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Effect of Phospholipases and Proteases on the [^{3}H]N^6-(R)-Phenylisopropyladenosine ([^{3}H]R-PIA) Binding to A_1 Adenosine Receptors From Pig Cerebral Cortex

Vicent Casadó, Josefa Mallol, Carmen Lluis, Enric I. Canela, and Rafael Franco

Departament de Bioquimica i Fisiologia, Facultat de Quimica, Universitat de Barcelona, 08071 Barcelona, Catalunya, Spain

Abstract The effect of phospholipases and proteases on the membrane-bound and solubilized A_1 adenosine receptor has been studied. Phospholipids modulate the [^{3}H]N^6-(R)-phenylisopropyladenosine binding to A_1 adenosine receptors in crude membranes and in soluble preparations, because changes in the phospholipid environment decrease both the binding capacity and the affinity for the ligand. It has become clear that 1) there is co-solubilization of receptor and phospholipids; 2) the phospholipid requirements are different for the coupled and the uncoupled receptor; 3) a net charge in the polar head produced by phospholipase D prevents the agonist binding to the receptor-G protein complex; alternatively, when the whole polar head is removed by phospholipase C the uncoupled receptor is altered; and 4) the protease action upon the receptor suggests that receptor coupled to G protein is more protected by the membrane than the uncoupled receptor. In kinetic experiments performed on membranes it was demonstrated that phospholipase C and trypsin increased the K_d value of the high-affinity state by modifying both k_1 and k_{-1}. In contrast they only modified the dissociation constant of the low-affinity state. In conclusion it should be noted that phospholipids play a key role for the binding of R-PIA to A_1 adenosine receptor. Also, a different disposition within the membrane of the coupled and uncoupled receptor is encountered.

Key words: brain cortical membranes, receptor modulation, trypsin, binding capacity, co-solubilization

Physiological, pharmacological, and biochemical studies suggest that adenosine may perform significant functions in the central nervous system via specific receptors (P_1 purinoceptors) [1–4] which are of two classes: A_1 which mediates decreases of (AMP_c) and A_2 which mediates increases of (AMP_c) [5,6].

Adenosine A_1 receptors which mediate inhibition of adenylate cyclase activity appear to be associated with regulatory G proteins. In intact brain membranes A_1 receptors display two different affinity states which depend upon the association between receptor and regulatory protein [6–8]. It seems that the coupled receptor displays high affinity for the agonist whereas the uncoupled receptor displays low affinity. One measure of this interaction that is demonstrable in vitro is the regulation of receptor binding by guanine nucleotides and bivalent cations [8–10]. Guanine nucleotides or analogues such as guanylylimidophosphate [Gpp(NH)p] cause transition from the high- to the low-affinity state [10–13]. From a recent study in our laboratory [14] it is suggested that the high- and the low-affinity states of the A_1 adenosine receptor are different conformations induced by the structure of the membrane. This suggestion is based upon the finding that, when adenosine receptors are solubilized from pig brain cortical membranes in which high- and low-affinity states coexist, all soluble receptor populations are converted into a single high-affinity class. A single high-affinity class is also found for the receptor remaining in the detergent-treated membranes. Furthermore Gpp(NH)p is still able to produce a conversion to all low-affinity states in the detergent-treated membranes and, moreover, it converts the high-affinity sites of the solubilized receptor into homogeneous sites having "anomalous" very-low-

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Address reprint requests to Rafael Franco, Department of Biochemistry and Physiology, School of Chemistry, University of Barcelona, Marti i Franques 1, 08071 Barcelona, Spain.

affinity behaviour with respect to [^{3}H]N^6-(R)-phenylisopropyladenosine ([^{3}H]R-PIA) binding. It can then be concluded that the loss of membrane integrity favours the interaction of the A_1 receptor molecule with G protein. These facts indicate a dramatic variation of the thermodynamics of the ligand-receptor interaction when membranes are disorganized and the receptor is solubilized. Besides a conformational change of the receptor molecule a key role of membrane phospholipids cannot be ruled out to explain such behaviour.

In current models of membrane structure [15,16] phospholipids, in addition to maintaining the structural integrity of the membrane, play a key role in the interaction with protein components and may even modulate the activity of such proteins. Thus phospholipids are intimately involved in many receptor systems as glucagon [17–19], gonadotropin [20], vasopressin [21], thyrotropin-releasing hormone [22], and insulin [23] receptors. There are no similar studies concerning adenosine receptors, although indirect evidence obtained by studying the solubilized A_1 adenosine receptors has suggested that lipids may prevent soluble receptor inactivation [24,25]. Although Anand-Srivastava and Johnson [26] did not find any effect of phospholipases on the coupling of adenosine to striatal adenylate cyclase, an understanding of the relationship between [^{3}H]R-PIA binding activity and the phospholipid environment of the receptor is nevertheless essential to elucidate its mechanism of action, physiological regulation, and the ways in which activity can be altered pharmacologically. In this paper we have analyzed the effects of phospholipases on A_1 adenosine receptor activity in intact brain membranes and soluble preparations. The effect of proteases has been analyzed in parallel for comparative purposes. Finally, the interconversion between the high- and the low-affinity states induced by the action of phospholipases and proteases has also been studied.

EXPERIMENTAL PROCEDURES

Materials

[Adenine-2, 8-^{3}H], ethyl-2-^{3}H]-N^6-phenylisopropyladenosine ([^{3}H]R-PIA; 42,5 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, MA, USA). N^6-(R)-phenylisopropyladenosine (R-PIA), 3[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), guanylyl-imidodiphosphate [Gpp(NH)p], adenosine deaminase (EC 3.5.4.4), phospholipase D (EC 3.1.4.4) from cabbage (1.5 Units/mg), proteinase K (EC 3.4.21.14) from *Tritirachium album* (20 Units/mg), trypsin (EC 3.4.21.4) from bovine pancreas (100 Units/mg), and chymotrypsin A_4 (EC 3.4.21.1) from bovine pancreas (90 Units/mg) were obtained from Boehringer Mannheim (FRG); 50% polyethylenimine, crystallized bovine serum albumin, polyethylene glycol 8000, bovine gammaglobulins, phospholipase A_2 (EC 3.1.1.4) from *Crotalus adamanteus* venom (40 Units/mg), and phospholipase C (EC 3.1.4.3) from *Clostridium perfringens* (6 Units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Phospholipase A_2, which is heat stable, was boiled for 5 min in order to avoid proteinase contamination. All other products were the best grade available and purchased from Merck (Darmstadt. FRG). De-ionized water further purified with a Millipore Milli-Q system was used throughout. In all cases buffer pH was measured at 25°C.

Membrane Preparations

Porcine brains were obtained from the local slaughterhouse. Cortices (2–6 brains) were dissected on ice and washed with 0.25 M sucrose containing 5 mM Tris-HCl buffer pH 7.4. Tissue homogenization was performed in 10 volumes of the above ice-cold sucrose buffer with a homogenizer (Polytron Kinematica, PTA 20TS rotor, setting 4) for two periods of 5 s separated by an interval of 15 s. The homogenate was centrifuged at 105,000*g* for 30 min at 4°C and the pellet was resuspended in 10 volumes of 50 mM Tris-HCl buffer pH 7.4 and re-centrifuged under the same conditions. The pellet was stored at −20°C and immediately before its utilization the pellet was washed as described above and suspended in the same buffer.

Receptor Solubilization

Receptor solubilization was performed as previously described [14]. Briefly, cortical membranes were suspended in a 50 mM Tris-HCl buffer pH 7.4 containing 0.5% (w/v) CHAPS and 0.5% (w/v) digitonin. Total detergent/protein ratio was 2. After incubation for 10 min at 22°C, the suspension was centrifuged at 80,000*g*, 90 min, 4°C. The supernatant filtered through 0.22 μm Millipore filters constituted the receptor-solubilized fraction.

Protein Determination

Protein was determined according to the method of Lowry et al. [27]. Bovine serum albumin was used as standard.

Phospholipid Determination

The phospholipid content of the samples was estimated by the determination of total phosphorus according to Rouser and Fleischer [28].

Radioligand Binding Experiments and Treatment With Phospholipases and Proteases

As a general method, [^{3}H]R-PIA binding to pig brain cortex adenosine receptors was measured after the incubation of the membranes or soluble extracts (0.7 to 0.8 mg protein/ml) with adenosine deaminase (0.2 Units/ml) and 2 mM $CaCl_2$ for 15 min at 25°C in 50 mM Tris-HCl buffer pH 7.4. One of the following enzymes, at the concentration (Units/ml) given in parentheses, was then added: phospholipase A_2 (0.10), phospholipase C (0.30), phospholipase D (0.50), proteinase K (1), trypsin (5), or chymotrypsin (4). After standing at 25°C, for 15 or 60 min, 6.5 nM [^{3}H]R-PIA was added and the incubation was prolonged for 30 min. In this latter incubation the enzyme concentration, in all cases, was 8% lower due to the volume of reagents added. In all cases, free and bound ligand were separated and the radioactivity was counted as indicated previously [14].

Agonist protective effect against proteases and phospholipases was tested by radioligand binding experiments performed as described above, except that 20 nM [^{3}H]R-PIA was incubated (30 min) with either membrane suspensions or soluble extracts before treatment for 90 minutes with proteases and phospholipases. In the final incubation period the radioligand concentration was 50% less, i.e., 10 nM.

Radioligand binding experiments performed to determine equilibrium binding isotherms were carried out as described above for the general method. After 30 min of treatment with phospholipases or proteases, [^{3}H]R-PIA was added in a concentration range of 0.1 to 36 nM and the incubation was prolonged enough time (4 h) to achieve the equilibrium for the lowest concentration.

Association-dissociation experiments were carried out as described above for the general method but introducing the following modifications. After 60 min of treatment of crude membranes (0.6 mg protein/ml) with phospholipase C (0.15 Units/ml) or trypsin (5 Units/ml), [^{3}H]R-PIA was added (1.1 nM final concentration). For association measures, aliquots of 500 μl were taken at the indicated times and filtered. After 90 min of association, dissociation was induced by addition of cold R-PIA to a final concentration of 500 nM; at different times, 500 μl aliquots were taken and filtered.

When necessary nonspecific binding was determined in the presence of 2 μM unlabelled R-PIA. In all cases controls were established by substituting enzymes by buffer. No differences were detected in controls carried out with or without 2 mM $CaCl_2$. As described above, in this work the protease and phospholipase action was stopped by rapid filtration through Whatman GF/C filters. We did not follow the classical way of stopping the reaction by addition of enzyme inhibitors such as EGTA or EDTA for phospholipases and PMSF for proteases. The use of chelating agents induces decreases in (Mg^{2+}) bound to membranes; this cation, as has been demonstrated [14,29], dramatically affects the kinetic behaviour of the receptor. In a control experiment, addition of EDTA at a concentration of 10 mM produced a 29% decrease in 1 nM [^{3}H]R-PIA binding. Also, we demonstrated that additions of PMSF at a final concentration of 1 mM, which is that used for inhibiting proteinases, produce a marked decrease (up to 21%) of 6 nM [^{3}H]R-PIA binding. In general terms, when membrane receptors are treated with phospholipases and proteases the separation of membranes from enzymes is performed via centrifugation [19,20,26]; however, this is not possible when there are soluble receptors in the assays [20]. Thus we decided to use a single procedure for membrane suspensions and for soluble extracts, since moreover the results obtained with both fractions were analyzed in comparative terms (see Results). For this reason membranes and enzymes were not separated prior to binding assays.

Analysis of Binding Data

Equilibrium binding data and association-dissociation data were routinely fitted to the equations described elsewhere [14,30]. The radioligand concentrations used had previously been determined by using a program for D-optimal discrete design of experiments [31]. The individual saturation isotherms and association-disso-

ciation experiments were analyzed by nonlinear regression by using the [R]ENZFITTER program (Elsevier Biosoft) or other available programs [32,33]. Other published programs may be also used for this kind of analysis [34]. Replicates of each point were performed and no more assumptions about errors were made.

Goodness of fit was tested according to the reduced χ^2 or SD values given by the program. Modified F test was used to analyze whether the fit to the two-states model significantly improved the fit to the one-state model [35]. In all cases it was considered that the two-sites model led to a significant improvement over the one-site model when $P < 0.001$. When no significant improvement over the one-site model was detected, the P values were greater than 0.30.

When means (± SEM or SD) are given, differences between groups were tested for significance by using Student's t-test for unpaired samples.

RESULTS

Solubilizing Effect of Phospholipase A_2 on A_1 Adenosine Receptors From Brain Membranes

Since phospholipase A_2 digestion products (lysophosphatides and fatty acids) are known to possess surface-active properties and to act as solubilizing agents [36], an attempt was made to quantify the receptor concentration in the supernatant following treatment of membranes with phospholipase A_2. No detectable solubilization of membrane-bound receptor was evident upon phospholipase A_2 treatment (data not shown). In comparison the CHAPS/digitonin mixture of detergents produces a significant solubilization of the receptor as was already demonstrated in our previous work [14]. Receptor solubilization by CHAPS/digitonin cocktails leads to the release of phospholipids from membranes (678 ± 6 μg P/g tissue, mean ± SEM n = 4). The question as to whether the solubilized receptor contains bound phospholipids is the object of our interest in this paper.

Effect of Phospholipases and Proteases in Membrane-Bound and Solubilized Adenosine Receptors

The effects of phospholipases and proteases obtained from different sources upon the [^{3}H]R-PIA binding capacity of brain membranes and soluble extracts were measured and analyzed. The results are presented in Table I. Phospholipase A_2, which did not affect the binding of [^{3}H]R-PIA to membranes, markedly decreased the binding in soluble extracts. In contrast, phospholipases C and D affected the binding of the radiolabeled compound in both fractions. However, the effect of phospholipase D was stronger than the effect of phospholipase C in soluble

TABLE I. Effect of Phospholipases and Proteases on Membrane-Bound and Soluble A_1 Adenosine Receptors†

Treatment	Time (min)	[^{3}H]R-PIA binding			
		Membranes		Solubilized receptors	
		pmol/mg protein	%	pmol/mg protein	%
Control	—	0.63 ± 0.03	100	0.88 ± 0.07	100
Phospholipase A_2 (0.10 Units/ml)	15	0.63 ± 0.03	100	0.41 ± 0.03***	46
Phospholipase C (0.30 Units/ml)	15	0.47 ± 0.03***	75	0.75 ± 0.05*	86
Phospholipase D (0.50 Units/ml)	15	0.36 ± 0.01****	58	0.26 ± 0.01****	30
Proteinase K	15	0.51 ± 0.01***	81	0.23 ± 0.02****	27
(1 Unit/ml)	60	0.47 ± 0.02***	76	0.01 ± 0.01****	1
Trypsin	15	0.55 ± 0.02**	88	0.59 ± 0.03***	67
(5 Units/ml)	60	0.49 ± 0.02***	79	0.14 ± 0.01****	16
Chymotrypsin	15	0.63 ± 0.01	100	0.88 ± 0.05	100
(4 Units/ml)	60	0.59 ± 0.01	95	0.32 ± 0.02****	36

†Phospholipases and proteases were incubated with membrane-bound or solubilized receptors (0.8 mg protein/ml) prior to the addition of [^{3}H]R-PIA (6.5 nM) as described in Methods. Values are mean ± SEM of four different experiments. Statistical significance vs. controls were calculated according to Student's t-test.

*$P < 0.1$.

**$P < 0.05$.

***$P < 0.005$.

****$P < 0.0001$.

extracts and in membranes. A more prolonged incubation (60 min) between phospholipase and either membranes or soluble extracts did not produce any quantitative change in the results obtained, 15 min being enough time to achieve the maximum effect. The explanation of the lack of effect of phospholipase A_2 in membranes but not in soluble extracts at the molecular level suggests that the enzyme cannot act upon the phospholipids which are in the vicinity of the receptor in the membrane.

In relation to the action of proteases, proteinase K, as expected due to its unspecificity, was the most effective in destroying the binding capacity in the soluble extracts. It was, however, much less effective in membranes, where it is observed that neither protease caused great decreases in the binding capacity of A_1 adenosine receptor. In the soluble extracts all proteases were able to degrade the receptor, the time of interaction being important to estimate the proportion of the adenosine receptor population which has lost the capacity for ligand recognition.

To assess the protective action of the ligand R-PIA upon the action of proteases and phospholipases the same type of incubation was performed but membranes or soluble extracts were incubated with [^{3}H]R-PIA before the addition of the enzymes (Table II). In the membrane fraction neither phospholipase A_2 nor chymotrypsin was tested since they did not affect the binding even in the absence of the agonist. [^{3}H]R-PIA did prevent the action of phospholipases and proteases in the membrane-bound receptor (Table II). In the solubilized receptor, the protective effect of R-PIA is relatively strong in the case of phospholipases C and D and of trypsin and chymotrypsin; the ligand did protect, but only weakly in the case of phospholipase A_2 and proteinase K.

Effect of Phospholipases and Proteases Upon the Equilibrium Binding Parameters of the Two Affinity States of the A_1 Adenosine Receptor

In intact pig brain cortex membranes A_1 adenosine receptors display two different states which possess high (K_d = 0.11 nM) and low (K_d = 1.1 nM) affinity for the agonist [^{3} H]R-PIA (Fig. 1, Table III). When the receptor is solubilized, a single K_d for R-PIA (K_d = 0.18 nM) appears (Fig. 1, Table III) in agreement with our previous work [14]. To investigate whether the effect of phospholipases and proteases is due to a change of maximum binding capacity or to a change in the receptor affinity, experiments which have led us to estimate the number of affinity sites and the equilibrium binding constants were carried out.

In Figure 1 binding isotherms and Scatchard plots corresponding to intact brain membranes (two affinity states) or solubilized receptors (one affinity state) are presented. In Figures 2 and 3, isotherm binding and Scatchard plots corresponding to either membranes or solubilized receptors treated with phospholipases and proteases are shown. The respective equilibrium parameters are indicated in Table III. In membranes the effect upon the equilibrium parameters of phospholipase A_2 and chymotrypsin was

TABLE II. Agonist Protective Effect on Protease and Phospholipase Action Upon A_1 Adenosine Receptor†

	[^{3}H]R-PIA binding			
	Membranes		Solubilized receptors	
Treatment	pmol/mg protein	%	pmol/mg protein	%
Control	0.61 ± 0.04	100	0.87 ± 0.05	100
Phospholipase A_2 (0.10 Units/ml)	n.d.	—	0.41 ± 0.02**	46
Phospholipase C (0.30 Units/ml)	0.61 ± 0.02	100	0.92 ± 0.05****	105
Phospholipase D (0.50 Units/ml)	0.62 ± 0.03	102	0.73 ± 0.03**	83
Proteinase K (1 Unit/ml)	0.58 ± 0.05	95	0.15 ± 0.04****	17
Trypsin (5 Units/ml)	0.64 ± 0.08	105	0.56 ± 0.06***	64
Chymotrypsin (4 Units/ml)	n.d.	—	0.75 ± 0.04**	86

†20 nM [^{3}H]R-PIA was incubated with either membrane or detergent extracts (1.5 mg protein/ml) prior dilution (1:1, v:v) with phospholipase or protease solutions. For more details concerning the treatments see text. Values are mean ± SEM of four different experiments. Statistical significanct vs. controls was calculated according to Student's t-test. n.d.: not determined.
**$P < 0.05$.
***$P < 0.005$.
****$P < 0.0001$.

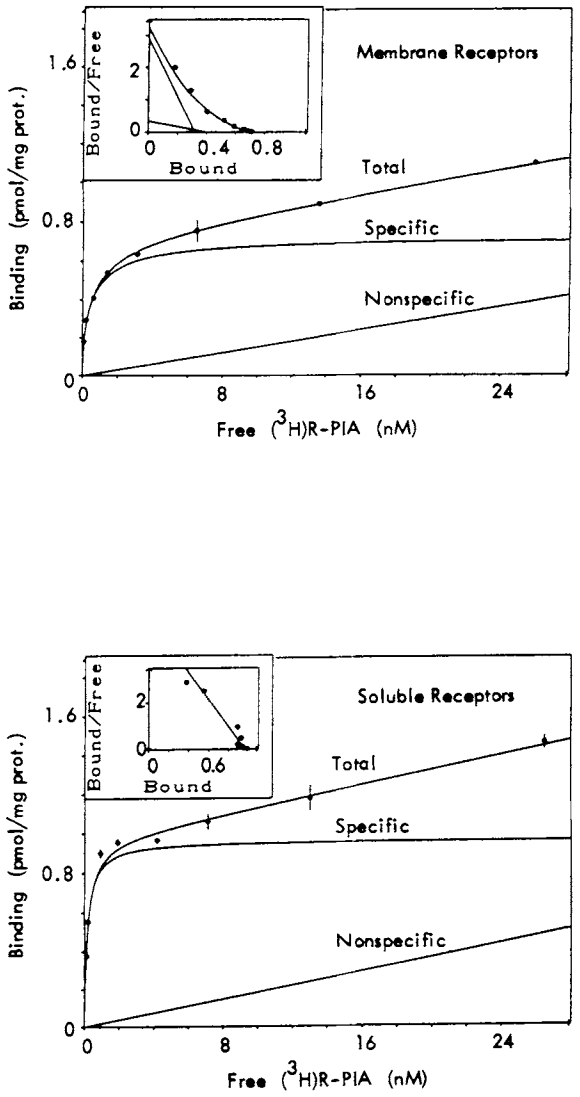

Fig. 1. Equilibrium isotherms of [^{3}H]R-PIA binding to crude membranes and detergent extracts. Radioligand binding was carried out as described in Methods. All points represent the mean ± SEM of five replicates. The data were adjusted by using a non-linear regression program to a two-sites model in the case of membranes and to a one-site model in the case of soluble extracts. In the latter case there was no significant improvement after considering the two-sites model (see Methods). **Inserts:** Scatchard plots of the computer-derived specific binding data.

not tested because these two enzymes did not change the agonist binding (Table I). In general, phospholipases C and D, proteinase K, and trypsin induced, on membranes, a decrease in the affinity of both high- and low-affinity states. Thus, a change of K_d (high affinity) from ≈0.1 nM to ≈0.3 nM and a change of the K_d (low affinity) from ≈1 nM to ≈2–6 nM are observed after treatment with these enzymes. There are interesting differences between the effect of phospholipases and proteases with respect to the loss of maximum binding capacity. The low-affinity state is more sensitive to proteinase K than the high-affinity state. This probably indicates that the high-affinity site is more protected by the membrane structure. On the other hand, phospholipase D, which did not modify the low-affinity binding, abolished the binding corresponding to the high-affinity state. The effect was the opposite when using phospholipase C which did not modify the maximum binding to the high-affinity site while it almost abolished the binding to the low-affinity site. In the case of solubilized receptor the action of proteases on equilibrium parameters was not tested because the reduction of binding is so great that the nonlinear regression analysis could not be performed on the small binding which remains in the treated extracts. Phospholipase A_2 led to the appearance of a second affinity state although the binding capacity of the high-affinity site was very depressed if compared with control soluble extracts. Furthermore the second affinity state produced by the enzyme had a K_d value (4 nM) which is greater than that of the low-affinity state. It should also be noted that phospholipase A_2 produced a marked decrease of the total binding (up to 56% of decrease) with respect to the control. With phospholipase D a state displaying an enlarged K_d value (5.1 nM) appeared with an R value 52% of the R of the control. This affinity state has a K_d value similar to the low-affinity state which appears in membranes after treatment with phospholipases and proteases (Table III). On the other hand, phospholipase C, which only slightly reduced the maximum binding, increased the K_d of the high-affinity state but did not induce the appearance of the low-affinity state.

Effect of Gpp(NH)p on the Agonist Binding of Membranes and Solubilized Receptors Treated With Trypsin or Phospholipases

In this paper the sensitivity of membranes to Gpp(NH)p was studied to assess whether the G protein remains functionally attached to the receptor after treatment with either phospholipases or proteases. The results appear in Table IV. In general, Gpp(NH)p sensitivity is demonstrated by a decrease of the binding due to the conversion of high-affinity to low-affinity states. Phospholipase C-treated membranes were sensitive to Gpp(NH)p; this confirms the results of Table III where it is indicated that phospholi-

TABLE III. Phospholipase and Protease Effect on A_1 Adenosine Receptor Equilibrium Parameters for [^{3}H]R-PIA Binding*

	Membranes			Solubilized receptors		
Treatment	Affinity states	R (pmol/mg protein)	KD (nM)	Affinity states	R (pmol/mg protein)	Kd (nM)
Control	2	0.32 ± 0.04 0.40 ± 0.04	0.11 ± 0.03 1.1 ± 0.2	1	0.96 ± 0.03	0.18 ± 0.03
Phospholipase A_2 (0.10 Units/ml)	—	n.d	n.d	2	0.06 ± 0.02 0.36 ± 0.02	0.12 ± 0.09 4 ± 1
Phospholipase C (0.30 Units/ml)	2	0.31 ± 0.06 0.09 ± 0.04	0.30 ± 0.06 4 ± 2	1	0.85 ± 0.02	0.43 ± 0.03
Phospholipase D (0.50 Units/ml)	1	0.46 ± 0.02	2.1 ± 0.1	1	0.50 ± 0.05	5.1 ± 0.7
Proteinase K (1 Unit/ml)	2	0.25 ± 0.04 0.27 ± 0.09	0.23 ± 0.05 7 ± 5	—	n.d.	n.d.
Trypsin (5 Units/ml)	2	0.26 ± 0.03 0.36 ± 0.03	0.35 ± 0.01 5 ± 1	—	n.d.	n.d.

*The values correspond to the plots shown in Figures 1–3. Values are mean ± SD of five separate experiments. R is the maximum number of binding sites and K_d is the equilibrium dissociation constant. n.d.: not determined.

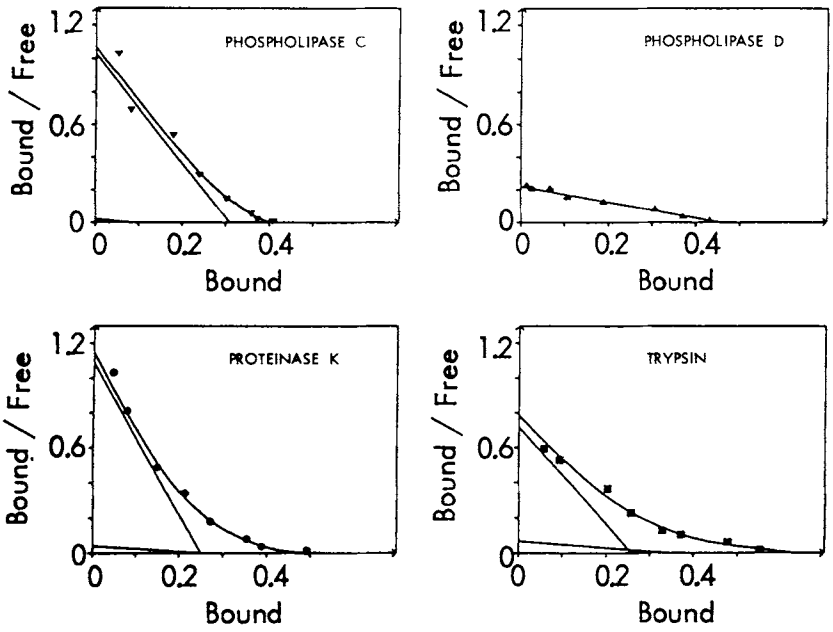

Fig. 2. Scatchard representations corresponding to membranes treated with phospholipases and proteases. Treatment with enzymes was carried out as described in Methods. All points represent the mean of five replicates. The data were adjusted by using a non-linear regression program to a two-sites model in the case of membranes treated with phospholipases C and D, and to a one-site model in the case of membranes treated with proteinase K and trypsin. In the latter case there was no significant improvement after considering the two-sites model (see Methods). Points are the computer-derived specific binding values for each concentration of the radioligand.

pase C produced a reduction in the binding of only the low-affinity state. In contrast the addition of Gpp(NH)p to phospholipase D-treated membranes, which lack the high-affinity center (see Table III), should not modify the binding in these membranes (Table IV). Membranes treated with trypsin or proteinase K are also insensitive to Gpp(NH)p. Notwithstanding, in both cases, two affinity states coexist which are similar to those found after treatment with phospholipase C (see Table III). This behaviour can only be explained by assuming that both proteases alter the center of G protein responsible for the recognition of Gpp(NH)p or alter the transduction

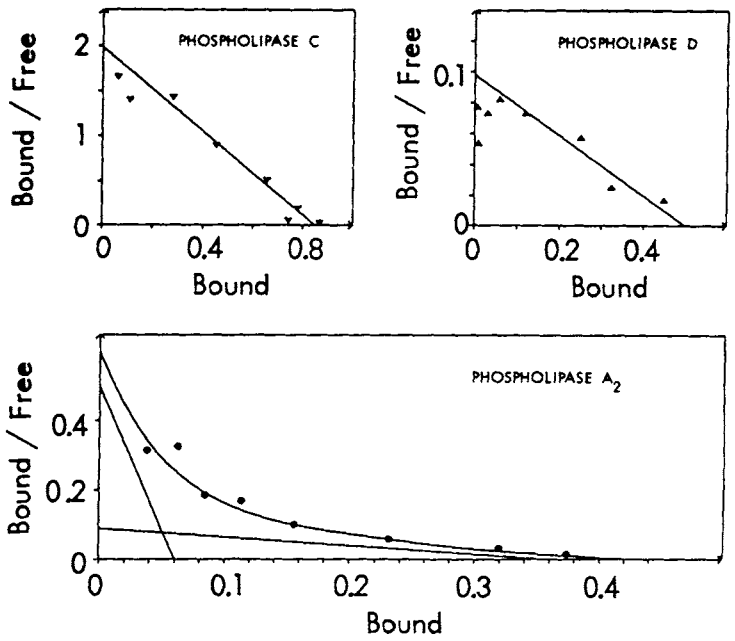

Fig. 3. Scatchard representations corresponding to soluble extracts treated with phospholipases. Treatment with enzymes and radioligand binding was carried out as described in Methods. All points represent the mean of five replicates. Binding data were adjusted by using a non-linear regression program to a two-sites model in the case of soluble extracts treated with phospholipase A_2, and to a one-site model in the case of soluble extracts treated with phospholipase C and phospholipase D. In the latter case there was no significant improvement after considering the two-sites model (see Methods). Points are the computer-derived specific binding values for each concentration of the radioligand.

TABLE IV. Effect of Gpp(NH)p on the Agonist Binding of Membranes and Solubilized Receptors Treated With Trypsin or Phospholipases†

	[^{3}H]R-PIA binding (pmol/mg protein)			
	Membranes		Solubilized receptors	
Treatment	Gpp(NH)p = 0	Gpp(NH)p = 100 μM	Gpp(NH)p = 0	Gpp(NH)p = 100 μM
Control	0.493 ± 0.009	0.334 ± 0.003****	0.733 ± 0.008	0.102 ± 0.007****
Phospholipase A_2 (0.10 Units/ml)	n.d.	n.d.	0.044 ± 0.004	0.004 ± 0.004****
Phospholipase C (0.30 Units/ml)	0.234 ± 0.005	0.117 ± 0.004****	0.57 ± 0.05	0.143 ± 0.008****
Phospholipase D (0.50 Units/ml)	0.132 ± 0.006	0.149 ± 0.003	0.140 ± 0.009	0.068 ± 0.008****
Trypsin (5 Units/ml)	0.248 ± 0.006	0.258 ± 0.006	n.d.	n.d.
Proteinase K (1 Unit/ml)	0.35 ± 0.04	0.31 ± 0.04	n.d.	n.d.

†Membranes or soluble extracts (0.7 mg protein/ml) were incubated with adenosine deaminase (0.2 Units/ml) and the enzyme indicated in 50 mM Tris-HCl buffer pH 7.4 containing 2 mM $CaCl_2$ at 25°C. After 60 min, either incubation buffer or Gpp(NH)p was added together with 1.08 nM [^{3}H]R-PIA. After 60 min of incubation, bound and free radioligand were separated as indicated in Methods. Nonspecific binding was determined in the presence of 0.8 μM cold R-PIA. Values are the mean ± SEM of four to six different experiments. Statistical significance of values vs. the controls without Gpp(NH)p was calculated according to Student's t-test. n.d.: not determined.
****$P < 0.0001$.

mechanism between Gpp(NH)p binding and receptor-G protein dissociation. In the case of soluble receptors the results are more easily interpreted since the control preparation exhibits a single high-affinity center which is sensible to Gpp(NH)p; in fact the active fraction which is solubilized is the receptor-G protein complex. This sensitivity to the guanine nucleotide analogue remains after incubation of the extracts with phospholipases (Table IV).

Effect of Phospholipase C and Trypsin on the Association-Dissociation Curves of [^{3}H]R-PIA to Membranes

The kinetic constants of [^{3}H]R-PIA association and dissociation to crude membranes and membranes treated with either phospholipase C or trypsin were determined as described in Methods. The results are presented in Figure 4 and the constants' values are given in Table V. Phospholipase C as well as trypsin induced slight decreases in the association constants and slight increases in the dissociation constants of the high-affinity state. This slowness of the association and quickness of the dissociation in turn provoke the increase of K_d values already patent in Table III. With respect to the low-affinity state, treatment with the enzymes did not produce any significant change in the association constant, whereas the dissociation constant increased markedly. This also explains why the treatment with either phospholipase C or trypsin leads to a loss of affinity which was noted in Table III.

DISCUSSION

The decrease of binding activity of the receptor caused by phospholipases strongly suggests that phospholipids interact with the amino acids of the protein molecule in the membrane and also in the extract obtained after treating the membranes with the CHAPS/digitonin mixture of detergents.

In agreement with our previous results [14], Table III shows that the soluble receptor exhibits a single affinity state ($K_d \approx 0.18$ nM) which is sensitive to Gpp(NH)p (Table IV). The breakdown of the fatty acid in position 2 of the phospholipids produces a change to a form of K_d = 4.2 nM which preserves a Gpp(NH)p sensitivity (Tables III, IV). The most probable explanation of this fact is a conformational change induced by the degradative action of phospholipase A_2 without the uncoupling of the receptor-G protein complex.

As shown in Table III, the high-affinity state present in membranes (which corresponds to the receptor-G protein complex) is lost after treatment with phospholipase D; however, the binding to the low-affinity site is retained, though a slight modification of the K_d occurs. In a sense, phospholipase C action is opposite that of phospholipase D. Taking the effects of phospholipases C and D into account it should be pointed out that some parts of the phospholipid molecule are important for the interaction of R-PIA with the binding site of A_1 adenosine receptors. It seems that the negative net charge produced in the polar part of the phospholipid when phospholipase D acts substantially modifies the bind-

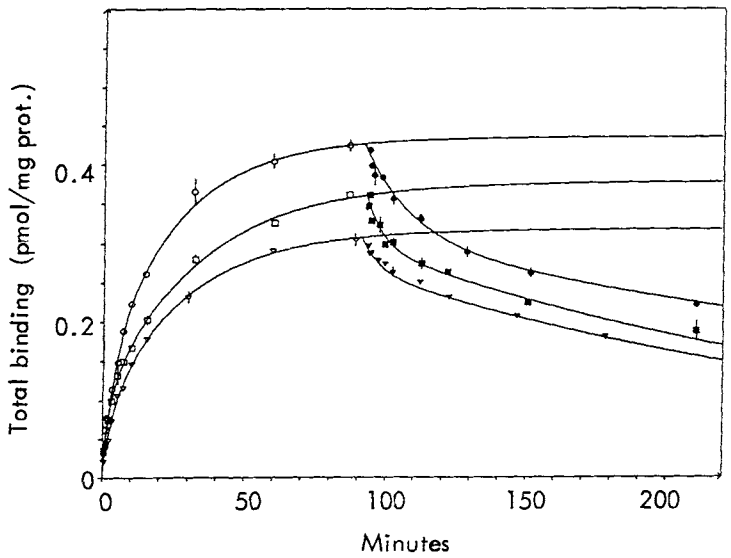

Fig. 4. Effect of phospholipase C and trypsin on the association-dissociation curves of [^{3}]R-PIA to membranes. The experiment was performed as indicated in Materials and Methods. At various incubation times, binding was assayed (open symbols). Dissociation (filled symbols) was induced after 90 min of association. All points represent mean ± SEM of three replicates. Lines drawn correspond to the best fit obtained (see Methods). (○, ●) Control membranes; (▽, ▼) membranes treated 60 min with 0.30 Units/ml of phospholipase C; (□, ■) membranes treated 60 min with 5 Units/ml of trypsin.

TABLE V. Association-Dissociation Parameters of Membranes Treated With Either Trypsin or Phospholipase C†

	High affinity		Low affinity	
Treatment	k_1 $min^{-1}\,nM^{-1}$	k_{-1} min^{-1}	k_1 $min^{-1}\,nM^{-1}$	k_{-1} min^{-1}
Control	0.047 ± 0.007	0.003 ± 0.001	0.08 ± 0.02	0.07 ± 0.02
Phospholipase C	0.035 ± 0.004***	0.005 ± 0.001**	0.07 ± 0.03	0.15 ± 0.04**
Trypsin	0.022 ± 0.005**	0.005 ± 0.001**	0.08 ± 0.03	0.16 ± 0.07**

†The values correspond to the plots shown in Figure 4. Values are mean ± SD of three separate experiments. k_1 and k_{-1} are the association and dissociation rate constants, respectively. Statistical significance vs. controls was calculated according to Student's t-test.
**$P < 0.05$.
***$P < 0.005$.

ing site of the receptor-G protein complex. Alternatively, when the whole polar head of the phospholipid is removed by phospholipase C, the receptor molecule which is most altered is that dissociated from G protein.

On the other hand, the same line of reasoning will lead us to conclude that membrane prevents proteolysis of the receptor. In the case of the very unspecific proteinase K this is obvious. In the case of trypsin the protective effect of the integrity of the membrane is evident when comparing the effect upon membranes and soluble extracts after 60 min of incubation (Table I). As expected, the receptor molecule in soluble form is very susceptible to the action of proteases. Chymotrypsin, however, produced the effect after 60 min of interaction time while the effect was null for the first 15 min. This behaviour can be explained by a cooperative variation of the tridimensional structure of the receptor after the first bonds cleaved, with the subsequent exposure of new groups originally placed in the hydrophobic interior of the molecule. The pattern of protease digestion is typical of an integral polytopic membrane protein. This type of protein which traverses the membrane a number of times is (in intact membranes) protected against proteases but may become accessible on prolonged proteolysis [37]. Quantitative and qualitative changes encountered by treating (15 and 60 min) soluble extracts by chymotrypsin and trypsin reveal a greater susceptibility of cleavage of the peptide bonds which are the target of trypsin: those whose carbonyl group is given by arginine or lysine. Those susceptible to the action of chymotrypsin (carbonyl groups given by aromatic amino acids) are less accessible.

The very unspecific proteinase K reduces the binding of the low-affinity state more than the binding of the high-affinity one. This suggests that the receptor-G protein complex is more protected by the membrane than the uncoupled receptor. As demonstrated in Table IV, membranes treated with either trypsin or proteinase K are not sensitive to Gpp(NH)p and display enlarged K_d values for both affinity states (Table III). This lack of sensitivity toward Gpp(NH)p which accompanies the high-affinity state (K_d values 0.23–0.35 nM) contrasts with the Gpp(NH)p sensitivity of the similar state (K_d 0.30 nM) produced after the treatment with phospholipase C. This strongly indicates that proteases, in addition to the modification of the receptor molecule, are able to modify the Gpp(NH)p binding site of G protein or alter the transduction mechanism between Gpp(NH)p binding and the uncoupling of the receptor-G protein complex.

As has been discussed, protease and phospholipase action upon A_1 adenosine receptors induces increases in K_d values of both high- and low-affinity states. In membranes association-dissociation experiments (Table V) indicate that the decrease of affinity of the high-affinity state induced by phospholipase C or by trypsin is due to a reduction in the rate of association of the ligand and to an increase in its rate of dissociation. On the other hand, the decrease of affinity observed for the low-affinity state is only due to an increase in the rate of dissociation.

In membranes the ligand R-PIA is able to protect the receptor completely from attack by phospholipases and proteases (Table II). In soluble extracts the protective effect is only important in the case of phospholipases C and D. Thus it is suggested that the binding of the agonist on the receptor located in the membrane hides some amino acid groups of the receptor as well as polar heads of phospholipids which are essential for the functionality of the receptor.

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