentrations of phenylephrine (A) or norepinephrine (B). Responses before POB (●, $n$ = 6 for A and B), after POB (1.0 μmol/l, 10 min) and 30-min washing (○, $n$ = 6 for A and B) and after POB and in the presence of prazosin (0.3 μmol/l) (△, $n$ = 6 for A and B). Arrows represent the cumulative effects of alkylation and prazosin. Broken line indicates the calculated shift of the dose-response curve in the presence of the given concentration of prazosin if the responses were mediated by an *alpha*-1 adrenoceptor. Bars represent S.E.M.

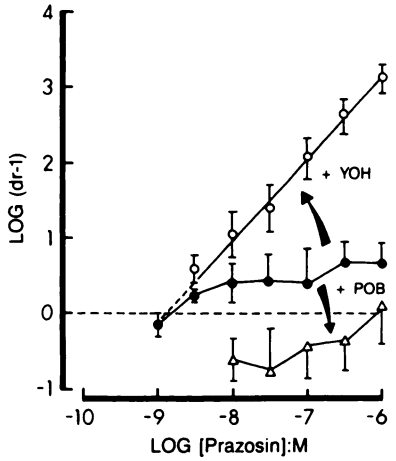


**Fig. 4.** The effects of yohimbine (YOH) and POB on the Schild regression for prazosin in normal rat splenic strips with norepinephrine as the agonist. Ordinates and abscissae as for figure 1. Schild regressions for prazosin with yohimbine compensation (same regression as that shown in fig. 2; ○, $n$ = 38), for prazosin alone (same as the regression shown in fig. 1; ●, $n$ = 24) and for prazosin in splenic strips alkylated with POB (0.3 μmol/l, 10 min; △, $n$ = 18). Arrows represent the effects of yohimbine and POB on the Schild regression for prazosin alone. Bars represent S.E.M.

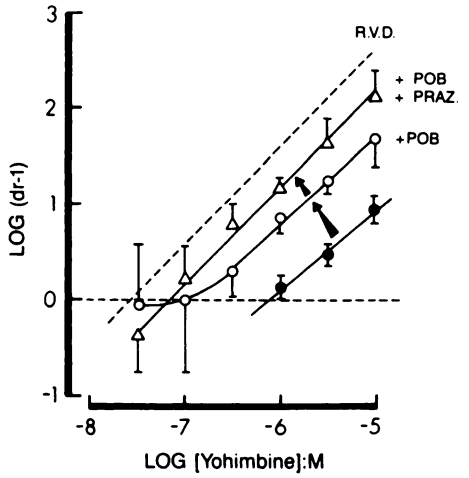


**Fig. 5.** Schild regressions for yohimbine antagonism of norepinephrine responses in rat splenic strips. Ordinates and abscissae as for figure 1. Schild regression for yohimbine in normal splenic strips (same as the regression shown in fig. 1; ●, $n$ = 8), in strips alkylated with POB(1.0 μmol/l, 10 min; ○, $n$ = 22) and in strips alkylated with POB (as above) and with added prazosin (0.3 μmol/l; △, $n$ = 22). Arrows indicate the cumulative effects of POB and POB + prazosin on the Schild regression for yohimbine. The broken line indicates the Schild regression for yohimbine antagonism of *alpha*-2 adrenoceptor responses of rat vasa deferentia (R.V.D.). Bars represent S.E.M.

tion of the responses to norepinephrine. These results strongly suggested that the responses to phenylephrine were mediated by *alpha*-1 adrenoceptors, whereas those to norepinephrine were mediated by both *alpha*-1 adrenoceptors and a receptor resistant to the effects of both POB and prazosin.

**Effect of prazosin of POB-treated splenic strips.** To investigate further the nature of the receptor mediating the contractions of splenic strips after alkylation of *alpha*-1 adrenoceptors with POB (see previous section), a Schild regression to prazosin was obtained on POB-alkylated spleen. Norepinephrine responses of these tissues were extraordinarily resistant to blockade by prazosin; the Schild regression for prazosin, shown in figure 4, had a slope of 0.4 (0.3–0.5). Figure 4 also shows previously shown data and thus summarizes the effects of yohimbine and POB on the potency of prazosin in rat spleen.

**Effects of yohimbine on POB-treated splenic strips.** A series of experiments were conducted in an attempt to characterize the POB- and prazosin-resistant norepinephrine effect. The working hypothesis, namely that this was due to activation of *alpha*-2 adrenoceptors, was tested further by measuring the equilibrium dissociation constant of yohimbine in POB-alkylated splenic strips. Figure 5 shows the Schild regression for yohimbine in POB-alkylated spleen; the regression was curvilinear and shifted to the left of the regression for yohimbine in normal nonalkylated spleen (shown in comparison in fig. 5). To accommodate the possibility that the curvature of the regression was due to a remnant population of *alpha*-1 adrenoceptors (*i.e.*, incomplete alkylation of *alpha*-1 adrenoceptors), 0.3 μmol/l) of prazosin was added to the medium. This concentration of prazosin is sufficient to occupy 99.8% of the remain-

ing *alpha*-1 adrenoceptors and thus produce a 475-fold shift to the right of a dose-response curve to *alpha*-1 adrenoceptor activation. Interestingly, the addition of prazosin had little effect on the dose-response curve to norepinephrine in splenic strips that had been alkylated by POB.

The addition of prazosin in POB-treated spleen caused a further shift to the left of the Schild regression for yohimbine. Thus, as shown in figure 5, after alkylation with POB and addition of 0.3 μmol/l of prazosin, the Schild regression for yohimbine was linear, had a slope not significantly different from unity (slope = 0.96, 0.9–1.05) and indicated a $pK_B$ for yohimbine of 7.25 (7.0–7.5).

**Classification of the POB/prazosin-resistant effect.** Experiments were initiated to determine whether or not the norepinephrine contractions of rat spleen, which were resistant to POB and prazosin, were mediated by a homogeneous population of receptors and also if this receptor was the previously defined *alpha*-2 adrenoceptor. The question of receptor homogeneity was addressed by the use of two agonists. The Schild regression for yohimbine in POB/prazosin-treated spleen was the same whether the agonist used was norepinephrine or cobefrin, a catecholamine with greater agonist activity for *alpha*-2 adrenoceptors (Kobinger and Pichler, 1981; Timmermans and van Zwieten, 1982). As given previously and shown in figure 6A, the Schild regression for yohimbine with norepinephrine as the agonist was linear, had a slope of 0.96 (0.90–1.05) and a $pK_B$ of 7.25 (7.0–7.5) and with cobefrin as the agonist, the regression also was linear, had a slope of 0.9 (0.7–1.1) and a $pK_B$ of 7.25 (6.9–7.55). An analysis of covariance of regression lines for these two Schild regressions indicated that they are not significantly different either with respect to slope or elevation.

The inhibition of field-stimulated twitch contraction of rat vas deferens was used as the standard assay for *alpha*-2 adrenoceptors. With clonidine as the agonist, the Schild regression for yohimbine was linear, had a slope not significantly different from unity (0.9, 0.8–1.0) and yielded a $pK_B$ of 7.7 (7.5–7.9) (see fig. 6B). An analysis of covariance indicated that the regression for yohimbine antagonism of *alpha*-2 adrenoceptors in rat vas deferens was not significantly different with respect to slope ($F = 0.6$, dF = 1, 59) but was significantly different from the regression for yohimbine antagonism in POB/prazosin-resistant rat spleen with respect to elevation of the regression lines ($F = 12.0$, dF = 1, 70; $P < .05$). The regression for spleen was taken as the mean regression from the data with norepinephrine and cobefrin (fig. 6A) inasmuch as these were not statistically different. These data indicated that the POB/prazosin-resistant splenic contractions to *alpha* adrenoceptor agonists could be mediated by a receptor distinct from the *alpha*-2 adrenoceptor with respect to yohimbine binding (see under "Discussion"). The data describing the Schild regressions are shown in table 1.

These experiments were repeated with the *alpha*-2 adrenoceptor antagonist idazoxan (Doxey *et al.*, 1983). Figure 7A shows Schild regressions for idazoxan in POB/prazosin-treated splenic strips with norepinephrine and cobefrin as agonists. As with yohimbine, there were no significant differences between these regressions with respect to either slope or elevation. Figure 7B shows the mean regression from all the data points in figure 7A and the Schild regression for idazoxan antagonism of clonidine activation of *alpha*-2 adrenoceptors in rat vas deferens. Again, as with yohimbine, the regression for idazoxan in rat spleen was not different with respect to slope from that in rat vas deferens ($F = 0.5$, dF = 1, 48) but did differ with respect to elevation ($F = 65$, dF = 1, 49; $P < .05$). Data describing these regressions are shown in table 1.

## Discussion

*Alpha* adrenoceptors have been subclassified on the basis of relative agonist and antagonist potency and also in terms of function and anatomical location. Thus the classification of postsynaptic *alpha*-1 and presynaptic *alpha*-2 adrenoceptors was established. Further studies have furnished abundant evidence that postsynaptic *alpha* adrenoceptors form a heterogeneous population and it has been suggested that *alpha*-2 adrenoceptors exist postsynaptically (for review see Timmermans and van Zwieten, 1981, 1982). The present studies on rat spleen were initiated in an attempt to obtain a simple isolated tissue preparation for the previously classified postsynaptic *alpha*-2 adrenoceptor. The finding that prazosin was a poor inhibitor of the contractions of rat spleen to norepinephrine and that it certainly did not produce simple competitive inhibition suggested that the spleen could possess a population of *alpha*-2 adrenoceptors in coexistence with *alpha*-1 adrenoceptors.

The detection and analysis of heterogeneous receptor populations is amenable to Schild analysis inasmuch as this method mathematically predicts the behavior of a competitive antagonist interacting with one receptor type; deviations from this behavior and the elimination of these deviations with secondary

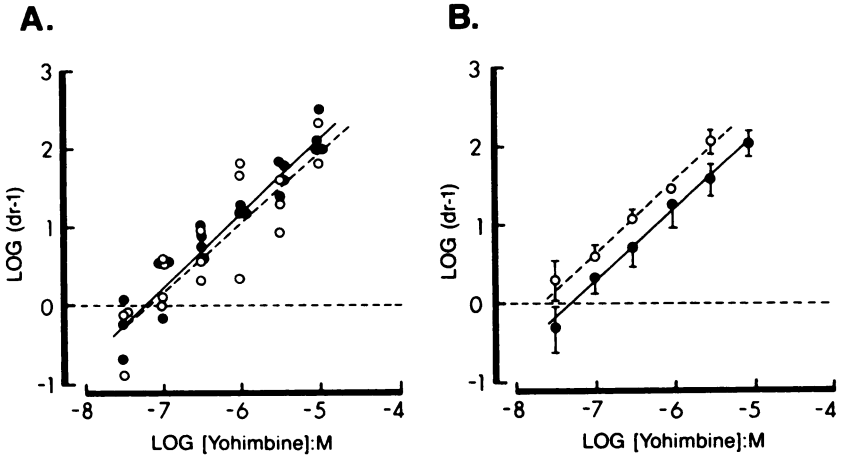


**Fig. 6.** Schild regressions for yohimbine in (A) rat-alkylated (POB 1.0 μmol/l, 10 min) splenic strips treated with prazosin (0.3 μmol/l) and (B) rat vasa deferentia and rat-alkylated splenic strips treated with prazosin. Ordinates and abscissae as for figure 1. A, Schild regression with norepinephrine (●, $n = 22$) and cobefrin (○, $n = 17$) as the agonists. B, Schild regression for yohimbine in normal rat vas deferens with clonidine as the agonist (○, $n = 11$) and rat-alkylated splenic strips (also treated with prazosin) with norepinephrine and cobefrin as the agonist (●, $n = 39$). This latter regression is the mean of the two regressions shown in A. Bars represent S.E.M.

drugs allows the definition of multiple receptor types, which subserve the same response. Analytical approaches to this problem with the use of Schild analysis have been published (Furchgott, 1981; Lemoine and Kaumann, 1983; Kenakin, 1984a, 1985, 1987).

The first observation was made with prazosin antagonism of responses to norepinephrine in normal rat splenic strips. The data with phenylephrine indicated that the rat spleen contained *alpha*-1 adrenoceptors. However, the highly atypical Schild regression for prazosin antagonism of norepinephrine responses indicated that this agonist activated a receptor that was highly resistant to prazosin; it was assumed as a working hypothesis that this second receptor was a postsynaptic *alpha*-2 adrenoceptor.

This working hypothesis was supported by the fact that the addition of yohimbine, in concentrations below those required to bind to *alpha*-1 adrenoceptors, greatly altered the atypical Schild regression for prazosin. In the presence of yohimbine, prazosin produced simple competitive antagonism of the norepinephrine response that was indistinguishable from the blockade of responses to phenylephrine. Still more support for this hypothesis was found in the data with POB, an alkylating agent selective for *alpha*-1 adrenoceptors (Constantine and Lebel, 1980; Minneman, 1983). Although POB alkylation all but eliminated the splenic response to the *alpha*-1 adrenoceptor-selective agonist phenylephrine, a POB-resistant response to norepinephrine was encountered. It is unlikely that this resistance to alkylation was the result of differential intrinsic efficacies of norepinephrine and phenylephrine (Kenakin, 1984b), since the intrinsic efficacies of these two agonists are nearly identical for *alpha*-1 adrenoceptors (Besse and Furchgott, 1976). Also, the fact that prazosin, in a concentration 475 times the equilibrium dissociation constant for *alpha*-1 adrenoceptors, produced a negligible inhibition of the POB-resistant response indicates that *alpha*-1 adrenoceptors do not mediate this contraction. In fact, the Schild regression for prazosin in POB-alkylated spleen was depressed further than in normal spleen.

TABLE 1

**Schild regression data for yohimbine and idazoxan in rat vas deferens and POB/prazosin-resistant rat spleen**

| Antagonist | Vas Deferens | | Rat Spleen | |
|---|---|---|---|---|
| | Slope | $pK_B$ | Slope | $pK_B$ |
| Yohimbine | 0.9 | 7.7 | 0.9 | 7.24 |
| | (0.8–1.0) | (7.5–7.9) | (0.8–1.0) | (6.9–7.6) |
| Idazoxan | 0.9 | 8.1 | 1.03 | 7.64 |
| | (0.7–1.1) | (7.85–8.35) | (0.9–1.1) | (7.3–8.0) |

Before the identity of the receptors mediating the POB/prazosin-resistant responses was pursued, studies were done to ensure that we were dealing with a homogeneous population of receptors and not a further mixture of more than one type. Except for the highly unlikely situation where two agonists have identical affinity and intrinsic efficacy for two receptor types, the Schild regression for an antagonist with differing affinity for two receptors will differ with different agonists. Therefore, the Schild regressions for yohimbine and idazoxan were obtained with norepinephrine and cobefrin as agonists. The analysis of covariance of regression lines indicated that for both antagonists, the Schild regressions were identical when norepinephrine and cobefrin were used to produce response. This was consistent with the hypothesis that the receptors mediating contraction in rat spleen after treatment with POB and prazosin were of a homogeneous type.

In terms of the working hypothesis, the POB/prazosin-resistant response would be attributed to the presence of postsynaptic *alpha*-2 adrenoceptors and thus should be as sensitive to blockade by yohimbine and idazoxan as previously classified *alpha*-2 adrenoceptors such as those found on presynaptic nerve terminals of rat vas deferens. This is where the data failed to support the hypothesis. In the case of both yohimbine and idazoxan, the Schild regressions in rat vas deferens (classical *alpha*-2 adrenoceptors) and POB/prazosin-resistant rat spleen were significantly different from each other with respect to elevation and the antagonists were more potent in rat vas deferens than in the spleen. This was sufficient evidence to reject the null hypothesis on which the Schild analysis is based, namely that the receptors in rat vas deferens and rat spleen are from a homogeneous population. Therefore with these data we were unable to classify the POB/prazosin-resistant adrenoceptor in rat spleen as an *alpha*-2 adrenoceptor.

The fact that the norepinephrine response was resistant to alkylation by POB and that it was blocked by drugs previously classified as selective *alpha*-2 adrenoceptor antagonists suggested that the receptor could be classified as an *alpha*-2 adrenoceptor. However, the Schild analysis presented a paradox in showing that the magnitude of the antagonism produced by the blockers was less than that found for the *alpha*-2 adrenoceptors in rat vas deferens. There are three possible reasons for this disparity. The first could be that in spite of the addition of

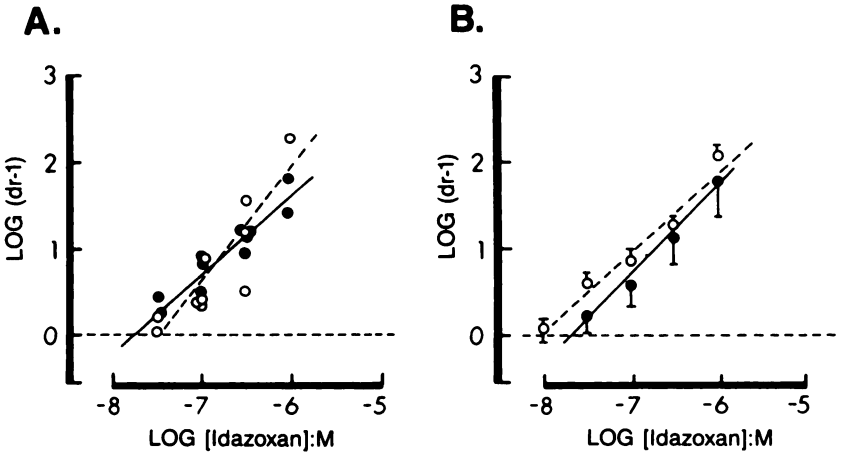


**Fig. 7.** Schild regressions for idazoxan in (A) rat-alkylated (POB 1.0 μmol/l, 10 min) splenic strips treated with prazosin (0.3 μmol/l) and (B) rat vasa deferentia and rat-alkylated splenic strips treated with prazosin. Ordinates and abscissae as for figure 1. A, Schild regression with norepinephrine (●, $n$ = 11) and cobefrin (○, $n$ = 11) as the agonists. B, Schild regression for idazoxan in normal rat vas deferens with clonidine as the agonist (○, $n$ = 12) and rat-alkylated splenic strips (also treated with prazosin) with norepinephrine and cobefrin as the agonist (●, $n$ = 22). This latter regression is the mean of the two regressions shown in A. Bars represent S.E.M.

uptake blockers to the organ baths, true equilibrium conditions may not have been achieved in the Schild analysis with splenic strips. Nonequilibrium steady states with respect to drug concentration in isolated tissues are known to produce artifactual data that closely resemble those due to receptor differences (Furchgott, 1972). Although all obvious precautions with respect to equilibrium conditions had been taken in these experiments, the small differences between the Schild regressions in rat vas deferens and POB/prazosin-resistant rat spleen suggests that an unforeseen technical factor may be present as an obfuscation.

The second possibility concerns the problem of receptor classification and nomenclature in general. In this analysis, the *alpha* adrenoceptor response of the rat vas deferens was arbitrarily chosen to represent the *alpha*-2 adrenoceptor; another tissue may not have demonstrated the small differences seen. In general, there is considerable variation in antagonist data describing *alpha*-1 and *alpha*-2 adrenoceptors (see following paragraph for references), which poses a practical problem of which tissue to use as a "standard" for these receptors. Therefore, the splenic contractions may indeed be due to an *alpha*-2 adrenoceptor but our choice of tissues generated the difference in Schild data. This possibility raises a more general question as to why there is such heterogeneity with respect to antagonism of *alpha* adrenoceptor responses and the spectre of the existence of another as yet unclassified *alpha* adrenoceptor or a receptor that is labile with respect to experimental conditions. The presence of such a receptor in POB/prazosin-resistant rat spleen would be a third possible reason for the difference in Schild data.

Prazosin-resistant *alpha* adrenoceptor responses in tissues that could not be classified as *alpha*-2 adrenoceptors have been described (*i.e.*, see McGrath, 1982 for review) and suggestions for the subclassification of postsynaptic *alpha*-1 adrenoceptors have been made (Flavahan and Vanhoutte, 1986). Compilations of the remarkable variation of $pA_2$ values for yohimbine and prazosin have been given by Drew (1985) and Agrawal *et al.* (1984); it is clear that even when experimental conditions are as near to equilibrium as is known to be possible, the potency of yohimbine and prazosin as *alpha* adrenoceptor blocking agents is quite variable. Studies on the affinity of norepinephrine on *alpha* adrenoceptors on various blood vessels have shown a striking 500-fold difference in affinity in different arterial preparations (Bevan *et al*, 1986). These data taken in total may indicate that postsynaptic *alpha* adrenoceptors are heterogeneous or that experimental conditions used in the *in vitro* procedures used to classify receptor events interact with a labile postsynaptic *alpha* adrenoceptor to change its binding characteristics with respect to agonists and antagonists. This latter hypothesis concerns intriguing data reviewed by McGrath (1982) (see also Medgett *et al.*, 1987) regarding changes in *alpha* adrenoceptor properties with the effects of acidosis and alkalosis.

The present data in rat spleen are not amenable to explanation with the existing classifications of postsynaptic *alpha*-1 and *alpha*-2 adrenoceptors and as such may represent an interesting isolated tissue system in which to explore possible further subclassification of *alpha* adrenoceptors. Further quantification of the potency of other agonists and antagonists in this preparation are needed to elucidate the nature of the receptor mediating the POB/prazosin-resistant responses to norepinephrine in this tissue.

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