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PROSTAGLANDIN $F_2\alpha$ RECEPTORS IN BOVINE CORPUS LUTEUM CELL MEMBRANES

EFFECT OF ENZYMES AND PROTEIN REAGENTS

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SUMMARY

Various enzymes and protein reagents inhibited [^{3}H]prostaglandin $F_2\alpha$ binding to bovine corpus luteum cell membranes. Studies were undertaken (a) to explore further on the dose response relationships with the above agents, (b) to investigate the mechanism of inhibition of binding with respect to receptor affinities and number and (c) to assess whether decreased binding reflected changes in receptors and/or other membrane components.

Preincubation of membranes with phospholipase A, trypsin, pronase, lipase, tetranitromethane, dinitrofluorobenzene, acetic anhydride and *N*-ethylmaleimide resulted in moderate to drastic inhibitions of [^{3}H]prostaglandin $F_2\alpha$ binding. The dose-dependent inhibition of binding by enzymes, but not by protein reagents (except for *N*-ethylmaleimide), exhibited a biphasic pattern: at lower concentrations, the loss of binding was low and relatively plateaued, but at higher concentrations, the losses were dramatic. The drastic reduction in binding by trypsin was due to destruction rather than solubilization of receptors from membranes. Phospholipase A was intrinsically more effective than phospholipases C and Ca^{2+} was not required for its inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding. Protein reagents inhibition of binding was differently influenced by added Ca^{2+} i.e., loss of binding increased with some (*N*-ethylmaleimide), decreased with others (tetranitromethane, dinitrofluorobenzene and azobenzene sulfenylbromide). These results are interpreted to indicate that Ca^{2+} induced conformational changes in membranes which may result in exposure of new groups and burying of already exposed modifiable groups.

Treatment of membranes with trypsin and *N*-ethylmaleimide selectively abolished high affinity prostaglandin $F_2\alpha$ receptors. The low affinity receptors were present but their numbers as well as their affinity were decreased. Lipase, phospholipase A, acetic anhydride, dinitrofluorobenzene and tetranitromethane appear to decrease binding by totally abolishing all prostaglandin $F_2\alpha$ receptors or by severely reducing their affinities.

The occupancy of receptors by prostaglandin $F_2\alpha$ afforded considerable pro-

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

tection against trypsin, phospholipase A, lipase and dinitrofluorobenzene. These data indicated that the inhibition of binding by the above agents, at least in part, can be attributable to changes in receptor sites alone.

INTRODUCTION

It has now been well demonstrated that corpora lutea of various species contain specific and high affinity receptors for human choriogonadotropin-lutropin [1–5], prostaglandin $F_2\alpha$ [6–12] and for prostaglandin Es [1, 11, 13]. All three of these receptors are discrete, not only in specificity of ligand binding but also in other properties [14–16]. All three receptors have been shown to be protein in nature requiring membrane lipids and specific phospholipids but not sialic acid residues for binding function (refs. 3 and 13, and Rao, Ch. V., submitted for publication). Protein functional groups such as tyrosyl, histidyl, tryptophan residues and amino groups (any one or all of them) but not sulfhydryl groups seem to be involved in binding interaction (refs. 3 and 13, and Rao, Ch. V., submitted for publication). In the above studies as well as in numerous others reported in the literature, a single concentration of different enzymes and protein reagents was used to investigate the molecular nature of ligand-membrane receptor interaction. Furthermore, whether these various treatments decreased binding by affecting the receptor affinities and/or number was not investigated. The use of enzymes and protein reagents to investigate macromolecular nature of membrane receptors is complicated by the possibility that the loss of binding could be secondary to the specific changes in other membrane components. The present studies were undertaken to throw some light on the above considered possibilities in the use of enzymes and protein reagents to investigate macromolecular nature of prostaglandin $F_2\alpha$-receptors interaction.

EXPERIMENTAL PROCEDURE

Unlabeled prostaglandin $F_2\alpha$ was generously donated by Dr. John Pike of the Upjohn Co., Kalamazoo, Mich. The following chemicals were purchased from the commercial sources indicated: [^{3}H]prostaglandin $F_2\alpha$ (178 Ci/mmol) from New England Nuclear Corp.; phospholipase A (*Crotalus terrificus terrificus*, 850.0 units/mg), phospholipase C (*Bacillus cereus*, 250 I.U./mg), lipase (Porcine pancreas, 81.0 units/mg), neuraminidase (*Vibrio cholerae*, 500.0 units/mg), from Calbiochem; trypsin (Bovine pancreas, 189.0 units/mg), from Worthington Biochemicals Corp; *N*-ethylmaleimide, tetranitromethane, 2,4-dinitrofluorobenzene, mercaptoethanol, iodoacetamide, dithiothreitol, ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) and phospholipase C (*Clostridium welchii*, 7.6 units/mg) from Sigma Chemical Co.; *p*-chloromercuribenzoate, azobenzene sulfenyl bromide and phospholipase C (*Clostridium perfringens*, 2.0 units/mg) from Nutritional Biochemical Corp.; EHWP Millipore filters (0.5 μm pore size) from Millipore Corp.

Procedures for checking purity of [^{3}H]prostaglandin $F_2\alpha$, its purification if needed, collection of bovine corpora lutea, isolation of the cell membrane fraction and performance of [^{3}H]prostaglandin $F_2\alpha$ binding assays were the same as described earlier [3, 9, 13, 17] (see table and figure legends for additional details). The composition of the homogenizing and incubation buffers was the same: 10 mM Tris · HCl,

pH 7.0 containing 250 mM sucrose, 1 mM $CaCl_2$, 1 mM dithiothreitol and 0.1 % gelatin. The protein content in an aliquot of the membrane fraction was determined by the method of Lowry et al. [18] using bovine serum albumin as the standard. In each experiment, the non-specific binding was determined using the same amounts of membrane protein and [^{3}H]prostaglandin $F_2\alpha$ as were used in the total binding tubes, in the presence of excess unlabeled prostaglandin $F_2\alpha$ ($2.8 \cdot 10^{-5}$ M). The specific binding which is presented in all the tables and figures was obtained by subtracting non-specific binding from total binding. Various enzymes and protein reagents essentially had no effect on non-specific binding component.

Phospholipases were boiled, unless stated otherwise, for 5 min at 95 °C before use. The membranes were treated with enzymes and protein reagents in one of the two following ways: (a) membranes were exposed to different agents for 1 h at 22 °C and then they were removed by centrifugation and washing ($6000 \times g$ for 15 min). The washed membranes were then incubated with [^{3}H]prostaglandin $F_2\alpha$ for 2 h at 22 °C (referred to as preincubation). In some experiments, following exposure of membranes to trypsin, the supernates were saved and recentrifuged at $48\,000 \times g$ for 30 min. Aliquots of supernates, along with the washed pellets, were reincubated with [^{3}H]-prostaglandin $F_2\alpha$. The bound and free [^{3}H]prostaglandin $F_2\alpha$ were separated by charcoal absorption [19] in the case of incubations with supernates. The membranes for control were handled similarly except for addition of different agents. (b) Aliquots of membrane fractions were incubated with [^{3}H]prostaglandin $F_2\alpha$ for 1 h at 22 °C and then various agents were added in 10 μl of 10 mM Tris · HCl, pH 7.0 and incubation continued for another 1 h at the same temperature (referred to as final incubation). In the control tubes, only 10 μl of Tris buffer was added.

RESULTS

Effect of phospholipases on [^{3}H]prostaglandin $F_2\alpha$ binding

It has been shown earlier that phospholipases, but not contaminating proteolytic enzymes, are resistant to boiling at 95 °C for 5 min and require Ca^{2+} for optimal enzyme activity [20, 21]. Therefore, boiled and non-boiled phospholipases were tested for their inhibitory effects on [^{3}H]prostaglandin $F_2\alpha$ binding (Table I). Pronase was included in this experiment to demonstrate that boiling indeed inactivates this proteolytic enzyme. Table I shows that non-boiled pronase virtually abolished [^{3}H]-prostaglandin $F_2\alpha$ binding and this ability was almost completely destroyed by prior boiling of the enzyme. When non-boiled phospholipases C from three different sources were used, the inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding was nearly complete. However, boiling these enzymes significantly reduced this ability. On the other hand, phospholipase A caused identical losses of [^{3}H]prostaglandin $F_2\alpha$ binding whether the enzyme was boiled or not. These results suggest that contaminating proteolytic enzymes in phospholipases C but not in phospholipase A were responsible for significant losses of [^{3}H]prostaglandin $F_2\alpha$ binding due to non-boiled enzymes. The moderate inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding by the boiled phospholipases C apparently reflects their intrinsic activity.

Phospholipase A cleaves β-ester bonds, whereas phospholipase C removes polar chains of lipids [22, 23]. As a result of β-ester bond cleavage, but not with the removal of exterior hydrophilic heads of phospholipids, membrane conformation has

TABLE I

EFFECT OF BOILED AND NON-BOILED ENZYMES ON THE [^{3}H]PROSTAGLANDIN $F_2\alpha$ BINDING TO BOVINE CORPUS LUTEUM CELL MEMBRANES

Boiled (95 °C 5 min) and non-boiled enzymes were added to aliquots of 1.9 mg membrane protein and preincubated (see experimental procedure). The control and treated membranes (389 μg protein) were then incubated with $5.8 \cdot 10^{-9}$ M [^{3}H]prostaglandin $F_2\alpha$. The loss of binding in control tubes was taken as 0 %. Enzyme to membrane protein ratios were 1 : 3.9 for pronase, phospholipase C (*Cl. perfringens* and *Cl. welchii*) and 12.5 units : 1.9 for phospholipase C (*B. cereus*) and phospholipase A. Each value in Tables I–IV, represents the mean and its standard error of quadruplicate determinations in two experiments.

Addition	% Inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding	
	Boiled	Non-boiled
Pronase	17.3 ± 5.0	94.8 ± 0.3
Phospholipase A	94.7 ± 1.9	99.5 ± 0.2
Phospholipase C (*B. cereus*)	25.2 ± 3.8	96.7 ± 0.2
Phospholipase C (*Cl. perfringens*)	25.3 ± 2.0	95.5 ± 0.2
Phospholipase C (*Cl. welchii*)	42.6 ± 0.8	96.7 ± 0.3

been shown to change [24]. Therefore, the greater effectiveness of phospholipase A over phospholipase C may be attributed to disruption of membrane conformation and/or to the inhibitory effects of released lysophospholipids.

Since phospholipase A but not phospholipases C markedly inhibits [^{3}H]-prostaglandin $F_2\alpha$ binding, the former enzyme was used in testing for Ca^{2+} requirements. Table II shows that phospholipase A treatment of the membranes resulted in a drastic reduction in binding. The concentrations of EGTA and EDTA which can readily chelate Ca^{2+} in the incubation media had virtually no effect on [^{3}H]prostaglandin $F_2\alpha$ binding. However, EDTA-Ca^{2+} but not EGTA-Ca^{2+} moderately reduced phospholipase A activity which may be due to inhibitory effects of EDTA-Ca^{2+} complexes formed. These results suggest that phospholipase A from *Crotalus*

TABLE II

EFFECT OF EGTA, EDTA AND CA^{2+} ON PHOSPHOLIPASE A INHIBITION OF [^{3}H]-PROSTAGLANDIN $F_2\alpha$ BINDING TO BOVINE CORPUS LUTEUM CELL MEMBRANES

Aliquots of membrane protein (1.9 mg) were preincubated with phospholipase A (9.8 units/mg membrane protein), EGTA (1.5 mM), EDTA (15.0 mM) and Ca^{2+} (10.0 mM) as indicated. The control and treated membranes (389 μg protein) were incubated with $5.9 \cdot 10^{-9}$ M [^{3}H]prostaglandin $F_2\alpha$. There was essentially no loss of binding in control tubes.

Addition	Percent inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding
Phospholipase A	95.6 ± 1.9
Phospholipase A + EGTA	95.2 ± 0.8
Phospholipase A + EGTA + $CaCl_2$	94.4 ± 0.0
Phospholipase A + EDTA	91.4 ± 0.4
Phospholipase A + EDTA + $CaCl_2$	72.8 ± 1.0

terrificus terrificus had no requirement for Ca^{2+} for its inhibitory action on [^{3}H] prostaglandin $F_2\alpha$ binding. These findings add to the already existing belief that not all phospholipases A require Ca^{2+} for their activity [25, 26].

Dose response relationships with various enzymes and protein reagents

Fig. 1 shows that trypsin, phospholipase A and lipase inhibited [^{3}H]prostaglandin $F_2\alpha$ binding in a dose dependent manner. However, the inhibition of [^{3}H]-prostaglandin $F_2\alpha$ binding by neuraminidase and phospholipase C was not marked and exhibited little, if any, dose dependency. Trypsin, phospholipase A and lipase inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding exhibited a biphasic pattern: at lower

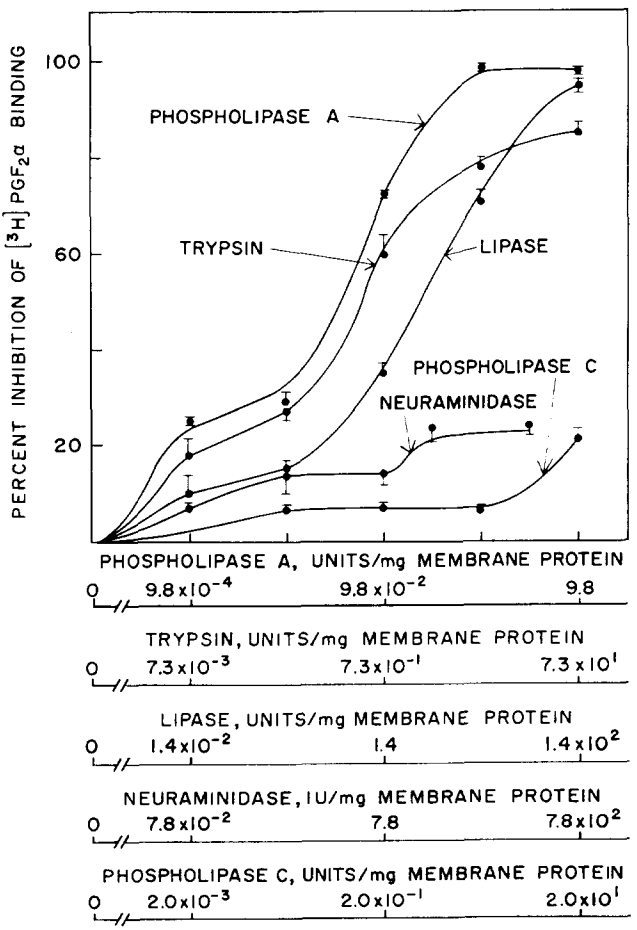

Fig. 1. Effect of increasing amounts of added enzymes on [^{3}H]prostaglandin $F_2\alpha$ binding to bovine corpora luteum cell membranes. Aliquots of 1.3 mg membrane protein were preincubated with the indicated amounts of different enzymes. Aliquots of control and treated membranes (255 μg protein) were then assayed for binding by incubating with $6.3 \cdot 10^{-9}$ M[^{3}H]prostaglandin $F_2\alpha$ ([^{3}H]$PGF_2\alpha$). Each value in Figs. 1 to 4A represents the mean and its standard error of quadruplicate determinations.

amounts of added enzyme, the loss of binding was small and relatively plateaued while in the presence of higher amounts the loss of binding was quite drastic. The biphasic enzyme effect was basically similar to that described by Banerjee et al. [27] except for the linear losses in binding at low amounts of added enzyme which plateaued with the increasing amounts added (opposite to that observed in the present study). The above differences between the studies may reflect the differences among the receptors. Although the reason for a biphasic enzyme effect is not known, it is quite likely that receptors and/or membranes may be insensitive up to certain concentrations of enzymes. When the enzyme levels exceed this threshold, membranes may become quite sensitive resulting in wide spread destruction of receptors and/or other membrane macromolecules.

It has been recently reported that reduced thyrotropin binding following trypsin treatment of thyroid membranes was due to solubilization of receptors from the membranes but not due to true destruction of the receptors [28]. Therefore, it was felt important to discriminate between these possibilities in the case of decreased [^{3}H]prostaglandin $F_2\alpha$ binding by trypsin. The concentrations of trypsin that drastically reduced [^{3}H]prostaglandin $F_2\alpha$ binding in membranes did not result in the appearance of binding macromolecule in the supernates (see experimental procedures). These results suggest that trypsin treatment of membranes resulted in the true destruction of prostaglandin $F_2\alpha$ receptors.

Fig. 2 shows the dose response relationships for various protein reagents. As shown in this figure, the inhibitory effects of protein reagents on [^{3}H]prostaglandin $F_2\alpha$ binding were apparent only at concentrations above 20 to 100 μM. Unlike with enzymes, no biphasic effects were observed in protein reagents inhibition of [^{3}H]-

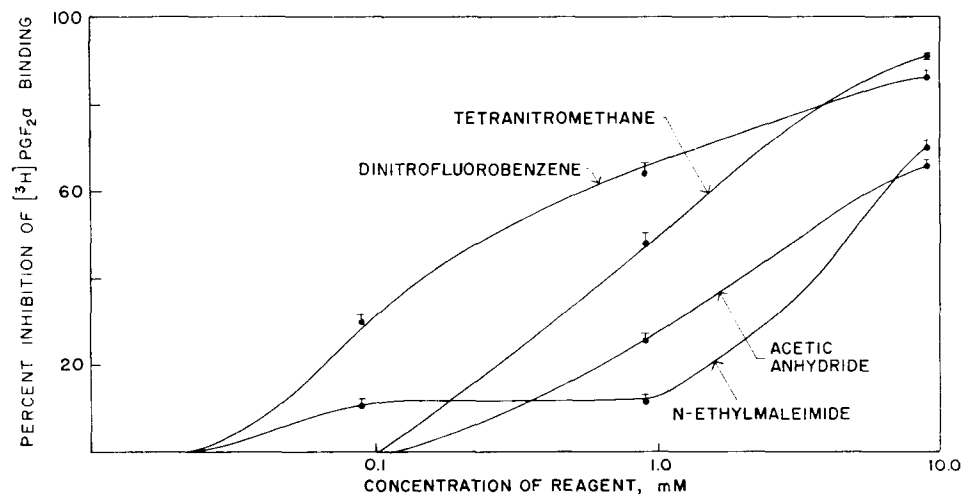

Fig. 2. Effects of increasing concentrations of different protein reagents on [^{3}H]prostaglandin $F_2\alpha$ binding to bovine corpus luteum cell membranes. Aliquots of 1.3 mg membrane protein were preincubated with indicated concentrations of different reagents. All reagents, except for *N*-ethylmaleimide (added in 10 mM Tris · HCl of pH 7.0), were added in ethanol so that the final concentration of ethanol was 9.0 %. Corresponding controls also contained 9.0 % ethanol. The control and treated membranes (255 μg protein) were incubated with $6.3 \cdot 10^{-9}$ M [^{3}H]prostaglandin $F_2\alpha$.

prostaglandin $F_2\alpha$ binding except for *N*-ethylmaleimide. 50 % inhibition of binding occurred at concentrations of 0.26 mM dinitrofluorobenzene, 1.0 mM tetranitromethane, 3.25 mM acetic anhydride and 4.90 mM *N*-ethylmaleimide. These differences in the effectiveness of various protein reagents in inhibiting binding may possibly reflect differences in the availability of modifiable functional groups in the membrane structure, involvement of some specific functional groups more than others in binding interaction and/or differences in optimal conditions for activity of different reagents. Inhibition of binding by *N*-ethylmaleimide may not indicate SH group involvement because various other SH group alkylating agents did not inhibit binding. *N*-Ethylmaleimide has been known to modify histidyl residues as well [29], therefore, inhibition of binding by this reagent may indicate possible involvement of histidyl residues in binding interaction. Other reagents, shown in Fig. 2, have been reported to modify tyrosyl, histidyl, tryptophan residues and amino groups [29]. Therefore, the loss of binding due to these reagents may reflect possible involvement of one or more of the above functional groups.

In the next experiment, the effect of a single effective concentration of protein reagents on [^{3}H]prostaglandin $F_2\alpha$ binding was examined in the presence and in the absence of added Ca^{2+}. The membrane preparations already contain 1 mM Ca^{2+} from the incubation buffer and probably some endogenous Ca^{2+} in the absence of added Ca^{2+}. In the absence of added Ca^{2+}, *p*-chloromercuribenzoate and mercaptoethanol had no effect on [^{3}H]prostaglandin $F_2\alpha$ binding, whereas iodoacetamide moderately enhanced binding (Table III). In the presence of added Ca^{2+}, *p*-chloro-

TABLE III

EFFECT OF PROTEIN REAGENTS, IN THE PRESENCE AND IN THE ABSENCE OF ADDED Ca^{2+}, ON [^{3}H]PROSTAGLANDIN $F_2\alpha$ BINDING TO BOVINE CORPUS LUTEUM CELL MEMBRANES

Aliquots of 1.1 mg membrane protein were preincubated for 10 min at 22 °C with and without added Ca^{2+}. Various protein reagents were then added and preincubation continued for another 1 h at the same temperature. The control and treated membranes were assayed for binding by incubating 194 μg protein aliquots with $6.1 \cdot 10^{-9}$ M [^{3}H]prostaglandin $F_2\alpha$.

Reagent	% Inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding	
	Ca^{2+} (17 mM)	$-Ca^{2+}$
P-Chloromercuribenzoate, 1.0 mM	28.3 ± 1.0	7.5 ± 4.5
Iodoacetamide, 10.0 mM	6.3 ± 1.6	0.0**
Mercaptoethanol, 10.0 mM	19.1 ± 2.0	3.6 ± 1.3
N-Ethylmaleimide, 10.0 mM	76.2 ± 2.3	68.9 ± 1.9
Azobenzene sulfenyl-bromide, 1.0 mM*	14.9 ± 2.3	34.3 ± 4.0
Tetranitromethane, 1.0 mM*	30.2 ± 4.9	53.5 ± 4.3
Dinitrofluorobenzene, 1.0 mM*	45.5 ± 2.1	63.9 ± 2.4
Acetic anhydride, 1.0 mM*	0.0***	22.4 ± 1.6

* Added in ethanol so that the final ethanol concentration was 9.0 %. Corresponding controls also contained the same concentration of ethanol. Other reagents were added in 10 mM Tris · HCl with a final pH of about 7.0.

** There was a 17.8 ± 4.9 % increase in binding in these tubes.

*** There was a 31.7 ± 3.2 % increase in binding in these tubes.

mercuribenzoate and mercaptoethanol caused moderate reductions in binding whereas iodoacetamide had very little effect. In the presence of added Ca^{2+}, *N*-ethylmaleimide inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding was increased whereas tetranitromethane, dinitrofluorobenzene and azobenzene sulfenylbromide inhibition of binding was decreased. Acetic anhydride, which inhibited binding in the absence of added Ca^{2+}, showed increased binding when Ca^{2+} was added. These differential effects of Ca^{2+} with respect to protein reagents inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding may be best explained by Ca^{2+} induced conformational changes in receptor and/or in other membrane components. These conformational changes may have resulted in exposure of previously unexposed protein functional groups and burying of previously exposed groups which were susceptible to modification by the above protein reagents. It would be of interest to point out a finding similar to the above by Simon and Groth [30]. They showed that the rate of opiate receptor inactivation by *N*-ethylmaleimide was markedly slowed down in the presence of added Na^+. They attributed this change to Na^+ induced conformational changes in the receptors.

Effect of increasing concentrations of added [^{3}H]prostaglandin $F_2\alpha$ to membranes treated with various enzymes and protein reagents

In order to examine whether various treatments of membranes altered the apparent number of available receptor sites or their affinities, aliquots of membrane protein were incubated with increasing amounts of [^{3}H]prostaglandin $F_2\alpha$. As shown in Fig. 3A, addition of increasing amounts of [^{3}H]prostaglandin $F_2\alpha$ to control membranes resulted in a dose dependent increase in [^{3}H]prostaglandin $F_2\alpha$ binding. Although enough [^{3}H]prostaglandin $F_2\alpha$ was not added (because of expense) to reach saturation, we have previously demonstrated that saturation indeed occurs at added concentrations of 10^{-7} M to 10^{-6} M. The addition of increasing amounts of [^{3}H]prostaglandin $F_2\alpha$ to membranes preincubated with lipase and phospholipase A resulted in no dose dependent increase in [^{3}H]prostaglandin $F_2\alpha$ binding. However, in the case of trypsin and *N*-ethylmaleimide (included in this group because of its addition in aqueous media), there was a dose dependent increase in [^{3}H]prostaglandin $F_2\alpha$ binding but it was markedly low when compared to the controls. These results demonstrate that the inhibition of binding by the above agents could not be overcome by increasing the amounts of [^{3}H]prostaglandin $F_2\alpha$ added which suggests that the losses of binding were irreversible. Similar results have been reported for some membrane receptors [22, 27, 31, 32] whereas for others [33, 34] it has been shown that enzyme inhibition of binding could be reversed by increasing hormone concentration.

Fig. 3B shows the Scatchard plot analysis [35] of the data from Fig. 3A. The plot for control membranes indicates the presence of high affinity-low capacity, low affinity-high capacity prostaglandin $F_2\alpha$ receptors. The apparent dissociation constants (K_d) and number of available receptors (N) were calculated from the reciprocal of the slopes and from the x-axis intercepts respectively and presented in this figure. As shown in this figure, *N*-ethylmaleimide and trypsin treatment of the membranes resulted in complete loss of high affinity prostaglandin $F_2\alpha$ receptors. The low affinity prostaglandin $F_2\alpha$ receptors were present in these membranes but their numbers as well as their affinity (especially with trypsin) were significantly reduced. The [^{3}H]-prostaglandin $F_2\alpha$ specific binding to lipase and phospholipase A treated membranes could not be used in Scatchard plot analysis (see discussion).

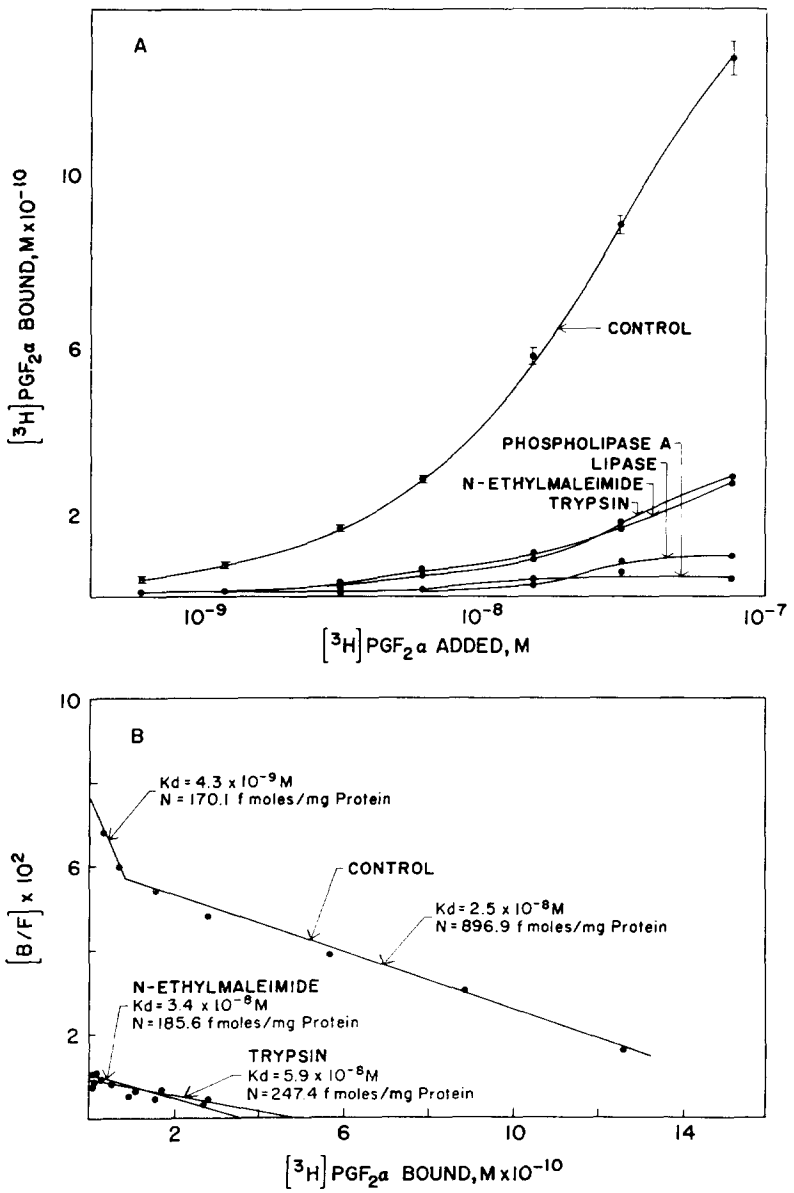

Fig. 3. (A) Dependence of [^{3}H]prostaglandin $F_2\alpha$ binding to bovine corpus luteum cell membranes, preincubated with various enzymes and *N*-ethylmaleimide, on [^{3}H]prostaglandin $F_2\alpha$ concentration in the medium. Membrane fractions were preincubated with various enzymes and *N*-ethylmaleimide: 73.0 units trypsin, 140.0 units lipase, 9.8 units phospholipase A per mg membrane protein; 9.1 mM *N*-ethylmaleimide. Aliquots of control and treated membranes (194 μg protein) were then incubated with increasing concentrations of [^{3}H]prostaglandin $F_2\alpha$. (B) The Scatchard plot analysis of [^{3}H]-prostaglandin $F_2\alpha$ binding data presented in A.

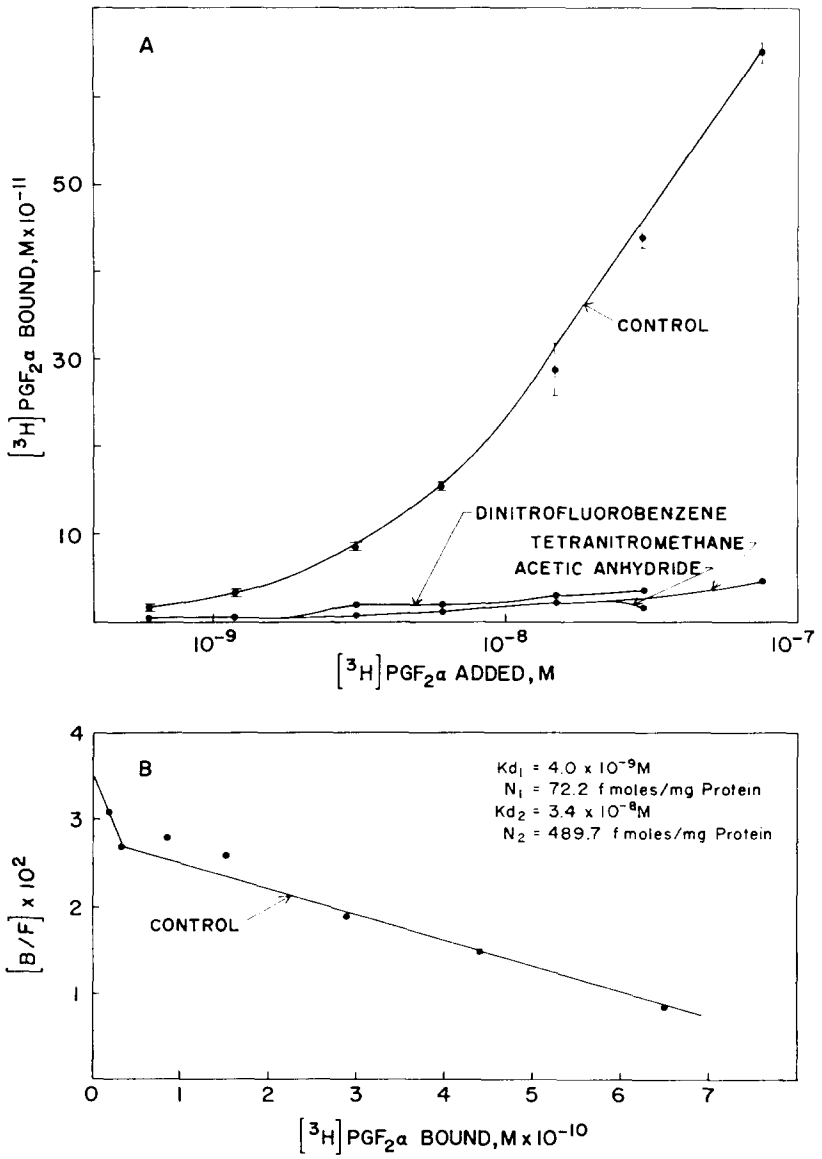

Fig. 4. (A) Dependence of [^{3}H]prostaglandin $F_2\alpha$ binding to bovine corpus luteum cell membranes, preincubated with various protein reagents, on [^{3}H]prostaglandin $F_2\alpha$ concentration in the medium. Aliquots of 5.4 mg membrane protein were preincubated with 9.1 mM acetic anhydride, dinitrofluorobenzene and tetranitromethane. Since the above reagents were added in ethanol with a final ethanol concentration of 9.0 %, the corresponding controls also contained the same concentration of ethanol. The control and treated membranes (194 μg protein) were then assayed for binding by incubating with increasing concentrations of [^{3}H]prostaglandin $F_2\alpha$. (B) The Scatchard plot analysis of [^{3}H]prostaglandin $F_2\alpha$ binding data presented in A.

TABLE IV

EFFECT OF ENZYMES AND PROTEIN REAGENTS, ADDED DURING PRE AND FINAL INCUBATIONS, ON [^{3}H]PROSTAGLANDIN $F_2\alpha$ BINDING TO BOVINE CORPUS LUTEUM CELL MEMBRANES

Various additions were made during pre or final incubations (see experimental procedure). Tubes containing 255 μg membrane protein were incubated with $5.8 \cdot 10^{-9}$ M [^{3}H]prostaglandin $F_2\alpha$. Appropriate controls were run for both pre and final incubations and the loss of binding in these tubes was taken as 0 %.

Addition	% Inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding	
	Preincubation	Final incubation
Trypsin, 740.0 units/mg membrane protein	89.4 ± 0.6	33.9 ± 0.7
Phospholipase A, 9.8 units/mg membrane protein	96.5 ± 1.9	63.9 ± 1.4
Lipase, 139.1 units/mg membrane protein	98.5 ± 1.0	80.1 ± 1.5
Dinitrofluorobenzene, 5.0 mM	82.5 ± 1.2	53.7 ± 1.8
N-ethylmaleimide, 5.0 mM	51.3 ± 0.8	51.9 ± 0.5

Fig. 4A shows that the addition of increasing amounts of [^{3}H]prostaglandin $F_2\alpha$ to control membranes resulted in a dose-dependent increase in [^{3}H]prostaglandin $F_2\alpha$ binding. On the other hand, similar addition to membranes preincubated with acetic anhydride, dinitrofluorobenzene and tetranitromethane resulted in no dose dependent increase in [^{3}H]prostaglandin $F_2\alpha$ binding.

The data of Fig. 4A was used in Scatchard plot analysis as shown in Fig. 4B. The control membranes in Fig. 4A bound significantly less [^{3}H]prostaglandin $F_2\alpha$ than the control membranes in Fig. 3A. Scatchard plot analysis (compare Fig. 3B to 4B) revealed that in the control membranes of Fig. 4A, there was a decrease in the number of available high and low affinity prostaglandin $F_2\alpha$ receptors. These decreases may perhaps be attributable to the presence of ethanol (9.0 %) during preincubation. The [^{3}H]prostaglandin $F_2\alpha$ specific binding in acetic anhydride, dinitrofluorobenzene and tetranitromethane treated membranes could not be used in Scatchard plot analysis (see discussion).

Effect of occupancy of receptors by prostaglandin $F_2\alpha$

In order to elucidate whether the occupancy of prostaglandin $F_2\alpha$ receptors afforded any protection from the inhibitory effects of various enzymes and protein reagents, these agents were added either during pre or final incubation. As shown in Table IV, preincubation of membranes with different enzymes and protein reagents resulted in a moderate to drastic reduction in [^{3}H]prostaglandin $F_2\alpha$ binding. When the same agents were added during final incubation, the loss of binding was significantly reduced except in the case of *N*-ethylmaleimide. These results suggest that occupancy of prostaglandin $F_2\alpha$ receptors in the membranes indeed affords partial protection against various enzymes and dinitrofluorobenzene.

DISCUSSION

The Scatchard plots were concave upwards when the saturation of [^{3}H]-prostaglandin $F_2\alpha$ binding data was used in Scatchard analysis [35]. This indicates that there was either negative cooperativity among prostaglandin $F_2\alpha$ receptors or simply reflects the presence of two groups of independent receptor sites. However, recent evidence from our laboratory suggests that the upward concavity in Scatchard plots was due to the presence of two groups of independent prostaglandin $F_2\alpha$ receptors with discrete affinities (see Rao, Ch. V., reference 36). The high but not low affinity prostaglandin $F_2\alpha$ receptors have been shown to exhibit dependency on cations [37]. Among the high and low affinity prostaglandin $F_2\alpha$ receptors, high affinity receptors appear to be more sensitive towards trypsin and *N*-ethylmaleimide. Though there was complete disappearance of high affinity receptors in the presence of the above two agents, low affinity receptors remain but their number (*N*-ethylmaleimide) as well as their affinity (trypsin) were decreased. The finding that trypsin selectively destroyed high affinity prostaglandin $F_2\alpha$ receptors is very similar to the observations of Glossman and Neville [34] on selective destruction of high affinity pholorizin receptors by proteolytic enzymes. The inhibition of binding by lipase, phospholipase A, acetic anhydride, dinitrofluorobenzene and tetranitromethane may be due to the complete abolishment of all of the prostaglandin $F_2\alpha$ receptors and/or due to severe losses in receptor affinities. Unfortunately, the present data do not discriminate between these two possibilities because the detection of sites with such severely lowered affinities would require the use of prohibitively high concentrations of [^{3}H]prostaglandin $F_2\alpha$. The findings of phospholipase A, pronase, trypsin and *N*-ethylmaleimide inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding were in excellent agreement with similar observations on ovine corpus luteum prostaglandin $F_2\alpha$ receptors [6].

The data presented in this paper demonstrate that preincubation of the membranes with various enzymes and protein reagents resulted in moderate to drastic inhibition of [^{3}H]prostaglandin $F_2\alpha$ binding. The loss of binding under these conditions may reflect changes in receptor sites alone and/or changes in other membrane macromolecules whose structural and functional integrity secondarily influence receptor binding. Since enzymes and protein reagents may not have selectivity towards any particular macromolecule within the membrane structure, both receptors and other macromolecules should be considered as targets for the changes induced by these agents. Addition of various enzymes and dinitrofluorobenzene during final as compared to pre-incubation resulted in much lower losses of binding. These results suggest that changes in receptor sites as well as in other membrane components were responsible for losses of binding. Nevertheless, the above data indicate that the loss of binding, at least in part, can be attributable to changes in the receptor sites alone. The observations that receptor occupancy had no protective effect against *N*-ethylmaleimide may suggest that the inhibition of binding by this reagent may reflect changes primarily in other membrane macromolecules and/or that ligand occupancy does not completely protect modifiable groups from this reagent.

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