INTERACCIÓN FUNCIONAL ENTRE LAS PROTEÍNAS IMPLICADAS
EN EL RECONOCIMIENTO MOLECULAR DE ADENOSINA.

Memoria presentada por el
Licenciado en Biología
FRANCISCO CIRUELA ALFÉREZ
para optar al grado de
Doctor en Biología

Barcelona, Noviembre de 1995.
B.3. Immunological identification of A₁ adenosine receptors in brain cortex.


*Journal Neuroscience Research (In the press).*
Immunological Identification of $A_1$ Adenosine Receptors in Brain Cortex

F. Ciruela, V. Casado, J. Mallol, E. I. Canela, C. Lluis, and R. Franco
Departament de Bioquímica i Biologia Molecular, Facultat de Química, Universitat de Barcelona, Barcelona, Catalunya, Spain

The $A_1$ adenosine receptor from pig brain cortex has been identified by means of two antipeptide antibodies against two domains of the receptor molecule: PC/10 antiserum was raised against a part of the third intracellular loop, and PC/20 antiserum was raised against a part of the second extracellular loop. PC/10 antibody was able to recognize a 39-kDa band that corresponded to the $A_1$ receptor, as demonstrated by immunoblotting and by immunoprecipitation of the molecule cross-linked to $^{[125]}$I(R)-2-azido-N2-p-hydroxy(phenylisopropyl)adenosine. Besides the 39-kDa band, PC/20 also recognized a 74-kDa form that does not seem to correspond to a receptor-G protein complex. The occurrence of the two bands was detected and analyzed in samples from different species and tissues showing a heterogeneous distribution of both. The 74-kDa form can be converted into the 39-kDa form by treatment with agonists or antagonists of $A_1$ adenosine receptors. These results suggest that $A_1$ adenosine receptor can occur in dimers and that the dimer—monomer conversion might be regulated by adenosine as the physiological ligand. Since the 74-kDa aggregates were not recognized by PC/10, it is likely that part of the third intracellular loop participates in the protein-protein interaction.

© 1995 Wiley-Liss, Inc.

Key words: photoaffinity labeling, antipeptide antibodies, immunoblotting

INTRODUCTION

Adenosine, acting outside the cell, exerts potent actions on a wide range of physiological systems, including nervous, cardiovascular, gastrointestinal, urogenital, respiratory, and lymphatic systems. Many of these actions are mediated via specific receptors named $P_j$ purinoceptors, which are linked to $G$-proteins (Daly et al., 1983). From studies on the variation of adenyly cyclase activity produced by adenosine analogues, and on rank order potency of agonists, $P_1$ purinoceptors were subdivided into two classes: $A_1$, which mediates decreases in cyclic AMP levels, and $A_2$, which mediates increases in cyclic AMP levels (Van Calker et al., 1979; Londos et al., 1980). The pharmacological characterization of the $A_2$ subtype in different tissues led to the finding of two different populations which were named $A_{2a}$ and $A_{2b}$, and had different affinities for the agonist CGS21680. It is now becoming clear that these subtypes of adenosine receptors can be linked to a variety of signal transduction systems apart from that of adenyly cyclase (Fredholm and Dunwiddie, 1988; Favors et al., 1990). The molecular characterization of these receptor subtypes ($A_1$, $A_{2a}$, and $A_{2b}$) is now possible, since they have been cloned (Tucker and Linden, 1993). A new subtype, $A_3$, which shares some sequence homologies with the others, has also been cloned (Zhou et al., 1992; Linden et al., 1993). They all belong to the family of receptor proteins with seven transmembrane domains.

Adenosine receptors present a heterogeneous distribution among brain regions. In rat brain (see Linden, 1994) $A_1$ is abundant in hippocampus, cerebellum, cortex, striatum, and thalamus, whereas $A_{2a}$ is found mainly in striatum but also in accumbens and olfactory bulb. On the other hand, $A_{2b}$ is found in pars tuberalis, and $A_3$ has low levels of expression in cortex, striatum, and olfactory bulb. Interestingly, in a neural model of cells such as bovine chromaffin cells, the only subtype of adenosine receptors present is the $A_{2b}$ (Cassadó et al., 1992).

The $A_1$ receptor molecule was first identified by Klotz et al. (1985) using a iodinated photoaffinity analog of the specific $A_1$ agonist R-PIA, $^{[125]}$Iazidohydroxy-R-PIA (R-AHPIA). A single specific band of 35 kDa was obtained from an analysis of rat brain membranes. Upon purification of the rat brain molecule, Nakata (1993) was able to raise a polyclonal antibody that recognized the purified 35-kDa band in immunoblot experiments. Here we describe the generation and characterization of two polyclonal antipeptide antibodies raised against different regions of the $A_1$ rat brain clone. By immunoblotting,
one of them identified the 39-kDa form of the receptor, whereas the other also recognized a second form of the receptor (74 kDa) that may correspond to a dimer. Since occur in vivo, and that aggregation has a physiological relevance.

**MATeRIALS AND METHODS**

**Materials**

\[^{[255]}\text{Na}\] (carrier free) and [adenine-2,8-\(^{3}\text{H}\), ethyl-2-\(^{3}\text{H}\)]-PIA ([\(^{3}\text{H}\)]RIA) (36.0 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA). (R)-N\(^{\text{3-}}\)-(phenylisopropyl)adenosine (R-PIA), N-ethylcarboxamidoadenosine (NECA), adenosine deaminase, \(\text{S}^{\text{guanylylimidodiphosphate}}\) (Gpp(NH)p), 3-\(3\)-cholamidopropyl)dimethylammonio)-1-propane-sulfonate (CHAPS), digitonin, bovine serum albumin, and electrophoresis reagents were obtained from Boehringer-Mannheim (Germany). [4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamido adenosine hydrochloride (CGS21680) and dipropylcyclopentyl xanthine (DPCPX) were from RBI (Research Biochemicals Incorporated, Natick, MA). Keyhole limpet hemocyanin was purchased from Calbiochem (La Jolla, CA). Phenylmethylsulfonyl fluoride (PMSF), pepstatin, aprotinin, leupeptin, 50% polyethylenimine, 2-chloroadenosine (CADO), Sephadex G-50 fine grade, \(m\)-maleimidobenzoyl-\(N\)-hydroxysuccinimide, and goat IgG anti-rabbit antibodies were purchased from Sigma (St. Louis, MO). Anti-Ga-common-antibody was purchased from New England Nuclear (Itisa, Madrid, Spain) and recognizes the sequence GAGESGKSTIVKQMK, which is common to \(\alpha_2\), \(\alpha_3\), and \(\alpha_2\) subunits of heterotrimeric G proteins. Western blotting reagents were from Schleicher \& Schuell (Germany). The ECL Western blotting detection system was from Amersham (Nuclear Ibérica, Spain). All other products were of the best grade available and were purchased from Merck (Darmstadt, Germany). Deionized water, further purified with a Millipore Milli-Q system, was used throughout.

**Membrane Preparations**

Pig and lamb tissues were obtained from the local slaughterhouse. Male rats, weighing 200–230 g, were of the Wistar strain. Tissues were dissected on ice and washed in 0.25 M sucrose containing Tris (5 mM)-HCl buffer pH 7.4. Tissue homogenization was performed in 10 volumes of the above sucrose buffer (ice cold) with a Polytron (Kinematica, PTA 20TS rotor, setting 4) for two periods of 5 sec separated by an interval of 15 sec. The homogenate was centrifuged at 105,000g for 30 min at 4°C, and the pellet was resuspended in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4 and recentrifuged under the same conditions. The pellet was washed once more as described above and resuspended in the same buffer for immediate use. No differences in the mobility of the bands detected by immunoblotting were found when a protease inhibitor cocktail (200 mM PMSF, 100 mM EDTA, 1 \(\mu\)M leupeptin, 1 \(\mu\)M pepstatin, and 1 \(\mu\)M aprotinin) was included in the assays.

**Receptor Solubilization**

Solubilization of cortical membranes from pig brain was performed as described by Casadó et al. (1990). Briefly, membranes obtained as described above were suspended in a 50 mM Tris-HCl buffer, pH 7.4, and then incubated for 1 hr at 25°C in the presence or in the absence of adenosine analogs. An equal amount of a solution containing 1% (w/v) CHAPS and 1% (w/v) digitonin in the same buffer, pH 7.4, was added. The total detergent/protein ratio was 2. After incubating for 10 min at 22°C, the suspension was centrifuged at 80,000g for 90 min at 4°C. The supernatant, passed through a 0.22-\(\mu\)M Millipore filter, constituted the solubilized receptor fraction.

**Protein Determination**

Protein was measured by the bicinchoninic acid method, BCA (Pierce), as described by Sorensen and Brodbeck (1986).

**Antibody Generation**

Antipeptide antisera were generated in New Zealand White rabbits by Bio-Kit (Barcelona). The peptides used for immunization were chosen from the proposed sequence of the cloned \(A_1\) adenosine receptor (Ma- han et al., 1991) and were selected on the basis of hydrophathy analysis (Kyte and Doolittle, 1982). The peptide KKVSASSGDPQKYYGKELK corresponds to part of the third intracellular loop (between transmembrane-spanning domains V and VI), and peptide GEP-VIKAEFEKVIS corresponds to part of the second extra-cellular loop (between transmembrane-spanning domains IV and V) (Fig. 1) except that the Cys in the original sequence was replaced by an Ala. This substitution was chosen to avoid the presence of two cysteines in the same peptide (see below), which would have led to undesired side reactions. It should be noted that in these circumstances the substitution C/A is considered as neutral in terms of raising antipeptide antibodies. In the case of the first peptide the homology between the rat clones of \(A_1\), \(A_2\), \(A_3\) is very low. The second peptide, in addition to a slight homology at the beginning with the \(A_2\) clone, has three amino acids (FEXV) in common addition to a slight homology at the beginning with the \(A_3\) subtypes of adenosine.
A$_1$ Adenosine Receptors in Brain

**EXTRACELLULAR**

Hum0

**Immunogenic Peptide:** G163-S176  
**Antiserum:** PC/20

**INTRACELLULAR**

Hum0

**Immunogenic Peptide:** K213-K231  
**Antiserum:** PC/10

---

**Fig. 1.** Scheme of the rat A$_1$ adenosine receptor. The predicted membrane-spanning topography of the rat A$_1$ adenosine receptors is shown. The amino acid sequences used for antibody generation are indicated. The cysteine 169 in the immunogenic peptide G163-S176 was replaced to alanine in order to avoid undesired side reactions before and during coupling to keyhole limpet hemocyanin (see Materials and Methods). The figure is adapted from the amino acid sequence reported by Mahan et al. (1991).

The peptides were synthesized by the Peptide Synthesis Service at the University of Barcelona, with an extra amino terminal cysteine residue, to facilitate conjugation with keyhole limpet hemocyanin via m-maleimidobenzoyl-N-hydroxysuccinimide coupling (Kitagawa and Aikawa, 1976). The composition of each peptide was verified by high-performance liquid chromatography. Each keyhole limpet hemocyanin-coupled peptide was injected into two rabbits using the immunization protocol method of Tanaka et al. (1985). The reactivities of the resulting antisera with the appropriate peptides, PC/10 in the case of the intracellular peptide and PC/20 in the case of the extracellular peptide (Fig. 1), were tested by enzyme-linked immunosorbent assay (Geerligs et al., 1988). In both cases, high affinity for their respective peptides was found (data not shown). The antisera (PC/10 and PC/20) were used directly for immunoblotting and immunoprecipitation experiments.
Radioligand-Binding Experiments

[^125]I-R-AHIPA or[^3H]R-PIA binding to intact tissues membranes was measured after incubating the membranes (final concentration 0.7–0.8 mg protein/mL) with adenosine deaminase (0.2 U/mL) for 30 min at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% CHAPS. The radioligand was then added, to give a final concentration in the range 0.1 to 0.35 nM for[^125]IR-AHIPA and 0.2 nM for[^3H]R-PIA. After standing at 25°C until equilibrium was achieved, usually 4 hr for[^125]IR-AHIPA and 1 hr for[^3H]R-PIA, free and bound radioligands were separated by rapid filtration of 500-μL aliquots through Whatman GF/C filters, which were subsequently washed in 10 mL of ice-cold Tris-HCl buffer. The filters were presoaked in 0.3% polyethyleneimine (2–4 hr, pH 10) to improve performance. Nonspecific binding was determined in the presence of an excess (200 to 500-fold) of R-AHIPA or R-PIA.

In all cases, after filtration, filters were placed in standard vials with 10 mL of Formula-989 liquid scintillation cocktail (New England Nuclear Research Products, Boston, MA). After shaking for a period of at least 1 hr, the vials were counted in a Packard TRI-CARB 1600TR liquid scintillation counter (for[^3H]) or in a Packard auto-gamma 5550 (for[^125]I).

Preparation of[^125]I-R-AHIPA

The product (R)-2-azido-N²-p-hydroxyphenylisopropyladenosine (R-AHIPA), whose synthesis has been described in detail elsewhere (Cristalli et al., 1984), was kindly provided by Dr. Ana Cubero (Department of Organic Chemistry and Biochemistry, University of Castilla-La Mancha, Ciudad Real, Spain). The iodination of the compound was carried out by the chloramine T method (Hunter and Greenwood, 1962). Briefly, 10 mL of Na[^125]I (1 mCi) was added to 50 mL of 0.1 M potassium phosphate buffer, pH 7.4, and the resulting solution was mixed with 20 μL of 100 μM R-AHIPA, followed by addition of 10 μL chloramine T (2 mg/mL). The test tube was vortexed for 30 sec, and the reaction was stopped by addition of 10 μL of sodium bisulfite (2 mg/mL). The reaction products were separated by gel filtration on Sephadex G-50 SF and eluted with 50 mM potassium phosphate buffer, pH 7.4, at a flow rate of 12 mL/h. Fractions of approximately 2 mL were collected, and 10-μL aliquots of the fractions were counted for radioactivity and tested for binding activity as described above. As[^125]I-R-AHIPA appears to stick to polypropylene, CHAPS (0.1% final concentration) was added in all aliquots. The same concentration of the detergent was also present in all experiments with the radioiodinated compound; this concentration does not alter the binding characteristics of A₁ adenosine receptor (data not shown). Total binding sites were calculated in radioligand-binding experiments comparing[^125]I-R-AHIPA and[^3H]R-PIA maximum binding. The measured specific radioactivity for[^125]I-R-AHIPA was 3,000 Ci/mmol, and the value of Kᵦ in the intact membranes was greater (2 ± 0.1 nM) than that shown for[^3H]R-PIA (1.2 ± 0.1 nM). Results were obtained by saturation isotherm analysis, as described below; values are mean ± SD of three independent experiments.

Data Analysis

Data from saturation isotherms were analyzed by non-linear regression using the ENZFITTER program (Elsevier Biosoft). Five replicates of each point were performed. Goodness of fit was tested according to the reduced χ² or SD values given by the program. Further statistical analysis was performed using the two-tailed Student’s t-test.

Photoaffinity Labeling of A₁ Adenosine Receptors

Membranes (0.7 mg/mL) were equilibrated in 50 mM Tris-HCl, 0.1% CHAPS buffer, pH 7.4, with 0.35 nM[^125]I-R-AHIPA in the absence or presence of different competing nonradioactive ligands for 4 hr at 25°C (2.5 ml final volume). Photolysis was then carried out in 3-mL perspex spectrophotometer cuvettes by 10-sec exposure to a 200-W mercury lamp (Applied Photophysics) at a distance of 4 cm from the lamp housing (Ciruela et al., 1994). After irradiation, an excess (500-fold) of unlabeled R-AHIPA was added to each aliquot of membranes, which was allowed to stand at 25°C for 4 hr before being filtered and washed in cool buffer to determine the covalently bound[^125]I-R-AHIPA; membranes subjected to the same treatment but not irradiated were used as control. Membranes were collected by centrifugation at 60,000g for 1 hr at 4°C.

ADP-Ribosylation of G Proteins

Pertussis toxin-catalyzed ADP-ribosylation of the α-subunit of heterotrimeric G proteins was performed as described by Ribeiro-Neto et al. (1987).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Photolabeled membranes were washed once in 50 mM Tris-HCl, 0.1% CHAPS buffer and dissolved in SDS-PAGE sample buffer (final concentration: 62 mM Tris-HCl, 1% SDS, 10% glycerol, 1% mercaptoethanol, 0.002% bromophenol Blue, pH 6.8) and incubated for 1 min at 100°C before loading of the protein (200 μg) onto the gel. Electrophoresis was performed according to the method of Laemmli (1970) using homogeneous slab gels containing 12.5% acrylamide separating and 4% acrylamide concentrative gel, which were run overnight at 80
V constant voltage. After running, the gel was fixed by immersion in 10% acetic acid, 10% methanol, and 80% water, dried at 80°C in a gel drier (Bio-Rad), and subjected to autoradiography at −80°C for 2–4 days.

**Immunoblotting**

Seventy-five micrograms of protein from membranes and solubilized receptor preparations (as indicated) was loaded onto polyacrylamide gels and run as described above. Proteins were transferred to polyvinyl difluoride (PVDF) membranes (Westran 0.2 μm PVDF membranes, Schleicher & Schuell) over 1 hr at a current of 15 mA, using a semi-dry Bio Rad Trans-Blot, in TOWbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS, pH 8.3) (Towbin et al., 1979). Nonspecific protein binding sites on the PVDF membranes were blocked by incubation overnight at 4°C in 5% (w/v) bovine serum albumin in 50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4, (TBS-TI) containing 0.1% NaN₃. After blocking, PVDF membranes were washed twice (10 min/wash) with 10 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.4, (TBS-TII) and incubated for 3 hr with specific antiserum (PC/10 or PC/20) diluted 1:3,000 in TBS-TI, containing 0.1% NaN₃. The filters were then washed three times with TBS-TII before incubation for 1 hr with horseradish-peroxidase-conjugated goat anti-rabbit IgG diluted 1:15,000 in TBS-TI. After washing the filters, Nonspecific immunoprecipitation was assessed by using the same amount of nonimmune rabbit serum. A goat IgG anti-rabbit Ig serum (5 uL of a solution of 10 mg protein/mL for each μL of specific antiserum or nonimmune serum) was added, and immune complexes were allowed to form overnight at 4°C, with constant rotation. Protein A-Sepharose CL4B (40 μL; 20 μL of packed gel) was then added, and the mixture was incubated for 2 hr, at 4°C, with constant rotation. Immunoprecipitates were washed twice with 1% Nonidet-P40 in TSA-buffer, twice with 0.1% Nonidet-P40 in TSA-buffer, and once with TSA-buffer alone. After that samples were dissolved with 60 μL of 2× SDS-PAGE sample buffer and incubated for 1 hr at 56°C and then 1 min at 100°C. SDS-PAGE was performed as described above. The gel was run, dried, and autoradiographed at −80°C for 5–7 days.

**RESULTS**

The A₁ receptor from pig brain was identified by binding of the specific ligand [¹²⁵I]R-AHPIA and by affinity cross-linking the same ligand. A specific band of approximate MW 39 kDa was labeled by affinity cross-linking (Fig. 2A). This band was displaced by R-PIA and NECA, but not by the selective A₂a agonist CGS21680. The results of displacement experiments at different ligand concentrations are shown in Figure 2B. The affinity-labeled band and the specific [¹²⁵I]R-AHPIA binding was displaced by nanomolar concentrations of DPCPX, R-PIA, and R-AHPIA and by low micromolar concentrations of CADO and NECA. A significant displacement using CGS21680 was achieved only at 100 μM. In cross-linking and binding experiments nonspecific labeling or binding was high, as is usual for iodinated ligands of A₁ receptors. These data are similar to those described in the literature for adenosine A₁ subclass and confirm that the cross-linking of [¹²⁵I]R-AHIPA was specific for the A₁ subtype of adenosine receptors present in pig brain cortex. The efficiency of the labeling using [¹²⁵I]R-AHIPA was 34 ± 5%. The ability of the antipeptide antibodies to immunoprecipitate the radiolabeled receptor was tested (Fig. 3). PC/10 and PC/20 antisera, two antipeptide antibodies directed, respectively, against an intracellular and an extracellular sequence of the A₁ receptor (see Fig. 1), were able to immunoprecipitate the affinity cross-linked receptor. Nevertheless, PC/10 immunoprecipitated the iodinated receptor more efficiently than PC/20 (see Fig. 3).

Western immunoblots of whole pig brain membranes performed using PC/10 antibody showed a 39-kDa specific band (Fig. 4, left). This band migrated at an...
Fig. 2. Specificity of the [125I]R-AHPIA labeling and of the [125I]R-AHPIA cross-linking to pig cortical brain membranes. Cross-linking experiments: In A, competitive inhibition of the cross-linking of 0.35 nM [125I]R-AHPIA into the 39-kDa band (arrow) was assayed in the absence (total) or in the presence of 50 nM of the above indicated ligand. In the foot of B the same experiments were performed in absence or presence of different ligands at the indicated concentrations. Samples were placed at 4 cm from the lamp and exposed to UV light for 10 sec. Membranes were washed with buffer to eliminate excess reagents and treated with SDS-PAGE sample buffer (1 min, 100°C). The gel was run, dried, and autoradiographed as described in Materials and Methods. The autoradiography is representative of those obtained in two different experiments. The specific band is indicated by an arrow. Binding experiments (top part of B): Competitive inhibition of the 0.1 nM [125I]R-AHPIA binding was determined in the absence (total) or in the presence of the above-indicated ligands by rapid filtration of 500-µL aliquots. Whatman GF/C filters were subsequently washed in 10 mL of ice-cold Tris-HCl buffer (see Materials and Methods). The graph shown is representative of three separate experiments, and all points represent the mean ± SD of five replicates.

identical MW to that observed by SDS-PAGE after affinity cross-linking of the receptor with [125I]R-AHPIA. The same 39-kDa band was obtained by immunoblotting detergent extracts. Interestingly, when using PC/20 antibody (Fig. 4, center panel), two specific bands (39 and 74 kDa) were clearly visible in whole membrane preparations. The low-molecular-weight band was the same as that obtained using PC/10 antibody for immunoblotting, and we assume it represents the 39-kDa A1 receptor. The high-molecular-weight band was not evident in the cross-linking experiment with [125I]R-AHPIA (Fig. 2). The 74-kDa band may represent a receptor–G protein complex, which may be recognized by PC/20 but not by PC/10 antibody. In order to test this possibility, the same immunoblots (after eliminating previous antibodies: see Materials and Methods) were developed using antibodies against a common sequence of α-subunits of G proteins.

Only a single broad band (38–42 kDa) corresponding to Gα subunits was detected with this antiserum (right hand lanes of Fig. 4). The absence of any G-protein recognition in the 74-kDa band provides evidence that this band might not correspond to a receptor–G protein complex and opens the possibility that the 74-kDa band could be a dimer of the 39-kDa receptor that is recognized by PC/20 antibody but not by PC/10.

On the other hand, reagents that are reported to dissociate receptor–G protein complexes such as Gpp(NH)p, N-ethylmaleimide, or pertussis toxin were tested for their ability to promote the conversion of the upper band to the lower band. It is well known that uncoupling of the G protein leads to a receptor of low affinity for the ligand. Thus, as indicated in Figure 5 all these compounds uncoupled the A1 receptors since they reduced the 0.1 nM [125I]AHPIA binding. Under the same con-
Fig. 3. Immunoprecipitation of pig cortical brain adenosine A<sub>1</sub> receptor. Crude membranes prepared from rat brain cortex were subjected to photoaffinity labeling with 0.35 nM of [125]<sup>I</sup>R-AHPIA in the absence (lane a) or in the presence (lane b) of 500-fold higher concentration of R-AHPIA. Labeled membranes were solubilized in TSA-buffer containing 1% Nonidet-P40 (1 hr on an ice bath) and centrifuged to sediment insoluble material. Immunoprecipitation was carried out in 500 μL of the supernatant (500 μg protein) using 5 μL of PC/10 (lane 1), PC/20 (lane 3), or the respective nonimmune serum (lanes 2,4) and to SDS/PAGE as described in Materials and Methods. Each panel is representative of, at least, three experiments performed with membrane preparations from different animals. Arrow: Band corresponding to A<sub>1</sub> adenosine receptor.

In order to search for the presence of 39-kD and 74-kDa forms in other sources and in different tissues, analysis by Western blotting was performed using membrane preparations from brain cortex, striatum, lung, and kidney of either pig, rat, or lamb (Fig. 6). For comparative purposes the specific binding of [125]<sup>I</sup>R-AHPIA was also measured in all samples. From a qualitative point of view, there is an excellent correlation in all sources assayed between [125]<sup>I</sup>R-AHPIA binding and the presence of the A<sub>1</sub> monomer in immunoblots. Thus, the level of A<sub>1</sub> receptors in lung, irrespective of the source, was undetectable by Western blotting and by [125]<sup>I</sup>R-AHPIA binding. In contrast, the highest levels of the monomer by immunodetection, in addition to the highest [125]<sup>I</sup>R-AHPIA binding, were found in brain cortex preparations. The quantitative differences that sometimes occurred (see Fig. 6) between [125]<sup>I</sup>R-AHPIA binding and immunoblotting might be due to both differences in affinity of the receptor for [125]<sup>I</sup>R-AHPIA and differences in recognition by the antibody used, which was raised against a peptide corresponding to the rat A<sub>1</sub> sequence. As an example of these apparent discrepancies, the binding of [125]<sup>I</sup>R-AHPIA to the receptor in pig kidney was relatively low even though the signal in the Western blotting was high for the monomer and for the aggregate (Fig. 6).

The aggregate detected by immunoblotting using PC/20 was present in all pig tissues. Interestingly, in rat brain the signal corresponding to the higher MW band was double; one was slightly higher than that obtained in rat tissues (MW 79 kDa), and the second had a lower MW (69 kDa). In lamb tissues the band of the aggregate was not clear (Fig. 6). Taken together these data show that the nonaggregated and the aggregated forms of the A<sub>1</sub> adenosine receptor are widely distributed.

**DISCUSSION**

In this report we present results obtained with two anti-peptide antibodies (PC/10 and PC/20) which recognize two different epitopes of the A<sub>1</sub> subtype of adenosine receptors. Although these antibodies were generated against peptide sequences derived from the cloned rat sequence (Mahan et al., 1991), the reactivity of these antibodies toward samples from rat, pig, and lamb was high probably because of the high degree of homology that these receptors share (Olah et al., 1992). The specificity of PC/10 and PC/20 antibodies was studied by immunoprecipitation of the receptor cross-linked to [125]<sup>I</sup>R-AHPIA followed by SDS-PAGE analysis. The band that immunoprecipitated had a MW around 39 kDa, suggesting that the complex in the 74-kDa band does not include a G protein (Table I).

**RESULTS**

Assuming that the band of higher molecular mass is a dimer, the dimer/monomer proportion was higher in CHAPS-detergent extracts than in whole membranes (Fig. 4; Table I). The dimer/monomer proportion markedly decreased in the acetone precipitate and upon treatment of membranes with either 50 nM R-AHPIA, 50 nM DPCPX, or 50 R-PIA plus 100 μM Gpp(NH)p (Fig. 4; Table I). Treatment with agonist or antagonist led to an increase in the intensity of the 39-kDa form, detected by the PC/10 antibody, to the detriment of the 74-kDa band (Table I). Neither agonists nor antagonists were able to increase the intensity of the single band obtained by immunoblotting with the anti-G<sub>α</sub> antibody, suggesting that the complex in the 74-kDa band does not include a G protein (Table I).

- **Fig. 3.** Immunoprecipitation of pig cortical brain adenosine A<sub>1</sub> receptor. Crude membranes prepared from rat brain cortex were subjected to photoaffinity labeling with 0.35 nM of [125]<sup>I</sup>R-AHPIA in the absence (lane a) or in the presence (lane b) of 500-fold higher concentration of R-AHPIA. Labeled membranes were solubilized in TSA-buffer containing 1% Nonidet-P40 (1 hr on an ice bath) and centrifuged to sediment insoluble material. Immunoprecipitation was carried out in 500 μL of the supernatant (500 μg protein) using 5 μL of PC/10 (lane 1), PC/20 (lane 3), or the respective nonimmune serum (lanes 2,4) and to SDS/PAGE as described in Materials and Methods. Each panel is representative of, at least, three experiments performed with membrane preparations from different animals. Arrow: Band corresponding to A<sub>1</sub> adenosine receptor.

- **Fig. 6.** Analysis by Western blotting was performed using membrane preparations from brain cortex, striatum, lung, and kidney of either pig, rat, or lamb. For comparative purposes the specific binding of [125]<sup>I</sup>R-AHPIA was also measured in all samples. From a qualitative point of view, there is an excellent correlation in all sources assayed between [125]<sup>I</sup>R-AHPIA binding and the presence of the A<sub>1</sub> monomer in immunoblots. Thus, the level of A<sub>1</sub> receptors in lung, irrespective of the source, was undetectable by Western blotting and by [125]<sup>I</sup>R-AHPIA binding. In contrast, the highest levels of the monomer by immunodetection, in addition to the highest [125]<sup>I</sup>R-AHPIA binding, were found in brain cortex preparations. The quantitative differences that sometimes occurred (see Fig. 6) between [125]<sup>I</sup>R-AHPIA binding and immunoblotting might be due to both differences in affinity of the receptor for [125]<sup>I</sup>R-AHPIA and differences in recognition by the antibody used, which was raised against a peptide corresponding to the rat A<sub>1</sub> sequence. As an example of these apparent discrepancies, the binding of [125]<sup>I</sup>R-AHPIA to the receptor in pig kidney was relatively low even though the signal in the Western blotting was high for the monomer and for the aggregate (Fig. 6).

The aggregate detected by immunoblotting using PC/20 was present in all pig tissues. Interestingly, in rat brain the signal corresponding to the higher MW band was double; one was slightly higher than that obtained in rat tissues (MW 79 kDa), and the second had a lower MW (69 kDa). In lamb tissues the band of the aggregate was not clear (Fig. 6). Taken together these data show that the nonaggregated and the aggregated forms of the A<sub>1</sub> adenosine receptor are widely distributed.

- **DISCUSSION**

In this report we present results obtained with two anti-peptide antibodies (PC/10 and PC/20) which recognize two different epitopes of the A<sub>1</sub> subtype of adenosine receptors. Although these antibodies were generated against peptide sequences derived from the cloned rat sequence (Mahan et al., 1991), the reactivity of these antibodies toward samples from rat, pig, and lamb was high probably because of the high degree of homology that these receptors share (Olah et al., 1992). The specificity of PC/10 and PC/20 antibodies was studied by immunoprecipitation of the receptor cross-linked to [125]<sup>I</sup>R-AHPIA followed by SDS-PAGE analysis. The band that immunoprecipitated had a MW around 39 kDa.
which was the same as that obtained in immunoblotting experiments using PC/10. In addition to this band of 39 kDa, PC/20 antibody also recognized a band of 74 kDa. The possibility that the higher-molecular-weight band was caused by association of the A_1 receptor with a G-protein was investigated. At the outset this seemed a likely explanation of the data because the G-protein may associate with the cytoplasmic domains of the receptor and preferentially displace the PC/10 antibody. The results shown in Figure 4 discount this explanation because blots show no Go subunits within the 74-kDa region. The antibody anti-Ga used may not be able to recognize the receptor-G protein complex because the epitope which is recognized by the antibody is hidden in the receptor-G protein complex. However this possibility seems unlikely since the common sequence of Go subunits does not interact with heptaspanning membrane receptors. The maintenance of the upper band in membranes treated with uncouplers of receptor-G protein interactions, such as Gpp(NH)p, N-ethylmaleimide, or pertussis toxin, also rules out the possibility of a receptor-G protein complex for the 74-kDa band. After discarding G-proteins as partners of A_1 adenosine receptors in the 74-kDa band, the search for the protein forming the aggregate is difficult. However it should be noted that there are some reports in the literature that indicate that the A_1 adenosine receptors might exist as dimers in vivo. In fact, radiation-inactivation studies in rat cerebral-cortical membranes have shown that the target size of the A_1 receptor is 63 kDa (Frame et al., 1986). This molecular mass is much higher than that predicted from the rat clone, and the authors of these studies suggest that A_1 receptors may be dimeric or composed of subunits other than those that can be photolabeled by adenosine analogs (Frame et al., 1986). Using the same techniques Reddington et al. (1987) reported a molecular mass of 79,500 ± 7,000 Da for the A_1 receptor of the same source. Higher-molecular-mass bands have also been identified for other G-protein-coupled receptors expressed in Sf9 cells (Parker et al., 1991; Mouillac et al., 1992; Ng et al., 1993). Receptor-dimer formation has been suggested for the muscarinic M1 and M2 receptors expressed in Sf9 cells (Parker et al., 1991) and for serotonin 1B receptors expressed in BHK 570 cells (Pickering et al., 1993). In view of these data, our results would agree with the existence of dimers of A_1 adenosine receptors and, significantly, in our study a conversion of dimers to monomers could be achieved using agonists and antagonists of the A_1 receptor (Fig. 4). This strongly suggests that aggregates exist in vivo and that aggregation may be regulated by adenosine as the physiological ligand. It remains to be demonstrated whether the form of higher molecular mass is a consequence of the processing of the samples in vitro. However, it should be noted that dimers already appear in less processed samples such as in crude membranes treated only with SDS-sample buffer containing mercaptoethanol (Fig. 4). Mouillac et al. (1992) report higher-molecular-

**Fig. 4.** Immunoblotting analysis of the pig brain adenosine A_1 receptor. Pig cortical brain membranes were photoaffinity labeled with 0.35 nM of [125I]R-AHPIA (as indicated in the legend to Fig. 2) in the absence (lane A) or in the presence (lane B) of an excess (500-fold) of unlabeled R-AHPIA. Seventy micrograms of protein from cortical pig brain crude membranes (lanes 1) or detergent extracts (lanes 2-6) were used. The detergent extracts were prepared from untreated membranes (lane 2) or from membranes treated with either 50 nM R-AHPIA (lane 4), 50 nM DPCPX (lane 5), or 50 nM R-PIA plus 100 μM Gpp(NH)p (Lane 6). These reagents were maintained along the solubilization with CHAPS-digitonin. In lane 3 the acetone precipitate of detergent extracts was applied. After transferring and blocking, the PVDF membrane was incubated with the indicated reagent: PC/10, PC/20, anti-Ga or nonimmune serum (1:3,000-fold dilution in all four cases). The immunoreactive bands were visualized by horseradish-peroxidase-labeled goat anti-rabbit IgG antibodies (1:15,000-fold dilution), followed by ECL chemiluminescence detection. Each panel is representative of at least three experiments performed on different membrane preparation and detergent extracts. Arrows: Bands corresponding to A, adenosine receptors; arrowhead: band corresponding to Gα subunit of heterotrimeric G proteins.
Fig. 5. Immunoblotting and radioligand binding experiments in pig cortical brain membranes under different conditions. Pig cortical brain membranes (5 mg/mL) were pretreated with either 2 mM N-ethylmaleimide (NEM) for 15 min, 10 μL/mL of pertussis toxin, or 50 mM Tris-HCl buffer pH 7.4. The samples were diluted in cold with the same buffer; they were centrifuged and washed twice. For binding measurements, membranes (0.7 mg protein/mL) were incubated in 50 mM Tris-HCl pH 7.4 containing 0.1% CHAPS and 0.1 nM [125I]R-AHPIA for 4 hr in the presence of 50 nM R-AHPIA (Control + R-AHPIA), 100 μM Gpp(NH)p (Control + Gpp(NH)p), or buffer (NEM- and pertussis-toxin-pretreated membranes). Nonspecific binding was determined in the presence 50 nM of R-AHPIA. Free and bound radioligands were separated by rapid filtration through Whatman GF/C filters. Specific binding was determined by subtraction of the nonspecific binding from the total binding. The graph shown is representative of three separate experiments, and all points represent the mean ± SD of five replicates. For immunoblotting with the PC/20 antiserum, membranes (75 μg) from each animal and tissue were subjected to SDS-PAGE, and proteins were transferred to PVDF membranes and treated as indicated in the legend to Figure 4. Each immunoblotting is representative of, at least, three experiments performed with membrane preparations derived from different animals. *P < .05.

Fig. 6. Immunoblotting and radioligand binding experiments of adenosine A1 receptor from different sources. Cortical brain, striatum, lung, and kidney membranes (0.7 mg protein/mL) from pig, rat, and lamb were incubated in 50 mM Tris-HCl, 0.1% CHAPS buffer, pH 7.4, with 0.1 nM of [125I]R-AHPIA for 4 hr. Nonspecific binding was determined in the presence 50 nM of R-AHPIA. Free and bound radioligands were separated by rapid filtration through Whatman GF/C filters. Specific binding was determined by subtraction of the nonspecific binding from the total binding. The graph shown is representative of three separate experiments, and all points represent the mean ± SD of five replicates. For immunoblotting with the PC20 antiserum, membranes (75 μg) from each animal and tissue were subjected to SDS-PAGE, and proteins were transferred to PVDF membranes and treated as indicated in the legend to Figure 4. Each immunoblotting is representative of, at least, three experiments performed with membrane preparations derived from different animals. *P < .05.

Analyzing the widespread occurrence of the two weight bands of the β2-adrenergic receptors expressed in SF9 cells that do not appear in the cross-linking to the specific ligand [125I]cyanopindolol diazirine as the monomer of 40- to 50-kDa band does. In our case, since the 74-kDa band does not appear in the cross-linking experiments performed using [125I]R-AHPIA (Fig. 2), it is possible that the iodinated ligand promotes the conversion from the 74-kDa species to the 39-kDa species as the nonradiolabeled ligands do (Fig. 4). Another explanation would be a lower affinity of the [125I]ligand for the 74-kDa band. If this were the case, the experimental conditions would allow a good cross-linking of [125I]R-AHPIA to the 39-kDa polypeptide but not to the 74-kDa complex.
forms of A₁ adenosine receptors in pig, rat, and to a lesser degree in lamb tissues, and their different proportion in the tissues assayed (see Fig. 6), it seems that each source possesses a specific pattern of distribution between nonaggregated and aggregated forms of A₁ adenosine receptors. The 74-kDa forms of the A₁ receptor are present in both membranes and CHAPS-digitonin-detergent extracts; since they are not susceptible to the SDS present in both membranes and CHAPS-digitonin-detergent extracts, this suggests that the in vivo conversion to the 39-kDa form to the 39-kDa form took place when the reagents were added to membranes but not when they were added to the soluble extracts. This suggests that the in vivo conversion needs the structural integrity of the membrane and may be mediated by other membrane components apart from the A₁ receptor.

ACKNOWLEDGMENTS

F.C. is the recipient of a fellowship for "Formación de Investigadores a Catalunya" from Comissió Interdepartamental de Recerca i Innovació Tecnològica (CIRIT) (Diari Oficial de la Generalitat Catalana 23/11/1990). This work was supported by grants QFN 89/4001 and QFN93/4423 from CIRIT-DGICYT (Direcció General de Investigaciones Científicas y Tecnológicas) and by grants PB91/263 from DGICYT.

We acknowledge the technical support given in the photoaffinity labeling experiments by Dr. F. Trull from the Organic Chemistry Department. We thank Dr. David Andreu and Cristina Carreño from the Peptide Synthesis Service and Catalina Relaño from the Cell Culture Service, for excellent technical assistance (both services are part of the University of Barcelona).

TABLE I. Densitometric Analysis of the Immunoblots of Figure 4*

<table>
<thead>
<tr>
<th>Antiserum used and band detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC10, 39 kDa</td>
</tr>
<tr>
<td>39 kDa</td>
</tr>
<tr>
<td>Crude membranes</td>
</tr>
<tr>
<td>Detergent extracts</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Acetone precipitated</td>
</tr>
<tr>
<td>+50 nM R-AHPIA</td>
</tr>
<tr>
<td>+50 nM DPCPX</td>
</tr>
<tr>
<td>+50 nM R-PIA + 100 μM Gpp(NH)p</td>
</tr>
</tbody>
</table>

*Quantitative densitometry of the profiles given in Figure 3 was performed using a densitometer from Molecular Dynamics. Values of relative absorbance are given as area of the corresponding peak in function of the protein applied in each lane. Values are mean ± SD of five different scannings.

REFERENCES


Hunter WM, Greenwood FC (1962): Radioimmunoelectrophoretic as...


B.4. Adenosine deaminase interacts with A₁ adenosine receptors in pig brain cortical membranes.


*Journal of Neurochemistry* *(In the press).*
RESULTADOS 90
Adenosine Deaminase Interacts with A₁ Adenosine Receptors in Pig Brain Cortical Membranes.

Carles Saura, Francisco Ciruela, Vicent Casadó, Enric I. Canela, Josefina Mallol, Carmen Lluís, Rafael Franco.

Abstract: Adenosine deaminase is an enzyme of purine metabolism that has largely considered to be cytosolic. A few years ago, adenosine deaminase was reported to appear on the surface of cells. Recently, it has been demonstrated that adenosine deaminase interacts with a type II membrane protein known as either CD26 or dipeptidylpeptidase IV. In this article, by means of immunoprecipitation and affinity chromatography it is shown that adenosine deaminase and A₁ adenosine receptors interact in pig-brain cortical membranes. This is the first report in brain demonstrating an interaction between a degradative ecto-enzyme and the receptor whose ligand is the enzyme substrate. By means of this interaction adenosine deaminase leads to the appearance of the high-affinity site of the receptor, which corresponds to the receptor-G protein complex. Thus, it seems that adenosine deaminase is necessary for coupling A₁ adenosine receptors to heterotrimeric G proteins. Key Words: Adenosine deaminase, A₁ adenosine receptors, pig brain, cerebral cortex, ligand binding, coprecipitation, protein-protein interactions, G proteins, receptor-G protein complex.

Adenosine, acting through specific receptors located on the surface of cells is capable of exerting multiple physiological actions in a variety of systems (Olah and Stiles 1993, Tucker and Linden 1993, Dalziel and Westfall 1994). In the nervous system, adenosine behaves as a neuromodulator, inhibiting neuronal firing (Phillis and Wu 1981) and synaptic transmission (Kuroda 1978) and altering cAMP levels (Daly 1977). Four receptor types (A₁, A₂a, and A₃) have been cloned, which are heptaspanning proteins coupled to G proteins. A₁ adenosine receptors (A₁R) are heterogeneously distributed throughout the central nervous system, the cerebral cortex being a zone where they concentrate (Fastbom et al., 1987 a,b). In brain membranes, as in membranes from other sources, A₁R present two different affinities for agonists that depend upon the coupling to heterotrimeric G proteins (Lohse et al., 1984); coupled receptor-G protein complexes display high affinity for R-phenylisopropyladenosine (R-PIA, Kᵢ=0.1-0.2nM), whereas uncoupled receptors display low affinity (Kᵢ=I-2nM) (Lohse et al., 1984; Casadó et al., 1990). Functional assays to study A₁R are usually performed using metabolically stable synthetic ligands which could mimic the effects of the physiological ligand, adenosine. In experiments of ligand binding to A₁R or of second messenger production via A₁R, ADA is always added to the incubates in order to degrade endogenous adenosine, which behaves as a competitor.

Adenosine deaminase (ADA, EC.3.5.4.4) catalyzes the hydrolytic deamination of adenosine to inosine. Although the location of the enzyme is mainly cytosolic, ADA is also found in association with membrane fractions. Thus, in membranes from cultured brain cells Trams and Lauter (1975) described a similar
enrichment of ADA and the ecto-enzyme 5'-nucleotidase, which produces adenosine from AMP. In 1986 we demonstrated that ADA was present on the surface of rat brain synaptosomes with the same topology as that of ecto-5'-nucleotidase (Franco et al., 1986). Since then, ADA has been implicated in the control of the extracellular concentration of adenosine together with the enzymes that produce the nucleoside transport systems present on the cell surface.

Although an obvious role for ecto-ADA is to degrade endogeneous adenosine, other possible roles are under scrutiny. Thus, Catania et al., (1991) have demonstrated that ADA is able to stimulate the release of excitatory amino acids through a mechanism that is independent of adenosine depletion. In this report we provide the evidence that ADA and A1R interact in pig brain membranes. Through this interaction, i.e., in a catalytic-independent manner, ADA modifies the binding of the agonist [3H]R-phenylisopropyladenosine ([3H]R-PIA) to A1R. Since ADA leads to the appearance of the high-affinity component for ligand binding, it seems that ADA is needed for an efficient coupling of A1R to heterotrimeric G proteins.

Abbreviations used: ADA: Adenosine deaminase; A1R: Adenosine receptor; R-PIA: R-Phenylisopropyl adenosine.

MATERIALS AND METHODS

Materials

[Adenine-2,8-3H, ethyl-2-3H]phenyl-isopropyl adenosine ([3H]R-PIA) (36Ci/mmol), Na125I (17 mCi/μg iodine) and ECL immunoblotting detection system were purchased from Amersham (Nuclear Iberica, Madrid, Spain). [3H]1,3-dipropyl-8-ciclopentylxantine ([3H]DPCPX) (108 Ci/mmol) was from New England Nuclear Research Products (Boston, MA, USA). DPCPX was from Research Biochemicals Inc. (RBI, Natick, MA, USA). N6-(R)-(phenylisopropyl)adenosine (R-PIA), R-deoxycoformycin, polyethyleneimine 50%, γ-globulins, bovine serum albumin and goat anti IgG anti-rabbit IgG were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Calf adenosine deaminase, which was filtered through Sephadex G-25 prior to all assays, and electrophoresis reagents were obtained from Boehringer Manheim (Barcelona, Spain). CNBr activated Sepharose and Sephadex G-25 fine grade and Protein A Sepharose CL4B were from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other products were of the best grade available and purchased from Merck (Darmstadt, Germany). Deionized water further purified with a Millipore Milli-Q system was used throughout.

Antibodies

Antipeptide antisera against A1R were generated by immunization of female New Zealand White rabbits (Ciruela et al., 1995). The peptides used for immunization were synthesized by Peptide Synthesis Service of the University of Barcelona (Spain). The peptide which corresponds to part of the third intracellular loop of rat A1R (to give PC11 antibody) was: KKVSASSGDPQKYYGKELK. The peptide which corresponds to part of the second extracellular loop of rat A1R (to give PC21 antibody) was: GEPVKAEFEKVISC. The composition of the peptide was determined by amino acid analysis. Purity of each of the peptides was greater than 85% as determined by reverse-phase HPLC. The antisera generated were purified by affinity chromatography using the specific peptide coupled to Sepharose. The specificity of anti-A1R antibodies was tested as described elsewhere (Ciruela et al., 1995) PC11 and PC21 were not able to recognize ADA from a calf intestine commercial soluble preparation by immunoprecipitation and western-blotting. Anti-ADA was affinity purified from specific antisera raised by immunization of female New Zealand White rabbits as described elsewhere (Aran et al., 1991).

RESULTADOS
Membrane Preparation and solubilization
Porcine brains were obtained from the local slaughterhouse. Brain cortices 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 Polytron (Kinematica, PTA 20TS rotor, setting 4) for two periods of 5 seconds. The homogenate was centrifuged at 105,000 x 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 recentrifuged under the same conditions. The pellet was washed once more as described above and resuspended in the same buffer solution for immediate use.

Receptor solubilization was performed as described by Casado et al. (1990). Cortical membranes were resuspended in a 50 mM Tris-HCl buffer, pH 7.4, containing 0.5% (w/v) CHAPS and 0.5% (w/v) digitonin (the detergent/protein ratio was 2). After incubating for 15 min at 25°C, the suspension was centrifuged at 105,000 x g, 90 min, 4°C. The supernatant filtered through 0.22 μm Millipore filters constituted the receptor solubilized fraction.

Enzyme Activities
Adenosine deaminase (E.C. 3.5.4.4.) and lactate dehydrogenase (E.C. 1.1.1.29) were assayed using, respectively, adenosine and pyruvate and NADH as substrates, as described elsewhere (Franco et al., 1986).

Protein Determination
Protein was measured by the bicinchoninic acid method, BCA (Pierce), as described by Sorensen and Brodbeck (1986).

Adenosine determination
Washed brain cortical membranes (1.2 mg/mL) were incubated for 3 h at 25°C in 50 mM Tris-HCl buffer, pH 7.4. Samples performed in triplicates were deproteinized by incubation with 1.2 M perchloric acid at 0°C during 10 min. Samples were then centrifuged and the supernatants were delipidized by addition of 1 vol. of a mixture of heptane/freon (1:4, v/v) and neutralized with 1.2 M KOH solution. 20 μL of the aqueous phase (or standard solution containing ATP, ADP, AMP, IMP, adenosine, inosine, hypoxanthine and xanthine) were analyzed by HPLC using a Shimadzu LC-6A system with a UV spectrophotometric detector SPD-6A.

Radioiodination of Calf Adenosine Deaminase
Calf intestinal adenosine deaminase filtered through Sephadex G-25 was radiolabeled with $^{125}$I by the chloramine-T method described by Daddona and Kelley (1978), except that the reaction product ($[^{125}$I]$ADA$) was separated from free $^{125}$I with a Sephadex G-50 fine grade column; elution was performed with 50 mM Tris-HCl buffer, pH 7.4. Aliquots displaying adenosine deaminase activity (1.8x10$^7$ dpm/U) were pooled and stored at 4°C. 75% of the enzyme activity was recovered.

Binding Assays
$[^{3}$H]$R$-PIA and $[^{3}$H]$D$PCPX binding assays were carried out after incubation of the pig brain cortical membranes and the solubilized receptor suspensions with 0-20 U/mL adenosine deaminase for 1 hr at 25°C in 50 mM Tris-HCl buffer, pH 7.4. After the incubates had stood at 25°C for long enough to achieve the equilibrium for each radioligand concentration (0.5 hr for the highest and 8 hr for the lowest concentration; 2 hr in the case of 2 nM $[^{3}$H]$R$-PIA), free and bound ligand were separated by rapid filtration. 500 μL of membrane suspensions was passed through Whatman GF/C filters presoaked in 0.3% polyethyleneimine (2hr, pH 10), which were subsequently washed with 10 mL of ice-cold Tris-HCl buffer for less than 8s. In the case of solubilized receptor, 500 μL was added to a mixture of polyethylene glycol 8000 and bovine

RESULTADOS 93
\(\gamma\)-globulins to a final concentration of 10% (w/v) and 1.3 mg/mL respectively. After further incubation for 15 min at 4°C the suspension was passed through Whatman GF/C filters (presoaked in 0.3% polyethyleneimine) and washed with 10 mL of ice-cold Tris-HCl buffer, pH 7.4, containing 8% polyethylene glycol 8000. Non specific binding was determined in the presence of an excess of R-PIA (300 fold) and DPCPX (500 fold) in binding assays corresponding to \([^3H]R\)-PIA and \([^3H]DPCPX\) respectively.

\([^{125}I]\)ADA binding assays were performed with 0.75 mg/mL pig brain membranes in 50 mM Tris-HCl buffer, pH 7.4. After standing at 25°C for long enough to achieve the equilibrium for each \([^{125}I]\)ADA concentration (0.5 hr for the highest and 2 hr for the lowest), aliquots of 200 \(\mu\)L were filtered through Whatman GF/C filters and subsequently washed in 10 mL of ice-cold Tris-HCl buffer.

In all cases, the filters were then transferred to scintillation vials and, after the addition of 10 mL of scintillation fluid (Formula-989, New England Nuclear Research Products), vials were shaked overnight and counted using a Packard 1600 TRI-CARB scintillation counter with 50% of efficiency.

**Analysis of Binding Data**

The individual saturation isotherms were analyzed by nonlinear regression as described elsewhere (Casado et al., 1990). The total binding data (specific binding to A\(_1\) adenosine receptor plus nonspecific binding) were fitted to one- and two-affinity states according to the equations:

**One affinity state:**

\[
\text{Total Binding} = (R\cdot L/(K_d+L)) + K_n\cdot L
\]

**Two affinity states:**

\[
\text{Total Binding} = (R_H\cdot L/(K_{d_H}+L)) + (R_L\cdot L/(K_{d_L}+L)) + K_n\cdot L
\]

where L represents free radioligand concentration calculated as radioligand added minus radioligand bound. \(K_n\) denotes the constant for nonspecific binding. \(R_H\) and \(R_L\) are the maximum number of binding sites in either the one-state or two-state (high \(R_H\) and low \(R_L\) affinity) model. \(K_{d_H}\), \(K_{d_L}\) and \(K_n\) are the equilibrium dissociation constants for individual receptor species in the two models. Three to five replicates of each point were performed. Goodness of fit was tested according to reduced \(\chi^2\) or SD values given by the regression program. A modified F-test was used to analyze whether the fit to the two-state model significantly improved on the fit to the one-state model. In all cases it was considered that the two-state model led to a significant improvement over the one-state model when \(P<0.001\). When no significant improvement over the one state model was detected, the \(P\) value was greater than 0.3 (Hoyer et al., 1984)

**Chromatography of A\(_1\) Adenosine Receptor using an ADA Affinity Column**

Solubilized extracts (2 mg protein/mL, 0.5% CHAPS, 0.5% digitonin in 50 mM Tris-HCl buffer, pH 7.4) obtained from pig cortical membranes were diluted (1:2) with 50 mM Tris-HCl buffer, pH 7.4 and applied to a 1 mL of ADA-affinity column was prepared by direct coupling of 10 mg of commercial calf intestinal adenosine deaminase (filtered through Sephadex G-25 and further purified to homogeneity by affinity chromatography (Aran et al., 1990) to a 1 g of cyanoen bromide-activated Sepharose as described by Trotta et al. (1979). After the application, the column was cooled to 4°C and washed with 15 volumes of 50 mM Tris-HCl, 0.1% CHAPS, 0.1% digitonin, pH 7.4, until no protein was detected in the eluates. All fractions were assayed for \([^3H]R\)-PIA binding, lactate dehydrogenase activity and protein.

**Sodium Dodecyl-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting**

Samples were treated with SDS-PAGE sample buffer (Tris-HCl 62 mM, 10% SDS, 10%
glycerol, 1% mercaptoethanol, 0.002% bromophenol blue, pH 6.8), 5 min at 100°C, before loading onto the polyacrylamide gels. Electrophoresis was performed according to the method of Laemmli (1970) using homogeneous slab gels containing 12.5% acrylamide separating and 4% acrylamide concentrative gel, which were running at constant voltage (200 V) using a mini-protean system (Bio-Rad Lab., AB, Sweden). Proteins were transferred to PVDF membranes (Immobilon-P, Millipore) over 1 hr at constant voltage (100 V), using a wet Bio-Rad Trans-Blot, in Towbin buffer (25 mM Tris, 192 mM Glycine, 20% methanol, 0.1% SDS, pH 8.3) (Towbin et al., 1979). Nonspecific protein binding sites on the PVDF membranes were blocked by incubation overnight at 4°C using 5% (w/v) bovine serum albumin (BSA) in 50 mM Tris-HCl buffer containing 150 mM NaCl, 0.05% Tween-20, pH 7.4 (TBS-TI) and 0.02% NaN₃. After blocking, PVDF membranes were washed twice (10 min/wash) in 10 mM Tris-HCl buffer containing 500 mM NaCl, 0.5% Tween-20, pH 7.4 (TBS-TII) and incubated for 3 hr with the specific purified anti-A₁R antibody, PC21 (10 mg/mL) in TBS-TI including 0.02% NaN₃. The filters were washed five times with TBS-TII before incubation for 1 hr with horseradish-peroxidase-conjugated goat anti-rabbit IgG diluted 1:15000 in TBS-TI. The filters were washed five times with TBS-TII as described above and incubated at equal volumes of ECL detection reagent 1 and 2 (Amersham). The reagent was drained off and the filters were placed in contact with a film (Agfa Curix-Rp2), which was developed by chemiluminescence. The usual time for visualizing immunoreactive bands was 1 minute.

Immunoprecipitation of ADA
Cortical membranes from pig brain were resuspended and solubilized in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, (TSA-buffer), 0.02% NaN₃, 1% (v/v) Nonidet P-40 (1 hr on an ice bath) in the presence of 2 U/ml of adenosine deaminase either fully active or inhibited using Hg²⁺ or deoxycoformycin. In the immunoprecipitation procedure CHAPS-digiotin detergent was discarded to avoid artefactual charge effects in protein-protein interactions. For the Hg²⁺-dependent blockade of the deaminase activity a preincubation of 1 hr with 0.1 mM HgCl₂ was performed; free Hg²⁺ was removed by gel filtration using Sephadex G-25. Deoxycoformycin (0.1 µM) was included in the solubilization mixture. The solubilized preparation was then centrifugated at 105,000 x g for 90 min to pellet insoluble materials. The solubilized material (1 mg protein/mL) was incubated with the specific purified antibodies PC11, PC21 and anti-adenosine deaminase (40 µg/mL) in TSA buffer which included 1% Nonidet P-40. Nonspecific immunoprecipitation was performed by using the same amount of a rabbit anti-mouse IgG. Immune complexes were allowed to form overnight at 4°C with constant rotation. Protein A-Sepharose CL-4B (40 µL) was then added and incubated for 2 hr, at 4°C with constant rotation. Immunoprecipitates were washed once with 1% Nonidet P-40 in TSA buffer and once with 0.1% Nonidet P-40 in TSA buffer. Samples were incubated with 60 µL of 10-fold concentrated SDS-PAGE sample buffer and treated for 10 min at 100°C. SDS-PAGE was performed as described above.

RESULTS
The modification by ADA of the binding of synthetic agonists to A₁R has been explained assuming that the enzyme degrades the endogenous ligand, adenosine (Linden 1989). The level of endogenous adenosine in a preparation of pig brain membranes, determined by HPLC (see Methods) is very low (less than 25nM). Therefore, in this system, the presence of ADA should not affect the binding of the agonist binding to the receptor. To analyze whether this was true, the effect of ADA upon agonist and antagonist binding to A₁R was
assayed in pig cerebral cortex membranes. Either the agonist ([3H]R-PIA) or the antagonist ([3H]DPCPX) binding was enhanced by ADA, with EC50 values of 0.67 mU/ml for agonist binding and 1.51 mU/ml for antagonist binding (Figure 1).

![Graph showing the effect of adenosine deaminase on agonist ([3H]R-PIA) and antagonist ([3H]DPCPX) binding to pig brain cortical membranes.](image)

The specificity of ADA in enhancing agonist binding was tested by determining the 1 nM [3H]R-PIA binding in the absence (0.18 pmol/mg protein) and in the presence of bovine γ-globulins (μg/mL): 0.13 (0.23 pmol/mg protein), 1.3 (0.22 pmol/mg protein) or 13 (0.19 pmol/mg protein). Thus the presence of γ-globulins in a concentration up to 100 fold greater than that of ADA, had any effect in agonist binding.

The equilibrium binding analysis of [3H]R-PIA to crude membranes in the absence of ADA was consistent with the existence of a single low-affinity state (Kd = 4.1 nM). Addition of ADA did not modify Bmax but resulted in the conversion of 37% of the low-affinity state into a high-affinity site (Kd = 0.22 nM, Table 1). The Scatchard plot of [3H]R-PIA binding in the absence of ADA is linear, whereas that obtained in the presence of the enzyme indicates the existence of two components for the binding (Figure 2). Thus, the classical distribution of A1R into two affinity sites, one uncoupled (low-affinity) and the other coupled (high-affinity) to heterotrimeric G proteins, is only evident when ADA is present in the assays. It is unlikely that endogenous adenosine is responsible for the disappearance of the high-affinity component of A1R. In fact, ADA was still able to induce a 29% increase in the binding of 0.2nM [3H]R-PIA to membranes which were preincubated with 0.2U/mL ADA and thoroughly washed afterwards; adenosine was not detected in the binding medium by HPLC.

![Graph showing the effect of adenosine deaminase on agonist ([3H]R-PIA) and antagonist ([3H]DPCPX) binding to pig brain cortical membranes.](image)

**TABLE 1. Effect of adenosine deaminase, active or inhibited by DCF and Hg2+, on the [3H]R-PIA equilibrium binding parameters.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>4.1±0.5</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>+</td>
<td>2.5±0.7</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>-</td>
<td>2.4±0.2</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>+</td>
<td>3.4±0.5</td>
<td>0.3±0.1</td>
</tr>
</tbody>
</table>

Binding parameters were derived from saturation experiments carried out with [3H]R-PIA in the absence or in the presence of 0.02 U/ml adenosine deaminase (active or inactivated by preincubation with 0.1 μM DCF or 10 μM Hg2+). Data were fitted to one or two affinity-state model by a non-linear regression program (see Materials and Methods). Values ± SD given by the regression program.
FIG. 2. Scatchard plots of the [3H]R-PIA computer-derived specific binding data to pig brain membranes in the presence or in the absence of adenosine deaminase. Binding of [3H]R-PIA to cortical membranes was performed without (A) or with (B) adenosine deaminase 0.02 U/ml as described in Materials and Methods. All points represent the mean of four replicateds. The data were fitted using a nonlinear regression program to one-site model (A) or two-sites model (B). In the first case there was no significant improvement after considering the two-site model (see Materials and Methods).

These results suggested that ADA might modulate agonist binding to pig brain membranes in a catalytic-independent manner. To analyze whether this explanation was correct, [3H]R-PIA binding experiments were carried out in the presence of either fully active ADA or enzymatically inactive ADA. The inhibitors used were, R-deoxycoformycin and Hg²⁺, each of which completely blocked the enzymatic activity at the concentrations used. The ADA-dependent increase of [3H]R-PIA binding was seen in the case of fully active ADA or enzymatically inactive ADA. The inhibitors used were, R-deoxycoformycin and Hg²⁺, each of which completely blocked the enzymatic activity at the concentrations used. The ADA-dependent increase of [3H]R-PIA binding was seen in the case of fully active enzyme and also in the case of Hg²⁺-inhibited ADA (Figure 3). In the presence of Hg²⁺-inhibited ADA it was possible to detect the two affinity states of A₁R, with equilibrium binding parameters similar to those obtained using active ADA (Table 1). Hg²⁺ by itself did not have any effect on [3H]R-PIA binding to membranes. Therefore, Hg²⁺ was able to block activity without affecting the ADA-induced appearance of the high-affinity form of A₁R.

Interestingly, R-deoxycoformycin, the irreversible inhibitor of the enzyme, prevented the appearance of the second affinity state since the results obtained were similar to those found in the absence of ADA (Table 1). Thus, R-deoxycoformycin blocked both the enzyme activity and the appearance of the high-affinity form of the receptor. This effect was probably due to the fact that deoxycoformycin prevents the interaction between ADA and membranes (See below). In order to modify agonist binding to A₁R, ADA must interact with membranes. Iodinated ADA binds to pig brain cortical membranes with a Kᵢ value of 230 nM and a Bₘₐₓ of 2.9 pmol/mg protein (Figure 4). In these membranes an interaction between ADA and A₁R occurred, as demonstrated by
**FIG. 4.** Equilibrium binding isotherm of [125I]ADA to pig brain cortical membranes. Membranes (0.75 mg/ml) were incubated with [125I]ADA in Tris-HCl 50 mM buffer, pH 7.4, at 25°C as described in Materials and Methods. Nonspecific binding was determined by the presence of 200 fold unlabelled adenosine deaminase. Data points are mean ± SEM of four replicates.

Insert: Scatchard plot of the computer-derived specific binding data. The plot was linear, with a \( K_d \) of 230±30 nM and a \( B_{max} \) of 2.9±0.2 pmol/mg protein. B means specific binding and F means free ligand concentration.

coimmunoprecipitation. We have developed two different antipeptide anti-A,R antibodies (PC11 and PC21) which immunoprecipitate pig brain cortex A,R photolabelled with [125I]R-AHPIA and recognize the 39 kDa band of the receptor by western-blotting (Ciruela et al., 1995). Immunoprecipitation of A,R from detergent extracts using a specific antibody (see Methods) also yielded the 44kDa band corresponding to ADA (Figure 5 A). The same kind of experiment performed with Hg\(^{2+}\)-inhibited ADA led to the coprecipitation of ADA and A,R. In contrast, the band of coprecipitated ADA disappeared when deoxycoformycin was used in the assays (Figure 5 B). Thus, deoxycoformycin prevents the interaction between the enzyme and the receptor. On the other hand, an ADA-Sepharose column retained 62% of the \( [3H]R\)-PIA binding sites of membrane detergent extracts, whereas there was no retention of lactate dehydrogenase, which was used as control protein (Figure 6 A). Elution of the A,R molecules retained in the column, as shown by immunoblotting (Figure 6 B), was only possible using SDS-containing buffers. These results clearly demonstrate that an interaction between ADA and A,R occurs in pig brain cortical membranes.

**DISCUSSION**

Reportedly, there are a number of membrane preparations that continuously produce endogenous adenosine (Linden 1989, Prater et al., 1992). The origin of this adenosine is not
known but it may come from the breakdown of endogenous adenine nucleotides bound to membranes or trapped in vesicles. For this reason, the experiments of ligand binding to adenosine receptors have always been performed in the presence of ADA. ADA should not be necessary in systems, such as the one described here, in which the endogenous adenosine concentration is very low. Thus, the ADA-induced modification of the equilibrium parameters of ligand binding to AₐR in brain membranes must be due to a catalytic-independent effect of the enzyme on the receptor. Indeed, in this report we have demonstrated, by coprecipitation and affinity chromatography, that ADA interacts with AₐR from pig brain cortical membranes and that the interaction results in the appearance of the high-affinity form of the receptor. The enzymatic activity of ADA was not involved in the appearance of this high-affinity form, since the same effect was achieved by Hg²⁺-inhibited ADA.

Thus, endogenous adenosine in this system does not play any role in the ADA-induced changes of AₐR equilibrium binding parameters. This may not be a particular case of the pig-brain preparation, since we have obtained very similar results working with the smooth muscle cell line DDT₄MF-2 (data in preparation).

It is interesting to note that, of the three potent inhibitors of the deaminase, Hg²⁺ was the only one capable of maintaining the ADA-induced potentiation of ligand binding to AₐR. R-deoxycoformycin is a transition-state analog of adenosine deaminase that alters the conformation of the enzyme. In fact, the X-ray structure of a complex between adenosine deaminase and the transition-state analog, 6-hydroxyl-1,6-dihydropurine ribonucleoside, indicates that the compound is almost completely inaccessible to the solvent. Thus, the movement of one or two loops of the enzyme is necessary for allowing access of the analog to and from the active site. On the other hand, Hg²⁺ probably blocks activity by reacting with some

FIG. 6. Chromatography of membrane detergent-extracts through an ADA-Sepharose column. Panel A. Pig brain detergent-extracts (1 mg protein/ml) were applied to an ADA-Sepharose column (570 U/ml) as described in Materials and Methods. After the application, the column was exhaustively washed and all fractions were assayed for [³H]R-PIA binding and lactate dehydrogenase activity. Data are given as percentage of the total enzyme activity (1.66 umol/min) or the specific [³H]R-PIA binding (4.4 pmol) applied to the column.

Panel B. Immunoblot after SDS-PAGE of the crude extract and of the retained fraction; the filter was developed using an antibody against AₐR (PC21). Arrow: AₐR.
sulfhydryl group, which is essential for enzyme activity but without affecting the conformation of the enzyme. This explanation fits with the results obtained from coprecipitation experiments (Figure 5 B) in which Hg\(^{2+}\) does not affect the interaction between ADA and A\(_R\), whereas deoxycoformycin prevents it. The permanent conformational change induced by the transition-state inhibitor of the enzyme would then result in the disruption of the ADA- A\(_R\) interaction. The same may be applied for the ground-state inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine.

An obvious role for ecto-ADA is to degrade the extracellular adenosine, thus controlling the amount of nucleoside available to A\(_R\). The demonstration of measurable effects due to the binding of ecto-ADA to cell surface proteins open new perspectives in the search for new, catalytic-independent roles for ecto-ADA. Catania et al., (1991) have demonstrated in cerebellar neurons that ADA, in a catalytic-independent manner, leads to large increases in the influx of \(^{45}\)Ca and hydrolysis of polyphosphoinositides. A\(_R\) were not involved since the effect, which might be secondary to a release of excitatory amino acids, was antagonized by glutamate receptor antagonists, but not by agonists or antagonists of adenosine receptors. Taking these data into account, it may be suggested that a role for ecto-ADA may be the facilitation of signalling events by a number of different pathways.

Aknowledgements: Supported by a joint (Echevarne Foundation and Spanish Ministry of Education) PETRI Grant (PTR92/0047) and by Grants from Fondo de Investigaciones Sanitarias de la Seguridad Social (no. 87/1389 and 91/0272), from CICYT (PB94/0941) and from CIRIT-DGICYT (QFN93/4423).

REFERENCES


Franco R, Canela EI, and Bozal J (1986):


Journal of Neurochemistry (In the press).
B.5. Adenosine deaminase affects signalling by interacting with cell surface adenosine receptors


*FEBS Letters* (Accepted).
Adenosine deaminase affects signaling by interaction with cell surface adenosine receptor.


Department de Bioquímica i Biologia Molecular. Facultat de Química. Univ. de Barcelona. Martí i Franquès 1. 08028 Barcelona. Catalonia. Spain.

Abstract: Adenosine deaminase (ADA) is not only a cytosolic enzyme but can be found as an ectoenzyme. At the plasma membrane, an adenosine deaminase binding protein (CD26, also known as dipeptidylpeptidase IV) has been identified but the functional role of this ADA/CD26 complex is unclear. Here by confocal microscopy, affinity chromatography and coprecipitation experiments we show that A₁ adenosine receptor (A₁R) is a second ecto-ADA binding protein. Binding of ADA to A₁R increased its affinity for the ligand, thus suggesting that ADA was needed for an effective coupling between A₁R and heterotrimeric G proteins. This was confirmed by the fact that ADA, independently of its catalytic behaviour, enhanced the ligand-induced second messenger production via A₁R. These findings demonstrate that, apart from the cleavage of adenosine, a further role of ecto-adenosine deaminase on the cell surface is to facilitate the signal transduction via A₁R.

Key words: Adenosine receptor. Adenosine deaminase. Protein-protein interaction. Signal transduction. Molecular recognition.

1. Introduction

Adenosine, acting through specific receptors on the cell surface, is capable of exerting multiple physiological actions in a variety of cell types [1-3]. All known adenosine receptors are heptaspanning macromolecules coupled to heterotrimeric G proteins. A₁ adenosine receptors (A₁R) display two different affinity states that depend upon the coupling to heterotrimeric G proteins; coupled receptor-G protein complexes display high affinity (Kd = 0.1-0.2 nM), whereas uncoupled receptors display low affinity (Kd = 1-2 nM) [4-6].

A₁R-mediated signaling depends on the effective concentration of extracellular adenosine, whose regulation involves a variety of nucleoside transport system and ectonucleotidases [7]. Since we discovered adenosine deaminase (ADA, E.C. 3.5.4.4.) on the surface of hematopoietic cells [8], ecto-ADA has also been implicated in controlling the extracellular concentration of the nucleoside. ADA binds to the cell surface of T lymphocytes through the activation marker CD26 [9], which is also known as dipeptidylpeptidase IV or ADA binding protein. In this report, a close interaction between ADA and the A₁ adenosine receptors (A₁R) present on DDT-MF-2 cells is demonstrated by immunoprecipitation, confocal microscopy and affinity chromatography. Here we also provide evidence that the interaction ADA-A₁R modulates ligand binding to A₁R and signaling via A₁R.

2. Materials and methods

2.1. Materials

[^3H]R-N⁴-(2-phenylisopropyl)-adenosine ([^3H]R-PIA) (36Ci/mmol), myo-[^3H]-inositol (85 Ci/mmol), and ECL immunoblotting detection system were purchased from Amersham (Nuclear Iberica, Madrid, Spain). Adenosine deaminase (ADA, E.C. 3.5.4.4.) from Sigma Chemical Co. (St. Louis, MO, USA) was chromatographed through a Sephadex G-25 column and the final preparation was homogeneous by electrophoresis. Rabbit anti-ADA antibody (Serotec,
FEBS Letters (Accepted).

Oxford, U.K.) has been developed in our laboratory [8]. Antibodies against A1 adenosine receptor, PC11 and PC21, are, respectively, affinity purified (chromatographed through specific peptide coupled to Shepharose) versions of antipeptide antisera PC10 and PC20 developed and characterized as described elsewhere [10]. The antibody against CD26 (H12 monoclonal) was purchased from Endogen Inc. DDT, MF-2 smooth muscle cells from hamster vas deferens were obtained from and cultured as recommended by the American Type Culture Collection.

2.2. Enzyme activities and protein determination

Ecto-adenosine deaminase (ADA, E.C. 3.5.4.4.) and dipeptidylpeptidase IV (CD26, E.C. 3.4.14.5.) activity were estimated as described by Franco et al., [11] (in the presence of 1 μM dipyridamole and 1 μM nitrobenzylthioinosine as adenosine transport inhibitors) and Nagatsu et al., [12] respectively. Protein was measured as described by Sorensen and Brodbeck [13].

2.3. Immunostaining experiments

For confocal microscopy analysis, cells were fixed (4% paraformaldehyde) for 15 min and washed in PBS-20 mM glycine. Double immunofluorescence staining was performed after 30 min incubation with PBS-20 mM glycine-1% bovine albumin serum (buffer B) by applying a mixture of antibodies (60 min, 37°C, buffer B): fluorescein-conjugated rabbit anti-A1R (PC21 antibody, 100 μg/mL) and rhodamine-conjugated rabbit anti ADA (40 μg/mL) or rabbit anti ADA (20 μg/mL) and monoclonal anti-CD26 (20 μg/mL) or rabbit PC21 (40 μg/mL) and monoclonal anti-CD26 (20 μg/mL). Rinsed coverslips were mounted with immunofluorescence medium. When fluorescence-conjugated antibodies were not used, a further incubation with the suitable secondary antibody (anti-rabbit IgG-fluorescein (1:50 in buffer B) or anti-mouse IgG.rhodamine (1:50 in buffer B)) was performed (45 min, 37°C). Observations were performed with a Leica TCS4D confocal scanning laser microscope.

Immunoprecipitation of A1R cross-linked to iodinated (R)-2-azido-N2-p-hydroxy-PIA (R-AHPIA), autoradiography and immunoblotting were performed as described by Ciruela et al., [10].

2.4. Radioligand binding assay

Saturation analysis of [3H]R-PIA binding to membranes (0.7 mg/mL) was performed as previously described [14]. Experiments using intact cells (1.5 million/mL), were performed at 4°C for 4 hr in serum-free DMEM buffered with 20 mM HEPES, pH 7.4. Saturation isotherms were obtained from five replicates for each [3H]R-PIA concentration (8 in the range 0.01-200 nM) and were fitted by non-linear regression analysis. The F-test was used to discriminate between two affinity states and one affinity state. The two-site model was selected when P<0.001 [14].

2.5. Second messengers determination

Ca2+ released from intracellular stores and the level of inositol phosphates (InsP) were measured as described elsewhere [15-17]. In cells (106 cells/mL) loaded with the fluorescent dye Fura 2-AM (5 μM, 30 min), the 50 nM R-PIA-induced Ca2+ mobilization, was determined in a dual-wavelength fluorimeter by using the ratio of excitation wavelengths 334/366 nm with emission cut off at 500 nm. For inositol phosphates determination, inositol-starved cells (0.5 million/mL) were incubated with DMEM containing myo[3H]inositol (5 μCi/mL) for 40-48 h. After addition of 10 mM LiCl (10 min), inositol phosphate production was induced (15 min) by 50 nM R-PIA. Cells were treated with perchloric acid and total [3H]InsP was isolated using a Dowex AG1-X8 column.

3. Results and discussion

DDT, MF-2 cells exhibit ecto-ADA. When 60 μM adenosine was used as substrate, ecto-ADA activity (see Methods) was 1.0 mU per million of intact cells. DDT, MF-2 cells also express on their surface binding sites for the A1 adenosine receptor specific agonist [3H]R-PIA (See table 1). These cells therefore provide a unique model to study potential interactions between the receptor for a ligand (adenosine) and the ecto-enzyme responsible for ligand degradation. As judged by cell surface immunofluorescence staining with rhodamine isothiocyanate-conjugated anti-ADA and fluorescein isothiocyanate-conjugated anti-A1R, the cell surface distributions of ADA (red fluorescence) and A1R (green fluorescence) on the surface of DDT, MF-2 cells appeared similar (Figure 1 A, B). Further analysis by confocal microscopy demonstrated that the degree of colocalization between ADA and A1R on the surface of DDT, MF-2 cells was very high. The intensity of white and its situation far from the axis origin of the cytofluorogram.
Fig. 1. Distribution of A,R, ADA and CD26 on the DDT,MF-2 cells surface. Cells were fixed (4% paraformaldehyde) for 15 min and washed in PBS-20mM glycine. Double immunofluorescence staining was performed as indicated in the Experimental Section.

Panel A. Surface expression of A,R (left), ADA (right). Vertical section (x-z), objective 1.4/100x.

Panel B. Surface expression of A,R (upper left), ADA (upper right).

Panel C. Cells on glass coverslips were preincubated with 2U/mL of exogenous ADA (30 min) before fixation (ADA from Sigma was chromatographed-Sephadex G-25; the final preparation was homogenous by electrophoresis). Surface expression of A,R (upper left) and ADA (upper right).

Panel D. Surface expression of CD26 (upper left), ADA (upper right).

Panel E. Surface expression of CD26 (upper left), A,R (upper right).

In B, C, D, E the lower left image corresponds to the superposition of the two fluorescences (yellow) whereas the bottom right image shows colocalisation in white (insert: multi-color analysis of confocal images). Scale bar: 10 μm.

Middle section (x, y), objective 1.4/100x.
indicated that the colocalization was quantitatively very high (Figure 1 B, insert of the bottom right image). Since DDTjMF-2 cells also express CD26 on their surface (dipeptidylepeptidase activity using 1mM gly-pro-paranitroanilide as substrate was 3.4mU per million cells), the codistribution of ADA (red fluorescence) and CD26 (green fluorescence) (Figure 1 D) and of CD26 (green fluorescence) and A, R (red fluorescence) (Figure 1 E) were studied. In contrast to the codistribution of ADA and A, R, ADA and CD26 or A, R and CD26 were distributed independently with a low degree of colocalization (in white). Taken together, these colocalization studies suggested that there were significantly more ADA molecules colocalizing with A, R than with CD26; therefore, A, R may act as a second receptor for ecto-ADA.

Next, we examined whether exogenous ADA could bind to cell surface A, R molecules that did not colocalize with endogenous ADA, i.e. those labelled green in the images of Figure 1B. Indeed, addition of exogenous ADA led to the complete disappearance of the green fluorescence and to an increase in white in the confocal images, which indicated increased colocalization (Figure 1C). Thus, all A, R molecules present on the surface of DDTjMF-2 cells can bind ADA. We conclude that an interaction ADA-A, R occurs at the cell surface level in intact cells. The ability of ADA to recognize A, R was confirmed by isolating the A, R from a membrane detergent extract (obtained as described in [14]) with an ADA-Sepharose affinity column. A, R was 67% retained within the column, whereas a control protein, lactate dehydrogenase, was completely eluted. The receptor, which was strongly bound to ADA-Sepharose, was eluted with a sodium dodecyl sulfate-containing buffer.

RESULTADOS 108
Conversely, ADA was coprecipitated from membrane extracts using two (PC 11 and PC21) different antipeptide anti-AjR antibodies (Figure 2 A). Both antibodies immunoprecipitated AjR photolabelled with $^{[125]}$I-R-PIA and both recognized the 39kDa band of the receptor by Western blotting (Figure 2 B). PC11 was more effective in immunoprecipitation and Western blotting experiments than PC21. For this reason the coprecipitated ADA band was stronger when PC 11 was used (Figure 2 A). These results support the notion that AjR binds ADA on the surface of DDT,MF-2 cells.

### TABLE 1.

Equilibrium parameters of $[^3H]$R-PIA binding to DDT,MF-2 cells and to cell membranes in the absence or presence of ADA.

<table>
<thead>
<tr>
<th>Presence of ADA</th>
<th>Affinity-state</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td>High-affinity</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>None</td>
<td>Low-affinity</td>
<td>50±10</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>0.2 U/mL</td>
<td>Low-affinity</td>
<td>8.7±1.6</td>
<td>0.15±0.05</td>
</tr>
<tr>
<td>High-affinity</td>
<td>0.79±0.1</td>
<td>0.28±0.03</td>
<td>—</td>
</tr>
<tr>
<td>0.2 U/mL</td>
<td>Low-affinity</td>
<td>8.7±1.6</td>
<td>0.15±0.05</td>
</tr>
<tr>
<td>High-affinity</td>
<td>1.5±0.5</td>
<td>0.25±0.05</td>
<td>—</td>
</tr>
<tr>
<td>0.2 U/mL</td>
<td>Low-affinity</td>
<td>9.2±2</td>
<td>0.13±0.07</td>
</tr>
<tr>
<td>High-affinity</td>
<td>1.5±0.5</td>
<td>0.25±0.05</td>
<td>—</td>
</tr>
<tr>
<td>0.2 U/mL plus Hg$^{2+}$</td>
<td>Low-affinity</td>
<td>10±6</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>Intact Cells</td>
<td>High-affinity</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>None</td>
<td>Low-affinity</td>
<td>40±10</td>
<td>0.1±0.03</td>
</tr>
<tr>
<td>0.2 U/mL</td>
<td>Low-affinity</td>
<td>10±6</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>High-affinity</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.2 U/mL plus Hg$^{2+}$</td>
<td>Low-affinity</td>
<td>10±3</td>
<td>0.15±0.02</td>
</tr>
</tbody>
</table>

*ADA in 50 mM Tris-HCl buffer, pH 7.4, was incubated for 2h with 100 μM HgCl$_2$. Free Hg$^{2+}$ was eliminated by gel filtration using Sephadex G-25. The eluted protein (Hg$^{2+}$-ADA) was devoid of deaminase activity.

The complete blockade of deaminase activity, by preincubating ADA with Hg$^{2+}$, did not produce any significant changes in the $K_d$ and $B_{max}$ values obtained in the presence of ADA (table 1). On the other hand, endogenous adenosine did not affect $[^3H]$R-PIA binding to AjR. First, the concentration of adenosine in the binding assays to membranes was very low (25-40 nM detected by HPLC). Second, IC$_{50}$ values for adenosine as displacer of $[^3H]$R-PIA binding to membranes in presence of Hg$^{2+}$-inhibited ADA were very high: 129 μM (high affinity) and 2.7 mM (low affinity). Thus, this alteration in the behavior of membrane AjR in the presence of ADA is not due to the degradation of the endogenous ligand, adenosine, but to a direct effect upon AjR.

Does ADA induce signal transduction events via AjR? To address this, DDT,MF-2 cells were treated with R-PIA with or without ADA (or Hg$^{2+}$-inhibited ADA) and the release
Fig. 3. R-PIA-induced calcium mobilization and total \([^{3}H]\)InsP production in DDT, MF-2 cells.

50 nM R-PIA induced calcium mobilization (A) and inositol phosphates production (15 min) (B). Cells were incubated (30 min) in the absence (-ADA) or presence of 0.2 U/ml of adenosine deaminase, enzymatically active (+ADA) or irreversibly inhibited by 100 μM HgCl₂ (2h, room temperature) and desalted through a Sephadex G-25 (+ADA-Hg²⁺).

of Ca²⁺ from intracellular stores and the level of inositol phosphates (InsP) were measured. As shown in Figure 3 A, the R-PIA-induced increase in intracellular Ca²⁺ in the presence of exogenous ADA was five-fold higher than in the absence of the enzyme. ADA by itself did not modify the level of these second messengers. In some experiments the effect of R-PIA upon Ca²⁺ mobilization was evident only in the presence of exogenous ADA. The increase in inositol phosphates was also amplified by external ADA (Figure 3 B). Induction of both second messengers was independent of the catalytic activity of the enzyme since a similar potentiation was obtained by Hg²⁺-inhibited ADA. Thus, ADA is necessary for an efficient coupling of A₁R with the signal transduction machinery. Signalling in the absence of ADA may be due to a small proportion of A₁R molecules interacting with endogenous ADA.

Taking into account that the changes of affinity in the case of A₁R reportedly reflect changes in the coupling to heterotrimeric G proteins, we postulate that ADA is required for coupling of the A₁R to heterotrimeric G proteins in DDT₁, MF-2 cells. This is the first report providing the evidence that a heptaspanning receptor requires a cell surface protein for efficient signalling. Modulation of A₁R-mediated signal transduction by ADA-A₁R interaction opens a new perspective in the regulation mechanism of heptaspanning receptors coupled to G proteins.

Acknowledgements: Supported by a joint (Echevarne Foundation and Spanish Ministry of Education) PETRI Grant (PTR92/0047) and by Grants from Fondo de Investigaciones Sanitarias de la Seguridad Social (no. 87/1389 and 91/0272), from CICYT (PB94/0941) and from CIRIT-DGICYT (QFN93/4423). We thank...
4. References


RESULTADOS 111
FEBS Letters (Accepted).

RESULTADOS 112
B.6. Ligand-induced clustering and desensitization of A1 adenosine receptors. Involvement of Ser/Thr phosphorylation


*British Journal of Pharmacology (Submitted).*
LIGAND-INDUCED CLUSTERING AND DESSENSITIZATION OF A₁ ADENOSINE RECEPTORS. INVOLVEMENT OF SER/THR PHOSPHORYLATION

Francisco Ciruela, Carles Saura, Enric I. Canela, Josefa Mallol, Carmen Lluïs, and Rafael Franco.
Department de Bioquímica i Biologia Molecular. Facultat de Química. Universitat de Barcelona. Martí i Franquès 1. 08028 Barcelona. Spain.

By immunocytochemistry using antibodies against A₁ adenosine receptors (A₁R) and confocal microscopy, we show that stimulation of A₁R by an agonist (R-phenylisopropyladenosine, R-PIA) caused a rapid (15 min) aggregation of receptor molecules on the cell surface of DDT,MF-2 cells. Internalization of the receptor was a slower event (t₁/₂ = 12 h) that correlated with a time-dependent decrease (50 %) of cell surface [³H]R-PIA binding sites (t₁/₂ = 16 h). The half reduction of binding sites was in part (30 %) due to internalization and in part (20 %) due to the presence of cell surface receptor molecules unable to bind the ligand. Chronic exposure of DDT,MF-2 cells to 50 nM R-PIA produced a functional desensitization as deduced from second messenger production assays. The agonist effect was counteracted by the antagonist 1, 3-dipropyl-8-cyclopentylxanthine (DPCPX) which itself (50 nM, 48 h) produced an increase of A₁R surface expression. This increase did not lead to potentiation of early signal transduction events. Quantification of the cellular content of A₁R by immunoblotting and by flow cytometry in cells pretreated with 50 nM R-PIA indicate that there was a time-dependent (t₁/₂ = 38 h) down-regulation of the receptor. A rapid agonist-induced enhancement of the basal phosphorylation of the receptor was demonstrated by immunoprecipitation and autoradiography. The ligand-induced aggregation and internalization was mimicked by treatment of cells with activators of protein kinase C or A. These results indicate that Ser/Thr phosphorylation of A₁R is involved in the ligand-induced aggregation of cell surface A₁R and in the homologous desensitization of these receptors.

Introduction

The ubiquitous nucleoside adenosine exerts multiple physiological actions via specific receptors (Olah and Stiles, 1992; Tucker and Linden, 1993; Dalziel and Westfall, 1994; Palmer and Stiles, 1995) four of which (A₁, A₂a, A₂b and A₃) have been cloned (Libert et al., 1991; Stehle et al., 1992; Maenhaut et al., 1990; Zhou et al., 1992). A₁ adenosine receptors (A₁R) are functionally coupled to members of the pertussis toxin-sensitive family of G-proteins, G₁₁, G₁₂, G₁₃ and G₆ (Freissmuth et al., 1991; Munshi et al., 1991). Activation of A₁ adenosine receptors regulates the activity of several membrane and intracellular proteins such as adenyate cyclase (Londos et al., 1980), Ca²⁺-channels (Dolphin et al., 1986; Mogul et al., 1993), K⁺-channels (Böhm et al., 1986) and phospholipase C (Gerwins and Fredholm, 1992). The smooth muscle cell line DDT,MF-2 from hamster vas deferens (Norris et al., 1974) expresses the A₁R in a stable manner (Gerwins et al., 1990). Equilibrium parameters for agonist binding to these cells are similar to those found in intact tissue preparations (William et al., 1991). On the other hand, the number of receptors for each cell is high (Bmax= 0.16 pmol/mg protein, which represents 100,000 receptors/cell). All this makes these cells a suitable model to study cell biology and cell biochemistry aspects of A₁R functioning.

Chronic agonist stimulation of certain receptors induces desensitization (an attenuated response to stimulation) and down-regulation (a marked reduction in the total number of receptor molecules per cell). Among G protein coupled receptors, desensitization and down-regulation have been extensively studied in cell systems expressing beta-adrenergic receptors (Lohse et al., 1992; Freedman et al., 1995). From these studies it has been suggested that, after a short period of agonist exposure, the receptors uncouple from G-proteins due to phosphorylation events catalyzed by receptor-specific kinases (e.g., beta adrenergic receptor associated receptor kinase-1 and -2) and/or kinases regulated by second messengers.
Phosphorylation of the receptor by specific kinases increases the affinity of the receptor for cytosolic factors (arrestins) inhibiting receptor binding to G-protein, whereas phosphorylation by protein kinase A directly impairs the ability of the receptor to interact with G-protein (Lohse et al., 1992; Okamoto et al., 1991). Receptor internalization or sequestration are phenomena by which a receptor translocates to intracellular compartments, which are still poorly characterized. Ligand-induced sequestration has been described for beta-adrenergic receptors in several cell lines but the molecular mechanisms of the event are not fully elucidated (Harden et al., 1980, 1983; Lohse et al., 1990; Longabaugh et al., 1989). Usually internalization is incomplete as occurs in the case of human m3 muscarinic receptors expressed in HEK 293 cells (Yang et al., 1995). However, by means of immunocytochemical techniques, Smit et al., (1995) have demonstrated that immunoreactivity against histamine H2 receptors tagged at the N-terminus completely disappears after one hour of agonist treatment. From these assays in stably transfected HEK-293 cells there is evidence that histamine causes internalization of the receptor by a cAMP-independent pathway.

Desensitization and down-regulation of A1 and A2a adenosine receptors follow two distinct pathways in DDT,MF-2 cells (Ramkumar et al., 1991). Whereas desensitization of A2a receptors results in no change in receptor number or affinity for the ligand, desensitization of A1 receptors follows a decrease in the number of receptors in membrane preparations and seems to be mediated by receptor phosphorylation (Ramkumar et al., 1991). Further characterization of desensitization mechanisms of canine A2a adenosine receptors indicates that agonist-stimulated receptor phosphorylation may induce short term desensitization by impairing receptor G, coupling. In addition, long term desensitization is mediated by down-regulation of receptor number and up-regulation of inhibitory G proteins (Palmer et al., 1994). Here, the distribution of A1 adenosine receptors in DDT,MF-2 cells has been studied by immunocytochemistry using two anti-peptide antibodies against A1 adenosine receptors (Ciruela et al., 1995 a). Desensitization mechanisms of A1 receptors have been accurately analyzed using a set of techniques which include, confocal microscopy, ligand binding, immunoblotting and receptor phosphorylation. The results obtained are very important since they provide evidence of the agonist induction of cell surface A1 receptor aggregation and subsequent receptor internalization and desensitization. All these events are mediated by a ligand-induced increase of the basal phosphorylation of the A1 adenosine receptor.

Abbreviations used: A1,R: A1 adenosine receptors; R-PIA: R-phenylisopropyladenosine; DPCPX: 1,3-dipropyl-8-cyclopentylxanthine; PMA: Phorbol 12-myristate 13-acetate; Clo-cAMP: Chlorophenyl-cyclicadenosine 3',5'-monophosphate.

Materials and Methods

Materials.

[Adenine-2,8-3H,ethyl-2-3H]phenyl-isopropyl 1 adenosine ([3H]R-PIA) (36Ci/mmole), myo-[3H]-inositol (85 Ci/mmole), and the ECL immunoblotting detection system were purchased from Amersham (Nuclear Iberica, Madrid, Spain). [32P]orthophosphate was from New England Nuclear Research Products (Boston, MA, USA). 1, 3-dipropyl-8-cyclopentylxanthine (DPCPX) was from Research Biochemicals Inc. (RBI, Natick, MA, USA). N6-(R)-(phenylisopropyl)adenosine (R-PIA), phenylmethylsulfonl fluoride (PMSF), pepstatin, aprotinin, leupeptin, fluorescein isothiocyanate, rhodamine isothiocyanate, phorbol 12-myristate 13-acetate (PMA), chlorophenyl-cyclic adenosine 3',5'-monophosphate (Clo-cAMP), and fura 2-AM were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Calf adenosine deaminase, which was filtered through Sephadex G-25 prior to all assays, and electrophoresis reagents were obtained from Boehringer.
Mannheim (Barcelona, Spain). Sephadex G-25 fine grade and Protein A Sepharose CL4B were from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other products were of the best grade available and purchased from Merck (Darmstadt, Germany). Deionized water further purified with a Millipore Milli-Q system was used throughout.

Antibodies.

Antipeptide antisera against \( \alpha \)R were generated by immunization of female New Zealand White rabbits and characterized as described previously (Ciruela et al., 1995 a). The peptides used for immunization correspond to a 19 amino acid sequence of the third intracellular loop of \( \alpha \)R (PC 11 antibody) and a 14 amino acid sequence of the second extracellular loop of \( \alpha \)R (PC21 antibody). The antiserum generated was purified by affinity chromatography using the specific peptide coupled to Sepharose CL4B. Sheep anti-rabbit IgG-fluorescein F(ab')2 fragment and horseradish-peroxidase-conjugated goat anti-rabbit IgG were purchased from Boehringer Mannheim (Barcelona, Spain).

Cell Cultures, Membrane Preparation and Protein determination.

DDTjMF-2 smooth muscle cells, originally isolated from a steroid-induced leiomyosarcoma of Syrian hamster vas deferens (Norris et al., 1974), were obtained from the American Type Culture Collection. Cells were cultured (37°C, 5% \( \text{CO}_2 \)) in Dulbecco's modified Eagle's medium (DMEM) (Whittaker, Walkersville, NY, USA) supplemented with 5% (vol/vol) fetal calf serum and 5% (vol/vol) horse serum (GIBCO, Grand Island, NY, USA), 1% nonessential amino acids, 2 mM L-glutamine, and 1 mM sodium pyruvate. For desensitization and sequestration experiments, cells were grown in the absence or in the presence of 50 nM R-PIA and/or 50 nM DPCPX, 50 mM Cl-\text{cAMP}, or 10 nM PMA. After the indicated incubation time, cells were washed twice in PBS at 4°C, and resuspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4 containing a cocktail of protease inhibitors (1 mM leupeptin, pepstatin, aprotinin, 200 mM PMSF, and 100 mM EDTA).

Protein was measured by the bicinchoninic acid method, BCA (Pierce), as described by Sorensen and Brodbbeck (1986). Radioligand-Binding Experiment and Analysis of Binding Data.

For agonist binding to intact cells, cells pretreated as indicated above were harvested and resuspended (1 x 10^6 cells/mL) in assay medium (serum-free DMEM buffered with 20 mM HEPES, pH 7.4). Cells were preincubated with 0.2 U/mL ADA for 30 min at 4°C. Aliquots (0.1 mL) were then added to 96-well microtiter plates containing \(^{[3]}\text{H}\)R-PIA (eight concentrations in the range 0.01-200 nM, performed in triplicate), in a final volume of 0.2 mL. Nonspecific binding was defined as that occurring in the presence of 500 fold excess of DPCPX. Binding was carried out at 4°C for 4 h.

Membranes (2 mg/mL) were obtained from cells pretreated as indicated above were resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing a cocktail of protease inhibitors, and preincubated with 0.2 U/mL ADA for 30 min at 25°C. Aliquots (0.1 mL, 200 \( \mu \)g of protein) were then added to 96-well microtiter plates containing \(^{[3]}\text{H}\)R-PIA (eight concentrations in the range of 0.01-200 nM, performed in triplicate), in a final volume of 0.2 mL. Nonspecific binding was determined as described above.

In all cases, binding was stopped by a rapid filtration over glass fiber filters (Whatman GF/C filters), in a Brandel cell harvester (Brandel, Biomedical Research and Development Laboratories Inc., MD, USA). Filters were then washed in 5 mL of ice-cold PBS (in the case of intact cells) or ice-cold Tris buffer (in the case of membranes). Filters were then transferred to scintillation vials and 10 mL of scintillation fluid (Formula-989, New England Nuclear Research Products) was added. Vials were shaken overnight and counted using a Packard 1600 TRI-CARB scintillation counter with 50% of efficiency.

Data from saturation isotherms were analyzed by
non-linear regression as described elsewhere (Casado et al., 1990). The F-test was used to discriminate between two or one affinity states. The two-site model was selected when \( P<0.001 \).

**Second messenger determination.**

For phosphoinositide assay, cells (0.5-1 x 10^5/mL) were incubated (40-48 h) in inositol-free medium (DMEM supplemented with 5% horse serum and 5% fetal calf serum, both dialyzed against inositol-free DMEM) with myo-[3H]inositol (5 mCi/mL) in the absence or the presence of 50 nM R-PIA or 50 nM DPCPX. Cells were then collected, washed twice in pre-warmed (37°C) PBS containing 0.5 mM MgCl\(_2\), 0.9 mM CaCl\(_2\), and incubated (5 x 10^6 cells/mL PBS) with 10 mM LiCl containing 0.2 U/mL ADA for 10 min. The phosphoinositide production was initiated by the addition of 0.1 mM of pre-warmed PBS containing R-PIA (concentrations between 1 nM and 10 mM, performed in triplicate) and 0.2 U/mL ADA. After 30 min, the assay was ended by perchloric acid protein precipitation (30 min on ice). Neutralized samples (4 M KOH, 1 M Tris, 60 mM EDTA) were centrifuged for 20 min at 3000 x g and the aqueous phase was loaded into a poly-prep chromatography column (Bio-Rad Lab., AB, Sweden) containing 1 g of an anion exchange resin (AG 1-X; 200-400 mesh; formate form). The total inositol phosphates pool was eluted according to the method of Berridge et al., 1983, and the radioactivity was measured. The data are expressed as percentage of increase above the basal level.

For intracellular calcium measurement, cells preincubated (48 h) in the absence or the presence of 50 nM R-PIA or 50 nM DPCPX were centrifuged, washed twice in pre-warmed (37°C) PBS containing 0.5 mM MgCl\(_2\), 0.9 mM CaCl\(_2\), and incubated (5 x 10^6 cells/mL PBS) with 10 mM LiCl containing 0.2 U/mL ADA for 10 min. The phosphoinositide production was initiated by the addition of 0.1 mM of pre-warmed PBS containing R-PIA (concentrations between 1 nM and 10 mM, performed in triplicate) and 0.2 U/mL ADA. After 30 min, the assay was ended by perchloric acid protein precipitation (30 min on ice). Neutralized samples (4 M KOH, 1 M Tris, 60 mM EDTA) were centrifuged for 20 min at 3000 x g and the aqueous phase was loaded into a poly-prep chromatography column (Bio-Rad Lab., AB, Sweden) containing 1 g of an anion exchange resin (AG 1-X; 200-400 mesh; formate form). The total inositol phosphates pool was eluted according to the method of Berridge et al., 1983, and the radioactivity was measured. The data are expressed as percentage of increase above the basal level.

Immunoblotting and immunostaining experiments.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described elsewhere (Ciruela et al., 1995). For flow cytometry measurements preteated cells were harvested, rinsed in PBS and then fixed in 4% paraformaldehyde in PBS for 15 min or fixed and permeabilized (4°C, 1 h) in 70% ethanol-30%PBS. Cells were washed in PBS containing 20 mM glycine (Buffer A) to quench the aldehyde groups. After 30 min incubation in buffer A containing 1% bovine serum albumin (BSA) (buffer B), cells were incubated first with anti-A,R (PC21 or PC11, 40 µg/mL) for 1 h at 37°C, washed and stained with fluorescein-conjugated anti-rabbit IgG antibody. As control, an irrelevant rabbit IgG was used. Stained cells were analysed on an EPICS Profile flow cytometer (Coulter, Hieleah, FL). Mean fluorescein fluorescence from 5000 cells was measured, and the mean value of triplicates was presented as % of control cells.

For immunofluorescence staining, cells growing on glass coverslips were incubated in the absence or the presence of 50 nM R-PIA, 50 mM C10-cAMP, or 10 nM PMA in complete medium for 15 and 45 min. They were then rinsed in PBS, fixed in 4% paraformaldehyde in PBS for 15 min and washed in Buffer A. Immunofluorescence staining was performed after 30 min incubation with buffer B by applying a fluorescein-conjugated rabbit anti-A,R (PC21 antibody, 100 µg/mL). The antibody was diluted in buffer B and the incubation was performed for 60 min at 37°C. The coverslips were rinsed for 30 min in buffer B and mounted with immunofluorescence medium (ICN Biomedicals Inc., Costa Mesa, CA, USA).

To analyze receptor internalization by immunocytochemistry, cells growing on glass coverslips in the presence of a fluorescein-conjugated rabbit anti-A,R (PC21 antibody, 5 µg/mL), were incubated with or without 50 nM R-PIA, and 50 mM C10-cAMP, or 10 nM PMA in a complete medium for 5, 12, 24, 48 and 72 h, and then rinsed in PBS. At this step it is important to note that fluorescein-conjugated PC21, which was interacting with membrane receptor in non-fixed cells, was washed up, and only internalized fluorescein-conjugated PC21 remained in the preparation. Cells
Figure 1: Modulation by R-PIA, PMA or Clom-cAMP of the distribution of cell surface A2,R.
Cells were treated with serum-free DMEM in absence (a,b) or presence of 50 nM R-PIA (c,d), 10 nM PMA (e,f) or 50 μM Clom-cAMP (g,h) for 15 min (a,c,e,g) or 45 min (b,d,f,h). After treatment, cells were fixed (4% paraformaldehyde) for 15 min and washed in PBS-20mM glycine. The immunofluorescence staining was performed as indicated in Materials and Methods using a fluorescein-conjugated rabbit anti-A2,R (PC21 antibody, 100 μg/mL). Scale bar: 10 mm. Middle section (x, y), objective 1.4/63x.
Figure 2. Internalization of cell surface A, R induced by R-PIA, PMA or Cl–cAMP.
Cells were grown in DMEM containing a fluorescein-conjugated rabbit anti-A, R (PC21 antibody, 5 μg/mL) in the absence (a,b) or the presence of 50 nM R-PIA (c,d), 10 nM PMA (e,f) or 50 μM Cl–cAMP (g,h) for 12 h (a,c,e,g) or 24 h (b,d,f,h). After treatment, cells were fixed and mounted in immunofluorescence medium and observed by confocal microscopy. Images correspond to a horizontal section at the middle of the cell. Scale bar: 10 mm. Middle section (x, y), objective 1.4/63x.
were then fixed in 4% paraformaldehyde in PBS for 15 min and washed twice in PBS containing 20 mM glycine. The coverslips were mounted as described above.

Confocal microscope observations were made on a Leica TCS 4D (Leica Lasertechnik GmbH, Heidelberg) confocal scanning laser microscope adapted to an inverted Leitz DM IRBE microscope.

Phosphorylation and Immunoprecipitation.

Cells (50 x 10⁶), harvested and washed twice in phosphate-free DMEM, were incubated at 37°C for 2 h with 10 mL phosphate-free DMEM, 20 mM HEPES, pH 7.4 containing 1 mCi of [³²P]orthophosphate/tube, to label the intracellular ATP pool, and then washed three times in DMEM to remove unincorporated radioactivity. Loaded cells were resuspended (1 x 10⁶ cells/mL) in DMEM, supplemented with 1% (vol/vol) fetal calf serum, 1% (vol/vol) horse serum in 20 mM HEPES, pH 7.4, and incubated (2 x 10⁶ cells) with the indicated reagents. Cells were harvested and washed twice in ice-cold PBS before their disruption and solubilization in 0.5 mL of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM Na₃VO₄, 50 mM NaF, 200 mM PMSF, 100 mM EDTA, 1 mg/mL leupeptin, pepstatin, chymostatin, and antipain) containing 1% (v/v) Nonidet P-40 (1 h on an ice bath). The solubilized preparation was centrifuged at 80,000 x g for 90 min and the solubilized material (1 mg protein/mL) was processed for immunoprecipitation, SDS-PAGE and autoradiography as described elsewhere (Ciruela et al., 1995 a).

Results

Agonist-induced clustering and internalization of cell surface A₁ adenosine receptors.

The distribution of A₁ adenosine receptor (A₁R) on the DDT MF-2 cell surface was determined by immunofluorescence staining using PC21 antibody, directed against a sequence of the second extracellular loop of A₁R. The receptor distribution in nonpermeabilized cells, fixed with paraformaldehyde, was analyzed by confocal microscopy. A model of dotted tackling membrane distribution of the receptors in these cells is shown in Figure 1a and b. Following 15-min of cell exposure to the A₁R agonist R-PIA (50 nM), the intensity of staining observed at the cell surface was the same as in untreated cells. The bright punctate accumulations of antigen, suggestive of receptor clustering in some parts of the membrane, began to appear at 15 min (Figure 1c) but were more apparent after 45-min of R-PIA treatment (Figure 1d). Confocal analysis of cells using fluorescein-conjugated PC21 antibody (See Methods) also indicated internalization of the receptor in response to a chronic exposure (up to 72 h) to 50 nM R-PIA (Figure 2). Confocal microscopy permits scanning of multiple thin (~1 mm) parallel sections through individual cells and facilitates optical separation of cytosolic from plasma membrane fluorescence. No significant receptor internalization was observed after 5 h of incubation with 50 nM R-PIA. However, after 12 or 24 h, cells showed a large amount of internalized receptors in small intracellular vesicles (Figure 2 c, d). No obvious difference in receptor internalization was observed after 24 h or 72 h of R-PIA treatment. Thus, the chronic exposure of cells to the adenosine agonist R-PIA led to a time-dependent (t₁/₂ > 12 h) translocation of A₁R's to intracellular stores.

Desensitization and down regulation of A₁ adenosine receptors.

To test if there was any relationship between clustering or internalization and receptor desensitization and down-regulation, binding experiments with radioligands, and quantification of A₁R molecules by immunoblotting and flow cytometry using antibodies against A₁R were performed. Preincubation of DDT MF-2 cells with 50 nM R-PIA resulted in a time-dependent (t₁/₂ = 16 h) reduction in [³²H]R-PIA specific binding sites. The loss was evident after 1 h of preincubation but reached a maximum (~50%) at 48 and 72 h (Figure 3A). In contrast, chronic exposure of the cells to the adenosine receptor antagonist DPCPX (50 nM) resulted in an
Figure 3. Ligand-induced modulation of the A1R cell surface expression.

Panel A: Binding experiments to cells preincubated with either 50 nM R-PIA or 50 nM DPCPX. The concentration of the ligand, [3H]R-PIA, was 5 nM.

Panel B: Flow fluorocytometry of surface A1R. Untreated cells (control) or cells preincubated with either 50 nM R-PIA or 50 nM DPCPX for 48 h were washed, fixed, and analyzed as described in Materials and Methods. As a control, an irrelevant rabbit IgG was used. Left panel: mean fluorescein fluorescence from 5000 cells was measured, and the mean value of triplicates was presented as % of control cells. Right panel: Intensity of A1R fluorescence in non permeabilized cells (representative experiments).

increase in [3H]R-PIA specific binding sites that peaked (~30%) at 24-48 h of treatment (Figure 3A). Furthermore, the antagonist DPCPX (50 nM) counteracted the agonist-induced loss of binding sites. To determine whether the variation of [3H]R-PIA binding sites correlated with the presence of receptor molecules at the plasma membrane, flow cytometry experiments using the PC21 antibody against an extracellular epitope of A1R were performed in nonpermeabilized cells pretreated with the agonist, the antagonist or both. The lack of label when using the PC11 antibody, which is directed against an intracellular epitope of A1R, indicates that the plasma membrane was intact; therefore the label obtained by using PC21 antibody is a direct measurement of the number of cell surface A1R (Figure 3 B). The 30% increase in [3H]R-PIA binding sites in cells pretreated for 48 h with 50 nM DPCPX correlated with the similar increase in intensity of fluorescence at the surface level (Figure 3B, left). This is a characteristic feature for an antagonist-induced up regulation of A1R. On the other hand, there is a lack of correlation between the 50% decrease in [3H]R-PIA binding sites in cells pretreated for 48 h with 50 nM R-PIA and the 30% decrease of fluorescence intensity (Figure 3B, left). These results indicate that, after R-PIA treatment of the cells, 30% of receptor molecules disappear from the cell surface, whereas 20% of the receptor molecules remain on the cell surface but in a desensitized form, which is unable to bind ligands.

In order to determine whether the R-PIA-induced disappearance of A1R from the cell surface was due to a down-regulation of the receptor, the total number of A1R molecules present in DDT1 MF-2 cells pretreated with 50 nM R-PIA or 50 nM DPCPX was quantified by immunoblotting. After 48 h of agonist treatment a 20% decrease in A1R label was observed, whereas a 30% decrease was seen after 72 h of treatment (Figure 4A). The agonist-induced reduction in receptor molecules detected by immunoblotting was slower (t_{1/2} = 38 h) than the reduction detected by [3H]R-PIA specific binding assays (t_{1/2} = 16 h, see above). On the other hand, pretreatment of cells with the antagonist had no effect on the total number of A1R molecules detected by immunoblotting. The 20% down-regulation (reduction in the number of receptor molecules per cell) of A1Rs after 48 h of 50 nM R-PIA treatment was confirmed by flow cytometry.

RESULTADOS 122
A
digm Anti-ARM antibody used for developing was PC11. The lower part shows the electrophoretic band (36 kDa) corresponding to the AR. Quantitative densitometry of blots was performed using a Computing Densitometer (Molecular Dynamics).

Panel B: Fluorocytometry of total AR in permeabilized cells. Untreated cells (control) or cells preincubated with either 50 nM R-PIA or 50 nM DPCPX for 48 h were washed, fixed, permeabilized and analyzed by flow cytometry as described in Materials and Methods. As a control, an irrelevant rabbit IgG was used. Left panel: mean fluorescein fluorescence from 5000 cells was measured, and the mean value of triplicates was presented as % of control cells. Right panel: Intensity of AR fluorescence in permeabilized cells (representative experiment).

using permeabilized cells and any of the two antibodies, PC21 and PC11, that recognize respectively, an extracellular and an intracellular epitope of AR (Figure 4B).

Next, we examined whether the functional receptors present on the cell surface after agonist or antagonist pretreatment had the same thermodynamic characteristics as in untreated cells. Analysis of the equilibrium binding of [3H]R-PIA to membranes of untreated DDT,MF-2 cells was consistent with two affinity states for AR (Table I). After 48 h exposure to 50 nM R-PIA or 50 nM DPCPX prior to the membrane preparation, a single affinity state arises for [3H]R-PIA with a Kd value which varies between 0.6 and 0.8 nM and a reduction in the maximum binding for R-PIA-treated cells but not for DPCPX-treated cells (Table I). For intact DDT,MF-2 cells, a single affinity state is detected with a Kd value for [3H]R-PIA binding very similar to the Kd slow observed in membranes. This equilibrium constant did not change after 48 h of pretreatment with either 50 nM R-PIA or 50 nM DPCPX (Table I). On the other hand, maximum binding decreased in R-PIA-treated cells whereas it increased in DPCPX-treated cells (Table I).

![Figure 4. Down-regulation of AR in DDT,MF-2 cells.](image)

**Table 1.** Dissociation constant and maximum binding of [3H]R-PIA to membranes of DDT,MF-2 cell line and intact cells after 48 h exposure to AR agonist and antagonist.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Affinity-state</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>High-affinity</td>
<td>0.43±0.1</td>
<td>0.86±0.1</td>
</tr>
<tr>
<td></td>
<td>Low-affinity</td>
<td>8.6±1.6</td>
<td>0.45±0.07</td>
</tr>
<tr>
<td>R-PIA</td>
<td>High-affinity</td>
<td>0.79±0.1</td>
<td>0.76±0.04</td>
</tr>
<tr>
<td></td>
<td>Low-affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPCPX</td>
<td>High-affinity</td>
<td>0.63±0.7</td>
<td>1.35±0.02</td>
</tr>
<tr>
<td></td>
<td>Low-affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>High-affinity</td>
<td>7.26±1.4</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td></td>
<td>Low-affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-PIA</td>
<td>High-affinity</td>
<td>7.56±1.1</td>
<td>0.1±0.01</td>
</tr>
<tr>
<td></td>
<td>Low-affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPCPX</td>
<td>High-affinity</td>
<td>7.88±1.6</td>
<td>0.22±0.02</td>
</tr>
<tr>
<td></td>
<td>Low-affinity</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Cells were incubated with the compound indicated for 48 hr before the membrane obtencion or binding experiment.*

RESULTADOS 123
Functional consequences of desensitization

Experiments of second-messenger production were carried out to analyze the effect of desensitization on signalling via A1R. In DDT,MF-2 cells, R-PIA increased the level of [3H]inositol-phosphates and mobilized intracellular calcium in a concentration-dependent manner, with an EC50 of 7.9±1.6 nM and 11.2±1.7 nM respectively (Table II). 50 nM R-PIA pretreatment (48 h) of cells reduced the agonist-induced [3H]inositol-phosphate accumulation and calcium peak by 35% without changing the EC50 values (Table II). On the other hand, no significant changes were observed in agonist-induced second messenger production after 50 nM DPCPX pretreatment of cells (Table II). These results indicate that the DPCPX-induced appearance of A1R on the cell surface is not related to a potentiation of signal transduction events in response to a saturating concentration of agonist.

Table 2.
Stimulation of [3H]inositol-phosphates accumulation and calcium peak induction by A1R agonist R-PIA in DDT,MF-2 cells.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>IP Accumulation</th>
<th>Calcium peak induction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (nM)</td>
<td>Emax (% of control)</td>
</tr>
<tr>
<td>None</td>
<td>7.9±1.6</td>
<td>100±9.2</td>
</tr>
<tr>
<td>R-PIA</td>
<td>10±2</td>
<td>67±10</td>
</tr>
<tr>
<td>DPCPX</td>
<td>7.5±1.2</td>
<td>110±7.2</td>
</tr>
</tbody>
</table>

*After 48 h pre-incubation of the cells with the medium in presence or absence of A1R ligand, [3H]inositol-phosphates and calcium determination were performed as described in Materials and Methods.

Correlation between desensitization and phosphorylation of A1R in DDT,MF-2 cells.

To analyze whether protein phosphorylation reactions are involved in A1R desensitization, the level of phosphorylation of A1R molecules was analyzed in naive cells or in cells pretreated with agonist. Phosphorylation was induced by 50 nM R-PIA in DDT,MF-2 cells metabolically labelled with 32P in serum-free media. After the assay, A1R molecules were immunoprecipitated using the affinity purified specific anti-A1R antibody, PC11, which does immunoprecipitate photoaffinity-labelled A1R in pig brain cortex (Ciruela et al., 1995 a) and in DDT,MF-2 cells (Ciruela et al., 1995 b). The immunoprecipitate from untreated cells, analyzed by SDS-PAGE and autoradiography, gave rise to a single phosphorylated band of ~36 kDa corresponding to the A1R. No phosphorylated material was immunoprecipitated using an irrelevant rabbit IgG. Under similar conditions R-PIA induced an increase in phosphorylation, which was 2-fold after 8 h of incubation (Figure 5).

![Figure 5. Increase of basal phosphorylation of A1R induced by ligand or by activators of protein kinases.](image-url)
These data clearly show that in control cells A₅Rs are phosphorylated but that the phosphorylation is enhanced by the agonist. The agonist-induced A₅R phosphorylation could be mimicked by a PKC activator, PMA, and by a PKA activator, Clo-cAMP (Figure 5), which suggest that A₅R is phosphorylated in Ser/Thr residues. The involvement of Ser/Thr residues in the receptor phosphorylation was indirectly confirmed since an antibody against phosphotyrosine was unable to immunoprecipitate, in untreated or treated cells, any phosphorylated protein having a molecular weight similar to that of A₅R (data not shown).

As receptor phosphorylation occurs even in the absence of the agonist but it is enhanced by it, a number of assays were carried out in order to test whether receptor phosphorylation could be related to receptor clustering. For this purpose immunocytochemical detection of A₅R was performed on the surface of non permeabilized cells treated with Clo-cAMP or PMA. Both PMA and Clo-cAMP induced cell surface clustering of A₅R. The Clo-cAMP and PMA modulation was slower than that exerted by R-PIA since 15-min of cell exposure had a small effect on the distribution of A₅R (Figure 1e, g). However, bright punctate accumulations of antigen began to appear after 45 min of treatment (Figure 1f, h). In experiments performed using fluorescein-conjugated PC21 antibody (see Methods) it was possible to detect receptor internalization caused by the protein kinase A or C activators (Figure 2e, f, g, h). This internalization was almost as effective as that due to R-PIA (compare images in figure 2) thus indicating that receptor internalization is directly related to its phosphorylation. Thus, both reagents were able to mimick the R-PIA modulation of the A₅R distribution on the cell surface.

**Discussion**

In the study of G-protein-coupled receptors, attenuation of agonist binding and agonist-induced signalling is classically observed after chronic agonist stimulation of the receptor (Sibley et al., 1987; Hausdorff et al., 1990; Lefkowitz et al., 1990; Pei et al., 1995). This attenuation is known by the term desensitization and is absolutely required to achieve the refractory period which follows chronical exposure to an agonist. Although the desensitization of A₅Rs has been well characterized by means of pharmacological and molecular approaches (Ramkumar et al., 1991), the agonist-induced redistribution of receptors between the cell surface and cellular compartments, which might be inherent to the desensitization process, had not been studied. The main reason for that has been the lack of specific anti-A₅R antibodies able to detect receptor molecules by immunostaining. In this report, by confocal analysis using antibodies against A₅R, we show that agonist saturation of A₅R induced a rapid receptor clustering on the DDT,MF-2 cell surface, followed by receptor sequestration to intracellular compartments. By agonist binding as well as by flow cytometry and immunoblotting analysis we provide evidence that clustering and internalization are events related with receptor desensitization and down-regulation.

Evidence that receptor phosphorylation has a role in the desensitization of the beta₂-adrenergic receptors is well documented. Functional uncoupling of the receptor from Gₛ proteins is triggered by phosphorylation (Benovic et al., 1985, 1989, 1991; Pitcher et al., 1992; Freedman et al., 1995). In addition to this rapid functional uncoupling there is a slower event, the spatial uncoupling, which is caused by internalization or sequestration of the receptor (Harden 1980; Stachelin and Simons, 1982; Zemcik and Strader, 1988). Similarly, studies performed with the canine A₂a adenosine receptor indicate that receptor phosphorylation may induce short term desensitization by impairing receptor-Gₛ coupling; also the A₂a receptor is subjected to long term down-regulation (Palmer et al., 1994). The results presented in this paper indicates that incubation of DDT,MF-2 cells with the A₅R agonist, R-PIA, induced an increase of receptor phosphorylation (Figure 5), already observed at 15 min of agonist.
treatment but which was more evident after 8 h. The basal phosphorylation detected in nontreated cells may be due to a tonic effect exerted by the physiological ligand, adenosine, present in the cell culture medium. It should be however noted that the constitutively activated serotonin 2C receptor has a basal phosphorylation that can still be enhanced by agonists (Westphal et al., 1995). As shown by the assays using specific protein kinase A or C activators, the agonist-induced increase of A1R phosphorylation is probably due to Ser/Thr kinases. Mutation of carboxyl-terminal threonine residues in human m3 muscarinic receptor modulates the extent of sequestration and desensitization (Yang et al., 1995). Freedman et al., (1995) have demonstrated that ligand-induced phosphorylation of beta2-adrenergic receptors is due, in intact cells, to protein kinase A and beta-adrenergic receptor kinase 1. Also, agonist-dependent phosphorylation of the mouse delta-opioid receptor is caused by G protein-coupled receptor kinases but not by protein kinase C (Pei et al., 1995). On the other hand, neither protein kinase A or C are involved in homologous desensitization of human 5-HT1A receptors expressed in Sf9 cells (Nebigil et al., 1995). The early increase in A1R phosphorylation correlates well with the ligand-induced clustering of the receptor at the cell surface of DDT1-MF2 cells (Figure 1B). In fact, even in the absence of ligand there is a correlation between clustering and phosphorylation of A1R made evident by the assays performed in cells pretreated with activators of protein kinase A or C (Figures 1 and 2).

The agonist-induced time-dependent disappearance of [3H]R-PIA binding sites from the DDT1-MF-2 cell surface \( t_{1/2} = 16 \) h, Figure 3A) correlated with the agonist-induced internalization of the receptors \( t_{1/2} = 12 \) h, Figure 2) and both events seem to begin 5-7 h after R-PIA treatment of cells. This contrasts with the 15 min required for clustering and enhancement of basal phosphorylation. Receptor internalization depends on phosphorylation since activation of protein kinase A and C in the absence of agonist mimic the R-PIA induced receptor internalization (Figure 2). The disappearance of binding sites from the cell surface after 48 h of agonist treatment was only partly due to the receptor internalization, since some of the receptors are present on the cell surface in an inactive, desensitized, form. In DDT1-MF-2 cells, A1R are coupled to the phosphatidylinositol breakdown and \([Ca^{2+}]\) mobilization pathways (Gerwins and Fredholm, 1992). The diminished response, in terms of inositol-phosphate accumulation and intracellular Ca2+ mobilization, found in cells treated with 50 nM R-PIA for 48 h is a clear signal of receptor desensitization. Taken together, these data indicate that the internalized and the inactivated receptor molecules can account for the attenuation of R-PIA-induced signalling (see Table II). The agonist-induced internalization was detected by the presence of small intracellular vesicles containing fluorescent-labelled anti-A1R antibody complexes. To know whether A1R sequestering into intracellular compartments led to receptor degradation, immunoblotting assays were employed to quantify the total content of the receptor in DDT1-MF-2 cells treated or not with R-PIA. Results presented in figure 4 indicate that there is an agonist-induced loss of A1R (down-regulation) which is slower \( t_{1/2} = 38 \) h than the agonist-induced internalization \( t_{1/2} = 12 \) h, Figure 2). These findings agree with the notion that part of desensitized G protein coupled receptors are directed to lysosomes for degradation whereas the rest are recycled back to the cell surface (Lohse, 1993). A recent report by Pippig et al., (1995) evidences that dephosphorylation and recycling of sequestered beta2-adrenergic receptors, may permit receptor resensitization.

Overall, these results obtained in DDT1-MF-2 cells indicate that chronic exposure of A1R to agonists gives rise to time-dependent events including rapid A1R phosphorylation (in Ser/Thr) and aggregation on the cell surface, followed by a desensitization of the receptor (inability to recognize the ligand) and a reduced cellular
response, which occur at the same time as receptor is directed to intracellular compartments. Although induction of A_1R phosphorylation and clustering on the cell surface are events that occur prior to receptor desensitization they seem to be required for progression towards desensitization.

**Acknowledgements:** Supported by a joint (Echevarría Foundation and Spanish Ministry of Education) PETRI Grant (PTR92/0047) and by Grants from Fondo de Investigaciones Sanitarias de la Seguridad Social (no. 87/1389 and 91/0272), from CICYT (PB94/0941) and from CIRIT-DGICYT (QFN93/4423). We thank Susana and Jaume Comas Castel from Servei Científic i Tècnic for their technical assistance.

**References**


SMIT, M.J., TIMMERMAN, H., ALEWIJNSE, A.E., PUNIN, A., VAN DEN NIEUWENHOF, I., BLAUW, J., VAN MINNEN & LEURS, R.


C. DISCUSIÓN.
C. DISCUSIÓN.

La adenosina es un autocoide capaz de ejercer gran número de efectos fisiológicos al interaccionar con receptores específicos de la membrana. Su función dependerá tanto de los sistemas de transducción de la señal a los que estén acoplados sus receptores como de la concentración extracelular del nucleósido que se alcance en unas condiciones determinadas. Por ello, es de primordial interés el conocimiento de los sistemas responsables de la aparición y desaparición de adenosina extracelular, así como de los mecanismos que los regulan. Por esta razón la primera parte del trabajo, objeto de esta memoria, corresponde a la caracterización molecular del transportador de nucleósidos presente en la membrana luminal de túbulo proximal de riñón, y al estudio de los mecanismos que regulan su función.

En el momento de iniciar esta Tesis, nuestro grupo de investigación había puesto de manifiesto la existencia de enzimas localizadas en la membrana luminal de tubuladura renal de cerdo que eran capaces de producir y degradar adenosina, lo que permitía predecir la aparición transitoria de este autocoide como producto del metabolismo nucleotídico extracelular (Blanco et al., 1990, 1993). Las acciones que puede ejercer la adenosina en la tubuladura renal son mediadas por dos sistemas distintos: uno, es la acción directa sobre sus receptores específicos y el otro es su transporte hacia el interior celular para contribuir a la recuperación de nucleósidos purínicos. Ambos aspectos constituían dos líneas de investigación de interés para nuestro equipo. El túbulo proximal renal posee dos tipos básicos de transportador de nucleósidos: el dependiente de Na\(^+\), que ya había sido caracterizado en tubuladuras renales (Franco et al., 1990 b) y el independiente de Na\(^+\), del que se ocupa este trabajo y del que se han descrito dos tipos, uno sensible a la inhibición por NBTI (forma es) y el otro resistente a este inhibidor (forma ei) (ver A).

Las BBM renales de cerdo mostraron un transporte de adenosina independiente de Na\(^+\) que era insensible al NBTI, por lo tanto cabía esperar que estas membranas se comportaran como un sistema carente de centros de unión de \(^3\)H-NBTI. Sin embargo, los resultados descritos en esta memoria ponen de manifiesto que las BBM renales de cerdo poseen la capacidad de unión específica de \(^3\)H-NBTI, a centros de alta afinidad. Los parámetros termodinámicos (K\(_T\) = 1 nM y B\(_{\text{max}}\) = 1 pmol/mg proteína en BBM de cerdo) y el orden de potencia como desplazantes de diferentes inhibidores y substratos es NBTI>dilazep>dipiridamol>inosína=adenosina>uridina, son los típicos para un transportador independiente de sodio y sensible a NBTI (tipo es) descrito por varios autores (Wu & Phillis, 1982; Geiger et al, 1985; Heaton & Clanachan, 1987; Marangos & Deckert, 1987). La identificación de estos centros de unión con receptores de adenosina se descartó al observar la poca afinidad que mostraban por diferentes antagonistas y agonistas de los receptores, observándose mayor afinidad para los ligandos del subtipo A\(_1\) del receptor, como se había descrito previamente para el transporte de \(^3\)H-uridina en eritrocitos de cobaya (Balwierczak et al, 1989). Por otra parte, la baja afinidad de los centros de unión de \(^3\)H-NBTI por la citocalsina B y la glucosa indica que el transportador de glucosa no interfere en la caracterización del transportador de adenosina, como se había sugerido en otros sistemas, en los que el \(^3\)H-NBTI presenta cierta afinidad por el transportador de glucosa. Nos encontrábamos, por lo tanto, ante la paradoja de la existencia de un transportador de adenosina que podía unir NBTI, pero que transportaba adenosina de manera insensible al NBTI. Cuando se analizó detenidamente la cinética del transporte de adenosina en las BBM renales de cerdo, se observó que el valor de K\(_T\) era superior a 700 μM. Este valor es muy elevado comparado con la cinética de incorporación de adenosina por los transportadores de tipo es (Plagemann et al., 1988). Ello
pone de manifiesto que el transportador que une NBNTI en BBM renales de cerdo no es funcional para el transporte de adenosina, y que la incorporación de $[\text{H}]$adenosina en las BBM es mediada por un proceso de difusión simple, como corresponde a la práctica linealidad de las curvas de saturación. Lógicamente, la difusión simple de adenosina no es inhibible por NBNTI.

Se procedió a la caracterización molecular del transportador para averiguar si presentaba alguna anomalía que pudiera explicar su incapacidad para transportar adenosina. La solubilización del transportador se ha llevado a cabo usando CHAPS como detergente en presencia de un 20% de glicerol y 20 μM de adenosina. La eliminación de los dos últimos compuestos mediante una columna de filtración molecular y la reducción de la concentración de detergente (CHAPS) por debajo de su concentración micelar crítica (CMC=8 mM) es esencial para la recuperación de niveles altos de unión de $[\text{H}]$NBNTI en la fracción solubilizada. El transportador solubilizado muestra un orden de potencia de los diferentes ligandos que desplazan la unión de $[\text{H}]$NBNTI, similar al encontrado en membranas intactas, si bien la afinidad por $[\text{H}]$NBNTI es, en promedio, unas tres veces menor. Por otro lado, tanto la glucosa como la citocalasina B, potente inhibidor del transporte de glucosa, poseen una $K_i$ elevada para el desplazamiento de $[\text{H}]$NBNTI, indicando de esta manera que el transportador de glucosa solubilizado conjuntamente al de adenosina no interfiere en la caracterización del transportador de nucleósidos.

El NBNTI es un compuesto fotoactivable con capacidad de unirse covalentemente a una amina primaria, por lo que resulta útil en estudios de marcoje por fotoafinidad. En BBM se produce la unión covalente específica de $[\text{H}]$NBNTI a una proteína de 65 kDa de peso molecular, determinado por electroforesis en SDS-PAGE y autorradiografía. Este peso molecular para el transportador es similar al descrito para el transportador de nucleósidos en eritrocitos de cerdo (Kwong et al, 1987), y superior al descrito para el transportador de eritrocitos humanos (40-55 kDa, Kwong et al, 1988; Kwong et al, 1992), células tumorales ascites Ehrlich (42 kDa, Hammond & Johnstone, 1989) o células cromafines (51 kDa, Torres et al, 1988; 1992). La banda de proteína fotomarcada con $[\text{H}]$NBNTI desaparece únicamente por competencia con adenosina, NBNTI, dipiridamol y uridina, mientras que ni la citocalasina B ni la glucosa tienen efecto sobre la incorporación específica de radiactividad en dicha banda, lo cual confirma que se trata del transportador de nucleósidos. La masa molecular del transportador nucleosídico de BBM de cerdo no depende de la presencia de inhibidores de proteasas, pero sí que depende del tratamiento térmico de las muestras previo a la electroforesis. Así pues, el tratamiento a 100°C durante un minuto rinde una única banda de 65 kDa, mientras que un tratamiento más suave, 37°C durante una hora, permite identificar, además, bandas cuya masa molecular corresponde a la presencia de dímeros y tetrámeros del transportador fotomarcado. La existencia de estas asociaciones oligoméricas ya había sido puesta de manifiesto en eritrocitos humanos por estudios de radiación-inactivación (Jarvis et al, 1984) y en células cromafines por estudios similares de fotomarcado (Torres et al, 1988; 1992). El tipo de tratamiento necesario para la aparición de dímeros indica que la interacción entre monómeros es de tipo hidrófobo. La importancia fisiológica de la existencia de estos dímeros o tetrámeros no ha sido explicada hasta el momento, si bien se sugiere que estas asociaciones pueden conferir algunas particularidades cinéticas al transportador (Torres et al, 1992).

La carga iónica del transportador se determinó analizando su comportamiento al cromatografiarlo a través de columnas de intercambio iónico previo fotomarquaje y solubilización con un detergente no iónico (Tritón X-100). Había sido descrito que el transportador de eritrocitos de cerdo eluye en una columna de DEAE-cellulose a una concentración salina de entre 80 y 110 mM (Kwong et al, 1986), mientras que el transportador procedente de eritrocitos humanos eluye en el volumen muerto de la columna (Jarvis & Young, 1981). El comportamiento DISCUSIÓN 132
cromatográfico del transportador fotomarcado de BBM renales de cerdo es, sorprendentemente, diferente del de eritrocitos de la misma especie y similar al de eritrocitos humanos, es decir, no queda retenido en una columna de DEAE-Sepharose a pH 8,3. Esta diferencia de comportamiento entre el transportador nucleósido de diferentes tejidos (riñón y eritrocito) de una misma especie (cerdo) puede ser debida a la existencia de dos genes diferentes, dos productos de diferentes splicings o modificaciones postranscripcionales selectivas de cada tipo celular tales como variaciones en la composición de carbohidratos de la glicoproteína. De acuerdo con la última hipótesis, la eliminación de los N-oligosacáridos por tratamiento del transportador fotomarcado de BBM renales de cerdo con endoglicosidasa F rinde, en ambos casos, riñón y eritrocitos, una única proteína de 57 kDa (Kwong et al 1986). La posibilidad de que existan diferencias entre la composición de las cadenas de oligosacáridos del transportador de ambas procedencias estaría de acuerdo con los datos reportados por Hammond & Johnstone (1989), que demuestran diferencias en la glicosilación para transportadores de diferentes tipos celulares. Ello podría explicar la diferencia de comportamiento entre el transportador de eritrocitos y el de la membrana luminal de células epiteliales de túbulo proximal que son polarizadas y presentan mecanismos postranscripcionales específicos (Simons & Wandinger-Ness, 1990).

La caracterización molecular de los centros de unión de NBTI en BBM renales de cerdo confirma que se trata del transportador de adenosina de forma es que no muestra ninguna anomalía aparente. Por lo tanto, continuábamos preguntándonos por qué este transportador es no funcional. Con objeto de investigar los motivos que provocan la aparición en BBM de transportadores capaces de unir NBTI, pero incapaces de transportar, se han elegido diferentes modelos de membranas luminales sobre los cuales se pueda estudiar, en una aproximación más "in vivo", la capacidad de inhibición que posee el NBTI sobre el transportador de nucleósidos independiente de Na⁺. Se ha tomado como modelo la línea celular LLC-PK1, procedente de túbulo proximal de riñón de cerdo. Estas células crecen en monocapa adheridas a la placa por la membrana basal, exponiendo la membrana luminal al medio de cultivo. A partir de cultivos de estas células se pueden obtener BBM, por lo que se pueden comparar los fenómenos de transporte en célula intacta y en vesículas de membranas luminales. Por otro lado, también se ha trabajado con cultivos primarios de células epiteliales de túbulo proximal de riñón de cerdo, modelo celular al que se pueden comparar los resultados obtenidos con BBM de riñón de cerdo. Tanto en BBM (obtenidas de riñón de cerdo y de células LLC-PK1) como en células intactas (LLC-PK1 y cultivos primarios) se puso de manifiesto la existencia de centros de unión de \[^3\]H\]NBTI de alta afinidad. La caracterización de estos centros de unión de \[^3\]H\]NBTI mediante los parámetros termodinámicos (\(K_D\) y \(B_{\text{max}}\)) y el orden de potencia de ligandos específicos indica que en todos casos, los centros de unión de \[^3\]H\]NBTI de alta afinidad se corresponden con el transportados de nucleósidos equilibrativo (tipo es). En células LLC-PK1 y cultivos primarios existe una correlación entre la unión de \[^3\]H\]NBTI y la inhibición del transporte de \[^3\]H\]adenosina independiente de Na⁺ por concentraciones nanomolares de NBTI. Esta correlación se pierde completamente en BBM obtenidas tanto a partir de riñón de cerdo como de células LLC-PK1, donde, a pesar de existir unión de NBTI, no hay inhibición del transporte de adenosina por este compuesto debido a la ausencia de transporte mediado de adenosina e incorporación de adenosina por difusión simple.

Otros hechos interesantes observados en estos estudios, es la pérdida concomitante de centros de unión para el \[^3\]H\]NBTI e incorporación de \[^3\]H\]adenosina en cultivos de células LLC-PK1 confluentes, de manera que las células confluentes no unen NBTI ni transportan adenosina. Lo que resulta aún más interesante, es que este fenómeno puede ser simulado por un análogo del AMPe (clorofenilAMPe), al actuar sobre células subconfluentes, pero no por un activador de la
proteína quinasa C como el PMA. Ello pone de manifiesto que el transportador presente en estas células se regula directa o indirectamente por un proceso de fosforilación dependiente de la proteína quinasa A. El transportador fosforilado pierde la capacidad de transporte de \(^{[\text{H}]}\)adenosina y de unión de \(^{[\text{H}]}\)NBTI. Una regulación por fosforilación-desfosforilación se ha descrito en otros sistemas, como células S49 (Nagy et al, 1991), fibroblastos transformados (Meckling-Gill & Cass, 1992) y células cromafines, donde la activación de las proteínas quinasa A o C provoca descensos de la capacidad de unión de \(^{[\text{H}]}\)NBTI y de la capacidad de transporte (Sen et al, 1990; Delicado et al, 1991; Miras-Portugal et al, 1991). Sorprendentemente, las BBM obtenidas a partir de células LLC-PK1 en cultivo celular confluen te, donde el transportador teóricamente estaría fosforilado, presentan centros de unión de \(^{[\text{H}]}\)NBTI de alta afinidad, por lo que se puede asumir que el transportador es desfosforilado durante el proceso de obtención de BBM, dando como resultado la reaparición de los centros de unión de \(^{[\text{H}]}\)NBTI. Los resultados obtenidos al introducir ortovanadato (inhibidor inespecífico de fosfatasa) o fosfatasa alcalina en la obtención de BBM, confirman esta hipótesis.

El proceso de fosforilación-desfosforilación comentado anteriormente podría explicar de forma satisfactoria la ausencia de unión de \(^{[\text{H}]}\)NBTI en células LLC-PK1 confluentes y la recuperación de la unión en BBM obtenidas a partir de estas células, pero no explica la falta de correlación entre la unión de \(^{[\text{H}]}\)NBTI y la no inhibición del transporte de \(^{[\text{H}]}\)adenosina en BBM obtenidas de riñón de cerdo o de células LLC-PK1. Una situación similar ha sido descrita por Gati et al, (1986), los cuales observaron que células Novikoff poseen centros de unión de \(^{[\text{H}]}\)NBTI de alta afinidad sin expresión detectable de transporte equilibrativo sensible a NBTI. Una posible explicación sería la de suponer un posible cambio en la molécula del transportador que le permitiera la unión de NBTI pero que provocase la pérdida de la capacidad de transporte de substratos. Esta hipótesis formulada por Gati et al., (1986) también puede asumirse a partir de los resultados obtenidos en este trabajo respecto al alto contenido de centros de unión de \(^{[\text{H}]}\)NBTI en BBM procedentes tanto de células confluentes como subconfluentes, donde no se observa transporte equilibrativo sensible a NBTI. El cambio en la molécula del transportador, que lo convierte en no funcional para el transporte de \(^{[\text{H}]}\)adenosina, deberá producirse probablemente en el proceso de obtención de las membranas (BBM). Este hipotético cambio, no parece implicar una acción proteolítica, puesto que, como ya se ha discutido anteriormente, no se observa ningún efecto de los inhibidores de proteasas sobre la masa molecular del transportador; además, tanto en BBM como en células LLC-PK1 el peso molecular determinada es la misma. Sin embargo, no puede quedar descartada una pequeña variación en el peso molecular no detectable mediante SDS-PAGE.

Recientemente, el grupo de Miras Portugal ha descrito que el ATP ejerce una regulación directa sobre el transportador nucleosídico incrementando la unión específica de \(^{[\text{H}]}\)NBTI y el transporte de \(^{[\text{H}]}\)uridina en células cromafines, siendo un efecto no ligado a la hidrólisis del propio ATP (Casillas et al, 1994). La falta de ATP en el interior de las vesículas de BBM (Sayós et al, 1994) puede ser la causa que origina la pérdida de capacidad de transporte. Para demostrarlo sería necesaria la medida de la unión de \(^{[\text{H}]}\)NBTI y del transporte de adenosina en presencia de ATP, pero dada la elevada actividad ATPasa de las BBM resulta prácticamente imposible realizar este experimento.

Estos resultados nos permiten concluir que las membranas de túmpulo proximal de riñón de cerdo disponen de un transportador de nucleósidos independiente de Na\(^+\) del tipo \(es\) que se regula por procesos de fosforilación-desfosforilación. Los resultados presentados completaban un objetivo del grupo de investigación que era la caracterización del conjunto de moléculas capaces de regular la concentración de adenosina presente en el medio luminal de las tubuladuras renales.

DISCUSIÓN 134
Por otra parte, es obvio que un papel fisiológico transcendental de la adenosina extracelular es el que ejerce a través de la interacción con sus receptores específicos y dentro del marco del reconocimiento molecular de la adenosina extracelular, nuestro grupo de investigación está interesado en la caracterización de receptores de adenosina. Se han descrito cuatro subtipos de receptores de adenosina: $A_1$, $A_2A$, $A_2B$ y $A_3$ (vease A). Aunque casi todos ellos están presentes en distintas zonas del riñón (Palacios et al., 1987; Weber et al., 1988; Blanco et al., 1992), las BBM renales de cerdo y la línea celular LLC-PK1 constituyen un mal sistema para estudiar receptores de adenosina desde el punto de vista farmacológico, ya que muestran muy poca afinidad por cualquiera de los ligandos agonistas y antagonistas de los distintos subtipos de receptores. Ello puso de manifiesto la necesidad de desarrollar anticuerpos que permitieran detectar la expresión de los receptores sin la necesidad de radioligandos. En el momento que se planteó esta necesidad sólo se había clonado el receptor $A_1$ de adenosina (Mahan et al., 1991), por lo que el segundo objetivo de esta Tesis fue la obtención y caracterización de anticuerpos antipeptídicos contra el receptor $A_1$.

La obtención de anticuerpos antipeptídicos contra el receptor $A_1$ ha sido un reto importante ya que, en aquel momento, no se había logrado la obtención de anticuerpos antipeptídicos de ningún receptor de la familia de receptores acoplados a proteína-G. Ello ha motivado que la caracterización de los anticuerpos se haya realizado en sistemas modelo donde se conocía la expresión elevada de receptor $A_1$ funcional, como en corteza cerebral de cerdo (Casado et al., 1990) y en la línea celular DDT,MF-2 de musculatura lisa de hamster (Gerwins et al., 1990). Los experimentos efectuados para la caracterización de los anticuerpos anti-$A_1$R en estos sistemas han dado lugar a tres hechos que consideramos de especial importancia. Uno es la existencia de forma dimérica del receptor. Otro es que la ectoadenosina desaminasa (enzima que metaboliza adenosina en el medio extracelular) es capaz de interaccionar con el receptor $A_1$ (interacción proteína-proteína) y facilitar tanto la unión de ligandos como la transducción de la señal. Finalmente, hemos puesto de manifiesto que los receptores $A_1$ de adenosina, en el proceso de desensibilización por saturación con el agonista, sufren un proceso de agregación en la superficie celular que está mediado por un proceso de fosforilación en Ser/Thr del receptor.

Basándonos en la secuencia aminoacídica del $A_1$R clonado en cerebro de rata (Mahan et al., 1991) generamos dos anticuerpos antipeptídicos (PC/10 y PC/20) los cuales reconocen dos epitopos diferentes del $A_1$R, uno localizado en el segundo "loop" extracelular del receptor y el otro en el tercer "loop" intracelular. A pesar de que estos anticuerpos fueron generados contra secuencias peptídicas derivadas de la secuencia del $A_1$R clonado en rata, el reconocimiento del $A_1$R presente en otras especies como rata, cerdo y cordero, resultó ser bastante elevado, debido en parte al elevado grado de homología que presentan estos receptores (Olah et al., 1992). La especificidad de los anticuerpos PC/10 y PC/20 se puso de manifiesto, en membranas de corteza cerebral de rata, mediante experimentos de inmuprecipitación del receptor fotomarcado con $^{[125]}$I-AHIPA y análisis por SDS-PAGE. Se observaba una banda de 39 kDa de peso molecular que también se pone de manifiesto en experimentos de western-blotting empleando el anticuerpo PC/10. Estos resultados concuerdan con la caracterización molecular del receptor descrita previamente. Klotz et al. (1985) empleando $^{[125]}$I-AHIPA obtuvieron por SDS-PAGE y autorradiografía una única banda proteica de 35 kDa de peso molecular correspondiente al receptor $A_1$ de adenosina presente en membranas de cerebro de rata. Con posterioridad, Nakata (1993) purificó a homogeneidad mediante cromatografía de afinidad el $A_1$R presente en membranas de cerebro de rata, rindiendo el purificado una única banda de 35 kDa de peso molecular que se ponia de manifiesto tanto por tinción total de proteínas en SDS-PAGE como por western-blotting empleando anticuerpos policlonales generados a partir del propio purificado.

DISCUSIÓN 135
Sorprendentemente, al emplear membranas de corteza cerebral de cerdo y el anticuerpo PC/20 en experimentos de western-blotting, a parte de la banda de 39 kDa aparecía otra banda de 74 kDa de peso molecular. Inicialmente, se pensó que la banda de elevado peso molecular era el resultado de la asociación del A,R con una proteína G, por el hecho de que el anticuerpo PC/10 no era capaz de reconocer esta banda de elevado peso molecular probablemente debido a que la proteína G estaría interaccionando con el dominio citoplasmático que reconoce el anticuerpo PC/10. Con el fin de determinar si existía algún tipo de proteína G acoplada a la banda de 74 kDa, se realizaron experimentos de western-blotting empleando anticuerpos anti proteína G, no observándose la presencia de proteínas G en esta banda de elevado peso molecular. La presencia de esta banda en membranas de córtex cerebral tratadas previamente con desestabilizadores de las interacciones receptor-proteína-G, tales como el Gpp(NH)p, la N-etilmaleimida o la toxina pertussis, elimina por completo la posibilidad de que esta banda de 74 kDa de peso molecular pueda tratarse de un complejo proteico A,R-proteína-G.

La posible naturaleza del complejo de 74 kDa empezó a vislumbrarse al conocer que algunos autores habían descrito la posible existencia "in vivo" de dímeros del receptor A t. Así, los estudios de radiación-inactivación en membranas de córtex cerebral de rata han demostrado que el tamaño del receptor A t es de 63 kDa (Frame et al, 1986), que es el doble de el peso molecular calculada a partir de la secuencia aminoacídica del receptor clonado. Empleando la misma técnica, Reddington et al. (1987) observaron que el A,R del mismo origen poseía un peso molecular de 79.500 ± 7.000 Da. Por otro lado, la formación de dímeros de receptores acoplados a proteína-G ha sido sugerida para los receptores muscarínicos M1 y M2 expresados en células SF9 (Parker et al, 1991) y para el receptor de serotonina 1β expresado en células BHK 570 (Pickering et al., 1993). Nuestros resultados están en concordancia con la existencia de dímeros de A,R, que pueden convertirse en monómeros vía la interacción con agonistas y antagonistas del receptor A, de adenosina. Todo esto sugiere la posibilidad de que estos dímeros puedan existir "in vivo" y que la agregación pueda estar regulada por la adenosina como ligando fisiológico. Sin embargo, no cabe descartar la posibilidad de que la generación de dímeros del receptor sea consecuencia del procesado de las muestras "in vitro". El análisis de la distribución de ambas formas del receptor A, en diferentes tejidos (córtex cerebral, estriado, pulmón y riñón) de diferentes especies (cerdo, rata y cordero), indica que cada fuente analizada posee un patrón característico de distribución y proporción entre la forma monomérica y dimérica del A,R.

La forma dimérica del receptor A (74 kDa), que no es reconocida por el anticuerpo PC/10 se encuentra presente tanto en las membranas de córtex cerebral como en extractos solubles de las mismas, y por el hecho de que esta forma dimérica sea resistente al tratamiento con SDS, podemos concluir que los monómeros o subunidades de receptor interaccionan por una región hidrofóbica que podría encontrarse próxima al tercer "loop" intracelular, ya que el epítopo reconocido por el anticuerpo PC/10 quedaría inaccesible en la forma dimérica del receptor. En presencia de ligando (agonista o antagonista) que estaría ocupando el centro de unión, podría inducir un cambio conformacional en los dominios transmembrana y citoplasmáticos del receptor que implicarían la conversión de la forma de 74 kDa a la de 39 kDa. Por otro lado, la conversión de la forma dimérica del receptor a la forma monomérica sólo tiene lugar cuando el ligando se añade a las membranas de córtex cerebral y no cuando se adiciona a una fracción soluble de estas membranas, indicando que la conversión precisa de la integridad estructural de la membrana y que podría estar mediada por otros componentes de la membrana aparte del receptor A,.

Sorprendentemente, al analizar la unión del agonista [3H]R-PIA o del antagonista [3H]DPCPX a las membranas de corteza cerebral de cerdo, se puso de manifiesto que la unión de
ambos ligandos depende de la presencia de la adenosina desaminasa (ADA) en el medio de incubación, siendo un efecto dosis-dependiente con valores de EC\textsubscript{50} de 0.67 mU/mL de ADA para la unión del agonista R-PIA y de 1.89 mU/mL ADA para la del antagonista DPCPX.

Clásicamente se sugiere que la presencia de adenosina en el medio de incubación es la responsable de la disminución de la unión de ligandos al receptor, al competir el ligando endógeno con los agonistas utilizados en esos experimentos. La presencia de ADA elimina la adenosina endógena. El origen de la adenosina endógena es desconocido pero podría proceder de los nucleótidos de adenosina unidos a la membrana o atrapados en vesículas, los cuales podrían ser degradados por ecto-nucleotidasas u otros enzimas del metabolismo purínico (Linden, J., 1989; Prater et al, 1992). Por esta razón, de manera general, los experimentos de unión de ligandos metabólicamente estables a los receptores de adenosina se realizan siempre en presencia de adenosina desaminasa, la cual transforma la adenosina endógena en inosina y no actúa sobre los ligandos del receptor (Cooper and Caldwell, 1990). En nuestro caso, utilizando membranas de corteza cerebral de cerdo, la ADA no debería tener efecto pues la concentración de adenosina en estas membranas no es superior a 25 nM, lo cual implicaría un desplazamiento mínimo de la \[^{3}H\]R-PIA (1 nM) unida al receptor A\textsubscript{1} de adenosina, considerando que la afinidad de \[^{3}H\]R-PIA es 1 nM y la de la adenosina superior a 100 nM. Por lo tanto, de forma indirecta podríamos concluir que la modificación que ejerce la ADA la unión de ligandos al receptor A\textsubscript{1}, podría ser debida a un efecto independiente de la actividad enzimática de la ADA sobre el AjR. Para confirmar esta hipótesis, se realizaron experimentos de unión de \[^{3}H\]R-PIA en presencia de ADA enzimáticamente activa o inactiva, usando como inhibidores la R-DCF, y el Hg\textsuperscript{2+}, los cuales, a las concentraciones utilizadas, bloquean completamente la actividad enzimática de la ADA sin alterar la unión de ligandos al receptor. Se observó un incremento en la unión de la \[^{3}H\]R-PIA a las membranas tanto en el caso de ADA enzimáticamente activa como en el caso de ADA completamente inhibida con Hg\textsuperscript{2+}. Además, en presencia de ADA inhibida con Hg\textsuperscript{2+} era posible detectar los dos estados de afinidad para la unión de agonistas al A\textsubscript{1}R, con \(K_{d}= 3.6\) nM para el estado de baja afinidad y \(K_{d}= 0.25\) nM para el de alta afinidad. Estos parámetros termodinámicos son muy similares a los obtenidos con ADA activa pero únicamente se detecta el estado de baja afinidad en ausencia de ADA. Por otro lado, el potente inhibidor de la ADA, la R-DCF, prevenía la aparición del estado de alta afinidad, siendo los parámetros cinéticos obtenidos muy similares a los hallados en ausencia de ADA (K\textsubscript{d}= 3.16 nM). Se puede concluir, que el efecto en la unión del agonista al A\textsubscript{1}R es independiente de la actividad catalítica de la enzima y que es necesaria la presencia de ADA (activa o no) para poder detectar el estado de alta afinidad de los receptores A\textsubscript{1} de adenosina.

Con el fin de determinar si realmente existe una interacción entre la ADA y el A\textsubscript{1}R se realizaron experimentos de coinmunoprecipitación. Se utilizaron extractos solubles de membranas de córtex cerebral y los anticuerpos anti-A\textsubscript{1}R purificados: PC11 y PC21 (inmunoglobulinas purificadas por cromatografía de afinidad a partir de los antisueros correspondientes PC/10 y PC/20 respectivamente). En los inmunoprecipitados del receptor, se detectó por western-blotting una banda de 44 kDa correspondiente a la ADA. Por otro lado, al pasar un extracto soluble de córtex cerebral de cerdo a través de una columna de atrapamiento de ADA-Sepharose se retuvo el 62% del receptor A\textsubscript{1} del extracto soluble aplicado, mientras que una proteína control utilizada, la lactato deshidrogenasa, no se retenía en la columna. Estos resultados demuestran claramente que existe una interacción entre la ADA y el A\textsubscript{1}R en membranas de cerebro de cerdo.

Resulta interesante destacar que de los dos potentes inhibidores de la ADA, el Hg\textsuperscript{2+} es el único capaz de mantener la activación inducida por ADA en la unión del agonista al RA\textsubscript{1}, mientras que el otro, la R-DCF, no permiten esta activación. Este último inhibidor es un análogo de la DISCUSIÓN 137
adenosina que mantiene a la enzima en el estado de transición, induciendo un cambio conformacional en la ADA que podría ser crítico para su acoplamiento con el receptor A<sub>1</sub> de adenosina en la membrana. En cambio, el Hg<sup>2+</sup> probablemente bloquea la actividad enzimática reaccionando con grupos sulfhidrilo esenciales en la actividad de esta enzima, sin ocasionar cambios conformacionales en la ADA que afectarían a la interacción con el A<sub>1</sub>R. Esta explicación queda hasta cierto punto corroborada por los experimentos de coinmunoprecipitación, donde el Hg<sup>2+</sup> no afecta para nada a la interacción entre la ADA y el A<sub>1</sub>R, mientras que la R-DCF elimina totalmente la coimmunoprecipitación de ambos. De aquí se puede extrapolar que el cambio conformacional inducido de manera permanente a la ADA resultaría en una pérdida total de la interacción entre la ADA y el A<sub>1</sub>R.

Un papel obvio para la ecto-ADA es la degradación de la adenosina extracelular, controlando de esta manera la cantidad de nucleósido disponible para interaccionar con el A<sub>1</sub>R. La demostración de que la ecto-ADA puede unirse a proteínas presentes en la superficie celular abre nuevas perspectivas acerca de la función no catalítica de la ADA. Para poder averiguar una función fisiológica a la interacción entre ADA y A<sub>1</sub>R es necesario poder constatar que esta interacción se produce en un sistema más próximo a "in vivo". Por ello, buscamos un modelo donde se encontrasen ambas proteínas en la superficie celular. Escogimos la línea celular DDT<sub>MF</sub>-2 de musculatura lisa de hamster que expresa de manera estable el receptor A<sub>1</sub> de adenosina (Gerwins et al., 1990), con unos parámetros termodinámicos para la unión de [³H]R-PIA muy similares a los encontrados en el tejido intacto, y con un número de receptores por célula bastante elevado (100.000 receptores/célula). Por otro lado, estas células también mostraban la presencia de ecto-ADA en su superficie celular (1 mU/10<sup>6</sup> células). Todas estas características hacían de estas células el modelo idóneo para estudiar la interacción entre el receptor A<sub>1</sub> de adenosina y la ADA.

Mediante experimentos de inmunocitoquímica utilizando los anticuerpos anti-A<sub>1</sub>R, PC11 y PC21 y anticuerpos anti-ADA, se comprobó que tanto el A<sub>1</sub>R como la ecto-ADA poseían un patrón de distribución bastante similar. El análisis por microscopía confocal ponía de manifiesto un elevado grado de colocalización entre ambas proteínas en la superficie celular. Se observó que la cantidad de receptor era superior a la de ADA y cuando se añadía ADA exógena a las células el grado de colocalización con el receptor era total. A partir de estos resultados, se puede concluir que todas las moléculas de A<sub>1</sub>R presentes en la superficie celular poseen la capacidad de unir ADA. Por otro lado, la capacidad de la ADA para reconocer al receptor A<sub>1</sub> de adenosina se demostró al quedar retenido específicamente el A<sub>1</sub>R en una columna de afinidad que contenía ADA. Así mismo, a igual que lo observado con membranas de corteza cerebral de cerdo, la ADA coinmunoprecipitaba con el receptor. Todos estos resultados corroboraban la idea de que el A<sub>1</sub>R está uniendo ADA en la superficie celular de las células DDT<sub>MF</sub>-2.

Ya que quedaba demostrada la interacción entre la ADA y el A<sub>1</sub>R en esta línea celular, el siguiente objetivo fue investigar si el ADA modulaba la unión de ligandos al receptor y si modulaba la transducción de señales. Se analizó el efecto de la ADA sobre los parámetros termodinámicos de la unión de la [³H]R-PIA al receptor. Las células DDT<sub>MF</sub>-2 presentaban, en ausencia de ADA exógena, un único centro de muy baja afinidad (K<sub>D</sub>= 40 nM) similar al encontrado, bajo las mismas condiciones, en la fracción de membrana de dichas células. En presencia de ADA exógena, la afinidad para la [³H]R-PIA se incrementa sustancialmente (K<sub>D</sub>= 10 nM). La presencia de ADA produce la conversión de un único estado de muy baja afinidad (K<sub>D</sub>= 50 nM) a dos estados, uno de alta afinidad (K<sub>D</sub>= 0.79 nM) y otro de baja afinidad (K<sub>D</sub>= 8.7 nM) cuando se utiliza la fracción de membrana celular. Por otro lado, la ADA inhibida con Hg<sup>2+</sup> produce las mismas variaciones cinéticas que la ADA activa enzimáticamente, lo que permite...

DISCUSIÓN 138
establecer que su efecto es independiente de la actividad enzimática.

Se analizó el efecto de la ADA sobre la capacidad de transmisión de señal a través del receptor A<sub>1</sub> de adenosina. Se analizó la capacidad del agonista R-PIA para generar un pico transitorio de calcio y la acumulación de inositolfosfatos en presencia o ausencia de ADA exógena. Cuando las células eran pretratadas con ADA activa o inactiva (ADA-Hg<sup>2+</sup>) la capacidad de generar ambos segundos mensajeros era mucho más evidente y cinco veces superior a la obtenida en la ausencia de enzima. Por ello parece que la ADA es necesaria para el correcto acoplamiento entre el receptor A<sub>1</sub> de adenosina y la maquinaria de transmisión de la señal. Teniendo en cuenta que los cambios en la afinidad del receptor reflejan cambios en la interacción con las proteínas-G, podríamos postular que la ADA es necesaria para acoplar el A<sub>1</sub>R a las proteínas-G en las células DDT<sub>MF-2</sub>.

Cuando se analizaba la expresión del receptor A<sub>1</sub> de adenosina en la superficie de las células DDT<sub>MF-2</sub> mediante experimentos de inmunocitoquímica empleando el anticuerpo PC21 y posterior análisis por microscopía confocal, se observaba que en las células tratadas con concentraciones saturantes de R-PIA durante 15 minutos, se inducía una rápida clusterización de los receptores presentes en la superficie celular, seguida de un proceso de secuestro, algo más lento, de estos receptores en un compartimento intracelular. Puesto que este fenómeno no se observaba en ausencia de agonista, se interpretó como un proceso de regulación homóloga del receptor en estas células. El análisis del proceso de desensibilización concomitante al proceso de clusterización e internalización ocupa la última parte del trabajo objeto de esta tesis. Es bien conocido que el tratamiento crónico con agonistas de un receptor puede inducir la desensibilización (disminución de la respuesta ante un estímulo) y down-regulation (una marcada reducción en el número total de receptores). Aunque la desensibilización del receptor A<sub>1</sub> de adenosina ha sido bien caracterizada a nivel farmacológico y molecular (Ramkumar et al., 1991), no existían hasta el momento datos acerca de la dinámica de movilización del receptor desde la membrana plasmática hasta compartimentos intracelulares, y de cuales eran los mecanismos que regían dicho proceso. En los modelos clásicos de receptores acoplados a proteína-G, se requiere de minutos a horas para observar una disminución en el número de centros de unión así como una atenuación en la capacidad de transmisión de la señal (Sibley et al., 1987; Hausdorff et al., 1990; Lefkowitz et al., 1990; Pei et al., 1995). Esta atenuación o desensibilización, está asociada normalmente a un proceso de fosforilación del receptor (Westphal et al., 1995). Existen evidencias probadas, como en el caso del receptor β<sub>2</sub>-adrenérgico (Pippig et al., 1995), donde se demuestra que la fosforilación del receptor juega un papel fundamental en el proceso de desensibilización, observándose que la estimulación del receptor β<sub>2</sub>-adrenérgico en células intactas induce, en primer lugar, un rápido desacoplamiento de la proteína-G<sub>S</sub>, el cual es mediado por la fosforilación del receptor, y en segundo lugar, acontece un proceso más lento de secuestro de receptores hacia un compartimento intracelular. Un tipo similar de desensibilización ha sido descrito para el receptor A<sub>2a</sub> de adenosina (Palmer et al., 1994). En el caso del receptor A<sub>1</sub> de adenosina no está nada claro si el proceso de fosforilación del receptor inducido por un agonista altera la capacidad de interacción de este receptor con la proteína-G<sub>S</sub> (Ramkumar et al., 1991). Nosotros hemos puesto de manifiesto que la incubación de las células DDT<sub>MF-2</sub> con el agonista del receptor de adenosina R-PIA, induce la fosforilación "in vivo" del receptor, observándose ya a los 15 minutos de incubación con este agonista y siendo mucho más evidente a las 8 h de tratamiento.

Los fenómenos de fosforilación y la clusterización inducidos por el ligando, ocurren en el mismo intervalo de tiempo (fenómenos tempranos) por lo que parecía probable que estuviesen fuertemente relacionados. En efecto, se pudo comprobar que ello era así ya que activadores tanto
de la proteína quinasa C como de la proteína quinasa A en ausencia de agonista inducían la fosforilación del receptor. No se observaba ninguna banda de fosforilación del mismo peso molecular que el receptor cuando se inmunoprecipitaba empleando un anticuerpo anti-Tyr-P, lo que confirma que el receptor se fosforila en Ser/Thr.

Si bien la clusterización y la fosforilación del receptor son fenómenos tempranos, la desensibilización (medida como pérdida de centros de unión de agonista en la superficie celular y decremento de la capacidad de transmitir la señal) es un proceso a más largo plazo ($t_{1/2} = 16$ h) y que correlaciona con la internalización del receptor hacia compartimentos intracelulares estudiada por técnicas de inmunocitoquímica ($t_{1/2} = 12$ h). Ambos procesos se producen entre las 5-7 h de tratamiento con el agonista R-PIA. Por otro lado, la internalización del receptor precisa de la fosforilación previa del mismo, pues las células preincubadas con activadores de la proteína quinasa C y proteína quinasa A presentan un mayor grado de internalización del receptor A1 de adenosina.

Mediante la técnica de citometría de flujo, se observa que la desaparición de los centros de unión de [3H]R-PIA en la superficie celular tras 48 h de incubación con el agonista R-PIA, es debida tan sólo en parte a la internalización del receptor, ya que parte de los receptores están presentes en la membrana plasmática en una forma inactiva. Así, los receptores internalizados y los inactivos en la membrana plasmática son los causantes de la disminución de la capacidad de transmisión de la señal por parte de los agonistas del receptor A1 de adenosina.

La internalización del receptor inducida por el agonista R-PIA que transcurre de manera paralela a la desensibilización, implica la aparición de pequeñas vesículas intracelulares que contienen el receptor. Una cuestión obvia que surge es saber si el receptor presente en estas vesículas sufre una inmediata degradación (down-regulation). Para responder a esta pregunta, realizamos experimentos de western-blotting con la idea de determinar el número total de receptores presentes en las células DDT1MF-2 incubadas en presencia o en ausencia de R-PIA. Así, determinamos que la degradación del receptor es un proceso dependiente del tiempo ($t_{1/2} = 38$ h) y más lento que el proceso de internalización ($t_{1/2} = 12$ h), observándose que para períodos largos de tratamiento con el agonista (72 h), los receptores internalizados (43%) son casi todos degradados (30%). Estos resultados corroboran la hipótesis formulada para la mayoría de receptores acoplados a proteína-G, donde se propone que parte de los receptores desensibilizados podrían ir directamente a los lisosomas para su degradación, mientras que otra parte podría ser reciclada hacia la membrana plasmática.

De toda esta serie de resultados podemos concluir que la saturación con el agonista de los receptores A1 de adenosina presentes en la superficie de las células DDT1MF-2 desencadenaría una serie de procesos dependientes del tiempo que implicaría la fosforilación del receptor en serinas o treoninas con la consecuente clusterización de los mismos en la superficie celular, estos procesos irían seguidos de la desensibilización de los receptores que vendría determinada por el secuestro o internalización de los receptores en compartimentos intracelulares.

DISCUSIÓN 140
D. CONCLUSIONES.
D. CONCLUSIONES.

A.- Las células LLC-PK1 así como cultivos primarios de células epiteliales y las vesículas
de membranas luminales (BBM) de corteza de riñón de cerdo, presentan centros de unión de
\[^3\text{H}\]NB\text{TI} de alta afinidad. Las características cinéticas, farmacológicas y moleculares de estos
centros permiten identificarlos como el transportador equilibrativo de nucleósidos sensible a NB\text{TI}
(fórmula es). Este transportador es una proteína de 65 kDa de peso molecular con capacidad de
presentar distintas asociaciones oligoméricas.

Las células LLC-PK1, cuando crecen hasta confluencia, inhiben el transporte de adenosina
por un mecanismo de fosforilación del transportador equilibrativo de nucleósidos, probablemente
mediante la proteína quinasa A. El transportador fosforilado no une \[^3\text{H}\]NB\text{TI} ni es capaz de
transportar adenosina. El proceso de obtención de BBM a partir de estas células, de cultivos
primarios o de tejido renal provoca la desfosforilación de este transportador, recuperando
la capacidad de unión de \[^3\text{H}\]NB\text{TI}, pero no la capacidad de transportar \[^3\text{H}\]adenosina.

B.- Se ha logrado la obtención de anticuerpos policlonales antipeptídicos que reconocen
especificamente al receptor A\textsubscript{1} de adenosina de diversas procedencias mediante técnicas de
western-blotting, inmunoprecipitación e inmunocitoquímica.

La utilización de estos anticuerpos en sistemas modelo, que muestran una expresión elevada
del receptor A\textsubscript{1}, ha dado lugar a las siguientes conclusiones:

1.- En corteza cerebral de cerdo, mediante western-blotting se observa una banda de 39
kDa de peso molecular que corresponde al receptor presente en dicho tejido, y se pone de
manifiesto la existencia de otra banda de 74 kDa de peso molecular que corresponde a dímeros
del receptor. Si bien el papel fisiológico de los dímeros es aún desconocido, se observa que la
conversión dímero/monómero está regulada por ligandos del receptor, así como por agentes que
afecten a la estabilidad de la membrana plasmática.

2.- La adenosina desaminasa aumenta la unión de ligandos al receptor A\textsubscript{1} de adenosina
presente en membranas de corteza cerebral de cerdo y en células DDT,MF-2. Esta acción es
independiente de la actividad catalítica del enzima. Los experimentos de coimmunoprecipitación
y cromatografía de afinidad demuestran que la adenosina desaminasa interacciona
molecularmente, directa o indirectamente, con el receptor. Además, por inmunocitoquímica y
análisis por microscopía confocal se demuestra un elevado grado de colocalización de la
adenosina desaminasa y del receptor A\textsubscript{1} en la superficie de las células DDT,MF-2. El hecho de que
esta interacción sea necesaria para que la unión de los agonistas del receptor induzca la
generación de segundos mensajeros, sugiere que la ADA es necesaria para conseguir el correcto
acoplamiento del receptor el receptor A\textsubscript{1} de adenosina y las moléculas responsables de la
aparición de la señal.

El conjunto de estos resultados es especialmente importante puesto que es la primera vez
que se describe la interacción molecular entre un receptor de la familia de receptores acoplados
a la proteína G y el enzima que degrada la hormona de este receptor.

CONCLUSIONES 141
3.- Mediante técnicas de inmunocitoquímica y microscopía confocal, se observa que la saturación con el agonista de los receptores A₁ de adenosina de la superficie de las células DDT₂MF-2 produce la agregación de las moléculas del receptor en la superficie de la célula seguida de un proceso de internalización del receptor. Se ha podido demostrar, mediante técnicas de unión de ligandos, citometría de flujo y western-blotting, que estos fenómenos están asociados a un proceso de desensibilización homóloga del receptor que implica una serie de sucesos consecutivos. El fenómeno se inicia con la saturación por agonista del receptor, la cual provoca su fosforilación en Ser/Thr, originando una agregación del receptor en la membrana plasmática de la célula. Estos hechos, que ocurren en un intervalo de tiempo corto, conducen a que el receptor presente en la membrana esté en parte inactivo y que una fracción del receptor se internalice y aparezca en vesículas intracelulares donde sólo en parte es degradado.