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INHIBITION OF LIGAND BINDING TO THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTORS BY DIETHYLPYROCARBONATE

PROTECTION BY RECEPTOR LIGANDS AND REVERSAL BY HYDROXYLAMINE

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Abstract—The potential of histidines to modulate the binding of agonists and antagonists to human platelet thromboxane A_2 (TXA_2) receptors was investigated. TXA_2 receptors were purified from crude platelet membranes via affinity and wheat germ lectin chromatography. Radioligand binding studies were conducted using the TXA_2, mimetic [^{125}I]BOP (I-BOP = [1*S*-(1α,2β(5*Z*),3α(1*E*,3R*),4α)]-7-[3-(3-hydroxy-4-(4′-iodophenoxy)-1-butenyl)7-oxabicyclo-[2.2.1]heptan-2-yl]-5-heptenoic acid) and the TXA_2 receptor antagonist [^{125}I]SAP (I-SAP = 7-[(1*R*,2*S*,3*S*,5*R*)-6,6-dimethyl-3-(4-iodobenzenesulfonylamino)-bicyclo-[3.1.1]hept-2-yl]-(5*Z*)-heptenoic acid). The histidine modifying reagent diethylpyrocarbonate (DEPC) produced a concentration (30–100 μM) dependent inhibition of binding of both [^{125}I]BOP and [^{125}I]SAP. DEPC treatment significantly ($P < 0.05$, $N = 6$) decreased the affinity of the receptor for [^{125}I]SAP (K_d = 2.4 ± 0.4 and 5.4 ± 0.4 nM, control and DEPC, respectively) without significantly decreasing the B_{max}. The effects of DEPC were reversed by hydroxylamine. The inhibition of [^{125}I]BOP and [^{125}I]SAP binding produced by DEPC was reduced significantly by prior incubation of the purified receptors with the TXA_2 receptor agonist U-46619 or the TXA_2 receptor antagonist SQ 29548. The results strongly support the notion that one or more histidines reside in a domain that can modulate ligand binding to the TXA_2 receptor.

Key words: thromboxane A_2 receptors; histidines; diethylpyrocarbonate

Occupation of platelet TXA_2/PGH_2† receptors by TXA_2, PGH_2 or more stable agonists, such as U-46619, results in platelet activation, including shape change, aggregation and secretion [1–3]. The human platelet TXA_2/PGH_2 receptor has been solubilized [4] and purified to apparent homogeneity [5], and its radioligand binding characteristics have been studied. TXA_2/PGH_2 receptors have been cloned from human placenta and mouse cDNA libraries and their amino acid sequences deduced [6, 7]. They belong to the large group of G-protein-linked receptor proteins that are monomeric and have seven transmembrane spanning domains.

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† Abbreviations: TXA_2, thromboxane A_2; PGH_2, prostaglandin H_2; I-BOP, [1*S*-(1α,2β(5*Z*),3α(1*E*,3*R**),4α)]-7-[3-(3-hydroxy-4-(4′-iodophenoxy)-1-buteny)-7-oxabicyclo-[2.2.1]heptan-2-yl]-5-heptenoic acid; I-SAP, 7-[(1*R*,2*S*,3*S*,5*R*)-6,6-dimethyl-3-(4-iodobenzenesulfonylamino)-bicyclo-[3.1.1]hept-2-yl]-(5*Z*)-heptenoic acid; I-SAP-N_3, [7-[(1*R*,2*S*,3*S*,5*R*)-6,6-dimethyl-3-(4-azido-3-iodobenzenesulfonylamino)-bicyclo-[3.1.1]-hept-2-yl]-(5*Z*)-heptenoic acid]; DEPC, diethylpyrocarbonate; MES, 2-[*N*-morpholino]-ethanesulfonic acid; and CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Knowledge of the amino acid sequences of TXA_2/PGH_2 receptors provides the necessary information to begin to clarify the role of selected amino acids and domains for receptor function. In this context, it is interesting to note that changes in pH have been reported to differentially influence agonist and antagonist binding to this receptor [8]. In washed human platelets and solubilized membranes, a change in pH from 7.4 to 6.0 results in an increased affinity for several agonists and no change or a decrease for antagonists [8]. In washed platelets, this is also accompanied by an apparent increase in receptor number [8]. The increased affinity is associated with an enhanced biological response, i.e. shape change, in washed platelets [8]. These observations raised the possibility that an amino acid(s) that is positively charged at acidic pH may confer a conformational change in the TXA_2/PGH_2 receptor responsible for the increase in affinity for agonists.

One possible amino acid candidate for this phenomenon is histidine. The imidazolium form of histidine has a pK_a of approximately 6.0. The deduced amino acid sequence of the TXA_2/PGH_2 receptor of the human placenta [6] and mouse lung [7] both contain two highly conserved histidines. One conserved histidine is located in the second extramembranous loop and the second is located in the third intracytoplasmic loop [6, 7].

DEPC has been used to specifically modify histidines [9]. Interestingly, treatment of washed platelets with DEPC resulted in a significant attenuation of the increased agonist affinity at pH 6.0, which was demonstrated by detection of agonist affinities similar to those at pH 7.4. There was also a significant reduction in total specific binding (B_{max}) by DEPC at pH 6.0, which was less pronounced at pH 7.4 [8]. However, since these studies were conducted in washed platelets and crude membranes, it is possible that the pH effect and the effect of DEPC were indirect and not direct effects on the receptor.

The present study was designed to analyze the potential influence of histidines on ligand binding to purified human platelet TXA_2/PGH_2 receptors. Equilibrium radioligand binding was performed using two structurally and functionally dissimilar ligands: [^{125}I]BOP, a high-affinity TXA_2/PGH_2 receptor agonist [10], and [^{125}I]SAP, a high affinity TXA_2/PGH_2 receptor antagonist that antagonizes the human platelet aggregation receptor but stimulates shape change [11].

MATERIALS AND METHODS

Materials. I-BOP, I-SAP and I-SAP-N_3 were synthesized and radiolabeled with ^{125}I as previously described [10–12]. The following were gifts: U-46619 [13] (Upjohn Co., Kalamazoo, MI), L 657925 [14] (Merck Frosst Canada Inc., Point Claire-Dorval, Quebec), and SQ 29548 (Squibb Pharmaceutical Co., Princetown). DEPC was purchased from the Aldrich Chemical Co. (Milwaukee, WI), hydroxylamine from Sigma (St. Louis, MO) and CHAPS from Boehringer–Mannheim (Indianapolis, IN).

Preparation of TXA_2/PGH_2 receptors. Human platelet membranes were prepared from outdated human platelets as previously described [4]. TXA_2/PGH_2 receptors were prepared from solubilized human platelet membranes, following the techniques described by Ushikubi *et al.* [5] with minor modifications. The crude membranes were solubilized in buffer, pH 7.4, containing Tris (20 mM), EGTA (5 mM), glycerol (0.25 mL/mL), phenylmethylsulfonyl fluoride (0.128 mg/mL), benzamidine (0.156 mg/mL) and CHAPS (10 mM). After centrifugation at 200,000 *g* for 1 hr, the supernatant was adjusted to 2.5 mM CHAPS and 0.5 M KCl.

An S-145 affinity gel column was prepared as previously described [5] and equilibrated with buffer, pH 7.4, containing: Tris (20 mM), EGTA (5 mM), glycerol (0.25 mL/mL), CHAPS (10 mM), KCl (1 M) and asolectin (0.2 mg/mL) at 4°. The equilibrated column was loaded with 120 mL of solubilized receptor solution per 20 mL affinity gel for 12–14 hr at a circulation rate of 1 mL/min. The void volume was removed by gravity, and the gel was washed with ten column volumes of elution buffer, pH 7.4, containing: Tris (20 mM), EGTA (0.2 mM), glycerol (0.25 mL/mL), CHAPS (10 mM), phenylmethylsulfonyl fluoride (0.128 mg/mL), benzamidine (0.156 mg/mL) and asolectin (1.0 mg/mL).

After washing, the affinity gel column was connected in series with a 2-mL wheat germ lectin Sepharose 6MB column that was pre-equilibrated with 100 mL of elution buffer. Receptor protein was displaced from the S-145 column with 100 μM SQ 29548, which was allowed to circulate at 0.1 mL/min for 3 days. The columns were then separated, and the wheat germ lectin column containing bound receptor protein was washed with 100 mL of elution buffer at 0.1 mL/min. The wheat germ lectin column was then washed with a 3 mM (6 mL) and then a 300 mM *N*-acetyl-D-glucosamine (NADG) buffer containing Tris (20 mM), EGTA (0.2 mM), CHAPS (10 mM), asolectin (1 mg/mL), pH 7.4, applied at a rate of 0.2 mL/min. The receptor was eluted with 300 mM NADG in 2-mL fractions (8 fractions). The fractions containing the receptor were identified using radioligand binding. These fractions were pooled and stored at −80°.

Protein determination. Protein content was determined by the Bio-Rad protein assay except for the protein content of the purified receptors, which was accomplished by the procedure of Kaplan and Pedersen [15].

Radioligand binding assays. The purified receptors (0.6 to 0.8 μg protein/mL) were thawed on ice and preincubated for 1 hr at 4° in pH 7.4 or pH 6.0 buffer. The incubation mixture (200 μL final volume) consisted of HEPES (25 mM), EDTA (2 mM), CHAPS (5 mM), asolectin (0.5 mg/mL), 60–80 ng protein, 50,000–70,000 cpm [^{125}I]BOP or [^{125}I]SAP and various concentrations of I-SAP or I-BOP (0.05 to 250 nM) and incubated for 1 hr at 30°. Assays were performed in silanized (12 × 75 mm) glass tubes. The mixture was then filtered rapidly through Whatman GF/C glass-fiber filters, presoaked with 0.3% polyethylenimine. This was followed by three additional washings with ice-cold HEPES (25 mM)/EDTA (2 mM)/CHAPS (0.1 mM). The filtration procedure was complete within 10 sec. Radioactivity was counted using an LKB gamma-counter. Nonspecific binding was defined as the amount of radioactivity bound in the presence of 10 μM L 657925 and was typically less than 10% of the total cpm bound.

Treatment with DEPC. Stock concentrations (300 mM) of DEPC were made fresh in ethanol, diluted with ice-cold HEPES/EDTA, pH 6.0 or pH 7.4, and immediately added to the purified receptors in the incubation buffer as described above at the appropriate pH. Final concentrations were 3, 10, 30 and 100 μM. DEPC incubation was performed on ice for 30 min prior to the experiment and was followed by another 30-min incubation at 30° with the radiolabeled ligand. The $T_{1/2}$ for DEPC at 40° is 10 min [16]; thus only about 10% of the original DEPC would remain at the end of the incubation period. The subsequent procedures were identical to those described for the radioligand binding studies.

Influence of hydroxylamine, SQ 29548 and U-46619 on DEPC treatment. Hydroxylamine (30 μM final concentration) was freshly prepared in ice-cold HEPES/EDTA, pH 6.0 or 7.4, and added to the purified receptors following a 10-min preincubation with DEPC and continued for 20 min. Alternatively, U-46619 (100, 300 or 1000 nM final concentration) or SQ 29548 (30, 100 and 300 nM final concentration)

Table 1. pH dependence of [^{125}I]BOP binding and its modification by DEPC

pH	Specific bound cpm			
	Vehicle	DEPC (10 μM)	DEPC (30 μM)	DEPC (100 μM)
6.0	3880 ± 100	3160 ± 70	1990 ± 70*	990 ± 50*
	(3)	(5)	(6)	(6)
	100%	88%	56%	28%
7.4	710 ± 140†	370 ± 40†	390 ± 50†	420 ± 40†
	(3)	(6)	(6)	(6)
	100%	51%	55%	58%

Data are presented as the means ± SEM of the number of experiments indicated in parentheses. The numbers below indicate the percent of specific bound counts compared with the vehicle group.

* $P < 0.05$ (DEPC vs no-DEPC at same pH).

† $P < 0.01$ (pH 7.4 vs pH 6.0).

(total volume of 20 μL) was added to the purified receptors for 10 min prior to the addition of DEPC and continued for another 20 min. At the end of that period, the volume was increased to 200 μL (10-fold dilution of the U-46619 and SQ 29548), the incubation was continued for another 30 min at 30° with the radiolabeled ligand, and the reaction was stopped as described above.

Photoaffinity labeling. Photoaffinity labeling of the purified TXA_2/PGH_2 receptor followed modifications of the procedures of Mais *et al.* [17]. An aliquot of an ethanolic stock of [^{125}I]SAP-N_3 was dried under N_2 in a silanized glass tube and resuspended into MES (62.5 mM) buffer containing CHAPS (5 mM) at pH 6.5. [^{125}I]SAP-N_3 (4×10^6 cpm) was mixed with an equal volume of purified receptor into individual wells of a 96-well micro-titer plate (Falcon Microtest III). Vehicle or competing antagonist, L657925 (20 μM final concentration), was added, and incubations were continued for 20 min at 30° in the dark. Photolysis was performed using a 254 nm UV-light source (Ultraviolet Products, Inc., San Gabriel, CA) at a distance of 1 cm for 10 sec. Incubations were terminated by the addition of a standard SDS-containing reducing buffer and heated at 100° for 10 sec. SDS–PAGE followed by autoradiography was then performed.

Data analysis. The equilibrium binding data for [^{125}I]BOP and [^{125}I]SAP were analyzed according to Scatchard [18] using the computer program LIGAND [19]. Student's two-tailed *t*-test for non-paired data was used for statistical evaluation of the results. P values of ≤ 0.05 were considered significant.

RESULTS

Receptor purification. The combined S-145 affinity and wheat germ lectin chromatography yielded a highly purified receptor preparation with a K_d for [^{125}I]SAP of 2.6 ± 0.3 nM and a B_{max} of 7.8 ± 2.1 nmol/mg protein (N = 7). Assuming a molecular mass of 57 kDa for the PGH_2/TXA_2 receptor [5], a theoretical value of 17.5 nmol/mg protein would correspond to a receptor population purified to homogeneity.

Effects of DEPC on [^{125}I]BOP binding at pH 6.0 and pH 7.4. The specific binding of [^{125}I]BOP was up to 5-fold greater at pH 6.0 as compared with pH 7.4 (Table 1). DEPC produced a concentration dependent inhibition of [^{125}I]BOP binding at pH 6.0. However, at pH 7.4 the inhibition was not concentration dependent. DEPC concentrations higher than 100 μM failed to produce any greater effect (data not shown). There was no effect of ethanol on binding up to 0.1%, the highest concentration used.

Effects of DEPC on [^{125}I]SAP binding. The specific binding of [^{125}I]SAP was also inhibited by DEPC treatment. At 30 μM DEPC, the specific binding of the ligand was reduced by 42 ± 4% (N = 6), at 60 μM by 62 ± 2% (N = 8), and at 100 μM by 68 ± 7% (N = 9). This last value was not significantly different ($P > 0.05$) from the 73 ± 3% inhibition of [^{125}I]BOP binding at pH 6.0 with 100 μM DEPC (N = 14).

Reversal of DEPC-induced inhibition of [^{125}I]-BOP and [^{125}I]SAP binding by hydroxylamine. Hydroxylamine has been shown previously to reverse the carbethoxylation of histidines by DEPC [16]. Therefore, we determined if hydroxylamine was capable of reversing the effect of DEPC on radioligand binding. Incubation of the purified receptors with 30 μM hydroxylamine resulted in a minor reduction (18 ± 6%; N = 6) of [^{125}I]BOP binding at pH 6.0 (Table 2). Hydroxylamine (30 μM) did not affect the minor reduction of binding produced by 10 μM DEPC. There was also no difference in [^{125}I]BOP binding between 10 and 30 min of preincubation with 100 μM DEPC, suggesting that the carbethoxylation reaction was complete within 10 min. However, there was a significant though incomplete reversal of DEPC-induced inhibition of [^{125}I]BOP binding with 30 and 100 μM DEPC. Equimolar concentrations of DEPC and NH_2OH (30 μM) produced about a 50% protection, whereas about 20% protection was seen with NH_2OH (30 μM) and 100 μM DEPC (Table 2). The reversal of the reduction in [^{125}I]BOP binding by NH_2OH induced by DEPC may actually be an underestimate since NH_2OH also caused some (18%) loss of specific binding.

Table 2. Effects of hydroxylamine on DEPC-induced reduction in [^{125}I]BOP binding

Treatment	% Inhibition of specific [^{125}I]BOP binding			
	Vehicle	DEPC (10 μM)	DEPC (30 μM)	DEPC (100 μM)
Vehicle		16 ± 10 (5)	45 ± 5 (7)	76 ± 4 (7)
NH_2OH (30 μM)	18 ± 6 (6)	14 ± 12 (5)	24 ± 7* (9)	60 ± 5* (9)

Data are presented as the means ± SEM of the number of experiments indicated in parentheses. The specific bound cpm for the vehicle group was 3950 ± 370.
* $P < 0.05$ (NH_2OH treated vs vehicle treated).

Table 3. Effects of U-46619 on DEPC-induced reduction in [^{125}I]BOP binding

Ligand	% Reduction of specific binding		
	U-46619 (0.3 μM)	DEPC (60 μM)	U-46619 + DEPC
[^{125}I]BOP	14 ± 6	64 ± 4	46 ± 3*

Data are presented as the means ± SEM for 6 experiments. Values represent the percent inhibition of specific binding compared with the vehicle-treated groups. The specific bound cpm for the vehicle group was 2820 ± 80.
* $P < 0.01$ (DEPC + U-46619 vs DEPC).

Inhibition of [^{125}I]BOP and [^{125}I]SAP binding by DEPC: Protection by U-46619. If DEPC was modifying an amino acid(s) within or close to the binding domain of the receptor, then preincubation with an agonist (U-46619) or antagonist (SQ 29548) should block its effect on binding. Incubation of purified TXA_2/PGH_2 receptors with U-46619 resulted in a concentration dependent competition with [^{125}I]BOP and [^{125}I]SAP binding. To determine the optimum concentration of U-46619 that has minimal residual effects on binding, we incubated the receptor preparation with various concentrations of U-46619 and [^{125}I]SAP. U-46619 (0.1 to 10 μM) produced 9 ± 8% reduction of binding of the radioligand at 0.1 μM U-46619 (N = 6), 24 ± 2% at 0.3 μM U-46619 (N = 8), and 47 ± 18% (N = 3) at 1.0 μM U-46619. Subsequent incubation with 30 μM DEPC did not result in any additional reduction of [^{125}I]BOP binding, producing 27 ± 6% (N = 5) at 0.1 μM U-46619 + 30 μM DEPC and 56 ± 6% inhibition (N = 3) at 0.3 μM U-46619 + 30 μM DEPC, respectively. Increasing DEPC to 60 μM reduced [^{125}I]BOP binding by 64 ± 4% (N = 6). This value was not affected by pretreatment with 0.1 μM U-46619 (62 ± 4% of control). However, pretreatment with 0.3 μM U-46619 significantly reduced (N = 6; $P < 0.01$) the inhibition of [^{125}I]-BOP binding to 46 ± 3% of control (Table 3).

Data similar to that with [^{125}I]BOP were obtained when [^{125}I]SAP was used as the radioligand. U-46619 (0.3 μM) reduced [^{125}I]SAP binding by 24 ± 2% of control (N = 6) and DEPC (60 μM) by 62 ± 2% (N = 6). Pretreatment with U-46619 (0.3 μM) resulted in significantly less inhibition by DEPC (53 ± 3%; N = 6; $P < 0.05$) than with DEPC alone. U-46619 at 0.1 μM (not shown) did not block the effect of DEPC.

Protection of [^{125}I]BOP binding from inhibition by DEPC following pretreatment with SQ 29548. As summarized in Table 4, pretreatment with the TXA_2 receptor antagonist SQ 29548 prevented DEPC-induced inhibition of [^{125}I]BOP binding. This effect was most pronounced after pretreatment with 100 nM SQ 29548 and resulted in a significantly higher I-BOP binding than seen after treatment with 60 μM DEPC. Similar results were obtained with [^{125}I]SAP as the radioligand (data not shown).

Effects of DEPC on photoaffinity labeling of the TXA_2/PGH_2 receptor. To further substantiate that DEPC was modifying a domain involved in ligand binding, its effects on photoaffinity labeling of the TXA_2/PGH_2 receptors were evaluated. L 657925 (20 μM), a TXA_2/PGH_2 receptor antagonist, completely abolished the incorporation of [^{125}I]SAP-N_3 into the receptor, demonstrating specificity of [^{125}I]-SAP-N_3 for TXA_2/PGH_2 receptors. A specifically labeled band corresponding to a molecular mass of 52 kDa was seen in the absence of competing antagonist, which corresponds to the known molecular mass of the purified TXA_2/PGH_2 receptor (Fig. 1). A concentration dependent inhibition of photoaffinity labeling was seen following pre-incubation of purified receptor with DEPC (Fig. 1). At the lower concentrations of DEPC (10 μM), a 28% inhibition of [^{125}I]SAP-N_3 binding was seen, whereas with higher concentrations of DEPC (30 and 100 μM) there was a 62 and 80% inhibition of photoincorporation. Hydroxylamine (30 μM) abolished the inhibition of ligand incorporation produced by 10 μM DEPC and partially reversed the effects of higher concentrations (30 and 100 μM), by 67 and 29%, respectively.

Scatchard analysis of [^{125}I]SAP binding in the absence and presence of DEPC and hydroxylamine. To determine if DEPC was affecting either the K_d, B_{max} or both, equilibrium binding was performed followed by Scatchard analysis. Scatchard analysis of [^{125}I]SAP revealed a single class of binding sites. The K_d values were 2.4 ± 0.4 and 5.4 ± 0.4 nM and the B_{max} values were 3.92 ± 0.70 and

Table 4. Effects of SQ 29548 on DEPC-induced reduction in [^{125}I]BOP binding

	Vehicle	% Reduction of [^{125}I]BOP binding DEPC (60 μM)	SQ 29548 + DEPC (60 μM)
SQ 29548 (30 nM)	13 ± 2	71 ± 2	66 ± 2*
SQ 29548 (100 nM)	22 ± 5	71 ± 2	56 ± 2†
SQ 29548 (300 nM)	43 ± 1	71 ± 2	54 ± 4*

Data are presented as the means ± SEM of duplicate or triplicate determinations of two separate experiments. Values represent the percent reduction in [^{125}I]BOP binding compared with the vehicle group. The specific bound cpm for the vehicle group was 3500.
* $P < 0.05$ (DEPC + SQ 29548 vs DEPC).
† $P < 0.01$ (DEPC + SQ 29548 vs DEPC).

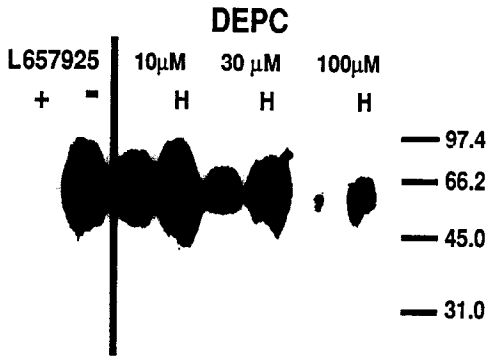

Fig. 1. Photoaffinity labeling of purified platelet TXA_2/PGH_2 receptors. Lanes 1 and 2 represent photoaffinity labeling of purified receptors in the absence or presence of L 657925 (10 μM), a TXA_2/PGH_2 receptor antagonist. Lanes 3, 5 and 7 represent labeling of the receptors in the presence of DEPC, and lanes 4, 6 and 8 represent labeling in the presence of DEPC and hydroxylamine.

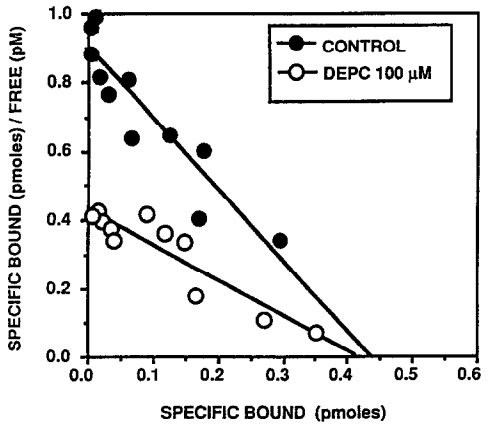

Fig. 2. Representative Scatchard analysis of equilibrium binding data with [^{125}I]SAP in the presence or absence of DEPC. DEPC significantly ($P < 0.05$) increased the K_d values without significantly changing the B_{max} values.

4.04 ± 0.35 nmol/mg protein (N = 6) in the absence or presence of 100 μM DEPC, respectively. The differences in K_d values were statistically significant ($P < 0.05$) (Fig. 2).

DISCUSSION

This study demonstrates that treatment of purified human platelet TXA_2/PGH_2 receptors with DEPC significantly decreased their affinity for I-BOP (agonist) and I-SAP (antagonist) binding. This effect was reversed by hydroxylamine and reduced by preincubation with either a TXA_2/PGH_2 receptor agonist or antagonist. In a previous study in crude platelet membranes, the effect of DEPC was assessed only on I-BOP binding; thus, its potential effects on antagonist binding were unknown. Indeed, it was inferred in that study that the effects of DEPC may be restricted to only agonist binding [8].

The biochemical and biological effects of DEPC result from reactions with functional groups in macromolecules, including aromatic amines, thiols, phenols, enolates and others [9, 20, 21]. Particular interest has been focused on the use of DEPC as a probe to detect functionally important histidines in a variety of proteins [20]. It has been suggested that DEPC reacts specifically and stoichiometrically with single histidyl residues in proteins, suggesting that in this respect it may be more selective than other acylating agents [16].

Previous studies from this laboratory showed that DEPC significantly reduces agonist binding to washed human platelet membranes at pH 6.0 [8] but fails to do so at pH 7.4. This was taken as evidence suggesting a potential involvement of histidine(s) in agonist binding to platelet TXA_2/PGH_2 receptors. The present study using a highly purified TXA_2/PGH_2 receptor preparation, confirms the observation that DEPC causes a concentration dependent inhibition of agonist binding at pH 6.0. Compared with its effects at pH 7.4, the inhibitory effect of DEPC at pH 6.0 was much greater and was concentration dependent at 10–100 μM. These concentrations are considerably lower than the millimolar concentrations of DEPC commonly used and suggest a potential specific interaction of the compound with its target amino acids. Also the use of highly purified receptors may have accounted for the need for lower concentrations of DEPC. The specificity was further demonstrated by the absence

of any greater inhibition at incubation times of more than 10 min.

DEPC does not always react specifically with histidyl residues in proteins. Carbethoxylation of α and ε-amino groups of amino acids, of thiol sulfurs and of phenolic oxygens of tyrosine may occur. However, these reactions are favored by higher pH, i.e. 7–9 [16], whereas in the present investigation the reaction in absolute terms was more pronounced at pH 6.0 and was attenuated at pH 7.4. Moreover, 50% inhibition of ligand binding was obtained with 30 μM DEPC. This is one argument against a reaction with the indole of tryptophan or lysine in proteins, which were found to require DEPC concentrations between 0.3 and 3 mM [21].

Another possibility to verify the interaction of DEPC with histidine(s) is the reversal of this reaction by hydroxylamine. Hydroxylamine has been found to remove the carbethoxy groups from modified histidyl residues and tyrosyl residues, but not that of modified lysyl or sulfhydryl residues [21]. Burstein and colleagues [20] have shown that 20 mM hydroxylamine removed the carbethoxy group from histidyl but not from tyrosyl residues when the hydroxylamine was added prior to the addition of DEPC. In the present study, 30 μM hydroxylamine reversed the inhibition of an equimolar concentration of DEPC by 50%, and a 3-fold higher molar ratio of DEPC/NH_2OH was also accompanied by a significant but less pronounced protection of the receptor binding site from inactivation. Furthermore, hydroxylamine produced some reduction in binding, which if this reduction was accounted for, the reversal of DEPC inhibition would have been greater.

In contrast to several previous investigations using crude membranes and intact cells, DEPC did not reduce receptor density significantly as assessed by [^{125}I]SAP binding. The receptor affinity was reduced markedly as seen from a 2-fold increase in the K_d (Fig. 2). A similar increase in K_d, though not significant, has been reported previously for crude platelet membrane preparations [8]. The differences in results reported in this study compared with those seen in crude membranes may reflect additional reactions or effects of DEPC in the crude membranes that are not seen in the purified receptors. We cannot exclude the possibility that some reduction in B_{max} has occurred; however, DEPC clearly produced a decreased affinity of the TXA_2 receptor for [^{125}I]-SAP binding.

If histidines are involved in modulating the ligand binding to the TXA_2/PGH_2 receptor, then inactivation of these amino acids may result in decreased affinity of the receptor, perhaps as the result of a change in the conformation of the agonist/antagonist binding domains. Pretreatment with either the agonist U-46619 or the antagonist SQ 29548 at concentrations previously shown to specifically bind to the TXA_2/PGH_2 receptor in washed human platelets [22, 23] protected the receptor from additional inactivation by DEPC at pH 6.0. That the effects of DEPC and hydroxylamine were in the binding domain region was further confirmed by photoaffinity labeling of the purified receptor preparation. DEPC (10–100 μM) produced a concentration dependent inhibition of [^{125}I]SAP-N_3 binding, which was reversed by hydroxylamine. Thus, inhibition of binding of three different ligands to the TXA_2/PGH_2 receptors by DEPC strongly supports the notion that the histidines are in or near to the binding domain.

There are two conserved histidines in the TXA_2/PGH_2 receptor at positions 84 and 225 in human placenta [6], which are also found in the mouse lung receptor at positions 85 and 226 [7]. These data suggest that these histidines may be highly conserved in TXA_2 receptors and play an important role in modulation of ligand binding. Indeed, histidines at similar steric positions are also found in many G-protein-coupled receptors [24]. Included among the group are adenosine (A_1 and A_2) [25, 26], $GABA_A$ [27], dopamine (D_2), β-adrenergic 2 and 3, muscarinic (M1), substance P and substance K, neuropeptide Y, cannabinoid, follicle-stimulating hormone (FSH), and opsin [28] receptors. These histidines are located in the second extramembranous loop and/or the third intracytoplasmic loop. The latter domain is thought to interact with the G-protein. The histidine in the second extramembranous loop may influence ligand binding since it is in close proximity to the third intramembranous domain, which is thought to participate in ligand binding. For several of these receptors it has been demonstrated that changes in pH affect ligand binding; indeed, in some cases the effect is specific for agonist binding [28–31].

DEPC has been used to modify binding to adenosine A_{2a} receptors [25] and muscarinic M1 receptors [32] in crude membrane preparations. In contrast to the present results, DEPC produced a significant decrease in the B_{max} without a change in K_d. In the case of the adenosine receptor, this effect was felt to be due to modification of histidine residues present in the sixth and seventh transmembrane domains [33]. Indeed, site-directed mutation of the histidine in the seventh transmembrane domain decreased both agonist and antagonist affinity. In contrast, a mutation of the histidine in the sixth transmembrane domain decreased antagonist affinity and B_{max} but did not affect agonist affinity. The histidines located in the second extramembranous loop and third intracytoplasmic loop were not mutated. The muscarinic receptors do not have histidines in the sixth or seventh transmembranous loops. Thus, the effects of DEPC previously reported for this receptor class may also be occurring as a result of modifications of the histidines in the second extramembranous and/or third intracytoplasmic loops.

Collectively, the current studies and previous data in the literature raise the possibility that the conserved histidines in the second extramembranous loop and/or the third intracytoplasmic loop play a modulatory role in ligand binding to G-protein-coupled receptors.

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