Activated Cardiac Adenosine A₁ Receptors Translocate Out of Caveolae

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The cardiacl effects of the purine nucleoside, adenosine, are well known. Adenosine increases coronary blood flow, exerts direct negative chronotropic and dromotropic effects, and exerts indirect anti-adrenergic effects. These effects of adenosine are mediated via the activation of specific G protein-coupled receptors. There is increasing evidence that caveolae play a role in the compartmentalization of receptors and second messengers in the vicinity of the plasma membrane. Several reports demonstrate that G protein-coupled receptors redistribute to caveolae in response to receptor occupation. In this study, we tested the hypothesis that adenosine A₁ receptors would translocate to caveolae in the presence of agonists. Surprisingly, in unstimulated rat cardiac ventricular myocytes, 67 ± 5% of adenosine A₁ receptors were isolated with caveolae. However, incubation with the adenosine A₁ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine. An adenosine A₂a receptor agonist did not alter the localization of A₁ receptors to caveolae. These data suggest that the translocation of A₁ receptors out of caveolae and away from compartmentalized signaling molecules may explain why activation of ventricular myocyte A₁ receptors are associated with few direct effects.

Adenosine has multiple and profound effects on cardiac function. Adenosine increases coronary blood flow and exerts direct negative chronotropic and dromotropic effects (1). There is also significant evidence that adenosine reduces both reversible and irreversible myocardial ischemic-reperfusion injury (2). The cardiac effects of adenosine are mediated by the activation of specific extracellular adenosine receptor subtypes, A₁, A₂a, and A₂b, located on vascular smooth muscle, coronary endothelial cells, and cardiac myocytes (1). The adenosine A₁ receptor subtype is localized primarily on cardiac myocytes (1).

The adenosine A₁ receptor is a 36-kDa, seven-transmembrane domain protein localized to the sarcolemma (3). Agonist binding induces a conformational change in the receptor, which facilitates its interaction with an inhibitory guanine nucleotide-binding protein (Gᵢ) (3). With the exception of the ferret, adenosine A₁ receptor occupancy does not significantly alter contractility, intracellular calcium, or cAMP concentration in normal mammalian ventricular myocardium (4). However, A₁ receptor occupation does reduce β-adrenergic receptor-induced increases in all of the above parameters. Although the anti-adrenergic effect of adenosine is well studied, evidence of other signal transduction mechanisms mediated by adenosine A₁ receptor occupation in ventricular myocardium is lacking. The fact that other receptors in ventricular myocytes, which presumably couple to the same Gᵢ protein, produce direct effects (5–8) raises the question of why adenosine A₁ receptor activation is associated with so few effects. One explanation for the lack of direct adenosine A₁ receptor effects may be the relatively low receptor density in ventricular myocardium compared with atrial tissue (9). Another possibility is that the receptor is inefficiently coupled to Gᵢ proteins and second messengers. Coupling efficiency may be due, in part, to the grouping of receptors and second messengers in microdomains for rapid and selective activation or deactivation of intracellular signaling mechanisms.

Caveolae are cholesterol/sphingomyelin-rich plasma membrane microdomains that have been shown to serve as a scaffold for receptors and second messengers (10). Caveolae are present in most cell types and are abundant in cardiomyocytes (11). There are several reports that G protein-coupled receptors redistribute to caveolae in response to receptor occupation (11–13). In addition, other studies have documented that specific second messenger systems, including heterotrimeric G proteins, are concentrated in caveolae (for review, see Ref. 10). Although the role of caveolae in cardiac signal transduction has not been well studied, it has been reported that muscarinic M₂ cholinergic receptors in rat ventricular cardiomyocytes localize to caveolae after agonist stimulation (11).

Because adenosine and acetylcholine exert similar effects in ventricular myocardium (14), we hypothesized that stimulation of adenosine A₁ receptors would promote translocation of the receptors into caveolae. Surprisingly, we found that in rat ventricular cardiomyocytes, adenosine A₁ receptors were associated with caveolae in unstimulated cells, whereas activation of the adenosine A₁ receptors induced the receptors to translocate out of caveolae.

EXPERIMENTAL PROCEDURES

Materials—Mouse IgGs directed against caveolin-3, clathrin, and eNOS were obtained from Transduction Laboratories (Lexington, KY). Rabbit IgGs directed against the adenosine A₁ receptor were supplied by Alpha Diagnostic International (San Antonio, TX) and Chemicon International (Temecula, CA). Mouse IgG directed against the human transferrin receptor was supplied by Zymed Laboratories Inc. (San Francisco, CA). Horseradish peroxidase-conjugated IgGs were supplied by Cappel (West Chester, PA). Super Signal® chemiluminescent substrate was purchased from Pierce. The cholesterol determination kit was from Wako (Richmond, VA). [³H]Acetate (5.21 Ci/mmol) were supplied by American Pharmaceuticals Biotech. Adenosine receptor agonists...
Redistribution of A1 Receptors

(4418) Characterization of rat ventricular cardiomyocyte subcellular fractions

Ventricular cardiomyocytes were subfractionated as described under “Experimental Procedures.” The amount of protein in each subcellular fraction was determined by Lowry assay. Galactosyltransferase and NADPH-cytochrome c reductase activities were measured in each fraction and normalized with respect to the values obtained for the post-nuclear supernatant fractions. ND, level of enzymatic activity was below the sensitivity of the assay. Presented data are the mean ± S.E. from three independent experiments, n = 3.

| Subcellular fraction | Protein (µg) | Galactosyltransferase | NADPH-cytochrome c reductase |
|----------------------|-------------|----------------------|----------------------------|
| Postnuclear supernatant | 3210 ± 123 | 100 ± 7.0             | 100 ± 9.3                  |
| Cytosol              | 1502 ± 63  | 8 ± 0.5              | 16 ± 7.4                   |
| Intracellular membranes | 1463 ± 86 | 91 ± 8.2             | 82 ± 9.6                   |
| Plasma membranes     | 192 ± 26   | 1.1 ± 0.2           | 1.8 ± 0.4                   |
| Caveolae membranes   | 9 ± 1.8     | ND                   | ND                         |

RESULTS

Isolation of Caveolae from Rat Ventricular Myocytes—We defined the presence of caveolae in ventricular myocytes by an established isolation procedure (16). In brief, ventricular myocytes were lysed and a postnuclear supernatant generated. The postnuclear supernatants were separated into cytosol, plasma membrane, and intracellular membrane (endoplasmic reticulum, Golgi, etc.) fractions by centrifugation in Percoll. The plasma membranes were then sonicated and fractionated by density gradient centrifugation to isolate caveolae. Similar to other cell type studies, the caveola fraction from ventricular myocytes contained less than 0.4% of the protein found in the initial postnuclear supernatant fraction (Table I).

The most probable contaminates of the plasma membrane and caveola fractions are the endoplasmic reticulum and Golgi. Therefore, we measured the amount of NADPH-cytochrome reductase (endoplasmic reticulum) and galactosyltransferase (Golgi) activity in each of the subcellular fractions (Table I). The majority of the activities were associated with the intracellular membranes. The plasma membrane contained less than 2% of the total postnuclear supernatant activities, and the caveola fraction did not contain any detectable NADPH cytochrome reductase or galactosyltransferase activity.

To ensure that the caveola fraction was not contaminated with other plasma membrane domains, 5 µg (Fig. 1A) or 3% (Fig. 1B) of each subcellular fraction was resolved by SDS-PAGE and immunoblotted for various caveola and non-caveola proteins. The equal protein loads in Fig. 1 illustrate the relative enrichment of each protein in the caveola fraction, whereas the proportional protein loads in Fig. 1B illustrate total protein distribution. Caveolin-3, a marker for muscle caveolae, was highly enriched in the caveola fraction. In addition, eNOS, which has been shown to directly interact with caveolin (21), was also enriched in the caveola fraction. The non-caveola proteins, clathrin and transferrin, were excluded from the caveola fraction. The yield of caveolin-3 and eNOS in the caveola fraction was determined by immunoblot analysis in the linear range of detection (data not shown). The estimated yield, with respect to the plasma membrane fraction, of caveolin-3 was 61 ± 6% and eNOS was 69 ± 8%.

Caveolae are highly enriched in cholesterol (16). To further characterize the myocyte caveola fraction, we used an enzymatic kit to determine the mass of cholesterol associated with each fraction. Fig. 2 demonstrates that the caveola fraction, which contained 24 µg of cholesterol, was 7.4-fold more enriched in cholesterol than the plasma membrane fraction, which contained 79 µg of cholesterol.

Subcellular Localization of Adenosine A1 Receptors—The subcellular distribution of A1 receptors under basal conditions was determined in ventricular myocytes incubated in HEPES buffer supplemented with adenosine deaminase. Three percent of each subcellular fraction was analyzed for the presence of A1 receptors by SDS-PAGE and immunoblotting. Under these conditions, A1 receptors were highly concentrated (67 ± 5% with respect to the plasma membrane) in the caveola fraction (Fig. 3A, Buffer). Similar results were obtained in two other groups of control myocytes.

Next, we determined the subcellular localization of A1 receptors after incubating myocytes with the A1 receptor agonist CCPA. Treatment with CCPA caused the quantitative removal of A1 receptors out of the caveola fraction (Fig. 3A, CCPA). Similar results were obtained when this treatment was repeated in additional myocytes. Quantification of the immunoreactive bands demonstrated that greater than 90% of the signal originally detected in the caveola fraction was now detected in the plasma membrane fraction (data not shown).
verify that this caveola to plasma membrane translocation was selective for A1 receptors, two additional protocols were performed. In the first, pretreatment of myocytes with the A1 receptor antagonist DPCPX prior to addition of CCPA prevented the redistribution of the receptor (Fig. 3A, CCPA + DPCPX). Finally, treatment of cells with the A2a agonist CGS 21680 did not affect the caveola localization of the A1 receptor (Fig. 3A, CGS). Immunoblots for caveola and non-caveola marker proteins demonstrated that CCPA treatment did not alter the yield or purity of caveola (Fig. 3B).

Recently, Stan et al. (22) suggested that subcellular fractionation methods to isolate caveolae are contaminated with vesicles with similar biophysical properties as caveolae. Therefore, to verify that A1 receptors are associated with caveolae, we used caveolin-3 IgG to immunoprecipitate caveola membranes from isolated caveola. Isolated ventricular myocytes were incubated in control or CCPA-supplemented HEPES buffer for 15 min, then processed to immunoprecipitate caveola with caveolin-3 IgG. The precipitated material and the remaining supernatants were resolved by SDS-PAGE and immunoblotted with IgGs for eNOS, A1 receptor, and caveolin-3. Caveolin-3 co-immunoprecipitated eNOS and the A1 receptor from caveola isolated from cells treated with buffer (Fig. 4, Buffer). However, caveolin-3 only co-immunoprecipitated eNOS from caveola isolated from cells treated with CCPA (Fig. 4, CCPA). Approximately 10% of caveolin-3, eNOS, and A1 receptor was not precipitated with caveolin-3 IgG.

We have shown by subfractionation and immunoprecipitation that A1 receptors are localized to caveolae. To confirm

**Fig. 1.** Characterization of rat ventricular cardiomyocyte caveolae. A, 5 μg of protein from each subcellular fraction was resolved by SDS-PAGE and immunoblotted for caveolin-3 and eNOS (caveola markers) and transferrin receptor and clathrin (non-caveola markers). B, equal proportions of each subcellular fraction (3%) were resolved by SDS-PAGE and immunoblotted as described above. PNS, postnuclear supernatant (98 μg); CYTO, cytosol (45 μg); IM, total intracellular membranes (43 μg); PM, total plasma membrane (6 μg); CM, caveola membrane (0.3 μg). The immunoblots were developed by the method of chemiluminescence. The caveolin-3 immunoblot was exposed for 10 s and the eNOS, transferrin receptor (TR), and clathrin immunoblots were exposed for 3 min. Representative data from four independent experiments are shown.

**Fig. 2.** Myocyte caveolae are highly enriched in cholesterol. The relative enrichment of cholesterol in each subcellular fraction (μg of cholesterol/mg of fraction protein) was determined using a commercially available kit (see “Experimental Procedures”). PNS, postnuclear supernatant; CYTO, cytosol; IM, total intracellular membranes; PM, total plasma membrane; CM, caveola membrane. The data are from three independent experiments, mean ± S.E., n = 3.

**Fig. 3.** CCPA induces the translocation of adenosine A1 receptors out of caveolae. A, ventricular cardiomyocytes were treated for 15 min with buffer, 200 nM CCPA, 200 nM CCPA + 200 nM DPCPX, or 100 nM CGS. The cells were then washed and processed to isolate caveola membranes. Equal proportions of each subcellular fraction (3%) were resolved by SDS-PAGE and immunoblotted for A1 receptor. PNS, postnuclear supernatant (101 μg); CYTO, cytosol (53 μg); IM, total intracellular membranes (41 μg); PM, total plasma membrane (7 μg); CM, caveola membrane (0.24 μg). The immunoblots were developed by the method of chemiluminescence (10-min exposures). A1 receptor was identified as a 37-kDa cross-reactive band. B, to confirm the fidelity of the caveola isolations in A, equal proportions of each subcellular fraction (3%) were resolved by SDS-PAGE and immunoblotted for caveolin-3, eNOS, transferrin receptor, and clathrin. Shown are data for cells treated with CCPA. Control immunoblots for the other treatments were similar (data not shown). Representative data from four independent experiments are shown.

**Fig. 4.** CCPA prevents the co-immunoprecipitation of adenosine A1 receptors with caveolin-3. Ventricular cardiomyocytes were incubated in control buffer or treated for 15 min with buffer containing CCPA (200 nM) at 37 °C. The cells were then processed to isolate caveola membranes. IgG directed against caveolin-3 (2 μg/ml) was used to immunoprecipitate caveola from the caveola enriched subcellular fraction. The entire precipitate and the entire supernatant was resolved by SDS-PAGE and immunoblotted for eNOS, A1 receptor, and caveolin-3. The immunoblots were developed by the method of chemiluminescence (4-min exposures). Representative data from three independent experiments are shown. PEL, pellet; SUP, supernatant.
these findings in an independent manner, A1 receptors were localized by immunoelectron microscopy. Cells were treated with buffer (Fig. 5A) or CCPA (Fig. 5B) as described above. The membranes were then processed to localize caveolin-3 (arrowheads) and A1 receptors (arrows). Caveolin-3 and A1 receptors were found over small invaginations on the membrane surface that had the characteristic appearance of caveolae in buffer-treated cells. However, after CCPA treatment, only caveolin-3 was associated with these small invaginations.

**DISCUSSION**

This is the first report of adenosine receptor association with caveolae. Under basal conditions, ventricular myocyte A1 receptors were localized primarily in caveolae. However, after agonist (CCPA) binding, A1 receptors translocated from the caveolae to the plasma membrane fraction. This effect was blocked by prior treatment with the selective A1 receptor antagonist DPCPX, indicating that this phenomenon is agonist-specific. This unique pattern of receptor localization may explain, in part, the lack of direct effects of A1 receptor in ventricular myocytes.

The A1 receptor is a member of the family of G protein-coupled receptors containing a seven-transmembrane-spanning domain. There are several reports that similar G protein-coupled receptors, e.g. angiotensin II (12), bradykinin B2 (13), and endothelin A (23), and muscarinic receptors (13) are present in caveolae. However, the present results indicate that, in contrast to these receptors, the A1 receptor translocates out of, rather than into, caveolae with receptor activation. There are at least two differences between ventricular myocyte A1 receptors and these other G protein-coupled receptors, which may explain this unique relationship of A1 receptors with caveolae. First, angiotensin II, bradykinin, and endothelin have been reported to increase intracellular calcium and exert direct inotropic effects in ventricular myocytes (5–7). In contrast, A1 receptor occupation is not associated with these effects (4). Another difference is the phenomenon of receptor desensitization. Angiotensin II, bradykinin B2, and endothelin A receptors all desensitize within minutes of receptor activation (24–26). In fact, it has been hypothesized that receptor movement into caveolae may play a role in this phenomenon (27). Cardiac A1 receptors desensitize only after several hours to days of continuous agonist treatment (28). It should be noted that movement out of caveolae is also associated with desensitization because agonist-induced translocation of epidermal growth factor receptor out of caveolae is associated with rapid desensitization (29).

Our observation of A1 receptor translocation from caveolae to sarcosome after agonist treatment is the opposite of that reported for the ventricular myocyte muscarinic M2 receptor (11). This is surprising, given the fact that A1 and muscarinic M2 receptors exert a nearly identical pertussis toxin-sensitive anti-adrenergic effect in ventricular myocytes. However, muscarinic receptors have several properties unique from A1 receptors, which may explain these different associations with caveolae. The muscarinic agonist carbachol, at doses similar to that used by Feron et al. (11), increases inositol phosphate turnover, intracellular Ca2+, and contractility in ventricular myocytes (5). In addition, it appears that muscarinic M2 receptors may exert direct effects in ventricular myocytes via coupling to eNOS (11). Similar to the observations of Feron et al. (11), we observed in the present study that eNOS is localized in caveolae. Finally, it has been reported that muscarinic M2 receptors can be desensitized with brief agonist exposure (30).

The A1 receptor antibody used in this study (Chemicon International) was raised against the third extracellular domain of the rat adenosine A1 receptor gene (amino acids 163–176). In addition to the 36-kDa band (the A1 receptor is a 36-kDa protein), the antibody also cross-reacted with a 74-kDa band. Ciruela et al. (31) also observed a 74-kDa band in several tissues (brain, kidney, lung) in several species (rat, pig, lamb) using a rabbit polyclonal antibody raised against the same peptide sequence of the rat A1 receptor. They reported that this band was converted to a 39-kDa form after agonist binding. We observed similar results in the present study, although agonist binding did not convert all of the higher molecular weight band to the lower molecular weight form. The mechanistic explanation for this observation is not known. Identical results were obtained with an Alpha Diagnostic International antibody generated against the same epitope (data not shown).

Several lines of evidence support the hypothesis that A1 receptor movement between caveolae and sarcosome is dependent on occupation of the receptor by an A1 agonist. First, all studies were performed in the presence of adenosine deaminase to degrade endogenously released adenosine. The subsequent addition of the A1 receptor agonist CCPA, which is relatively selective for A1 receptors, resulted in the movement of the receptor from caveolae to sarcosomal membranes. At the concentration used in the present study, there is no evidence that CCPA activates cardiac myocyte A2a or A2 receptors. In addition, the A2a agonist CGS 21680 did not induce A1 receptor translocation from caveolae. Finally, DPCPX, which is a highly selective adenosine A1 receptor antagonist (32), completely blocked the effects of CCPA.

Since this is the first report of the localization of the A1 receptor in caveolae, it is not known whether A1 receptor translocation out of caveolae activates or terminates signaling. There are reports that inhibitory G protein subunits are localized in caveolae (33), and cardiac A1 receptors couple primarily to Go subunits (34). Two components of the only known pathway that A1 receptor activation modulates in ventricular myocardium, β-adrenergic receptors and adenyl cyclase, have been reported to be localized in light vesicular fractions which may be caveolae (35, 36). Furthermore, cardiac adenyl cyclase can be inhibited in vitro by the caveolin-3 scaffolding domain peptide (37). Therefore, the location of A1 receptors is likely to significantly influence the ability of the receptors to couple to downstream signaling events. It has recently been reported that stimulation of rat cardiac myocytes with endothelin is associated with the recruitment of PKC-α and -ε isoforms to the caveolae fraction (38). Henry et al. (39) previously reported that A1 receptor stimulation of ventricular myocytes induced a transient cytosol to membrane translocation of PKC-δ. Since endothelin, but not A1 receptor stimulation is associated with direct effects in myocytes, it is possible that A1 receptor stimulation may recruit PKC-δ out of caveolae, where PKC signaling may be physiologically important.

In summary, we have demonstrated that, in contrast to other G protein-coupled receptors, A1 receptors translocate out of...
caveolae with agonist binding. The molecular consequences of this translocation are not known. Future studies will elucidate the relationship between A1 receptors, caveolae, and signal transduction.

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