Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors

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Abstract Adenosine deaminase (ADA) is not only a cytosolic enzyme but can be found as an ecto-enzyme. 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 A_1 adenosine receptor (A_1R) is a second ecto-ADA binding protein. Binding of ADA to A_1R increased its affinity for the ligand thus suggesting that ADA was needed for an effective coupling between A_1R and heterotrimeric G proteins. This was confirmed by the fact that ASA, independently of its catalytic behaviour, enhanced the ligand-induced second messenger production via A_1R. 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 A_1R.

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. A_1 adenosine receptors (A_1R) display two different affinity states that depend upon the coupling to heterotrimeric G proteins; coupled receptor-G protein complexes display high affinity (K_d = 0.1–0.2 nM), whereas uncoupled receptors display low affinity (K_d = 1–2 nM) [4–6].

A_1R-mediated signalling depends on the effective concentration of extracellular adenosine, whose regulation involves a variety of nucleoside transport systems and ecto-nucleotidases [7]. Since we discovered adenosine deaminase (ADA, EC 3.5.4.4) on the surface of hematopoietic cells [8], ecto-ADA has also been implicated in controling 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 A_1 adenosine receptor (A_1R) present on DDT_1MF-2 cells is demonstrated by immunoprecipitation, confocal microscopy and affinity chromatography. Here we also provide evidence that the interaction ADA-A_1R modulates ligand binding to A_1R and signalling via A_1R.

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2. Experimental

2.1. Materials

[^{3}H]*R*-N^6-(2-phenylisopropyl)-adenosine ([^{3}H]R-PIA) (36 Ci/mmol), myo-[^{3}H]inositol (85 Ci/mmol) and ECL immunoblotting detection system, were purchased from Amersham (Nuclear Iberica, Madrid, Spain). Adenosine deaminase (ADA, EC 3.5.4.4) from Sigma Chemical Co (St. Louis, MO, USA) was chromatographied through a Sephadex G-100 column and the final preparation was homogenous by electrophoresis. Rabbit anti-ADA antibody (Serotec, Oxford, UK) has been developed in our laboratory [8]. Antibodies against A_1 adenosine receptors, PC11 and PC12, are, respectively, affinity purified (chromatographied through specific peptide coupled to Sepharose) versions of antipeptide antisera PC10 and PC20 developed and characterized as described elsewhere [10]. The specificity of anti-A_1R antibodies was tested as described elsewhere [10]. PC11 and PC21 were not able to recognize ADA from DDT_1-MF2 cell extracts by immunoblotting (Fig. 3B) and from a calf intestine commercial soluble preparation by immunoprecipitation or immunoblotting. The antibody against CD26 (H12 monoclonal) was purchased from Endogen Inc. DDT_1MF-2 smooth muscle cells from hamster vas deferens were obtained from and cultured as recomended by the American Type Culture Collection.

2.2. Enzyme activities and ADA inhibition by Hg^{2+}

Adenosine deaminase (ADA, EC 4.5.4.4) and lactate dehydrogenase (EC 1.1.1.29) activities were estimated as described by Franco et al. [11] (in the presence of 1 μM dipyridamole and 1 μM nitrobenzylthioinosine as adenosine transport inhibitors). Dipeptidylpeptidase IV (CD26, EC 3.4.14.5) activity was assayed as described by Nagatsu et al. [12]. Calf ADA activity (50 U/ml) was completely abolished after preincubation with 100 μM $HgCl_2$ (2 h) and removal of free Hg^{2+} by gel filtration using Sephadex G-25.

2.3. Protein determination

Protein was measured as described by Sorensen and Brodbeck [13].

2.4. Immunostaining experiments

For confocal microscopy analysis, nonpermeabilized 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 serum albumin (buffer B) by applying a mixture of antibodies (60 min, 37°C, buffer B): fluorescein-conjugated rabbit anti-A_1R (PC21, 100 μg/ml) and rhodamine-conjugated rabbit anti-ADA (40 μg/ml), or rhodamine-conjugated rabbit anti-ADA (40 μg/ml) and fluorescein-conjugated anti-CD26 (20 μg/ml), or rhodamine-conjugated PC21 (100 μg/ml) and fluorescein-conjugated anti-CD26 (20 μg/ml). Rinsed coverslips were mounted with immunofluorescence medium. Observations were performed with a Leica TCS 4D confocal scanning laser microscope.

Immunoprecipitation of A_1R cross-linked to iodinated (*R*)-2-azido-*N*2-*p*-hydroxy-PIA(R-AHPIA), autoradiography and immunoblotting were performed as described by Ciruela et al. [10].

2.5. Radioligand binding assay

Saturation analysis of [^{3}H]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 h in serum-free DMEM buffered with 20 mM HEPES, pH 7.4. Saturation isotherms were obtained from five replicates for each [^{3}H]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

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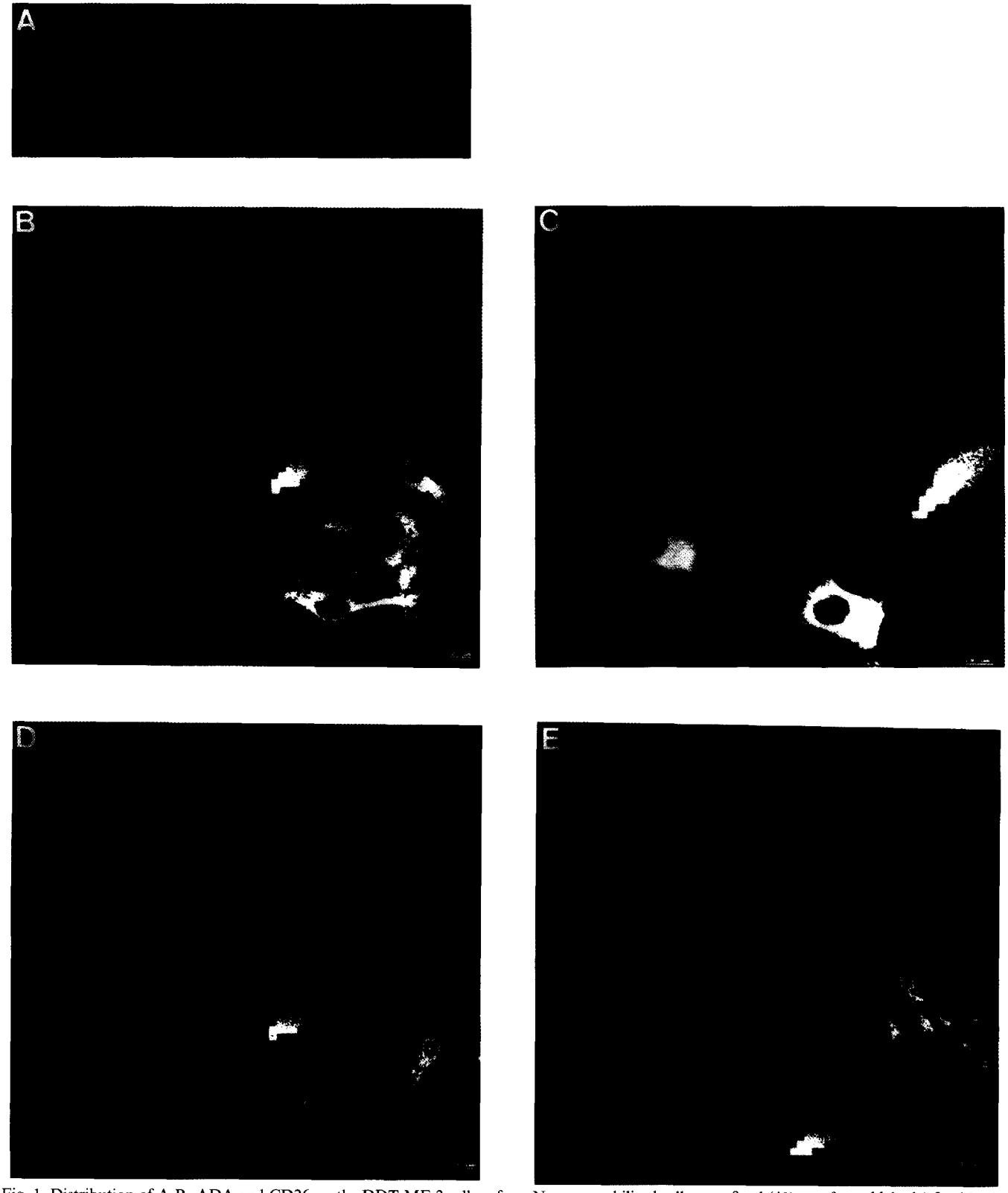

Fig. 1. Distribution of A_1R, ADA and CD26 on the DDT_1MF-2 cell surface. Nonpermeabilized cells were fixed (4% paraformaldehyde) for 15 min and washed in PBS-20 mM glycine. Double immunofluorescence staining was performed as indicated in section 2. Panel A. Vertical section of cells labeled for A_1R (left) and ADA (right). Images are at the same scale than in panel B. Panel B. Surface expression of A_1R (upper left) and ADA (upper right). Panel C. Cell on glass coverslips were preincubated with 2 U/ml of exogenous ADA (30 min) before fixation (ADA from Sigma was chromatographed on Sephadex G-100; the final preparation was homogenous by electrophoresis). Surface expression of A_1R (upper left) and ADA (upper right). Panel D. Surface expression of CD26 (upper left) and ADA (upper right). Panel E. Surface expression of CD26 (upper left) and A_1R (upper right). In B, C, D and E, the lower left image corresponds to the superposition of the two fluorescences (yellow) whereas the bottom right image shows colocalization in white (insert: multi-color analysis of confocal images). Scale bar = 10 μm.

and one affinity state. The two-site model was selected when $P < 0.001$ [14].

2.6. 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 DDT_1MF-2 cells were applied to a 1 ml of ADA-affinity column at a flow rate of 2.5 ml/h at room temperature. The 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) to a 1 g of cyanogen bromide-activated Sepharose. 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 [^{3}H]R-PIA binding, lactate dehydrogenase activity and protein.

2.7. Second messengers determination

Ca^{2+} release from intracellular stores and the level of inositol phosphates (InsP) were measured as described elsewhere [15–17]. In cells (10^6 cells/ml) loaded with the fluorescent dye Fura 2-AM (5 μM, 30 min), the 50 nM R-PIA-induced Ca^{2+} 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[^{3}H]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 [^{3}H]InsP was isolated using a Dowex AG1-X8 column.

3. Results and discussion

DDT_1MF-2 cells exhibit ecto-ADA. When 60 μM adenosine was used as substrate, ecto-ADA activity (see section 2) was 1.0 mU per million of intact cells. DDT_1MF-2 cells also express on their surface binding sites for the A_1 adenosine receptor specific agonist [^{3}H]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-A_1R, the cell surface distributions of ADA (red fluorescence) and A_1R (green fluorescence) on the surface of DDT_1MF-2 cells appeared similar (Fig. 1A,B). The label was indeed corresponding to a cell surface staining as judged by the lack of intracellular stain (Fig. 1A). Further analysis by confocal microscopy demonstrated that the degree of colocalization between ADA and A_1R on the surface of DDT_1MF-2 cells was very high. The intensity of white and its situation far from the axis origin of the cytofluorogram indicated that the colocalization was quantitatively very high (Fig. 1B, insert of the bottom right image). Since DDT_1MF-2 cells also express CD26 on their surface (dipeptidylpeptidase activity using 1 mM gly-pro-paranitroanilide as substrate was 3.4 mU per million cells), the codistribution of ADA (red fluorescence) and CD26 (green fluorescence) (Fig. 1D) and of CD26 (green fluorescence) and A_1R (red fluorescence) (Fig. 1E) were studied. In contrast to the codistribution of ADA and A_1R, ADA and CD26 or A_1R and CD26 had a different distribution pattern with a moderate degree of colocalization (in white). Taken together, these colocalization studies suggested that there were significantly more ADA molecules colocalizing with A_1R than with CD26; therefore, A_1R may act as a second receptor for ecto-ADA.

Next we examined whether exogenous ADA could bind to cell surface A_1R molecules that did not colocalize with endogenous ADA, i.e. those labelled green in the images of Fig. 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 (Fig. 1C). Thus, all A_1R molecules present on the sur-

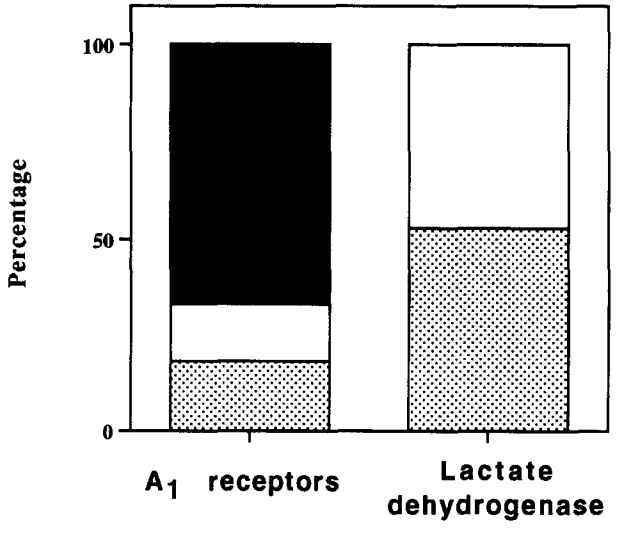

Fig. 2. Chromatography of cell extracts through an ADA-Sepharose column. The 1 nM [^{3}H]R-PIA binding to A_1 adenosine receptors and the lactate dehydrogenase activity was determined in the different fractions: not retained (dotted), wash (white) and retained (black). Values are given in percentage respect to the total amount applied: 4 pmol for A_1 adenosine receptors and 0.36 μmol/min for lactate dehydrogenase activity.

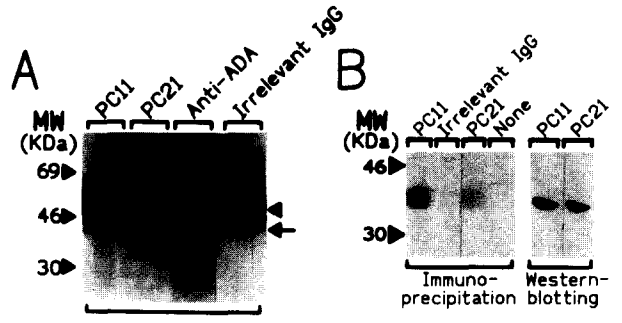

Fig. 3. Immunochemical detection of A_1R and ADA. Panel A. Coprecipitation of ADA using antibodies against A_1R. DDT_1MF-2 cell membranes were solubilized using 1% Nonidet-P40; immunoprecipitation using PC11 and PC12 (or anti-ADA and irrelevant IgG as controls) (40 μg/ml of each antibody) and immunoblotting (PVDF membranes) were performed as indicated in section 2. PVDF membranes were treated with anti-ADA antibody (5 μg/ml) and immunoreactive bands were visualized by horseradish-peroxidase-labelled goat anti-rabbit IgG antibodies (1:15000-fold dilution), followed by ECL chemiluminiscence detection. Arrow: ADA Arrow head: IgG heavy chain. Panel B. A_1R recognition by PC11 and PC21. Immunoprecipitation of A_1R cross-linked to iodinated (*R*)-2-azido-*N*2-*p*-hydroxy-PIA (R-AHPIA) (left image). Labelled membranes (0.5 mg protein/ml) were immunoprecipitated in the absence or in the presence of 40 μg/ml of either PC11, PC21 or an irrelevant IgG. Immunoprecipitated material was analyzed by SDS- PAGE. The gel was run, dried and autoradiographed. Immunoblotting of A_1R using PC 11 and PC21 antibodies (right image). In DDT_1MF-2 cells the 74 kDa band that PC21 recognizes in pig brain membranes [10], was not detected.

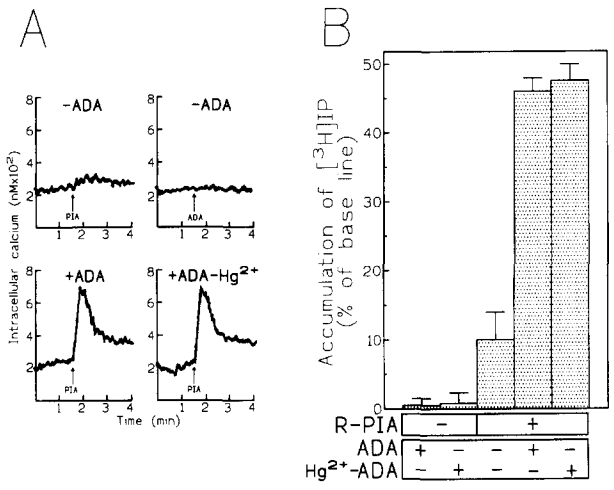

Fig. 4. R-PIA-induced calcium mobilization and total [^{3}H]InsP production in DDT$_1$MF-2 cells. 50 nM R-PIA induced calcium mobilization (A) and inositol phosphates production (15 min) (B). Cells were incubated (30 min) in the absence (−ADA) or presence of 0.2 U/ml of adenosine deaminase, enzymatically active (+ADA) or irreversibly inhibited by 100 μM $HgCl_2$ (2 h, room temperature) and desalted through a Sephadex G-25 (+ADA-Hg^{2+}).

face of DDT$_1$MF-2 cells can bind ADA. We conclude that an interaction ADA-A$_1$R occurs at the cell surface level in intact cells.

The ability of ADA to recognize A$_1$R was confirmed by isolating the A$_1$R from a detergent extract (obtained as described in section 2) with an ADA-Sepharose affinity column. A$_1$R was 67% retained within the column whereas a control protein, lactate dehydrogenase, was completely eluted (Fig. 2). Conversely, ADA was coprecipitated from membrane extracts using two (PC11 and PC21) different antipeptide anti-A$_1$R antibodies (Fig. 3A). Both antibodies immunoprecipitated A$_1$R photolabelled with [^{125}I]R-PIA (with an efficiency that ranges from 15 to 25% [10]) and both recognized the 39 kDa band of the receptor by Western blotting (Fig. 3B). However, PC11 was more effective in immunoprecipitation and Western blotting experiments than PC21. For this reason the coprecipitated ADA band was stronger when PC11 was used (Fig. 3A). These results support the notion that A$_1$R binds ADA on the surface of DDT$_1$MF-2 cells.

Because ADA and A$_1$R interacted on the surface of DDT$_1$MF-2 cells, the influence of ADA on the thermodynamic behavior of the agonist [^{3}H]R-PIA binding to A$_1$R was analyzed. Addition of ADA reportedly results in an increase in [^{3}H]R-PIA binding to A$_1$R as a consequence of the degradation of endogenous adenosine [18,19]. The effect of exogenous ADA on [^{3}H]R-PIA binding to intact DDT$_1$MF-2 cells and to membranes from these cells was analyzed. A$_1$R in intact cells displayed, in the absence of external ADA, a very low affinity state (K_d = 40 nM) similar to that found in membrane preparations (Table 1). In the presence of exogenous ADA the affinity for [^{3}H]R-PIA increased significantly (K_d = 10 nM). The effect of ADA in membranes was more pronounced since it converted the single very-low affinity state of A$_1$R (K_d = 50 nM) into two states (one of high affinity, 0.79 nM, and another of low affinity, 8.7 nM). 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 [^{3}H]R-PIA binding to A$_1$R. 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 [^{3}H]R-PIA binding to membranes in presence of Hg^{2+}-inhibited ADA were very high: 129 μM (high-

Table 1
Equilibrium parameters of [^{3}H]R-PIA binding to DDT$_1$MF-2 cells and to cell membranes in the absence or presence of ADA

	Presence of ADA	Affinity-state	K_d (nM)	B_{max} (pmol/mg prot.)
Membranes	None	High-affinity	–	–
		Low-affinity	–	–
		Very low-affinity	50 ± 10	0.4 ± 0.1
	0.2 U/ml	High-affinity	0.79 ± 0.09	0.28 ± 0.03
		Low-affinity	8.7 ± 1.6	0.15 ± 0.05
		Very low-affinity	–	–
	*0.2 U/ml plus Hg^{2+}	High-affinity	1.5 ± 0.5	0.25 ± 0.05
		Low-affinity	9 ± 2	0.13 ± 0.07
		Very low-affinity	–	–
Intact cells	None	High-affinity	–	–
		Low-affinity	–	–
		Very low-affinity	40 ± 10	0.10 ± 0.03
	0.2 U/ml	High-affinity	–	–
		Low-affinity	10 ± 6	0.11 ± 0.02
		Very low-affinity	–	–
	*0.2 U/ml plus Hg^{2+}	High-affinity	–	–
		Low-affinity	10 ± 3	0.15 ± 0.02
		Very low-affinity	–	–

*ADA in 50 mM Tris-HCl buffer, pH 7.4, was incubated for 2 h 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.

affinity) and 2.7 mM (low-affinity). Thus, this alteration in the behavior of membrane A_1R in the presence of ADA is not due to the degradation of the endogenous ligand, adenosine, but to a direct effect upon A_1R.

Does ADA induce signal transduction events via A_1R? To address this, DDT_1MF-2 cells were treated with R-PIA with or without ADA (or Hg^{2+}-inhibited ADA) and the release of Ca^{2+} from intracellular stores and the level of inositol phosphates (InsP) were measured. As shown in Fig. 4A, the R-PIA-induced increase in intracellular Ca^{2+} 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 Ca^{2+} mobilization was evident only in the presence of exogenous ADA. The increase in inositol phosphates was also amplified by external ADA (Fig. 4B). Induction of both second messengers was independent of the catalytic activity of the enzyme since a similar potentiation was obtained by Hg^{2+}-inhibited ADA. Thus, ADA is necessary for an efficient coupling of A_1R with the signal tranduction machinery. Signalling in the absence of ADA may be due to a small proportion of A_1R molecules interacting with endogenous ADA. It should be noted that signalling via A_1 adenosine receptors has been always investigated in the present of exogenous ADA. This practice used in principle to eliminate the endogenous ligand, adenosine, has lead to believe that exogenous ADA had no effect on ligand-induced signalling. However, as evidenced here, ecto-ADA has a key role in A_1R functioning.

Taking into account that the changes of affinity in the case of A_1R reportedly reflect changes in the coupling to heterotrimeric G proteins, we postulate that ADA is required for coupling of the A_1R to heterotrimeric G proteins in DDT_1MF-2 cells. This is the first report providing evidence that a heptaspanning receptor requires a cell surface protein for efficient signalling. Modulation of A_1R-mediated signal transduction by ADA-A_1R interaction opens a new perspective in the regulation mechanism of heptaspanning receptors coupled to G proteins.

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