

Adenosine Deaminase and A₁ Adenosine Receptors Internalize Together following Agonist-induced Receptor Desensitization*

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A₁ adenosine receptors (A₁Rs) and adenosine deaminase (ADA; EC 3.5.4.4) interact on the cell surface of DDT₁MF-2 smooth muscle cells. The interaction facilitates ligand binding and signaling via A₁R, but it is not known whether it has a role in homologous desensitization of A₁Rs. Here we show that chronic exposure of DDT₁MF-2 cells to the A₁R agonist, N⁶-(R)-(phenylisopropyl)adenosine (R-PIA), caused a rapid aggregation or clustering of A₁ receptor molecules on the cell membrane, which was enhanced by pretreatment with ADA. Colocalization between A₁R and ADA occurred in the R-PIA-induced clusters. Interestingly, colocalization between A₁R and ADA also occurred in intracellular vesicles after internalization of both protein molecules in response to R-PIA. Agonist-induced aggregation of A₁Rs was mediated by phosphorylation of A₁Rs, which was enhanced and accelerated in the presence of ADA. Ligand-induced second-messenger desensitization of A₁Rs was also accelerated in the presence of exogenous ADA, and it correlated well with receptor phosphorylation. However, although phosphorylation of A₁R returned to its basal state within minutes, desensitization continued for hours. The loss of cell-surface binding sites (sequestration) induced by the agonist was time-dependent ($t_{1/2} = 10 \pm 1$ h) and was accelerated by ADA. All of these results strongly suggest that ADA plays a key role in the regulation of A₁Rs by accelerating ligand-induced desensitization and internalization and provide evidence that the two cell surface proteins internalize via the same endocytic pathway.

Adenosine is an autacoid that exerts its physiological actions through specific cell surface receptors. Four adenosine receptors (A₁, A_{2A}, A_{2B}, and A₃), belonging to the family of G protein-coupled receptors, have been cloned and pharmacologically characterized (1). Acting through different adenosine receptors, adenosine is a neuromodulator in both the central and peripheral nervous systems (2, 3). Via A₁ adenosine receptors (A₁Rs),¹ adenosine reduces heart rate (4),

glomerular filtration rate, and renin release in the kidney (5); it induces bronchoconstriction (6, 7) and inhibits lipolysis. A₁Rs can be coupled to different pertussis toxin-sensitive G proteins (8–10), which mediate inhibition of adenylate cyclase (11) and regulate Ca²⁺ and K⁺ channels and inositol phosphate metabolism (1). A₁R displays two different affinities for agonist, which have classically been attributed to a different coupling to heterotrimeric G proteins (12). According to this two-independent site model, coupled receptor-G protein complexes display high affinity for agonists ($K_d = 0.1$ – 0.2 nM), and uncoupled receptors display low affinity (1–2 nM) (12, 13). The recently reported cluster-arranged cooperative model predicts that the high and low affinity sites are a consequence of the negative cooperativity of agonist binding and do not seem to be related to the content of free and G protein-coupled receptors (14). According to the cluster-arranged cooperative model, the agonist-induced conversion of the high to the low affinity state may partially explain the ligand-induced desensitization of A₁R (14, 15).

Receptors belonging to the G protein-coupled receptor family are desensitized and down-regulated in response to agonist stimulation. β_2 -Adrenergic receptors (β_2 -ARs) constitute the best characterized system within the G protein-coupled receptor family. Agonist-induced β_2 -AR desensitization is caused by a conformational change of the agonist-occupied receptor that facilitates receptor phosphorylation by second messenger-activated kinases or G protein-coupled receptor kinases (16, 17). Following β_2 -AR phosphorylation, β -arrestin binds to the phosphorylated receptor and uncouples the receptor from the heterotrimeric G proteins (18, 19). β -Arrestin not only desensitizes the receptor but also functions as a clathrin adaptor, mediating receptor sequestration, *i.e.* receptor internalization toward intracellular compartments (20, 21). Although sequestration is not required for phosphorylation and desensitization, it appears to be necessary for dephosphorylation and resensitization of β_2 -ARs (22, 23).

Like other G protein-coupled receptor members, A₁R expression is regulated in response to agonist or antagonist stimulation. Desensitization of A₁R has been described in intact animals and in cell cultures. Prolonged administration of A₁R agonists to animals leads to functional desensitization of A₁R in guinea pig heart (24), rat adipocytes (25), rat atrial muscle (26), and rat brain (27, 28). The reduced functional response is due to a net loss of A₁Rs or down-regulation, a decrease in the proportion of A₁R displaying the high affinity state for agonists and a decrease in the content of G_i proteins. Growth of rat adipocytes or smooth muscle DDT₁MF-2 cells in the presence of N⁶-(R)-(phenylisopropyl)adenosine (R-PIA)

adenosine deaminase; R-PIA, N⁶-(R)-(phenylisopropyl)adenosine; FITC, fluorescein isothiocyanate; TRITC, rhodamine isothiocyanate; β_2 -AR, β_2 -adrenergic receptor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution.

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¹ The abbreviations used are: A₁R, A₁ adenosine receptor; ADA, aden-

leads to the desensitization and down-regulation of A₁R (29) along with G_i α -subunits (30, 31). We have recently shown that treatment of DDT₁MF-2 cells with the agonist R-PIA induces a rapid phosphorylation and clustering of A₁Rs. The loss of binding sites on the cell membrane due to internalization of A₁R is a slower event occurring within hours. Since activators of protein kinase A or C mimicked the effect of the ligand, Ser/Thr phosphorylation seems to be related to short term clustering and desensitization as well as to long term internalization of A₁R (32).

A new perspective on the coupling of A₁R to G proteins has recently been provided. Ecto-adenosine deaminase (ecto-ADA; EC 3.5.4.4), which is capable of degrading adenosine to inosine on the cell surface (for a review, see Ref. 33), was shown to modulate ligand binding and signaling through A₁Rs on DDT₁MF-2 cells (34). Irrespective of its catalytic activity, ADA seems to be necessary for a high affinity binding of agonists to A₁R (35).

Here the effect of ADA on the ligand-mediated regulation of A₁R in DDT₁MF-2 cells has been studied. ADA affected both desensitization and internalization of A₁Rs. Moreover, by means of immunocytochemistry, confocal microscopy, and flow cytometry using antibodies against A₁R and ADA, we found that A₁R and ADA colocalize and that, after receptor activation, they cluster on the cell surface and internalize via the same pathway. All of these results provide evidence for concerted modulation of ADA and A₁R in response to receptor agonists.

EXPERIMENTAL PROCEDURES

Materials—[Adenine-2,8-³H,ethyl-2-³H]phenylisopropyladenosine (³H]R-PIA) (36 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Nuclear Iberica, Madrid, Spain). [³²P]orthophosphate (³²P_i) was from NEN Life Science Products. 1,3-Dipropyl-8-cyclopentylxanthine was from Research Biochemicals (Natick, MA). R-PIA, pepstatin, leupeptin, chymostatin, antipain, phenylmethylsulfonyl fluoride, Fura-2/AM, fluorescein isothiocyanate, rhodamine isothiocyanate, and calf adenosine deaminase (ADA) were purchased from Sigma. ADA was desalted with a Sephadex G-25 column (Amersham Pharmacia Biotech) prior to all assays. A unit of enzyme activity corresponds to 130 nmol of ADA protein. Electrophoresis reagents were obtained from Boehringer Mannheim. Sephadex G-25 and protein A-Sepharose CL4B were from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other products were obtained from Merck.

Antibodies and Fluorescent Probes—Antisera against purified calf ADA and against peptides of A₁ adenosine receptor (PC10 and PC20) were generated by immunization of female New Zealand White rabbits by the Biokit Company (Barcelona, Spain). Antibodies against A₁Rs (PC11 and PC21) were purified from serum by affinity chromatography (peptide coupled to thiol-Sepharose 4B (Amersham Pharmacia Biotech)). Anti-ADA antibody was purified using ADA coupled to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech). The specificity of antibodies for their respective antigens and extensive characterization of anti-ADA and anti-A₁R antibodies (PC11 and PC21) have been previously reported (36, 37). PC11 and PC21 antibodies do not recognize ADA from DDT₁MF-2 cell extracts by immunoblotting, immunoprecipitation, or immunocytochemistry (34). PC21 and PC11 A₁R antibodies were shown to be specific for cells expressing A₁Rs (32).

Fluorescence probes were obtained by reacting (for 2 h at 20 °C) 0.1 mg of fluorescein or rhodamine isothiocyanate with 1 mg of purified protein in coupling buffer (50 mM H₃BO₃, 200 mM NaCl, pH 9.2). Unbound fluorochrome was removed by rapid filtration through a Sephadex G-25 column (Amersham Pharmacia Biotech), and fractions were tested for protein content. Aliquots were stored at -80 °C.

Cell Cultures and Protein Determination—DDT₁MF-2 smooth muscle cells derived from a steroid-induced leiomyosarcoma of Syrian hamster vas deferens were obtained from the American Type Culture Collection. Cells were cultured (at 37 °C, 5% CO₂) in Dulbecco's modified Eagle's medium (DMEM) (Whittaker, Walkersville, NY), 1% nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 5% (v/v) horse serum, and 5% (v/v) fetal calf serum (Life Technologies, Inc.). Desensitization and internalization assays were performed with cells growing in the absence or in the presence of 65 nM ADA and/or 50 nM

R-PIA. At the indicated incubation times, cells were harvested and washed exhaustively in cold phosphate-buffered saline (PBS) before binding, immunocytochemistry, and intracellular calcium mobilization assays.

Protein content was measured by the BCA method (Pierce), as described by Sorensen and Brodbeck (38).

Radioligand Binding Assays—For binding assays, untreated or treated DDT₁MF-2 cells (1 mg/ml) were resuspended in serum-free DMEM buffered with 20 mM HEPES, pH 7.4, and preincubated with 65 nM ADA for 30 min at 4 °C. Radioligand binding was performed in the presence of 5 nM of [³H]R-PIA for 4 h at 4 °C. Nonspecific binding was performed in the presence of a 400-fold excess of R-PIA. Nonspecific binding with a 500-fold excess of the selective A₁R antagonist 1,3-dipropyl-8-cyclopentylxanthine resulted in similar displacement of [³H]R-PIA binding. When equilibrium had been reached, incubates were filtered through glass fiber filters (GF/C filters, Whatman, Kent, United Kingdom) in a Brandel cell harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD). Filters were washed in 5 ml of ice-cold PBS and transferred to vials containing 10 ml of scintillation solution EcocintH (National Diagnostics, Atlanta, GA). After overnight shaking, vials were counted using a Packard 1600 TRI-CARB scintillation counter with 50% efficiency. All points represent the mean of 3–5 independent experiments performed in triplicate.

Phosphorylation Assays—A₁R phosphorylation was induced by 65 nM ADA and/or 50 nM R-PIA treatment after the cellular ATP pool had been labeled with [³²P]orthophosphate. Briefly, DDT₁MF-2 cells washed twice and resuspended in phosphate-free DMEM (buffered with 20 mM HEPES, pH 7.4) were incubated (10⁶ cells/ml, 37 °C, 2 h) with 0.1 mCi/ml [³²P]orthophosphate. Loaded cells were resuspended (1 × 10⁶ cells/ml) in serum-free DMEM and incubated with the indicated reagents at 37 °C. After the corresponding incubation times, cells were centrifuged (10 s, 14,000 rpm) in a microcentrifuge and washed twice in 1 ml of ice-cold PBS before disruption and solubilization (1 h at 4 °C) in 0.5 ml of lysis buffer (20 mM HEPES, pH 7.4; 1% (v/v) Nonidet P-40; 100 mM NaCl; 1 mM Na₃VO₄; 50 mM NaF; 1 mM phenylmethylsulfonyl fluoride; 10 μ g/ml leupeptin, pepstatin, chymostatin, and antipain). The solubilized preparation (0.2 mg/ml) was immediately processed for immunoprecipitation with the anti-A₁R antibody (PC11, 50 μ g/ml) and protein A-Sepharose beads. Immunoprecipitated material was washed four times in lysis buffer (1 × 1%, 2 × 0.1%, and 1 × 0% Nonidet P-40) before resuspension in 50 μ l of SDS sample buffer. SDS-polyacrylamide gel electrophoresis and autoradiography were performed as described elsewhere (37). Phosphorylated bands were quantified with a computing densitometer (Molecular Dynamics, Inc.).

Immunostaining and Immunofluorescence Assays—For immunofluorescence staining, treated and untreated cells were washed in PBS and fixed in 4% paraformaldehyde in PBS, pH 7.4, for 15 min at room temperature. After two washes in PBS containing 20 mM glycine (buffer A) and in buffer A containing 1% bovine serum albumin (buffer B), cells were incubated with the rhodamine-conjugated anti-A₁R antibody (PC21-TRITC, 75 μ g/ml) and the fluorescein-conjugated anti-ADA antibody (anti-ADA-FITC, 75 μ g/ml) or, alternatively, with the fluorescein-conjugated anti-A₁R antibody (PC21-FITC, 75 μ g/ml) for 1 h at 37 °C. Three washes in buffer B were performed before mounting the samples in immunofluorescence medium (ICN Biomedical Inc., Costa Mesa, CA).

Internalization was analyzed by immunocytochemistry and confocal microscopy. To follow dynamic internalization, unfixed cells growing on glass coverslips were incubated (37 °C, 20 min) with 65 nM bovine serum albumin-FITC, with PC21-FITC (10 μ g/ml), and with 65 nM ADA-FITC and/or PC21-TRITC (10 μ g/ml) and then treated with 50 nM R-PIA at 37 °C for 24–72 h. Cells were washed twice in PBS, fixed in 4% paraformaldehyde in PBS for 15 min, and again washed twice in PBS 20 mM glycine. Glass coverslips were mounted as described above. Cells were examined with a LEICA TCS confocal scanning laser microscope attached to an inverted Leitz DMIRBE microscope (Leica Lasertechnik GmbH, Heidelberg). Images shown are representative of at least three experiments.

Internalization measurements for A₁Rs and cell surface ADA (ecto-ADA) were performed by immunostaining and flow cytometry analysis. Cells were grown in complete medium in the absence or presence of 50 nM R-PIA for 0–48 h. In some experiments, ADA (65 nM) was prebound by incubation for 2 h at 4 °C; unbound enzyme was eliminated by washing twice in PBS. Cells were fixed using 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were washed in buffer A and buffer B for 20 min before staining with anti-ADA-FITC (75 μ g/ml) or PC21-FITC (75 μ g/ml). Three washes in buffer B were performed before analyzing the mean fluorescence intensity from 5000–10,000 cells on an EPICS Profile flow cytometer (Coulter, Hialeah, FL). Similar assays

were performed for cells incubated (30 min to 48 h at 37 °C) with 65 nM fluorescein-conjugated ADA (ADA-FITC) in the absence or presence of 50 nM *R*-PIA. In order to remove as much of the cell surface-bound ADA as possible, cells were washed once in PBS and once in PBS/HCl, pH 2, for 15 min according to the method of Yang *et al.* (39). Cells were fixed and processed for flow cytometry as described above. For establishing comparisons in internalization studies, the mean of fluorescence intensity taken as reference was that found in cells incubated with 65 nM ADA-FITC for 15 min at 37 °C. This fluorescence intensity was similar to that found in cells incubated with 65 nM ADA-FITC at 4 °C for 2 h, which was considered as the background signal. Higher intensities would reflect internalization of ADA-FITC.

Measurement of Intracellular Free Calcium—Cells were washed and resuspended (5×10^6 cells/ml) in Hanks' balanced salt solution (HBSS containing 1.2 mM CaCl₂ and 20 mM HEPES, pH 7.4) and loaded with 5 μM Fura-2/AM for 30 min at 25 °C. Cells were washed twice and resuspended in HBSS (1×10^6 cells/ml) for 20 min at room temperature. Cells were placed in a cuvette (10⁶ cells/2 ml of HBSS, 25 nM ADA), and calcium peak induction was achieved by treatment with 50 nM *R*-PIA at 30 °C. For short term desensitization assays (1–15 min), cells were loaded and washed before resuspension in prewarmed (37 °C) serum-free DMEM (1×10^6 cells/ml) and incubated with the indicated reagents. Cells were washed three times in HBSS buffer to remove reagents before resuspension in the same buffer and *R*-PIA induction of calcium mobilization as indicated above. After these three washes [Ca²⁺] base-line levels were similar irrespective of the type of pretreatment. Therefore, differences in calcium mobilization were not a consequence of different calcium base-line levels in response to pretreatments. For long term desensitization (12, 24, and 48 h), incubations were performed before loading with Fura-2/AM. Calcium concentration was determined in a dual wavelength Shimadzu RF-5000 spectrofluorophotometer by using the ratio of excitation wavelengths 334/366 nm with emission cut-off at 500 nm. Free calcium concentration was calculated as described previously (40).

Statistical Analysis—Time course curves were fitted to a single-phase exponential decay equation, in the case of [³H]*R*-PIA binding. Statistical comparisons were made using the two-tailed Student's *t* test. Differences were considered significant when *p* was <0.05.

RESULTS

Effect of ADA on Cell Surface Distribution of A₁Rs in the Absence and in the Presence of Agonist—A₁ adenosine receptor (A₁R) distribution on the surface of DDT₁MF-2 cells was examined by immunocytochemistry and confocal analysis. Untreated and *R*-PIA-treated cells were fixed, incubated with fluorescein-conjugated rabbit anti-A₁R antibody (PC21-FITC), and analyzed by confocal microscopy. The PC21 antibody is directed against the second extracellular loop of A₁R and its specificity for cell surface-expressed A₁R has been previously demonstrated (32). Receptor distribution in untreated cells revealed homogenous bright staining on the surface of DDT₁MF-2 cells (Fig. 1A). No major differences in receptor distribution were observed in the presence of exogenous ADA (65 nM) for 15 min (Fig. 1B). A 5-min exposure of cells to the A₁R agonist *R*-PIA (50 nM) induced the appearance of bright accumulations of antigen on the plasma membrane, suggesting aggregation or clustering of receptor molecules (Fig. 1C). Clustering of A₁Rs was more evident after 15 min of *R*-PIA exposure, when brighter clusters were found (Fig. 1, compare C and E). Incubation of DDT₁MF-2 cells with ADA and *R*-PIA changed the pattern of cell surface A₁R distribution dramatically. In the presence of ADA, clusters of A₁R were already evident at 2 min, and the label was found as intense bright punctate accumulations at 5 or 15 min of *R*-PIA treatment (Fig. 1, D and F). This clearly shows the involvement of ecto-ADA in the regulation of the *R*-PIA-induced aggregation of cell surface A₁R molecules. All images shown in Fig. 1 represent reconstructions of multiple sections at different levels. When middle sections were analyzed, only the cell surface staining was observed, thus ruling out intracellular staining (data not shown; see Ref. 32).

To analyze the codistribution of A₁R and ADA on the plasma

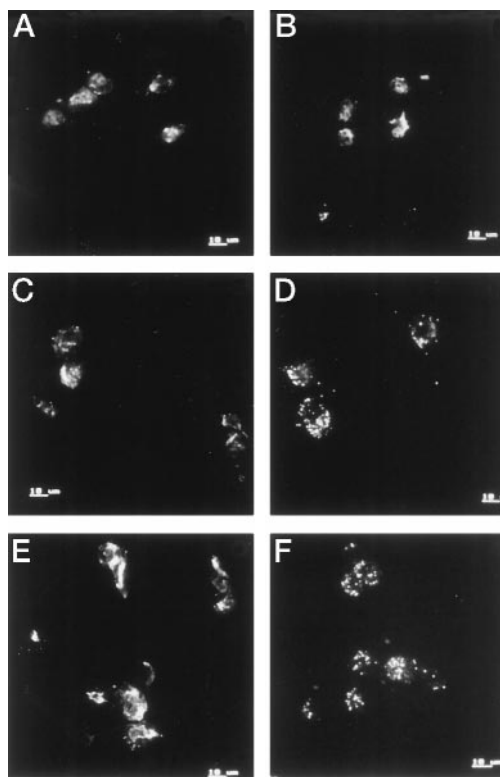


FIG. 1. Cell surface A₁R redistribution in response to ADA and/or *R*-PIA. DDT₁MF-2 cells were untreated (A) or treated with 65 nM ADA (B), 50 nM *R*-PIA (C and E), or 65 nM ADA plus 50 nM *R*-PIA (D and F) for 5 (C and D) or 15 min (A, B, E, and F) at 37 °C. Cells were fixed, processed for immunofluorescence staining with the use of a fluorescein-conjugated anti-A₁R antibody (PC21-FITC, 75 μg/ml), and observed by confocal microscopy. The images shown correspond to reconstructions of several horizontal sections of representative cells. Scale bar, 10 μm.

membrane of DDT₁MF-2 cells, ADA-treated cells were incubated with or without 50 nM *R*-PIA for 15 min. Cells were fixed, washed, and stained by double immunofluorescence using the fluorescein-conjugated anti-ADA antibody (anti-ADA-FITC) and the rhodamine-conjugated anti-A₁R antibody (PC21-TRITC). Homogenous distribution of ADA and A₁R on the cell surface was observed in untreated cells (Fig. 2, top). Superimposition of images by confocal analysis showed extensive colocalization (yellow) of both proteins on the plasma membrane. When cells were treated (15 min) with *R*-PIA, a redistribution of ADA and A₁Rs, which is consistent with clustering, was observed. *R*-PIA induced aggregation of both proteins in clusters seen as punctate accumulations of fluorescence and where colocalization between A₁R and ecto-ADA was very high (Fig. 2, bottom).

ADA Affects Ligand-induced Phosphorylation of A₁Rs—In a previous study, we have shown that ligand-induced phosphorylation of A₁R correlates with the clustering of receptors on the DDT₁MF-2 cell surface (32). To determine whether the potentiation of the clustering by ADA could involve changes in receptor phosphorylation, we immunoprecipitated the receptor from cells metabolically labeled with [³²P]orthophosphate. Exogenous ADA by itself had only a slight effect on A₁R phosphorylation. *R*-PIA (50 nM) induced, in the absence of ADA, a steady increase of A₁R phosphorylation from basal levels to a 2.1-fold increase at 15 min. Interestingly, in the presence of exogenous ADA (65 nM), *R*-PIA induced a rapid increase in A₁R phosphorylation with a maximum rise (3-fold increase) after 1 min; thereafter (5 and 15 min), the level of phosphorylation declined (Fig. 3). In the presence of ADA, receptor phosphorylation

FIG. 2. Colocalization between A₁R and ADA on the cell surface. Cells were treated with 65 nM ADA in the absence (*top*) or presence (*bottom*) of 50 nM R-PIA for 15 min at 37 °C. After treatment, cells were washed, fixed, and processed for immunostaining as described under "Experimental Procedures." Staining was performed with the fluorescein-conjugated anti-ADA antibody (anti-ADA-FITC, 75 µg/ml) and the rhodamine-conjugated anti-A₁R antibody (PC21-TRITC, 75 µg/ml). Cells were analyzed by double immunofluorescence with a confocal microscope to detect ADA (*green images*) and A₁R (*red images*). Superimposition of images reveals ADA/A₁R colocalization in *yellow*. Scale bar, 10 µm.

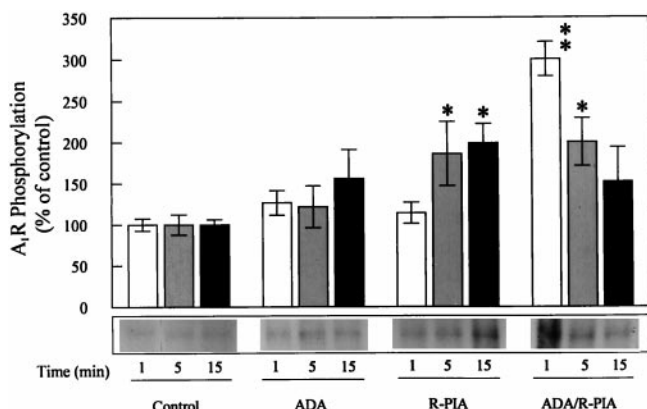
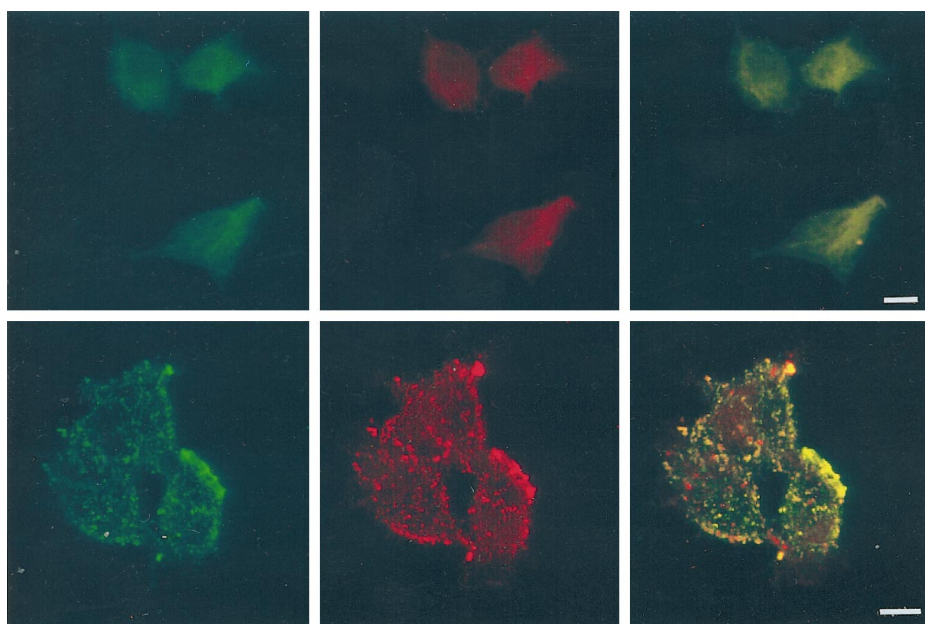


FIG. 3. Agonist-induced phosphorylation of A₁R in the absence or presence of ADA. DDT₁MF-2 cells were metabolically labeled with ³²P_i and stimulated by means of vehicle (control), 65 nM ADA, and/or 50 nM R-PIA for 1 (*white bars*), 5 (*gray bars*), or 15 min (*black bars*) at 37 °C. A₁R were immunoprecipitated and resolved by SDS-polyacrylamide gel electrophoresis. Gels were autoradiographed as described under "Experimental Procedures," and bands of phosphorylated A₁R were quantified with a computing densitometer (Molecular Dynamics). Data are expressed as percentages of the basal level found in control cells. Data are mean ± S.E. of four separate experiments performed in duplicate. *, *p* < 0.05; **, *p* < 0.005 with respect to the corresponding control at 1, 5, or 15 min. The electrophoretic band (36 kDa) corresponding to the A₁R in a representative experiment is shown at the *bottom*.

ation diminished to basal level after 15–30 min, whereas for cells treated with R-PIA in the absence of the enzyme the return to basal levels of phosphorylation was slower.

Agonist-induced Desensitization of A₁R in the Presence of ADA—Activation of A₁R with adenosine or analogs leads to both Ins(1,4,5)P₃ formation and intracellular calcium mobilization in DDT₁MF-2 cells (41, 42). To correlate changes in agonist-induced A₁R phosphorylation with possible changes in second messenger production, we analyzed the effect of ADA and/or R-PIA on agonist-induced mobilization of intracellular calcium. Cells were exposed to vehicle (control) or to 65 nM ADA and/or 50 nM R-PIA. After extensive washes, calcium mobilization was induced by 50 nM R-PIA. R-PIA caused a transient Ca²⁺ peak in naive (vehicle-treated) cells (Fig. 4A). Desensitization of the [Ca²⁺] response in R-PIA- or R-PIA/ADA-treated cells occurred rapidly (in minutes) and was sustained for at

least 48 h (Fig. 4B). R-PIA pretreatment reduced the maximum intracellular calcium concentration by 2, 30, and 44% at 1, 5, and 15 min, respectively. This effect was, however, potentiated in the presence of ADA, since the maximum desensitization (60%) was achieved at 5 min and maintained for up to 48 h (Fig. 4, A and B). No significant differences in maximum effect were observed when R-PIA pretreatment was done in the presence or in the absence of ADA (Fig. 4B). It should be noted that ADA pretreatment in the absence of R-PIA led to a significant and sustained desensitization (25–45% reduction of the response).

Loss of A₁R from the Cell Surface—Characterization of A₁R by radioligand binding using the agonist [³H]R-PIA indicated the presence of two affinity states of the receptors (*K*_{high} = 0.8–2 nM and *K*_{low} = 10–100 nM) in cell membrane preparations, whereas only the low affinity state was detected in intact cells (43). Ligand binding assays were performed in intact cells preincubated with vehicle (control) or with 50 nM R-PIA and/or 65 nM ADA. Cells were extensively washed and assayed for [³H]R-PIA binding at 4 °C. The results indicated a time-dependent loss of binding sites on the cell membrane of treated cells (Fig. 5). The loss of binding sites after incubation of cells with ADA in the absence of R-PIA was small. In contrast, preincubation of cells with 50 nM R-PIA with or without ADA resulted in a time-dependent reduction of [³H]R-PIA binding sites. The presence of ADA accelerated the reduction of binding sites, from *t*_{1/2} = 10 ± 1 h to *t*_{1/2} = 2.9 ± 0.5 h. The maximum loss of cell surface binding sites (around 41%) induced by agonist was not modified by the presence of ADA (Fig. 5).

Disappearance of A₁R from the cell surface of DDT₁MF-2 cells in response to agonist has been described elsewhere (32). Interestingly, the effect of ADA was evident even when the protein was prebound to the cell surface and the unbound enzyme was washed away (Fig. 6). On the other hand, the disappearance of A₁R from the cell surface was reversible. Thus, when chronically stimulated cells were further cultured for 24 h in the absence of R-PIA, the level of cell surface expression was recovered. These results indicate that, in the presence of ADA, R-PIA induces the sequestration of A₁R in intracellular compartments (see below).

Internalization of A₁R Together with ADA—In order to determine whether agonist-induced disappearance of binding sites affects the expression of cell surface ADA (ecto-ADA), fluorocytometry analysis was performed using nonpermeabi-

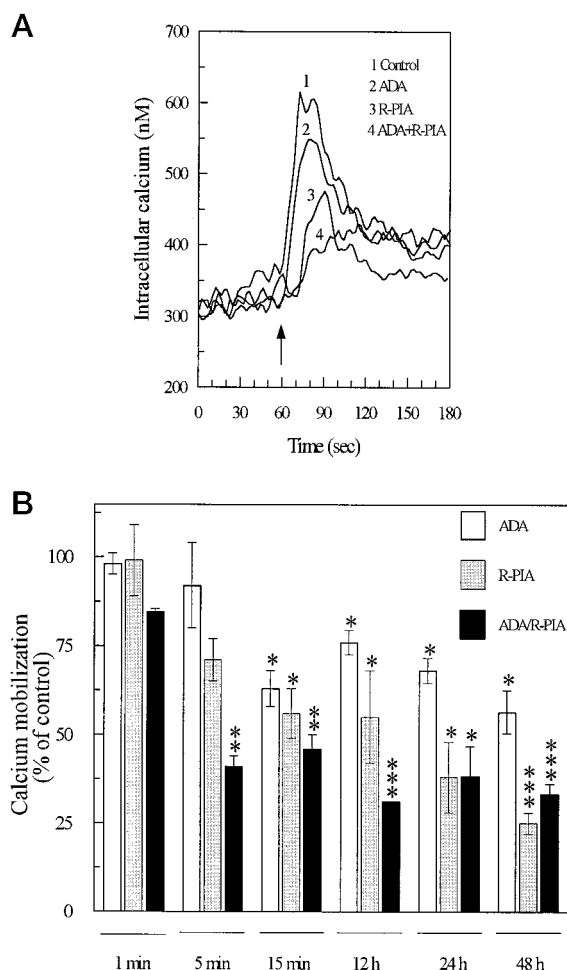


FIG. 4. Agonist-induced functional desensitization of A₁R in the absence or presence of ADA. A, cells were loaded with Fura-2/AM (30 min, 25 °C), resuspended in serum-free DMEM, and preincubated with vehicle (control), 65 nM ADA, 50 nM R-PIA, or 65 nM ADA plus 50 nM R-PIA for 5 min at 37 °C. After extensive washing, intracellular calcium mobilization was achieved by 50 nM R-PIA as described under "Experimental Procedures." Traces correspond to a representative experiment. Panel B, time course of functional desensitization, measured as R-PIA-induced intracellular calcium mobilization, after pretreatment of cells with ADA and/or R-PIA. Intracellular calcium mobilization was achieved as described in A. For long term desensitization (12, 24, and 48 h), cells were grown in the presence of the reagents prior to the loading with Fura-2/AM (see "Experimental Procedures"). Data are presented as percentage of the maximal response obtained for control cells stimulated with 50 nM R-PIA. Data are expressed as mean ± S.E. of three separate experiments. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.005 with respect to the untreated cells.

lized cells. After pretreatment with medium (control) or 50 nM R-PIA, cells were fixed, and ecto-ADA was labeled using the anti-ADA-FITC antibody. A significant time-dependent decrease in fluorescence intensity was observed after 8 h of R-PIA treatment, and a maximum decrease (40%) was obtained at 24 h, which suggests A₁R agonist-induced internalization of ecto-ADA (Fig. 7).

To follow ADA internalization, DDT₁MF-2 cells were labeled with ADA-FITC, which binds to cell surface A₁Rs (34). The labeling was performed in the presence or in the absence of 50 nM R-PIA. After extensive washes in acid buffer, cells fixed in paraformaldehyde were analyzed by flow cytometry. The acidic wash was necessary to remove ADA-FITC remaining on the cell surface; therefore, quantification of intracellular fluorescence is a direct measure of internalized ADA (see "Experimental Procedures"). A progressive time-dependent incorporation of fluorescence into the cells, in the period 2–48 h, was observed

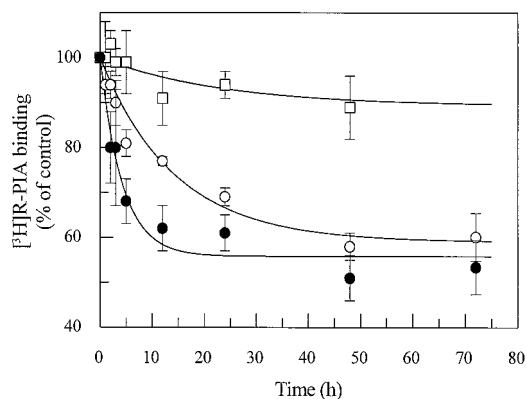


FIG. 5. Loss of cell surface [³H]R-PIA binding sites in response to ADA and/or R-PIA treatment. Cells were incubated with vehicle (control), 65 nM ADA (□), 50 nM R-PIA (○), or 65 nM ADA plus 50 nM R-PIA (●) for the indicated time at 37 °C. After pretreatment, cells were washed and incubated for 4 h at 4 °C with 5 nM [³H]R-PIA. Nonspecific binding was assessed in the presence of a 400-fold excess of unlabeled R-PIA. Data (mean ± S.E. of five experiments performed in quadruplicate) are presented as percentages of the specific binding with respect to untreated cells. *, *p* < 0.005; **, *p* < 0.001.

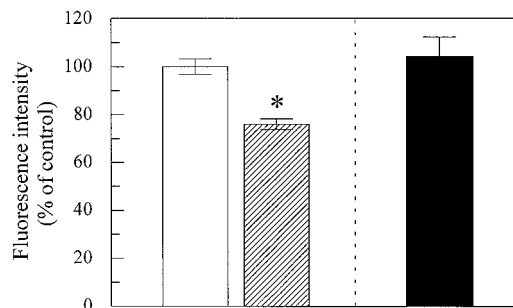


FIG. 6. Internalization of A₁Rs in cells preincubated with ADA. DDT₁MF-2 cells were treated with 65 nM ADA for 2 h at 4 °C. After binding of ADA to the cell surface, unbound enzyme was washed away, and cells were treated without (open bar) or with (striped bar) 50 nM R-PIA for 24 h at 37 °C. A sample of R-PIA-treated cells was washed twice in PBS and further cultured for 24 h in absence of the compound (solid bar). After treatment, cells were harvested, rinsed in PBS, fixed using 4% paraformaldehyde in PBS, and prepared for immunostaining using the fluorescein-conjugated anti-A₁R antibody (PC21-FITC, 75 μg/ml). Fluorescence quantification was performed on a EPICS Profile flow cytometer as indicated under "Experimental Procedures." Data are presented as percentages with respect to untreated cells. Values represent the mean ± S.E. from three separate experiments. *, *p* < 0.05.

in the absence of R-PIA. Maximal incorporation of ADA-FITC, which occurred at 48 h, increased (37%) in the presence of R-PIA. The differences between control and R-PIA-treated cells were significant between 8 and 48 h. Internalization of ADA-FITC into the cells was also analyzed by immunofluorescence and confocal assays. Cells maintained in the presence of ADA-FITC for 24–72 h showed accumulations of labeling in intracellular vesicles. R-PIA treatment for 24–72 h resulted in a marked increase in intracellular labeling in brighter and larger intracellular vesicles (Fig. 8A). These variations were not due to fluid phase internalization, since R-PIA did not affect the extent of fluorescein-conjugated bovine serum albumin internalization (Fig. 8B).

As shown in Figs. 5 and 9A and as described elsewhere (32), R-PIA induces the disappearance of cell surface A₁Rs even in the absence of exogenous ADA. Since cell surface ADA and A₁R colocalize, these results indicate that R-PIA might induce internalization of A₁R together with ADA. We tested this hypothesis by comparing the distribution of A₁Rs and ADA in double immunofluorescence staining experiments analyzed by confocal microscopy. Double immunofluorescence staining with the

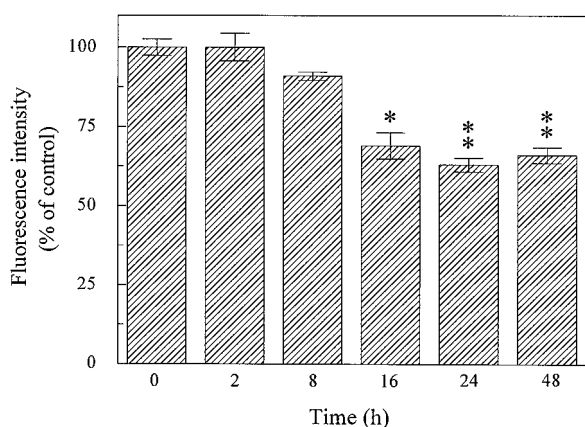


FIG. 7. Cell surface expression of ADA (ecto-ADA) in response to R-PIA. Cells were untreated (control) or treated with 50 nM R-PIA for 0–48 h at 37 °C. Cells were then washed once in PBS, fixed using 4% paraformaldehyde in PBS, and prepared for immunostaining with the fluorescein-conjugated anti-ADA antibody (ADA-FITC, 75 µg/ml) as indicated under “Experimental Procedures.” Fluorescence intensity was quantified on an EPICS Profile flow cytometer. Data are presented as percentages with respect to untreated cells. Values represent the mean ± S.E. from three separate experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$.

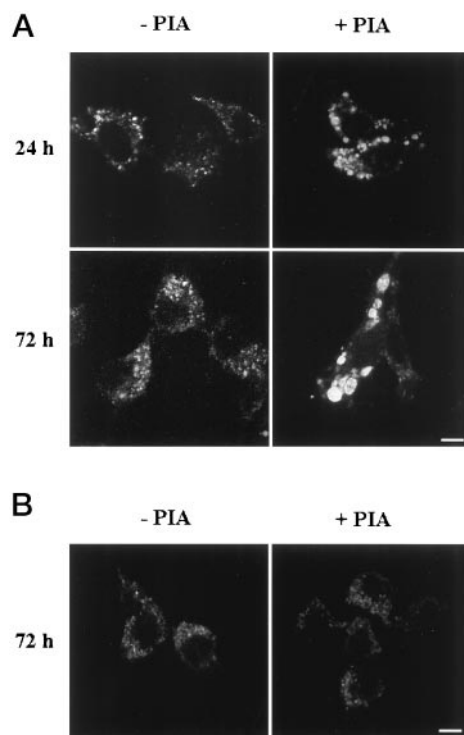


FIG. 8. Internalization of fluorescein-conjugated ADA or bovine serum albumin in the absence or presence of R-PIA. A, cells growing on glass coverslips in the presence of 65 nM fluorescein-conjugated ADA were incubated (37 °C) in the absence (–PIA) or presence (+PIA) of 50 nM R-PIA for 24 or 72 h. Glass coverslips were washed in PBS, fixed, mounted, and analyzed by confocal microscopy as described under “Experimental Procedures.” Panel B, cells were grown in the presence of 65 nM fluorescein-conjugated bovine serum albumin in the absence (–PIA) or presence (+PIA) of 50 nM R-PIA for 72 h. Cells were processed as indicated in A. Images correspond to a middle section of representative cells. Scale bar, 10 µm.

rhodamine-conjugated rabbit anti-A₁R (PC21-TRITC) and fluorescein-conjugated ADA (ADA-FITC) was performed in control and R-PIA-treated cells. Incubation of cells with ADA-FITC and PC21-TRITC for 24 h showed staining for ADA and A₁R in intracellular vesicles (Fig. 9B, top). Superimposition of images revealed a high degree of colocalization between the two

proteins (Fig. 9B, yellow). After 24 h of chronic exposure to 50 nM R-PIA, perinuclear staining and a marked decrease in the number of intracellular vesicles containing ADA and A₁Rs, accompanied by a drastic increase in size and brightness, were observed (Fig. 9B, bottom). Colocalization of both proteins (yellow) was confirmed in numerous cells analyzed at various planes, indicating that A₁Rs and ADA internalize together via the same pathway of endocytosis.

DISCUSSION

This study provides new insights into ligand-mediated mechanisms involved in the regulation and trafficking of A₁ adenosine receptors. Several studies have demonstrated agonist desensitization by means of a reduced inhibition of adenylate cyclase associated with a net loss of binding sites and G_i proteins (29, 30, 31). However, little was known about the biochemical mechanisms involved in desensitization and down-regulation or the intracellular pathways involved in the trafficking of A₁Rs. Another interesting question concerns the role of ecto-ADA in the desensitization of A₁R due to the fact that A₁R and ADA interact on the cell surface (34). The high expression (100,000 receptors/cell) of A₁Rs (43), capable of interacting with ADA (34) makes DDT₁MF-2 cells a suitable model to study the role of ADA in the regulation of A₁R expression.

By means of immunofluorescence staining using antibodies against A₁R and ADA, a nearly homogenous distribution of A₁R and ADA over the plasma membrane of DDT₁MF-2 cells was observed. Confocal analysis demonstrated a high degree of colocalization between ADA and A₁R, which was maintained in the cell surface clusters formed after R-PIA treatment. The appearance of clusters was accelerated when cells were incubated simultaneously with R-PIA and ADA. This suggests an ADA-mediated regulation of ligand-induced redistribution of A₁Rs. Prolonged agonist stimulation induced the disappearance of membrane A₁R binding sites on DDT₁MF-2 cell surface, as previously shown (29). This phenomenon was also accelerated in the presence of ADA, although the maximum effect was the same as that achieved by R-PIA alone.

Ecto-ADA also underwent R-PIA-induced internalization. Internalized ADA appeared in relatively small intracellular vesicles, where it colocalized with A₁R. This may be an agonist-independent process representing the natural recycling of the ectoenzyme, or it may be due to the presence of endogenous adenosine. This latter possibility seems unlikely, since no internalization of A₁R occurred in the absence of R-PIA. Although ADA internalization may be agonist-independent, long term agonist treatment increased the degree of ADA internalization. Double immunofluorescence assays analyzed by confocal microscopy showed colocalization of ADA and A₁Rs in the same intracellular vesicles. All these findings suggest that ADA and A₁Rs participate in the regulation of each other and that this mutual regulation includes internalization via the same endocytic pathway. To our knowledge, this is the first report describing the trafficking of a purine ectoenzyme and demonstrating a common internalization pathway for A₁R and the ectoprotein (ecto-ADA) interacting with it.

During the agonist-mediated redistribution (clustering) of A₁Rs on the cell surface, a time-dependent phosphorylation of A₁Rs occurs. Basal A₁R phosphorylation increased rapidly between 5 and 15 min as a consequence of agonist stimulation. Agonist-induced A₁R phosphorylation was accelerated and enhanced in the presence of exogenous ADA. Taking into account these data and the fact that ADA increased ligand-induced A₁R aggregation, the involvement of phosphorylation in the clustering of A₁ receptors is highly probable. In fact, clustering and internalization of A₁Rs were mimicked by activators of protein

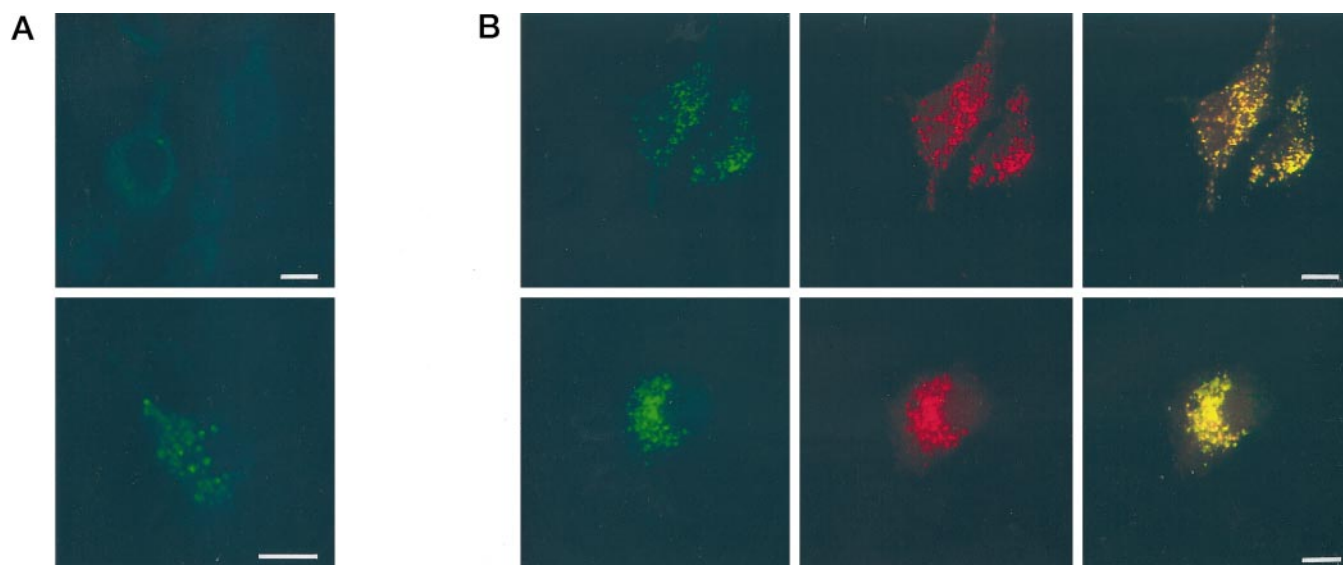


FIG. 9. Colocalization of internalized ADA and A₁ in the absence or presence of R-PIA. A, DDT₁MF-2 cells were grown in DMEM containing fluorescein-conjugated anti-A₁R antibody (PC21-FITC, 10 μg/ml) in the absence (*top*) or in the presence (*bottom*) of 50 nM R-PIA for 24 h. After treatment, cells were fixed, washed, and analyzed by confocal microscopy. Images correspond to a middle section of representative cells. Scale bar, 10 μm. B, DDT₁MF-2 cells were grown in DMEM containing fluorescein-conjugated ADA (65 nM) and rhodamine-conjugated anti-A₁R antibody (PC21-TRITC, 10 μg/ml) in the absence (*top*) or in the presence (*bottom*) of 50 nM R-PIA for 24 h. After treatment, cells were fixed, washed, and analyzed by confocal microscopy to detect ADA (*green images*) and A₁R (*red images*). Superimposition of images reveals the intracellular colocalization of ADA and A₁R in *yellow*. Scale bar, 10 μm.

kinase A and protein kinase C (32), thus showing the need for Ser/Thr phosphorylation in these events. Simultaneous to ligand-induced receptor phosphorylation and aggregation, a decrease in ligand-induced second messenger response, *i.e.* a functional desensitization, was observed. Functional desensitization was a rapid time-dependent process that occurred within minutes but remained for hours. This contrasts with data found in Chinese hamster ovary cells transfected with the human A₁R. Overexpressed A₁Rs were neither phosphorylated nor desensitized after 10 min of R-PIA treatment. Interestingly, a stably expressed chimeric A₁-A₃ adenosine receptor, in which the C-terminal domain of A₁R distal to its predicted palmitoylation site was replaced by the corresponding region of the A₃ subtype, was able to undergo functional desensitization and agonist-stimulated phosphorylation (44). Differences in amino acid sequence of the C-terminal domain of A₁R and A₃R can account for the distinct desensitization pathways found in Chinese hamster ovary cells. However, the short term desensitization and phosphorylation reported here for A₁R in DDT₁MF-2 cells demonstrate that the cellular environment near the plasma membrane is important for receptor regulation. The regulation of A₁R phosphorylation and desensitization by cell surface ADA is an example of the relevance of the membrane components for receptor regulation. To our knowledge, this is a novel finding, since no other receptor whose function is modulated by an ectoenzyme has been described.

Rapid ligand-induced desensitization of G protein-coupled receptors such as β₂-ARs (45), angiotensin II receptor (46), cholecystokinin receptor (47), δ-opioid receptor (48), and neurokinin 1 receptor (49) correlates with receptor phosphorylation and uncoupling from G proteins. Several reports have provided evidence that receptor desensitization is short term and does not require endocytosis (45, 50, 51). An exception is the agonist-induced desensitization of the secretin receptor, which occurs in the absence of receptor phosphorylation and is basically induced by receptor internalization (52). β₂-AR (22, 23) and neurokinin 1 receptor (49) resensitization seems to be a consequence of receptor dephosphorylation in endosomes and their recycling back to the plasma membrane. Our data indicate that although receptor phosphorylation returns to basal

level, desensitization of A₁R continued for hours (see Figs. 3 and 4). In a recent study, desensitization of the cholecystokinin receptor occurred in both rat pancreatic acinar cells and transfected Chinese hamster ovary cells, although receptor dephosphorylation was evident only in acinar cells. Moreover, cholecystokinin receptor in Chinese hamster ovary cells maintained its phosphorylation state throughout the time of internalization, whereas the acinar cell receptor was dephosphorylated to its basal state while remaining on the cell surface (47). All of these data suggest differences in regulation of G protein-coupled receptors, depending on the receptor subtype and the cell system in which the receptor is expressed.

The results provide new insights into the regulation and trafficking of A₁Rs in response to agonist stimulation. Moreover, it is shown that ecto-ADA, besides its role in regulating the extracellular concentration of adenosine, modulates all the regulatory mechanisms involved in desensitization of A₁R. It is remarkable that ADA and A₁R colocalize on the cell surface and in intracellular vesicles after internalization. During the agonist-dependent internalization process, ADA and A₁R follow a common endocytic pathway. This is probably the molecular mechanism underlying the recent discovery of increased plasma adenosine levels found after caffeine or sulfophenyltheophylline consumption (53). Although it does not occur in DDT₁MF-2 cells, there are cell types whose A₁Rs internalize in response to xanthine antagonists.² The probable internalization of ADA and A₁R from the cell surface in those antagonist-treated cells would lead to a decrease in adenosine deamination and, subsequently, to an increase in the extracellular levels of the nucleoside. As pointed out by Conlay *et al.* (53), alteration of adenosine levels by sudden changes in methylxanthine consumption could affect the physiology of many organ systems and provoke bronchospasm, alter blood pressure, change cardiac rhythms, or influence seizure thresholds. Confirmation that internalization of ecto-ADA is the cause of these alterations would be very valuable from a physiological and therapeutic point of view.

² A. Navarro, C. A. Saura, J. Mallol, E. I. Canela, C. Lluís, and R. Franco, manuscript in preparation.

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Adenosine Deaminase and A₁ Adenosine Receptors Internalize Together following Agonist-induced Receptor Desensitization

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