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SODIUM REGULATION OF α_2-ADRENORECEPTORS OF HUMAN PLATELETS: INACTIVATION BY 4,4'-DIISOTHIOCYANO-2,2'-STILBENE DISULFONIC ACID (DIDS)

S.M. Periyasamy and P. Somani

Departments of Pharmacology and Medicine,
Medical College of Ohio,
C.S. 10008,
Toledo, OH 43699

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In the present study, we investigated the effect of DIDS on Na^+ regulation of α_2-adrenoreceptors in human platelets. Pretreatment of platelet membranes at 23°C for 60 min with DIDS produced a reduction in the affinity of the receptors for both antagonist and agonist in a concentration related manner; however, there was a marked difference in the degree of reduction in the affinity of the receptors for antagonist and agonist. Thus, at 1 mM concentration of DIDS, the affinity of the receptors for antagonist was reduced by 2-fold while the affinity of the receptors for agonist was reduced by 14-fold. Furthermore, this concentration of DIDS abolished the ability of Na^+ in reducing the affinity of the receptors for agonist. We suggest that the effect of DIDS is via Na^+ binding component of the α_2-adrenoreceptor-adenylate cyclase complex.

The membrane-bound receptor-adenylate cyclase complex consists of at least three macromolecules, namely the receptors (R) located at the external surface of the plasma membrane, the catalytic unit of the enzyme (C) anchored in the inner surface of the plasma membrane and the regulatory proteins (G) acting as couplers between the receptors and the catalytic unit of the enzyme (1). Several receptor-adenylate cyclase complexes where activation of receptors leads to inhibition of adenylate cyclase are regulated by divalent (Mg^{2+}) and monovalent (Na^+) cations and by guanine nucleotide (GTP) (2). The molecular structure of the family of G-proteins involved in coupling between receptors and adenylate cyclase has been elucidated (3). Although earlier studies suggested that Na^+ regulates the receptors by binding to GTP-protein based on the observations that the effects of Na^+ are similar to those of GTP (4,5), recent

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data from several laboratories, including our own, have demonstrated that Na^+ and GTP mediate their effects through two distinct proteins (6,7). Michel et al (8) proposed that the Na^+ binding component in rabbit platelets may be a part of the α_2-adrenoreceptor protein. A similar proposal was made by Larsen et al (9) for opiate receptors. These proposals are contrary to the findings of Motulsky and Insel (10) who showed in human platelets, that intraplatelet Na^+ concentration regulates the α_2-adrenoreceptors. However, Michel et al (8) and Larsen et al (9) have not ruled out the possibility of Na^+ binding to other membrane components other than the GTP or receptor proteins. In the present study we report the effect of DIDS on Na^+ regulation of α_2-adrenoreceptors in human platelet membranes.

MATERIALS AND METHODS

MATERIALS: Yohimbine, epinephrine, DOWEX-50W (hydrogen form) and sodium salt of 4,4'-Diisothiocyano-2,2'-stilbene disulfonic acid (DIDS) were purchased from Sigma. [Methyl-^{3}H]-yohimbine [86.1 Ci/mmol] was obtained from NEN.

METHODS: Platelet membranes were prepared according to the method of Periyasamy and Somani (11).

Treatment of platelet membranes with DIDS

Sodium free DIDS was prepared by passing the sodium salt of DIDS through DOWEX-50W cation ion exchange column and the pH of the resulting acid form of DIDS was adjusted to pH 7.5 with Tris and stored at -60°C. Platelet membranes (1.0 mg/ml) were incubated with varying concentrations of DIDS at 23°C for 60 min. At the end of 60 min, the membranes were diluted with 15.0 ml of ice-cold 50 mM Tris-HCl + 1 mM EDTA pH 7.5. The diluted membranes were centrifuged at 40,000 x g for 30 min. This procedure was repeated one more time. The DIDS-treated membranes were suspended in assay buffer and used for the ligand binding studies. The control membranes were treated exactly in the same manner without treating with DIDS.

Ligand binding assay

Binding of [^{3}H]yohimbine to platelet membranes was carried out at 23°C for 40 min in a total volume of 0.5 ml containing 20 mM Tris-HCl (pH 7.5) plus 0.5 mM EDTA and 180-220 µg of membrane protein. Ascorbic acid (0.01% w/v final concentration) was used as an antioxidant in experiments in which catecholamines were included. This concentration of ascorbic acid had no effect on the binding of [^{3}H]yohimbine. The binding was initiated by adding the membranes to the incubation medium and terminated by the addition of 5.0 ml of ice-cold assay buffer followed by vacuum filtration over Whatman GF/C filters. The filter was then rapidly washed twice with 5 ml of ice-cold assay buffer. The whole process of filtration and washing was completed within 20 sec. The radioactivity bound to the membrane trapped by the filter was extracted into an Aqueous Counting Scintillent (Amersham) and counted in a Beckman Scintillation Counter with an efficiency of 40%. Nonspecific binding was determined in each assay by running parallel samples containing 10^{-6} M unlabelled yohimbine. Specific binding was obtained by subtracting nonspecific from total binding.

RESULTS

Inhibition of [^{3}H]yohimbine binding by unlabeled yohimbine

The displacement of specific [^{3}H]yohimbine binding by unlabeled yohimbine to human platelet membranes treated with or without DIDS is shown in Fig. 1. Yohimbine displaced [^{3}H]yohimbine from the binding sites in a concentration dependent manner in control as well as DIDS-treated membranes. However, the ability of yohimbine in displacing [^{3}H]yohimbine was reduced in DIDS-treated membranes as reflected by a shift of the yohimbine displacement curve to the right compared to control. Furthermore, this effect was directly related to the concentration of DIDS used. IC_{50} value for yohimbine was 4.0 ± 0.5 nM in control membranes and 8.0 ± 1.5 nM in membranes treated with 1.0 mM DIDS.

Inhibition of [^{3}H]yohimbine binding by epinephrine

The inhibitory effect of epinephrine on specific [^{3}H]yohimbine binding to platelet membranes treated with varying concentrations of DIDS is shown in Fig. 2. As expected, epinephrine displaced [^{3}H]yohimbine binding in both preparations in a concentration-dependent manner. In DIDS-treated membranes, the

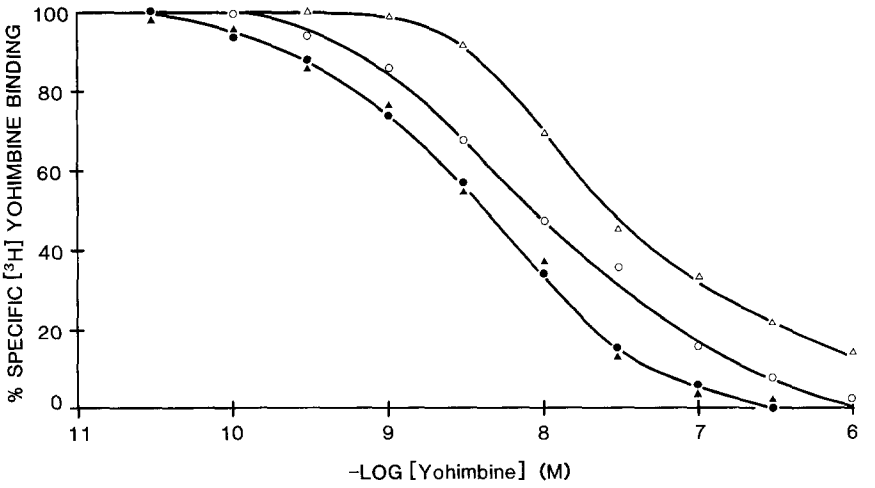

Fig. 1. Effect of yohimbine on specific [^{3}H]yohimbine binding to human platelet membranes treated without (●) or with DIDS 0.5 mM (▲), 1.0 mM (o) and 5.0 mM (△). Specific [^{3}H]yohimbine binding was determined as described in Materials and Methods and specific binding in the absence of unlabeled yohimbine was represented as 100%. The final concentration of [^{3}H]yohimbine in the binding assay medium was 5 nM. The data are the mean of three experiments conducted in duplicate.

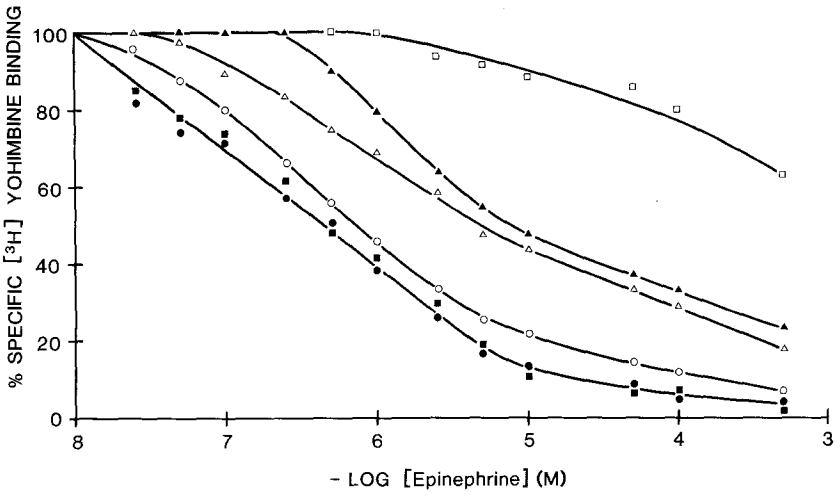

Fig. 2. Specific [^{3}H]yohimbine binding to human platelet membranes treated without (●) or with DIDS 0.01 mM (■), 0.1 mM (o), 0.5 mM (△), 1 mM (▲) and 5 mM (□) as a function of epinephrine. Platelet membranes were treated with DIDS as decribed in the text. Specific [^{3}H]yohimbine binding in the absence of epinephrine was represented as 100%. The final concentration of [^{3}H]yohimbine in the binding assay medium was 5 nM. The data are the mean of three experiments conducted in duplicate.

displacement curves were shifted to the right and the degree of shift was related to the concentrations of DIDS used. In the presence of 5 mM DIDS, not only was the curve shifted further to the right but higher concentrations of epinephrine were unable to displace [^{3}H]yohimbine binding completely. IC_{50} value for epinephrine was 0.5 ± 0.03 μM in control membranes and 7.0 ± 0.5 μM in membranes treated with 1.0 mM DIDS. Thus, while the affinity of the receptors to the antagonist was reduced by 2-fold, a 14-fold reduction was observed with the agonist after pretreatment with 1 mM DIDS.

Steady-state binding of [^{3}H]yohimbine

Figure 3 describes the Scatchard plot of [^{3}H]yohimbine binding to platelet membranes treated with or without DIDS. As is evident from the figure, the chemically modified receptors showed less affinity for yohimbine compared to control. The reduction in the affinity of the receptors for yohimbine was also reflected in the reduction in the number of [^{3}H]yohimbine binding sites from 361 ± 20 to 250 ± 25 fmoles/mg protein. The results of this study are in agreement with the findings obtained from the [^{3}H]yohimbine displacement study.

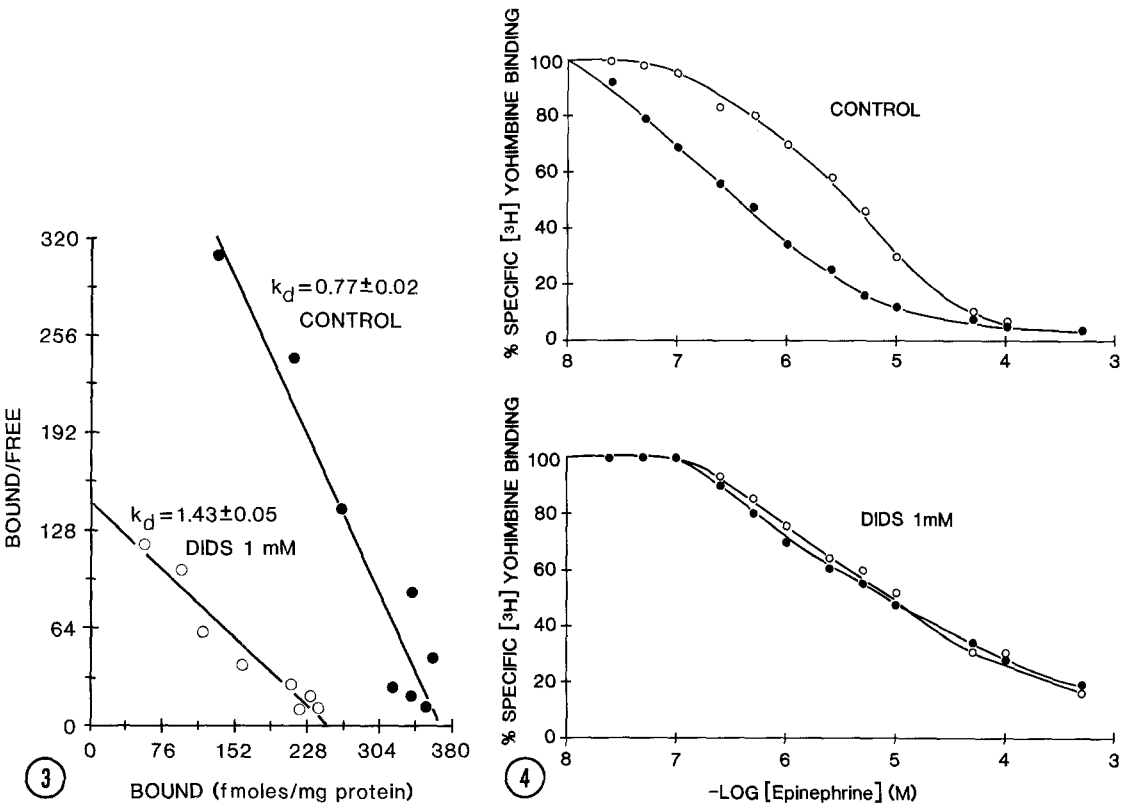

Fig. 3. Equilibrium binding data of specific [^{3}H]yohimbine binding to human platelet membranes treated without or with DIDS. The data were converted into Scatchard plot and were fitted by straight lines using linear regression analysis. The data are the mean of three experiments conducted in duplicate.

Fig. 4. Na^+ effect on epinephrine induced displacement of [^{3}H]yohimbine binding to human platelet membranes treated with DIDS. Specific [^{3}H]yohimbine binding to platelet membranes as a function of epinephrine alone (●) and epinephrine + 100 mM NaCl (o). The final concentration of [^{3}H]yohimbine in the binding assay medium was 5 nM. Specific binding in the absence of epinephrine was taken as 100%. The data are the mean of four experiments conducted in duplicate.

Effect of Na^+ on the affinity of α_2-adrenoreceptors for agonist

Figure 4 illustrates the displacement of specific [^{3}H]yohimbine by epinephrine in control as well as DIDS-treated membranes in the absence and presence of 100 mM NaCl. In the presence of 100 mM NaCl, the epinephrine displacement curve was shifted to the right by 10-fold in the untreated membranes. On the other hand, the epinephrine displacement curve was shifted to the right by 17-fold in DIDS-treated membranes compared to control. However, inclusion of 100 mM NaCl in DIDS-treated membranes did not shift the epinephrine dis-

placement curve further indicating that the Na^+ effect has been abolished by prior treatment of membranes with DIDS.

DISCUSSION

The major findings of the present study are that 1) pretreatment of membranes with DIDS, an anion transport inhibitor, reduced the affinity of the receptors for antagonist as evident from the yohimbine displacement and equilibrium binding studies; 2) it reduced the affinity of the receptors for agonist; the reduction in the affinity of the receptors for antagonist and agonist induced by DIDS was directly related to its concentration; 3) the reduction in the affinity of the receptors produced by DIDS for agonist was several fold greater than for antagonist. For instance, pretreatment of membranes with 1 mM DIDS produced 2-fold reduction for antagonist and 14-fold reduction for agonist; 4) DIDS abolished the ability of Na^+ in reducing the affinity of the receptors for agonist. To our knowledge, this is the first study reporting the abolition of Na^+ effect on receptor regulation.

Although DIDS altered the properties (affinity) of the native α_2-adrenoreceptors, this change in the properties of the receptors alone cannot explain the several fold difference in the affinity of the receptors for antagonist and agonist between control and DIDS-treated membranes. It is well documented that agonist forms a ternary complex with receptors and G-proteins resulting in high affinity state of the receptors (12). Furthermore, it has been shown in several receptor systems that N-ethylmaleimide uncouples the G-proteins from the receptors resulting in the conversion of receptors from high affinity to low affinity state and reduction in the affinity of the receptors for agonist. However, this uncoupling does not affect the affinity of the receptors for antagonist (6,9,13). Our results with DIDS for the agonist can be interpreted in a similar way and, indeed, GTP failed to produce an effect on epinephrine induced displacement of [^{3}H]yohimbine binding in DIDS-treated membranes (data not shown). Furthermore, DIDS treatment also abolished the Na^+ effect in reducing the affinity of the receptors for agonist. In our experimental condi-

tions, inactivation of GTP-binding protein produced consistently a 2-4 fold decrease in the affinity of the receptors for agonist compared to control membranes; whereas pretreatment of membranes with 1 mM DIDS produced a 14-fold decrease in the affinity of the receptors for agonist compared to control. It has been proposed that Na^+ reduces the affinity of the receptors for agonist allosterically by binding to the receptor proteins (9). If this is true, DIDS might bind to the Na^+ binding sites on the receptor molecule evoking Na^+-like effect. Alternatively, DIDS might uncouple a protein component, analogous to G-proteins, interacting with receptor protein, resulting in further reduction in the affinity of the receptors for agonist. We tend to favor the latter explanation because, in human platelets, Na^+ exerts its effect by binding to the inner aspect of the plasma membranes (10).

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