

# Involvement of Caveolin in Ligand-Induced Recruitment and Internalization of A<sub>1</sub> Adenosine Receptor and Adenosine Deaminase in an Epithelial Cell Line

SILVIA GINÉS, FRANCISO CIRUELA, JAVIER BURGUEÑO, VICENT CASADÓ, ENRIC. I. CANELA, JOSEFA MALLOL, CARME LLUÍS, and RAFAEL FRANCO

*Departament de Bioquímica i Biologia Molecular, Facultat de Química. Universitat de Barcelona, Spain*

Received June 14, 2000; accepted January 29, 2001

This paper is available online at <http://molpharm.aspetjournals.org>

## ABSTRACT

Chronic exposure of A<sub>1</sub> adenosine receptors (A<sub>1</sub>R) to A<sub>1</sub>R agonists leads to activation, phosphorylation, desensitization, and internalization to intracellular compartments of the receptor. Desensitization and internalization of A<sub>1</sub>R is modulated by adenosine deaminase (ADA), an enzyme that regulates the extracellular concentration of adenosine. ADA interacts with A<sub>1</sub>R on the cell surface of the smooth muscle cell line DDT1 MF-2, and both proteins are internalized following agonist stimulation of the receptor. The mechanism involved in A<sub>1</sub>R and ADA internalization upon agonist exposure is poorly understood in epithelial cells. In this report, we show that A<sub>1</sub>R and ADA interact in LLC-PK<sub>1</sub> epithelial cells. Exposure of LLC-PK<sub>1</sub> cells to A<sub>1</sub>R agonists induces aggregation of A<sub>1</sub>R and ADA on the cell surface and their translocation to intracellular compartments. Biochemical and cell biology assays were used to characterize the intracellular vesicles containing both proteins after

agonist treatment. A<sub>1</sub>R and ADA colocalized together with the rafts marker protein caveolin. Filipin, a sterol-binding agent that disrupts rafts (small microdomains of the plasma membrane), was able to inhibit A<sub>1</sub>R internalization. In contrast, acid treatment of the cells, which disrupts internalization via clathrin-coated vesicles, did not inhibit agonist-stimulated A<sub>1</sub>R internalization. We demonstrated that A<sub>1</sub>R agonist N<sup>6</sup>-(R)-phenylisopropyl adenosine promotes the translocation of A<sub>1</sub>R into low-density gradient fractions containing caveolin. Furthermore, a direct interaction of the C-terminal domain of A<sub>1</sub>R with caveolin-1 was demonstrated by pull down experiments. These results indicate that A<sub>1</sub>R and ADA form a stable complex in the cell surface of LLC-PK<sub>1</sub> cells and that agonist-induced internalization of the A<sub>1</sub> adenosine receptor and ADA is mediated by clathrin-independent endocytosis.

Desensitization is a ubiquitous phenomenon in which a cell attenuates its response to prolonged or excessive agonist stimulation. Following interaction with agonists, many receptors undergo a process of internalization and down-regulation with a net loss of binding sites on the cell surface (Benovic et al., 1988).

Different pathways for internalization of G-protein-coupled receptors have been reported. The  $\beta_2$ -adrenergic receptor in 293 cells, the human chorionic gonadotropin hormone/luteinizing hormone receptor, the thrombin receptor, and others are internalized by the classical endocytic pathway initiated at clathrin-coated pits (Von Zastrow and Kobilka, 1992; Hoxie et al., 1993). On the other hand, the cholecysto-

kinin receptor has been described to internalize through two pathways, one clathrin-dependent and another clathrin-independent (Roettger et al., 1995).

Caveolae and rafts are small microdomains of the plasma membrane characterized by high levels of cholesterol and glycosphingolipids (Sargiacomo et al., 1993). These structures have been detected in most cells but are particularly abundant in adipocytes, endothelial, and muscle cells. Caveolae are specialized structures involved in signal transduction (Lisanti et al., 1994), in internalization of small molecules by a process called potocytosis (Anderson et al., 1992; Anderson, 1993a) and in transcytosis (Anderson, 1993b). A role for caveolae in signal transduction was proposed following the identification of some extracellular mediators, receptors, and effectors in these structures. Caveolae were found to contain endothelin and its receptor (Chun et al., 1994), atrial natriuretic peptide (Page et al., 1994), platelet-derived growth

This research was supported by Spanish Comisión Interministerial de Ciencia y Tecnología R+D program (Grants PB97-0984 and BIO1999-0601-C02).

<sup>1</sup> Present address: Molecular Neurogenetics Unit, Massachusetts General Hospital, 149 13th St., Charlestown, MA 02129.

**ABBREVIATIONS:** ADA, adenosine deaminase; A<sub>1</sub>R, adenosine A<sub>1</sub> receptor(s); TRITC, rhodamine isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; GST, glutathione S-transferase; PVDF, polyvinylidene fluoride; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; Mes, 4-morpholineethanesulfonic acid; R-PIA, N<sup>6</sup>-(R)-phenylisopropyladenosine.

factor (Liu et al., 1996), subunits of G-proteins (Sargiacomo et al., 1993; Chang et al., 1994; Smart et al., 1995), the endothelial isoform of nitric-oxide synthase (Garcia-Cardena et al., 1996; Shaul et al., 1996), Src family kinases (Glenney, 1989; Arreaza et al., 1994), an inositol 1,4,5-triphosphate receptor (Fujimoto et al., 1992), and B2 bradykinin receptor (De Weerd and Leeb-Lundberg, 1997). The significance of the association of each of these proteins with caveolae is not clear. One hypothesis is that caveolae serve as compartments for recruitment of components in a signaling pathway to increase efficient and rapid coupling of receptors to more than one effector system (Lisanti et al., 1994).

Adenosine, acting through specific receptors located on the cell surface, exerts potent actions on a wide range of physiological systems, including nervous, cardiovascular, gastrointestinal, urogenital, respiratory, and lymphatic systems (Olah and Stiles, 1992; Tucker and Linden, 1993; Dalziel and Westfall, 1994). The agonist-induced desensitization of  $A_1$  adenosine receptor has been studied in a variety of cells and tissues such as adipose (Longabaugh et al., 1989) or the smooth muscle cell line DDT<sub>1</sub>MF-2 (Ramkumar et al., 1991; Ciruela et al., 1997). Chronic exposure of DDT<sub>1</sub>MF-2 cells to 50 nM R-PIA produces a functional desensitization and causes a rapid (15 min) aggregation, phosphorylation, and a slower internalization of  $A_1$  adenosine receptor. On the other hand, extracellular adenosine concentration is modulated by cell surface ADA. Recently, we reported, in these cells, that cell surface ADA interacts with  $A_1$ R and modulates ligand-induced signaling, desensitization, and internalization of  $A_1$ R (Saura et al., 1996). Although the biochemical mechanisms of  $A_1$ R desensitization have been established, the intracellular pathways and compartments involved in the sequestration and internalization of this G-protein-coupled receptor are less known. Although it is accepted that the clathrin pathway is the predominant pathway for agonist-induced internalization of many G-protein-coupled receptors (Böhm et al., 1997), alternative routes involving noncoated vesicles must be taken into account.

In this report, we show that  $A_1$ R and ADA interact on LLC-PK<sub>1</sub> cell surface. The activation of  $A_1$ R by an agonist induces clusters of both molecules on the cell surface followed by their translocation to intracellular compartments. To gain more insight about the intracellular pathways involved in the trafficking of  $A_1$ R and ADA, the cointernalization of both proteins after receptor activation was studied by means of electron and confocal microscopy. Our results show that  $A_1$ R internalizes using the same route as ADA and that both proteins use a clathrin-independent pathway.

## Materials and Methods

**Cell Cultures.** LLC-PK<sub>1</sub> pig kidney epithelial cells were provided by Dr. Rolf Kinne (Max Planck Institut für Molekularphysiologie, Dortmund, Germany). Cells were cultured (37°C, 5% CO<sub>2</sub>) in Dulbecco's modified Eagle's medium (DMEM; Life Technologies SA, Barcelona, Spain) supplemented with 5% (v/v) horse serum (Life Technologies SA), 1% nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.5 mM 2-mercaptoethanol.

**Antibodies.** Polyclonal antisera against purified calf ADA and against the peptides corresponding to part of the second extracellular loop (PC21) or part of the third intracellular loop (PC11) of  $A_1$ R, were generated by immunization of female New Zealand White rabbits

by Bio-Kit Company (Barcelona, Spain). PC21 or PC11 were purified by affinity chromatography using the specific peptide coupled to Sepharose CL4B (Pharmacia LKB Biotechnology, Uppsala, Sweden). Anti-ADA antibody was purified using ADA coupled to cyanogen bromide-activated Sepharose (Pharmacia LKB Biotechnology). Specificity and an extensive characterization of anti-ADA and anti- $A_1$ R antibodies (PC21 and PC11) have been previously reported (Aran et al., 1991; Ciruela et al., 1995). Rabbit anti-caveolin-1 antibody (anti-caveolin C13630) was obtained from Transduction Laboratories (Mamhead, UK). Horseradish-peroxidase-conjugated goat anti-rabbit IgG was purchased from Boehringer-Mannheim (GmbH, Germany). Protein A-gold (20 nm and 10 nm) was generously provided by Servicios Científico Técnicos (Universidad de Barcelona, Spain).

**Generation and Expression of the GST-Fusion Protein.** To produce the glutathione *S*-transferase fusion protein containing the C-terminal domain of adenosine  $A_1$  receptor (GST-CTA<sub>1</sub>R), the C-terminal tail of the full-length human  $A_1$ R in pcDNA3 (kindly given by Dr. S. A. Rivkees, Department of Pediatrics, Yale University, New Haven, CT) was amplified using proofreading *Pfu* DNA polymerase and primers F-CTA<sub>1</sub>R (5'-CGGCCATGGAATTCATTGTCTATGCCTTCCGCATCCAGAAGTTCGCG-3') and R-CTA<sub>1</sub>R (5'-CTCTAGACCTCGAGTCCCTCCGTAGGACCCTTGCCCTCCAGGCC-3'). Amplification conditions were as follows: 1 cycle of 1 min at 95°C, 45 s at 95°C, 45 s at 62°C, 2 min at 72°C; 29 cycles of 45 s at 95°C, 45 s at 62°C, 2 min at 72°C; 10 min at 72°C. The amplified product was subcloned into the *Eco*RI and *Xho*I sites of the *Escherichia coli* expression vector pGEX-4T-1 (Pharmacia Biotech). The sequence and orientation of the DNA construct was verified by sequencing using ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems). Recombinant fusion proteins GST and GST-CTA<sub>1</sub>R were expressed and purified on glutathione-Sepharose (Pharmacia Biotech) as described previously (Ciruela and McIlhinney, 1997). Briefly, bacterial overexpression of GST and GST-CTA<sub>1</sub>R was facilitated in the *Escherichia coli* BL21 strain (Promega, Madison, WI). The fusion proteins expression was performed with 0.1 mM isopropyl- $\beta$ -D-thio-galactopyranoside (Sigma, St. Louis, MO) for 3 h at 37°C.

**Pull-Down Assays.** GST and GST-CTA<sub>1</sub>R proteins (5  $\mu$ g each) were coupled to 120  $\mu$ l of a 50% suspension (v/v) of glutathione-agarose beads in buffer A (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 500 mM NaCl, 50 mM NaF) for 1 h at 4°C. GST-fusion protein-agarose slurries were preblocked in buffer A containing BSA (1 mg/ml). Cell membranes, prepared as previously described (Ciruela et al., 1997), were solubilized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 500 mM NaCl, 50 mM NaF, 60 mM *n*-octylglucoside, 1% Triton X-100) containing 1 mg/ml of BSA, for 30 min at 4°C. The solubilized material was centrifuged at 15,000 rpm for 20 min, and the supernatant was precleared with 120  $\mu$ l of the 50% suspension (v/v) of glutathione-agarose beads for 1 h with constant rotation at 4°C. After the preclearing, supernatants were transferred to a clean tube containing GST or GST-CTA<sub>1</sub>R coupled to the glutathione-agarose and incubated overnight with constant rotation at 4°C. Subsequently, the beads were washed twice with ice-cold lysis buffer, twice with ice-cold lysis buffer containing 6 mM *n*-octylglucoside and 0.1% Triton X-100, and twice with buffer A. Then 60  $\mu$ l of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to each sample. GST and GST-CTA<sub>1</sub>R bound proteins were dissociated by heating to 100°C for 5 min and resolved by SDS-PAGE in 12% gels (Laemmli, 1970). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech), using a semi-dry transfer system (Molnar et al., 1993). After blocking with 5% (w/v) dry milk in PBS (phosphate-buffered saline) containing 0.05% Tween-20 (PBS-T), PVDF membranes were washed in PBS-T and incubated overnight at 4°C with the affinity purified anti-caveolin antibody (Transduction Laboratories, Lexington, KY) in PBS-T-milk. Immunoreactive bands were detected with goat anti-rabbit antibody conjugated to horseradish-peroxidase

followed by development using chemiluminescence detection (Pierce).

**Immunostaining and Confocal Microscopy.** Cells growing on glass coverslips were incubated (4°C, 2 h) with PC21-TRITC (10 µg/ml) or anti-ADA-TRITC (10 µg/ml) in absence or presence of 100 nM R-PIA. Cells were washed twice with serum-free medium followed by incubation at 37°C for 0 min, 30 min, or 24 h. Cells were washed in PBS (pH 7.4), fixed (15 min) in 4% paraformaldehyde in PBS, and permeabilized with 0.02% saponin. After a 30-min incubation with 1% BSA in PBS, cells were labeled with fluorescein isothiocyanate-conjugated anti-caveolin antibody (dilution 1:75) at 37°C for 1 h. Coverslips were mounted using immunofluorescence medium (ICN Biomedical Inc., Costa Mesa, CA), and internalization was analyzed by confocal microscopy (LEICA TCS 4D confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope; Leica Lasertechnik GmbH, Heidelberg). When indicated, cells growing on glass coverslips were previously treated with 5 mM acetic acid at 37°C for 5 min or with 5 µg/ml filipin at 37°C for 30 min (the filipin was maintained in the medium during washes and internalization process). In contrast to what happens in other cell types, cell viability was not affected by treatment with either acetic acid or filipin. In all assays, cell viability was higher than 90%. Rinsed cells were then incubated with PC21-fluorescein isothiocyanate (10 µg/ml) in the absence or presence of 100 nM R-PIA or with transferrin-Texas red (20 µg/ml) at 4°C for 2 h. The internalization process at 37°C was performed for 30 min and fixed cells (4% paraformaldehyde) were analyzed by confocal microscopy.

**Immunoelectron Microscopy.** Cells in DMEM containing 20 mM Hepes and 1% BSA (buffer A) were incubated (4°C, 2 h) with an anti-ADA antibody (20 µg/ml) or an anti-A<sub>1</sub>R antibody (PC21; 10 µg/ml) in the absence or presence of 100 nM R-PIA. Cells were washed in cold buffer A and incubated with gold-conjugated protein A (20 nm) at 4°C for 45 min. Cells were washed in buffer A before induction of internalization at 37°C for 15 min, 30 min, or 24 h, then cells were rinsed with PBS (pH 7.4) and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h. Samples were treated with 1% OsO<sub>4</sub> in PBS for 45 min, dehydrated in ethanol, and embedded in Spurr's resin (Sigma). Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed in an electron microscope (Hitachi H-600, Philips, Amsterdam, The Netherlands).

For double labeling with an anti-ADA and an anti-A<sub>1</sub>R antibody, cells were incubated (2 h, 4°C) with an anti-ADA antibody (20 µg/ml) in buffer A in the absence or presence of 100 nM R-PIA. Cells were washed and stained (45 min, 4°C) with gold-conjugated protein A (10 nm) and then rinsed and incubated in buffer A at 37°C for 30 min. Cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M cacodylate buffer for 1 h. Infiltration and embedding in Lowicryl HM 20 was performed at -45°C and Lowicryl was polymerized with UV light. Ultrathin sections were rinsed twice (5 min/wash) in PBS-Gly 0.1 M and permeabilized with 0.02% saponin for 20 min. Sections were incubated at room temperature for 1 h with PC21 antibody (20 µg/ml) in PBS-1% BSA, washed three times with the same buffer, and incubated with protein A-gold (20 nm) for 45 min. Sections were rinsed once with PBS buffer and distilled water. Quantitative analysis was performed by counting on electron micrographs the totality of gold particles associated with the cell surface (randomly distributed versus clusters of ≥3 particles) or present in vesicles.

**Purification of Caveolin-Rich Membrane-Fractions.** Confluent LLC-PK<sub>1</sub> cells were incubated in the absence or presence of 100 nM R-PIA for 30 min. From these cells, caveolin-enriched membrane fractions were obtained according to the method of Song et al. (1996). LLC-PK<sub>1</sub> cells were washed with ice-cold PBS, scraped, and placed into 2 ml of 500 mM sodium carbonate, pH 11.0. Homogenization was carried out sequentially using a loose-fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts; Kinematica GmbH, Brinkmann Instruments, Westbury, NY), and a sonicator

(three 20-s bursts; Branson Sonifier 250, Branson Ultrasonic Corp., Danbury, CT). The homogenate was then adjusted to 45% sucrose by addition of 2 ml of 90% sucrose prepared in MBS (25 mM Mes, 0.15 M NaCl, pH 6.5) and placed at the bottom of an ultracentrifuge tube. A 5 to 35% sucrose gradient was formed above (4 ml of 5% sucrose/4 ml of 35% sucrose; both in MBS containing 250 mM sodium carbonate) and centrifuged at 105,000g for 16 to 20 h in a SW41.Ti rotor (Beckman Instruments, Palo Alto, CA). A light-scattering band confined to the 15 to 20% sucrose was observed. This band contained caveolin but excluded most of other cellular proteins. Gradient fractions of 1 ml were collected and treated with SDS-PAGE sample buffer (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 the protein (10 µg) onto the gel. Electrophoresis was performed according to the method of Laemmli (1970) using homogeneous slab gels containing a 15% acrylamide separating gel and 6% acrylamide concentrative gel, using the Mini-Protean system (Bio-Rad, AB, Sweden). Proteins were transferred (1 h, 90 V constant voltage) to a PVDF membrane (Immobilon-P; Millipore Corporation, Bedford, MA) using a semidry Bio-Rad Trans-Blot, in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Nonspecific protein binding sites on the PVDF membranes were blocked by overnight incubation at 4°C with 10% (w/v) nonfat dry milk in PBS, pH 7.4. After blocking, PVDF membranes were washed three times (10 min/wash) with TBS-TII (10 mM Tris-HCl, 500 mM NaCl, 0.5% Tween-20, pH 7.4) and incubated for 2 h with a rabbit anti-caveolin-1 (1:500) or an anti-A<sub>1</sub>R antibody (PC11; 10 µg/ml) diluted in TBS-TI (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) containing 0.1% NaN<sub>3</sub>. PVDF membranes were then washed three times with TBS-TII before incubation with horseradish peroxidase-goat anti-rabbit IgG diluted 1:15,000 in TBS-I for 1 h. Membranes were washed five times with TBS-TII as described above and then incubated in equal volumes of ECL detection reagent 1 and ECL detection reagent 2. The detection reagent was drained off, and filters were placed in contact with Hyperfilms ECL (Amersham Pharmacia Biotech). Films were developed using a standard photographic procedure.

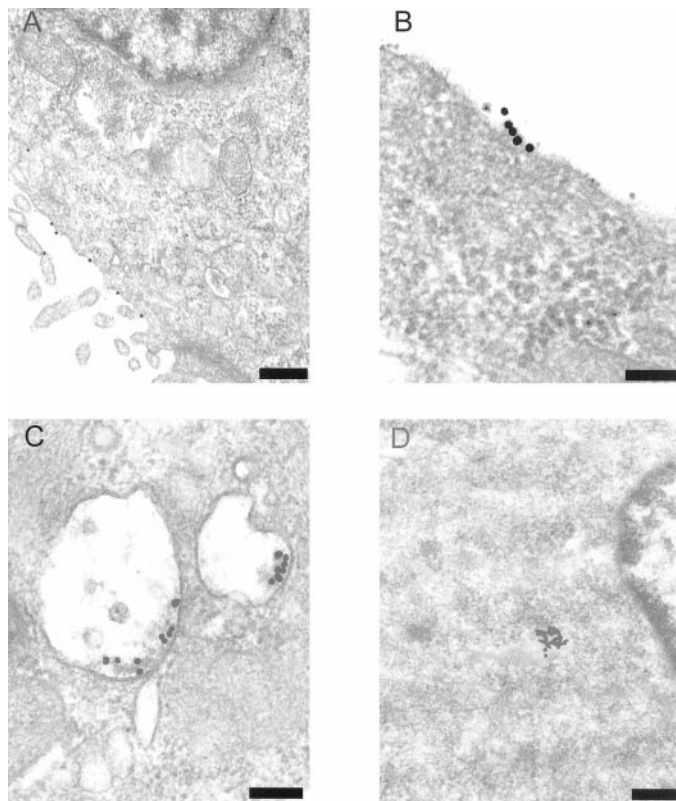
**Coimmunoprecipitation Assays.** Cell membranes from LLC-PK<sub>1</sub> cells were obtained after cell disruption in 50 mM Tris-HCl, pH 7.4, with a Polytron homogenizer (Kinematica, PTA 20TS rotor; Brinkmann Instruments) and centrifugation (105,000g, 45 min at 4°C). Membranes were solubilized in ice-cold lysis I buffer [PBS, pH 7.4, containing 1% Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS] for 1 h on ice and then centrifuged at 80,000g for 90 min. Supernatants (1 mg of protein/ml) were precleared by incubation (6 h) with protein A-Sepharose beads, transferred to a clean tube containing 12 µl of affinity purified anti-A<sub>1</sub>R antibody (PC11) or rabbit IgG antibody covalently coupled to protein A-Sepharose (Schneider et al., 1982), and incubated at 4°C overnight. Immunoprecipitates were washed twice in ice-cold lysis buffer II [PBS, pH 7.4, containing 0.1% Nonidet P-40, 0.05% (w/v) sodium deoxycholate and 0.01% (w/v) SDS] and once in ice-cold PBS, pH 7.4. Immunoprecipitates were resuspended in 60 µl of SDS-PAGE sample buffer (62 mM Tris-HCl, 1% SDS, 10% glycerol, 0.002% bromophenol blue, pH 6.8) and solubilized at 37°C for 15 min. After centrifugation to 10,000g for 1 min, proteins were resolved by 12.5% SDS-PAGE gels as described above. Immunoreactive bands were detected with the specific purified anti-A<sub>1</sub>R antibody PC11 (10 µg/ml) or anti-ADA antibody (10 µg/ml) diluted in TBS-TI containing 0.1% NaN<sub>3</sub>.

## Results

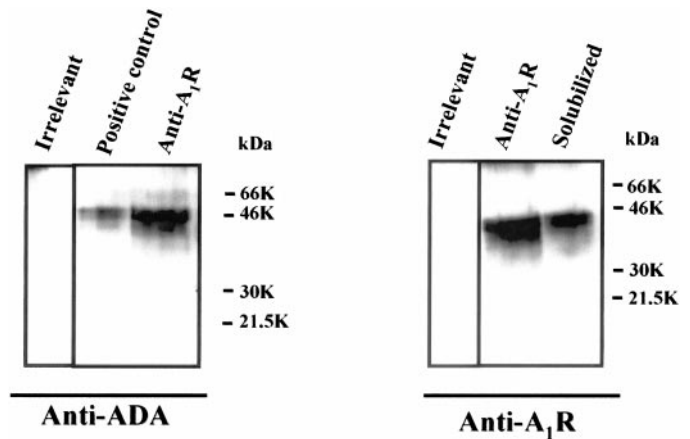
**Agonist-Induced Internalization of A<sub>1</sub>R and Cell-Surface ADA.** To investigate the internalization of A<sub>1</sub>R in response to the agonist R-PIA, LLC-PK<sub>1</sub> cells were incubated (4°C, 2 h) with anti-A<sub>1</sub>R antibody in the absence or presence of 100 nM R-PIA. After extensive washes, cells were incu-

bated with gold-conjugated protein A (4°C, 45 min). Internalization was induced by placing the samples at 37°C for 15 min, 30 min, or 24 h and processed for electron microscopy as indicated under *Materials and Methods*. No staining was observed in the absence of primary antibody (data not shown). Control cells showed dispersed gold particles exclusively on the cell surface (Fig. 1A). Even after 24 h at 37°C, A<sub>1</sub>R were mainly located on the surface of nontreated (control) cells (data not shown). Exposure of LLC-PK<sub>1</sub> cells to the agonist R-PIA induced an aggregation of more than 70% of A<sub>1</sub>R in clusters along the membrane (Fig. 1B). Internalization of A<sub>1</sub>R was evident after 30 min at 37°C. Gold particles were found in early endosomes and in developing late endosomes (Fig. 1C). The number of engulfed gold particles increased with the time of incubation being almost all the particles inside the cell after 40 min (data not shown). A time-dependent down-regulation or decrease in the total number of receptors in the cell has been described for the A<sub>1</sub>R when cells are chronically treated with an agonist (Fernandez et al., 1996; Ruiz et al., 1996; Ciruela et al., 1997). In agreement with those studies, A<sub>1</sub>R were accumulated mainly in vesicles near the nuclear membrane after 24 h of agonist exposure (Fig. 1D).

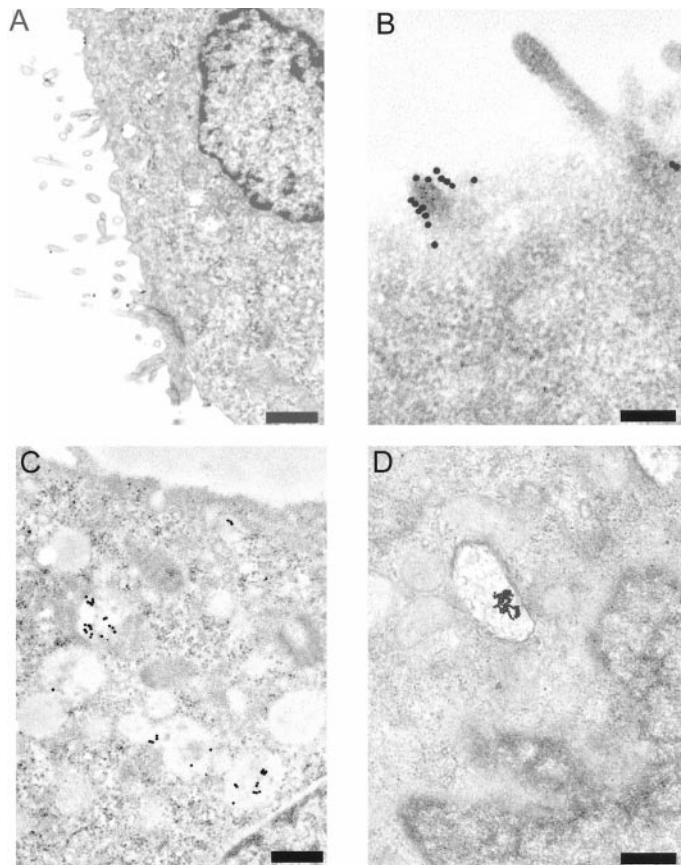
Agonist-induced desensitization and internalization of A<sub>1</sub>R is modulated by several cell factors, including ADA.



**Fig. 1.** Ultrastructural study of agonist-induced A<sub>1</sub>R internalization. Cells were incubated (2 h at 4°C) in DMEM-Hepes-BSA containing anti-A<sub>1</sub>R antibody (10 µg/ml) in the absence (A) or presence (B, C, and D) of 100 nM R-PIA. Rinsed cells were incubated (45 min at 4°C) with 20-nm gold-conjugated protein. Cells were washed in cold buffer and incubated for 15 min (B), 30 min (C), or 24 h (D) at 37°C. Cells were fixed, embedded in Spurr's resin, and ultrathin sections were analyzed by electron microscopy as described under *Materials and Methods*. In panel A, results corresponding to 15 min of internalization in the absence of R-PIA are shown, but no significant differences were observed after 15 min or 24 h of internalization. Scale bar: 0.4 µm (A); 0.2 µm (B and C); 0.4 µm (D).



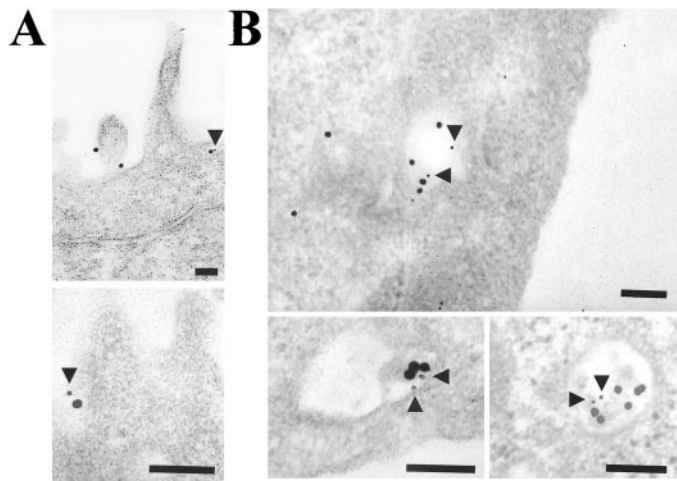
**Fig. 2.** Coimmunoprecipitation of adenosine A<sub>1</sub>R and ADA. LLC-PK<sub>1</sub> cell membranes were solubilized as described under *Materials and Methods*. Solubilized extracts (1 mg of protein/ml) were incubated with the specific anti-A<sub>1</sub>R (PC11) or an irrelevant goat anti-rabbit IgG both covalently coupled to protein A-Sepharose. SDS-PAGE and immunoblotting of the extracts (solubilized) and immunoprecipitates (irrelevant and anti-A<sub>1</sub>R) were performed to detect A<sub>1</sub> adenosine receptors (right panel) and to detect ADA (left panel). The positive control represents commercially available purified bovine ADA.



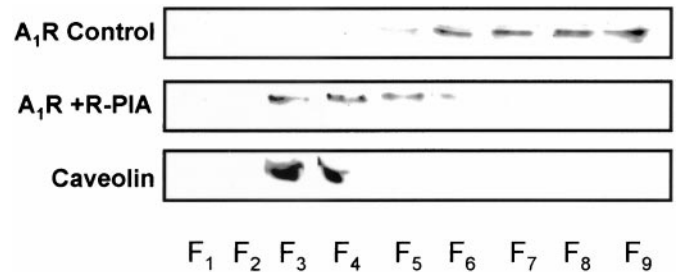
**Fig. 3.** Ultrastructural study of agonist-induced ADA internalization. Cells were incubated (2 h at 4°C) in DMEM-Hepes-BSA containing anti-ADA antibody (20 µg/ml) in the absence (A) or presence (B, C, and D) of 100 nM R-PIA. Rinsed cells were incubated (45 min at 4°C) with 20-nm gold-conjugated protein. Cells were washed in cold buffer and incubated for 15 min (B), 30 min (C), or 24 h (D) at 37°C and processed for electron microscopy as described under *Materials and Methods*. In panel A, results corresponding to 15 min of internalization in the absence of R-PIA are shown but no significant differences were observed after 15 min, 30 min, or 24 h of internalization. Scale bar: 0.5 µm (A); 0.2 µm (B); 0.5 µm (C and D).

We have described that ADA can interact with  $A_1R$  in brain cortex and DDT<sub>1</sub>MF-2 cells. Cell-surface ADA, independently of its catalytic activity, affects ligand-induced signaling through  $A_1R$  and accelerates agonist-induced phosphorylation, desensitization and internalization of the receptor (Saura et al., 1996, 1998; Ciruela et al., 1997). To investigate whether ADA and  $A_1R$  interact on the surface of LLC-PK<sub>1</sub> cells, a set of coimmunoprecipitation experiments were performed. Western blotting of membrane lysates showed a high expression of  $A_1R$  in membranes of LLC-PK<sub>1</sub> cells (Fig. 2, right panel). Immunoprecipitation of  $A_1R$  from detergent extracts yielded a 40-kDa band corresponding to  $A_1R$  (right panel) and a 44-kDa band corresponding to ADA (Fig. 2, left panel). This result indicates that  $A_1R$  and ADA form a complex in LLC-PK<sub>1</sub> cells. Thus the effect of  $A_1R$  agonist in the expression of cell-surface ADA on LLC-PK<sub>1</sub> cells was studied. Staining of LLC-PK<sub>1</sub> cells with an anti-ADA antibody showed predominantly cell surface ADA (Fig. 3A). There are a few particles in vesicles near the plasma membrane that reflect basal or antibody-induced internalization. A time-dependent movement of cell-surface ADA from the cell surface to intracellular compartments was induced after exposure of cells to the  $A_1R$  agonist. R-PIA induced a clustering of 50 to 65% cell-surface ADA molecules (15 min, Fig. 3B) as well as translocation of cell-surface ADA to early endosomes and multivesicular bodies (30 min, Fig. 3C), and internalization after 24 h to late endosomes and other types of vesicles near the nuclear membrane. The pattern and kinetics of internalization of cell-surface ADA were almost identical with those of  $A_1R$  (see Fig. 1).

The results described above suggest that chronic exposure of cells to the  $A_1R$  agonist R-PIA induces a time-dependent translocation of  $A_1R$  together with ADA to in-

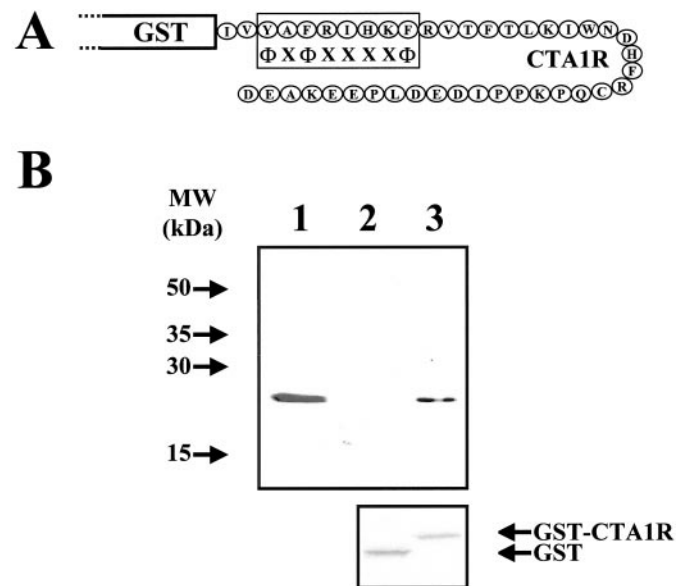


**Fig. 4.** Colocalization of ADA and  $A_1R$  at the ultrastructural level. Cells were incubated (2 h at 4°C) in DMEM-Hepes-BSA containing anti-ADA antibody (20  $\mu$ g/ml) in the absence (A) or presence (B) of 100 nM R-PIA. Rinsed cells were incubated (45 min at 4°C) with 10-nm gold-conjugated protein A. Cells were washed in cold buffer and incubated for 30 min at 37°C. Cells were then processed and embedded in Lowicryl resin as described under *Materials and Methods*. Ultrathin sections were washed, permeabilized, and stained with anti- $A_1R$  antibody (20  $\mu$ g/ml) for 1 h at room temperature. The samples were rinsed, incubated with 20-nm gold-conjugated protein A for 45 min at room temperature and treated as described under *Materials and Methods* for electron microscopy analysis. Different images for each treatment are shown; arrowheads point to the 10-nm gold particles labeling ADA. Scale bar: 0.2  $\mu$ m.



**Fig. 5.** Western blotting of fractions from LLC-PK<sub>1</sub> cells. Cells were preincubated in the absence (control) or presence of 100 nM R-PIA for 30 min at 37°C. The cells were lysed using a loose-fitting Dounce homogenizer, a Polytron tissue grinder, and a sonicator in sodium carbonate buffer and subjected to sucrose gradient centrifugation as described under *Materials and Methods*. Equal amounts of protein (10  $\mu$ g) from each of nine fractions (F<sub>1</sub>–F<sub>9</sub>) were separated by SDS-PAGE 15% acrylamide gel and transferred to PVDF membranes, which were blotted using anti- $A_1R$  antibody (PC11; 10  $\mu$ g/ml) or rabbit anti-caveolin-1 (1:5000). The immunoreactive bands were visualized by horseradish peroxidase-conjugated goat anti-rabbit IgG, followed by ECL chemiluminescence detection.

tracellular stores. We tested this hypothesis by comparing the distribution of  $A_1R$  and ADA in double immunogold staining and ulterior analysis by electron microscopy. LLC-PK<sub>1</sub> cells were surface-labeled at 4°C with an anti-ADA antibody and 10-nm gold-conjugated protein A in the absence (Fig. 4A) or presence (Fig. 4B) of R-PIA. Cells were warmed at 37°C for 30 min. After fixation, ultrathin sections cells were permeabilized and the intracellular  $A_1R$  was labeled with an anti- $A_1R$  antibody and protein A-gold (20 nm). As shown in Fig. 4A, cell-surface ADA (10-nm gold



**Fig. 6.** Interaction of GST-CTA<sub>1R</sub> with caveolin. Panel A, schematic representation of the GST-CTA<sub>1R</sub> fusion protein. The putative caveolin-binding domain present in the C-terminal tail of adenosine  $A_1$  receptor is depicted as  $\Phi$ X $\Phi$ XXXX $\Phi$ , where  $\Phi$  represents an aromatic amino acid and X represents any amino acid. Panel B, GST and GST-CTA<sub>1R</sub> pull-down experiment. Cells were solubilized in ice-cold lysis buffer as described under *Materials and Methods*. The solubilized proteins (lane 1) and proteins bound to GST (lane 2) or to GST-CTA<sub>1R</sub> (lane 3) after the pull-down experiments described under *Materials and Methods* were resolved by SDS-PAGE and immunoblotted using a polyclonal anti-caveolin antibody (1:5,000). The primary bound antibody was detected using a goat anti-rabbit antibody (1/60,000). The bottom panel shows the relative amount of GST and GST-CTA<sub>1R</sub> fusion proteins used in the pull-down stained with Coomassie Blue.

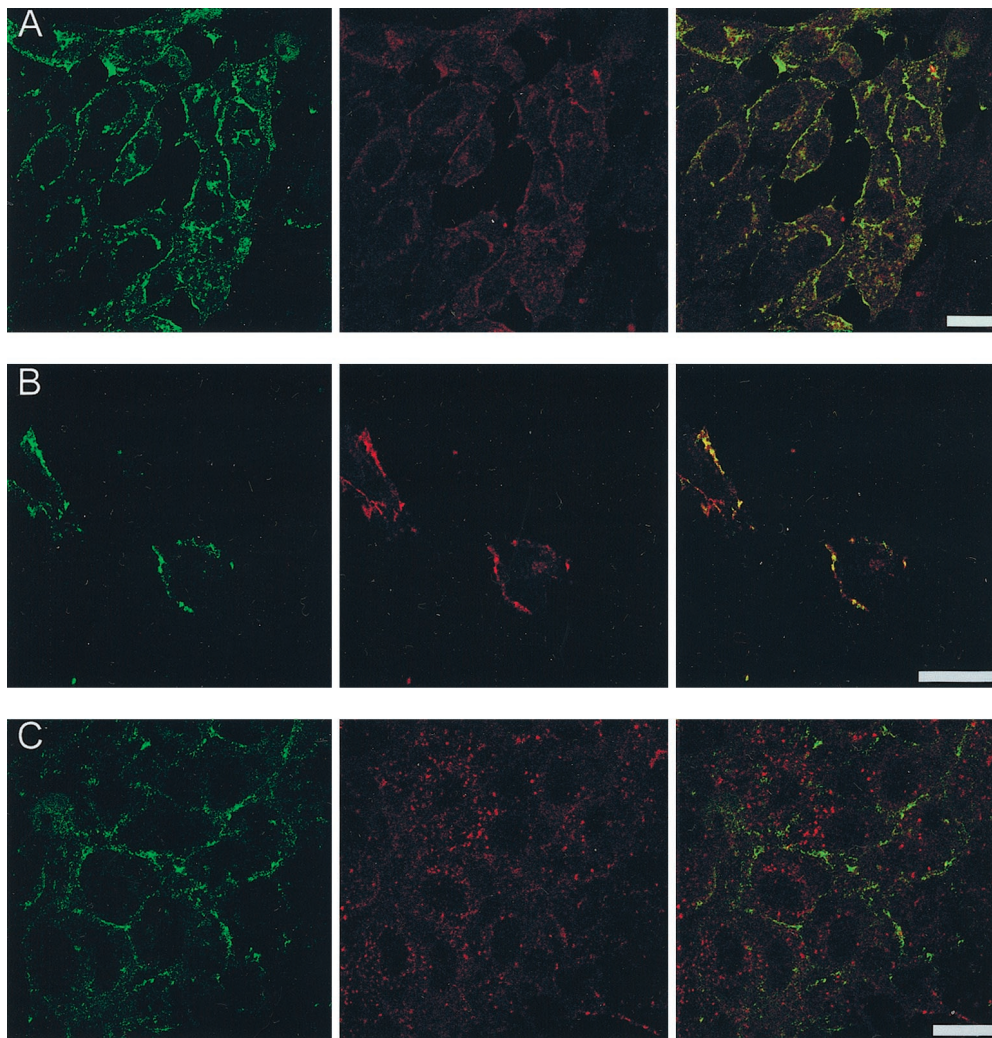
particles) codistributes with A<sub>1</sub>R (20-nm gold particles) on the plasma membrane of LLC-PK<sub>1</sub> cells. In the conditions of inclusion using Lowicryl, the number of particles labeling ADA is smaller than the number of particles labeling A<sub>1</sub>R. The colocalization is, however, very high, being 75% of the 10-nm gold particles together with 20-nm gold particles. When cells were warmed for 30 min, A<sub>1</sub>R and cell-surface ADA translocated to the same intracellular vesicles (Fig. 4B). More than 90% of 10-nm gold particles in endosomes were codistributing with 20-nm gold particles.

**A<sub>1</sub>R Are Enriched in Caveolin-Rich Membrane Domains and Interact with Caveolin-1.** A detergent-free method was used for purifying caveolae/rafts from LLC-PK<sub>1</sub> cells. An established protocol for separating rafts from the bulk of cellular membranes and cytosolic proteins is based on the specific buoyant density of rafts-rich membrane domains and their resistance to solubilization by the nonionic detergent Triton X-100 at low temperatures (Sargiacomo et al., 1993; Chang et al., 1994; Lisanti et al., 1994; Scherer et al., 1994, 1995; Smart et al., 1994; Corley-Mastick et al., 1995; Li et al., 1995; Robbins et al., 1995). Chang et al., (1994) have indicated that the inclusion of detergent in initial homogenization step results in the loss of some rafts-associated proteins, such as G<sub>βγ</sub> subunits. For this reason, we used a detergent-free method for the purification of caveolin-rafts.

This procedure replaces the detergent Triton X-100 by sodium carbonate, followed by progressive disruption of cellular membranes (see *Materials and Methods*). The lysate was adjusted to 45% sucrose and placed at the bottom of a 5 to 35% sucrose gradient for an overnight ultracentrifugation. Aliquots of the different fractions were collected and separated by SDS-PAGE and immunoblotted with anti-caveolin and anti-A<sub>1</sub>R antibodies. Figure 5 shows that endogenous caveolin was recovered quantitatively in fractions 3 and 4 whereas 90% of the receptor was present in fractions 6 to 9. The remaining was found in fractions 4 and 5.

We next explored in these cells the effect of R-PIA on the distribution of A<sub>1</sub>R using the protocol described above. The cells were treated with 100 nM R-PIA (30 min) and were extensively washed, lysed, and ultracentrifuged on a sucrose gradient. In R-PIA-treated cells, the receptor was mainly detected in fractions 3 and 4 in which the marker, caveolin, is present (Fig. 5). Receptor present in fractions 5 and 6 probably comes from intracellular pools where the receptor is not associated to rafts. Thus, R-PIA shifted A<sub>1</sub>R from high-density to low-density fractions in which caveolin was also present.

To address the question of whether the receptor and caveolin-1 interact, a GST-fusion protein, containing the C-terminal domain of the A<sub>1</sub> receptor (Fig. 6A), was prepared. This



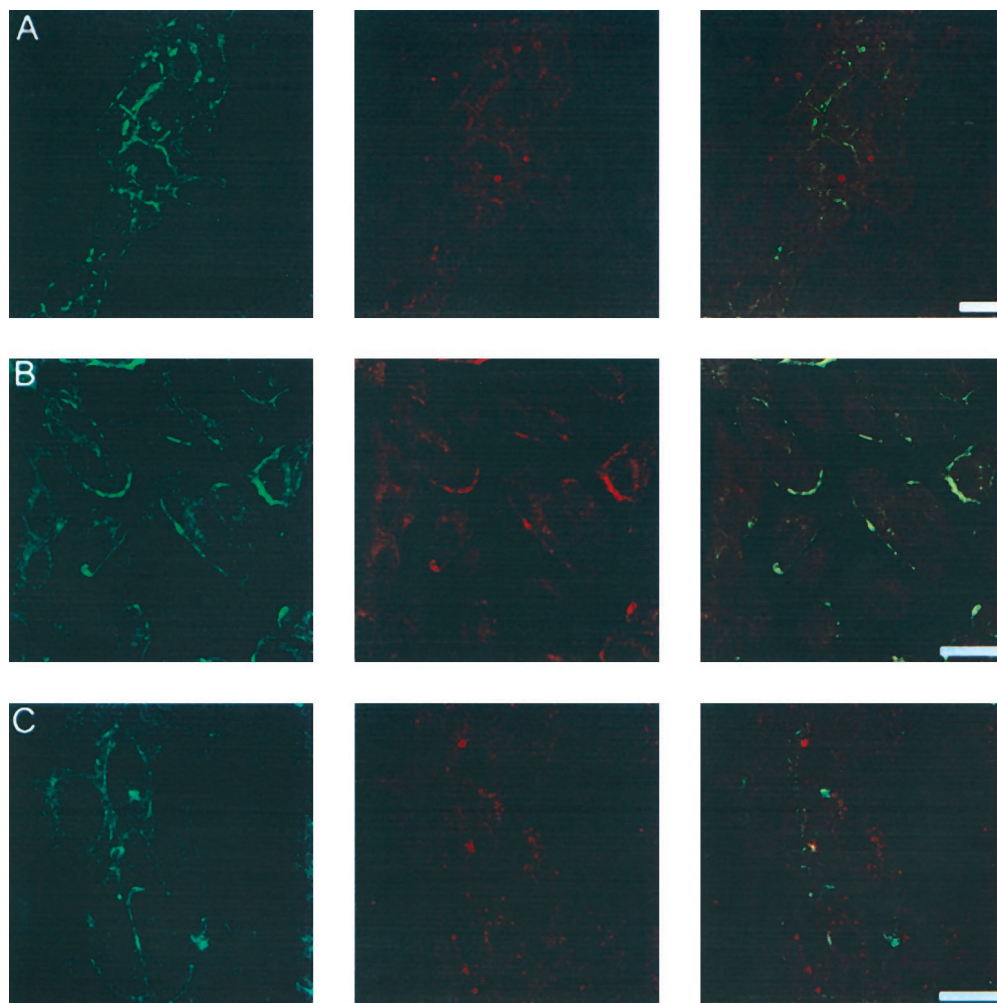
**Fig. 7.** Colocalization of adenosine A<sub>1</sub>R and caveolae. Cells were incubated (2 h at 4°C) in DMEM containing a rhodamine-conjugated rabbit anti-A<sub>1</sub>R antibody (PC21; 10 µg/ml) in the absence (A) or presence (B and C) of 100 nM R-PIA. Cells rinsed were incubated 0 min (A), 30 min (B), or 24 h (C) at 37°C. Cells were fixed, permeabilized, and stained with fluorescein-conjugated anti-caveolin antibody (1:75 dilution). The cells were then mounted in immunofluorescence medium, and observed with confocal microscopy. Colocalization is indicated in yellow. Image corresponds to a horizontal section at the middle of the cell. Scale bar, 10 µm.

C-terminal domain includes a stretch of amino acids that is similar to a caveolin binding motif ( $\Phi\text{X}\Phi\text{XXXX}\Phi$ , being  $\Phi$  an aromatic residue). Pull-down experiments using GST or GST-CTA<sub>1</sub>R were performed using cell extracts as indicated under *Materials and Methods*. As shown by Western blotting using an anti-caveolin-1 antibody (Fig. 6B), GST-CTA<sub>1</sub>R was able to interact with caveolin-1 whereas GST did not interact with the protein at the same concentration (Fig. 6B, bottom). This indicates that the C-terminal domain of A<sub>1</sub>R interacts specifically with caveolin-1.

**A<sub>1</sub>R and ADA Internalize via a Clathrin-Independent Pathway.** To confirm the potential role of caveolin in A<sub>1</sub>R and ADA internalization, colocalization studies by confocal microscopy were performed. Cells were incubated with rhodamine-conjugated anti-A<sub>1</sub>R antibody (PC21-TRITC) or rhodamine-conjugated anti-ADA antibody (anti-ADA-TRITC) in the presence or absence of 100 nM R-PIA. Cells were washed and permeabilized with 0.02% saponin and stained by double immunofluorescence using a fluorescein-conjugated anti-caveolin antibody. A bright linear staining for caveolin along portions of the cell borders was clearly observed (Figs. 7A and 8A). A similar pattern has previously been described for anti-caveolin-1 staining of fibroblasts (Smart et al., 1994, 1995; Roettger et al., 1995; Garcia-Cardena et al., 1996). In the absence of agonist treatment, A<sub>1</sub>R was diffusely located at the cell surface as well as ADA. After warming at 37°C for 30 min, the agonist R-PIA induced

colocalization of A<sub>1</sub>R and caveolin (Fig. 7B, yellow indicates colocalization). However, after 24 h at 37°C, A<sub>1</sub>R staining was observed in perinuclear vesicular compartments where the degree of colocalization between A<sub>1</sub>R and caveolin was very low. (Fig. 7C). The results obtained for ADA and caveolin-staining were similar to these obtained for A<sub>1</sub>R and caveolin-labeling (Fig. 8). In fact, colocalization between ADA and caveolin was found at 30 min (Fig. 8B), whereas at 24 h ADA did not colocalize with caveolin (Fig. 8C).

It has been described that acidification of the cytosol selectively inhibits endocytosis via clathrin-coated vesicles (Sandvig et al., 1987; Tolbert and Lameh, 1996). On the contrary, caveolae/rafts formation is blocked by filipin, a sterol-binding agent (Schnitzer et al., 1994). To confirm whether R-PIA-stimulated internalization involves caveolae/rafts, epithelial cells were pretreated for 5 min with 5 mM acetic acid or 30 min with 5  $\mu\text{g}/\text{ml}$  filipin at 37°C prior to treatment with 100 nM R-PIA. After 30 min of incubation with the agonist R-PIA, cells showed a large number of internalized receptors (Fig. 9B). In contrast, with filipin treatment the punctate staining disappeared (Fig. 9C), indicating that the A<sub>1</sub>R internalization was disrupted. The acetic acid treatment was not able to inhibit the A<sub>1</sub>R internalization (Fig. 9D), whereas it did prevent transferrin uptake (Fig. 9, D and F). In contrast to what happens in other cell types, cell viability was not affected by treatment with either acetic acid or filipin. In all assays, cell viability was higher than 90%. The fact that

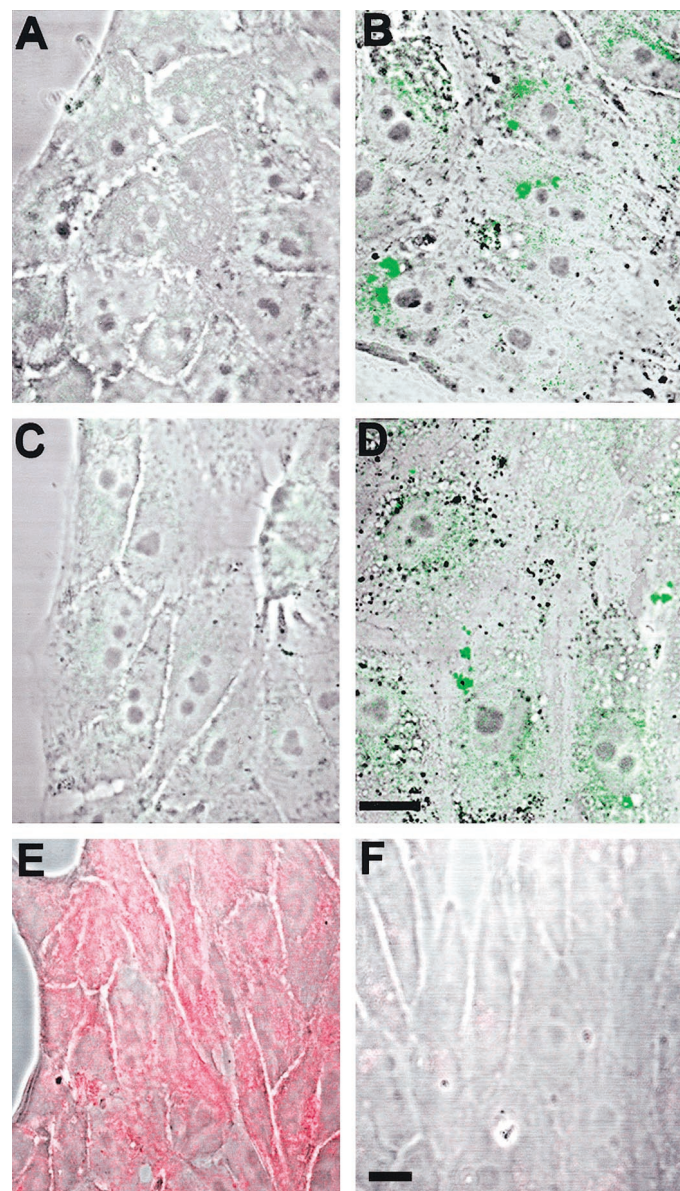


**Fig. 8.** Colocalization of ADA and caveolae. Cells were incubated (2 h at 4°C) in DMEM containing a rhodamine-conjugated rabbit anti-ADA antibody (10  $\mu\text{g}/\text{ml}$ ) in the absence (A) or presence (B and C) of 100 nM R-PIA. Cells rinsed were incubated 0 min (A), 30 min (B), or 24 h (C) at 37°C. Cells were fixed, permeabilized, and stained with fluorescein-conjugated anti-caveolin antibody (1:75 dilution). The cells were then mounted in immunofluorescence medium, and observed with confocal microscopy. Image corresponds to a horizontal section at the middle of the cell. Colocalization is indicated in yellow. Scale bar, 10  $\mu\text{m}$ .

internalization is blocked by filipin but not by acetic acid provides further evidence that rafts, and not clathrin-coated vesicles, are involved in A<sub>1</sub>R internalization.

## Discussion

Agonist-induced receptor internalization, as a mechanism of receptor regulation, remains a poorly understood phenomenon for G-protein-coupled receptors, specially for A<sub>1</sub>R. The first step toward a clearer understanding of the mechanism of internalization of A<sub>1</sub>R is to define the pathway by which the receptor is transported into the cell after activation by agonists.



**Fig. 9.** Effect of filipin and acetic acid treatment on A<sub>1</sub>R internalization. Cells were treated without (A, B, and E) or with 5 mM acetic acid (D and F) for 5 min or 5 μg/ml filipin (C) for 30 min and then with either fluorescein-conjugated anti-A<sub>1</sub>R antibody (10 μg/ml, 4°C for 2 h) in the absence (A) or presence (B, C, and D) of 100 nM R-PIA, or with transferrin-Texas red (20 mg/ml, 4°C for 2 h) (E and F). Cells were washed and incubated at 37°C for 30 min in DMEM. Cells were processed for confocal microscopy as described under *Materials and Methods*. Images correspond to the superposition of a middle section of the green fluorescence (internalized A<sub>1</sub>R) and cell phase contrast. Scale bar, 10 μm.

Ruiz et al. (1996) investigated in brain the mechanisms of A<sub>1</sub>R internalization during a desensitization process. Chronic treatment of rats with R-PIA desensitized A<sub>1</sub>R, and this loss of response was accompanied with a significant decrease in the total number of A<sub>1</sub>R in synaptic plasma membranes. Concurrently, a significant increase of A<sub>1</sub>R was observed in microsomes and coated vesicles. These data agreed with the fact that the predominant pathway described for G-protein-coupled receptor internalization was the classical endocytic pathway, initiated at the level of clathrin-coated pits and vesicles. However, Raposo et al. (1987) reported that muscarinic acetylcholine receptors internalize through noncoated vesicles in human fibroblasts. Moreover, Feron et al. (1997) have described a dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptors to caveolae in cardiac myocytes by isopycnic centrifugation and immunoprecipitation. Raposo et al. (1989) demonstrated by electron microscopy that in A431 cells, β-adrenergic receptors were mainly internalized via noncoated vesicles. In these experiments coated pits and coated vesicles were always devoid of gold particles suggesting that β-adrenergic receptors are excluded from coated vesicles. In these cells, the clathrin-dependent pathway was functional since the transferrin receptor was endocytosed by coated pits (Daukas and Zigmond, 1985). In other cell lines or in transfected cells, internalization of muscarinic or β-adrenergic receptors follows the clathrin-dependent pathway (Von Zastrow and Kobilka, 1992; Schnitzer et al., 1994).

In this study, we show that A<sub>1</sub>R internalize in LLC-PK<sub>1</sub> cells in response to agonist treatment. The involvement of rafts in endocytosis of this subtype of adenosine receptor was supported by biochemical analysis using a detergent-free method for purifying rafts-rich membrane domains from cultured cells. Using this procedure it was shown that the rafts marker, caveolin, copurifies with A<sub>1</sub>R after agonist treatment. Thus R-PIA stimulates the accumulation of a portion of the A<sub>1</sub>R receptors to caveolin-enriched fractions. To strengthen the conclusion that A<sub>1</sub>R translocate to rafts enriched in caveolin upon agonist exposure, a pull-down experiment with recombinant GST-CTA<sub>1</sub>R proved that the C-terminal portion of A<sub>1</sub>R interact with caveolin-1. In fact, the C-terminal part of A<sub>1</sub>R contains a motif, which shares homology with that found in caveolin-interacting proteins. The motif [ΦXΦXXXX and ΦXXXXΦXXΦ (Φ:Trp, Phe, or Tyr)] has been defined by Couet et al. (1997). Caveolin binding motifs are present in the epidermal growth factor receptor, platelet-derived growth factor receptor, fibroblast growth factor receptor, and nerve growth factor receptor (Corley-Mastick et al., 1995; Liu et al., 1996; Mineo et al., 1996; Wu et al., 1997). The A<sub>1</sub>R adenosine receptor presents in its cytoplasmic tail a sequence that resembles this caveolin binding motif (YAFRIHKF).

Recently, we have reported that cell-surface ADA interacts with A<sub>1</sub>R in brain cortex and DDT<sub>1</sub>MF-2 cells and that the enzyme is able to modulate ligand binding and signaling through A<sub>1</sub>R (Saura et al., 1998). In a catalytically independent fashion ADA seems to be necessary for a high-affinity binding of agonist to A<sub>1</sub>R (Ciruela et al., 1996; Saura et al., 1996). In this study, we demonstrate by coimmunoprecipitation that in the epithelial cell line, LLC-PK<sub>1</sub>, A<sub>1</sub>R is also a binding protein for cell-surface ADA. Furthermore, cell-surface ADA also suffered an R-PIA-induced internalization pro-

cess. Internalized ADA appeared in intracellular vesicles where it colocalized with A<sub>1</sub>R. The biochemical and confocal results presented here indicate that the common endocytic pathway described for R-PIA-induced A<sub>1</sub>R/ADA internalization (Saura et al., 1998) starts at the level of rafts in LLC-PK<sub>1</sub> cells. This is furthermore suggested by the fact that R-PIA-stimulated internalization of A<sub>1</sub>R is blocked by filipin, which inhibits rafts formation (Schnitzer et al., 1994), but not by acetic acid, which prevents internalization via clathrin-coated vesicles (Sandvig et al., 1987). On the other hand, double immunostaining assays analyzed by confocal or electron microscopy showed colocalization of ADA and caveolin in the same intracellular vesicles.

The occurrence of agonist-related internalization of A<sub>1</sub>R (and ADA) via noncoated vesicles constitutes an important advance toward understanding the mechanisms involved in endocytosis of G-protein-coupled receptors. Taken together, these results and those already reported in the literature suggest that the mode of receptor compartmentation in response to agonist stimulation may be governed by both the receptor subtype and the cell type in which it is expressed.

## References

- Anderson RG (1993a) Plasmalemmal caveolae and GPI-anchored membrane proteins. *Curr Opin Cell Biol*, **5**:647–652.
- Anderson RG (1993b) Caveolae: Where incoming and outgoing messengers meet. *Proc Natl Acad Sci USA* **90**:10909–10913.
- Anderson RGW, Kamen BA, Rothberg KG and Lacey SW (1992) Potocytosis: Sequestration and transport of small molecules by caveolae. *Science (Wash DC)* **255**, 410–411.
- Aran JM, Colomer D, Matutes E, Vives-Corrons JL and Franco R (1991). Presence of adenosine deaminase on the surface of mononuclear blood cells: Immunohistochemical localization using light and electron microscopy. *J Histochem Cytochem* **39**:1001–1007.
- Arreaza G, Melkonian KA, LaFevre-Bernt M and Brown DA (1994) Triton X-100 resistant membrane complexes from cultured kidney epithelial cells contain the Src family protein tyrosine kinase p62yes. *J Biol Chem* **269**:19123–19127.
- Benovic JL, Bouvier M, Caron MG and Lefkowitz RJ (1988) Regulation of adenylyl cyclase-coupled beta-adrenergic receptors. *Annu Rev Cell Biol* **4**:405–428.
- Böhm SK, Grady EF and Bunnett NW (1997) Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem J* **322**:1–18.
- Chang WJ, Ying Y, Rothberg K, Hooper N, Turner A, Gambliel H, De Gunzburg J, Mumby S, Gilman A and Anderson RGW (1994) Purification and characterization of smooth muscle cell caveolae. *J Cell Biol* **126**:127–138.
- Chun M, Liyanage U, Lisanti MP and Lodish HF (1994) Signal transduction of a G protein-coupled receptor in caveolae: Colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci USA* **91**:11728–11732.
- Ciruela F, Casadó V, Mallol J, Canela EI, Lluís C and Franco R (1995) Immunological identification of A<sub>1</sub> adenosine receptors in brain cortex. *J Neurosci Res* **42**: 818–829.
- Ciruela F and McIlhinney RAJ (1997) Differential internalisation of mGluR1 splice variants in response to agonist and phorbol esters in permanently transfected BHK cells. *FEBS Lett* **418**:83–86.
- Ciruela F, Saura C, Canela EI, Mallol J, Lluís C and Franco R (1996) Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. *FEBS Lett* **380**:219–223.
- Ciruela F, Saura C, Canela EI, Mallol J, Lluís C and Franco R (1997) Ligand-induced phosphorylation, clustering and desensitization of A<sub>1</sub> adenosine receptors. *Mol Pharmacol* **52**:778–797.
- Corley-Mastick C, Brady MJ and Saltiel AR (1995) Insulin stimulates the tyrosine phosphorylation of caveolin. *J Cell Biol* **129**:1523–1531.
- Couet J, Sargiacomo M and Lisanti MP (1997) Interaction of a receptor tyrosine kinase, EGF-R with caveolins. *J Biol Chem* **272**:30429–30438.
- Dalziel HH and Westfall DP (1994) Receptors for adenine nucleotides and nucleosides: Subclassification, distribution, and molecular characterization. *Pharmacol Rev* **46**:449–466.
- Daukas G and Zigmond SH (1985) Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leucocytes. *J Cell Biol* **101**:1673–1679.
- De Weerd WF and Leeb-Lundberg LM (1997) Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Galpha subunits Galpha2 and Galpha1 in caveolae in DDT1 MF-2 smooth muscle cells. *J Biol Chem* **272**:17858–17866.
- Fernandez M, Svenningsson O and Fredholm BB (1996) Adaptive changes in adenosine receptors following long-treatment with the adenosine receptor agonist R-PIA. *Life Sci* **58**:769–776.
- Feron O, Smith TW, Michel T and Kelly RA (1997) Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J Biol Chem* **272**:17744–17748.
- Fujimoto T, Nakade S, Miyawaki A, Mikoshiba K and Ogawa K (1992) Localization of inositol 1,4,5-triphosphate receptor-like protein in plasmalemmal caveolae. *J Cell Biol* **119**:1507–1513.
- Garcia-Cardena G, Oh P, Liu J, Schnitzer JE and Sessa WC (1996) Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation implications for nitric oxide signaling. *Proc Natl Acad Sci USA* **93**:6448–6453.
- Glenney JR Jr (1989) Tyrosine phosphorylation of a 22-kDa proteins is correlated with transformation by Rous sarcoma virus. *J Biol Chem* **264**:20163–20166.
- Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton R and Brass LF (1993) Internalization and recycling of activated thrombin receptors. *J Biol Chem* **268**:13756–13763.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227**:680–685.
- Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH, Nishimoto I and Lisanti MP (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *J Biol Chem* **270**:15693–15701.
- Lisanti MP, Scherer PE, Vidugiriene J, Tang ZL, Hermanoski-Vosatka A, Tu YH, Cook RF and Sargiacomo M (1994) Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source implications for human disease. *J Cell Biol* **126**:111–126.
- Liu P, Ying Y, Ko Y-G and Anderson RGW (1996) Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. *J Biol Chem* **272**: 6525–6533.
- Longabaugh JP, Didsbury J, Spiegel A and Stiles GL (1989) Modification of the rat adipocyte A<sub>1</sub> adenosine receptor-adenylate cyclase system during chronic exposure to an A<sub>1</sub> adenosine receptor agonist: Alterations in the subunit of G<sub>sa</sub> and G<sub>ia</sub> are not associated with changes in their mRNAs. *Mol Pharmacol* **36**:681–688.
- Mineo C, James GL, Smart EJ and Anderson RGW (1996) Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. *J Biol Chem* **271**:11930–11935.
- Molnar E, Baude A, Richmond SA, Patel PB, Somogyi P and McIlhinney RAJ (1993) Biochemical and immunocytochemical characterization of antipeptide antibodies to a cloned GluR1 glutamate receptor subunit—cellular and subcellular distribution in the rat forebrain. *Neuroscience* **53**:307–326.
- Olah ME and Stiles GL (1992) Adenosine receptors. *Ann Rev Physiol* **54**:211–225.
- Page E, Upshaw-Earley J and Goings GE (1994) Localization of atrial natriuretic peptide in caveolae in situ atrial myocytes. *Circ Res* **75**:949–954.
- Ramkumar V, Olah ME, Jacobson KA and Stiles G (1991) Distinct pathways of desensitization of A<sub>1</sub> and A<sub>2</sub> adenosine receptors in DDT<sub>1</sub>MF-2. *Mol Pharmacol* **40**:639–647.
- Raposo G, Dunia I, Delavier-Klutchko C, Kaveri S, Stroberg AD, Benedeth EL and Hoebeke J (1987) Redistribution of muscarinic acetylcholine receptors on human fibroblasts induced by regulatory ligands. *Biol Cell* **60**:117–124.
- Raposo G, Dunia I, Delavier-Klutchko C, Kaveri S, Stroberg AD and Benedeth EL (1989) Internalization of beta-adrenergic receptor in A431 cells involves non-coated vesicles. *Eur J Cell Biol* **50**:340–352.
- Robbins SM, Quintrell NA and Bishop MJ (1995) Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachment to membranes and association with caveolae. *Mol Cell Biol* **15**:3507–3515.
- Roettger BF, Rentsch RU, Pinon D, Holicky E, Hadac E, Larkin JM and Miller LJ (1995) Dual pathways of internalization of the cholecystokinin receptor. *J Cell Biol* **128**:1029–1041.
- Ruiz A, Sanz JM, Gonzalez-Calero G, Fernandez M, Andrés A, Cubero A and Ros M (1996) Desensitization and internalization of adenosine A<sub>1</sub> receptors in rat brain by in vivo treatment with R-PIA: Involvement of coated vesicles. *Biochim Biophys Acta* **1310**:168–174.
- Sandvig K, Olsnes S, Petersen OW and Van Deurs B (1987) Acidification of the cytosol inhibits endocytosis from coated pits. *J Cell Biol* **105**:679–689.
- Sargiacomo M, Sudol M, Tang ZL and Lisanti MP (1993) Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK. *J Cell Biol* **122**:789–807.
- Saura C, Ciruela F, Casadó V, Canela EI, Mallol J, Lluís C and Franco R (1996) Adenosine deaminase interacts with A<sub>1</sub> adenosine receptors in pig brain cortical membranes. *J Neurochem* **66**:1675–1682.
- Saura C, Mallol J, Canela EI, Lluís C and Franco R (1998) Adenosine deaminase and A<sub>1</sub> adenosine receptors are internalized together following agonist-induced receptor desensitization. *J Biol Chem* **273**:17610–17617.
- Scherer PE, Lisanti MP, Baldini G, Sargiacomo M, Corley-Mastick C and Lodish HF (1994) Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J Cell Biol* **127**:1233–1243.
- Scherer PE, Tang ZL, Chun M, Sargiacomo M, Lodish HF and Lisanti MP (1995) Caveolin isoforms differ in their N-terminal protein sequence and subcellular distribution. Identification and epitope mapping of an isoform-specific monoclonal antibody probe. *J Biol Chem* **270**:16395–16401.
- Schneider C, Newman RA, Sutherland DR, Asser U and Greavens MF (1982) A one step purification of membrane proteins using a high efficiency immunomatrix. *J Biol Chem* **257**:10766–10769.
- Schnitzer JE, Oh P, Pinney E and Allard J (1994) Filipin-sensitive caveolae-mediated transport in endothelium: Reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol* **127**: 1217–1232.
- Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RGW and Michel T (1996) Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem* **271**:6518–6522.
- Smart EJ, Ying Y-S and Anderson RGW (1995) Hormonal regulation of caveolae internalization. *J Cell Biol* **131**:929–938.
- Smart EJ, Ying YS, Conrad PA and Anderson RGW (1994) Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. *J Cell Biol* **127**:1185–1197.
- Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M and Lisanti M (1996)

- Co-purification and direct interactions of Ras with caveolin, an integral membrane protein of caveolae microdomains. *J Biol Chem* **271**:9690–9697.
- Tolbert LM and Lameh J (1996) Human muscarinic cholinergic receptor Hm1 internalizes via clathrin-coated vesicles. *J Biol Chem* **271**:17335–17342.
- Tucker AL and Linden J (1993) Cloned receptors and cardiovascular responses to adenosine. *Cardiovascular Res* **27**:62–67.
- Von Zastrow M and Kobilka BK (1992) Ligand-regulated internalization and recycling of human  $\beta_2$ -adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J Biol Chem* **267**:3530–3538.
- Wu C, Burtz S, Ying Y and Anderson RGW (1997) Localization of epidermal-growth-factor stimulated Ras/Raf1 interaction to caveolae membrane. *J Biol Chem* **272**:3554–3559.

---

**Send reprint requests to:** Rafael Franco, Dept. Bioquímica i Biologia Molecular, Universitat de Barcelona, Martí i Franquès 1, 08028 Barcelona, Spain. E-mail address: r.franco@sun.bq.ub.es

---