The Adenosine 5′,5‴,P_1,P_4-Tetraphosphate Receptor Is at the Cell Surface of Heart Cells†

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ABSTRACT: We have previously demonstrated the existence of an adenosine 5′,5‴,P_1,P_4-tetraphosphate (Ap_4A) receptor in mouse hart membrane fractions [Hilderman, R. H., Martin, M., Zimmerman, J. K., & Pivorun, E. P. (1991) *J. Biol. Chem. 266*, 6915–6918]. However, we did not determine the cellular localization or distribution of the receptor. In this report, the Ap_4A receptor is shown to be on the cell surface of individual mouse heart cells by the following four methods: (1) intact cells show specific, saturable, and reversible binding of Ap_4A; (2) monoclonal antibodies (Mabs) raised against the Ap_4A receptor inhibit Ap_4A binding to its receptor on intact heart cells; (3) bound Mabs are shown to be at the outer cell surface via reaction with a alkaline phosphatase co njugated goat anti-rat IgG; (4) when intact cells are labeled with the impermeable cell surface labeling reagent, (sulfosuccinimido)biotin, labeled receptor is immunoprecipitated with Mabs. Furthermore, subcellular fractionation of mouse hearts demonstrates that virtually all of the Ap_4A receptor is associated with a membrane fraction with at least 77% of the active receptor on plasma membranes.

It has been hypothesized that diadenylated nucleotides may represent a new class of neurotransmitters which interact with receptors distinct from the classical P_1 nucleoside (adenosine) and P_2 nucleotide (ATP) purinoceptors (Hoyle, 1990). Adenosine 5′,5‴,P_1,P_4-tetraphosphate (Ap_4A) is the major representative of this type of nucleotide.

Ap_4A affects nuclear and cytosolic enzyme activities. It is an activator of DNA polymerase α (Rapaport et al., 1991) and is an effective inhibitor of adenosine kinase and adenylate kinase (Grummt et al., 1979; Grummt, 1988). Furthermore, Ap_4A is released from cells and acts as an extracellular signal. Chromaffin granules costore Ap_4A and Ap_5A as well as catecholamines, ATP, and peptides (Rodriguez et al., 1988). In addition, the presence of Ap_4A in brain synaptosomes and its release by depolarizing agents emphasize the importance of Ap_4A as a possible neurotransmitter (Pintor et al., 1992). In this neural model, the release of Ap_4A after carbachol stimulation allowed its quantification in the extracellular media at concentrations in the micromolar range (Pintor et al., 1992). Extracellular Ap_4A increases the basal secretion of catecholamines from chromaffin cells (Castro et al., 1990) and induces calcium flux (Castro et al., 1992).

Ap_4A and Ap_3A are also stored in dense secretory granules of platelets, and on release inhibit platelet aggregation (Zamecnik et al., 1992). Extracellular Ap_4A also has pronounced effects on the smooth muscle tone of rabbit arteries (Harrison et al., 1975) and on hepatic parenchymal cell function (Busshardt et al., 1989). Furthermore, the activation of protein kinase C in chromaffin cells by extracellular Ap_4A suggests that Ap_4A binding to its receptor triggers a second messenger (Sen et al., 1993).

We have demonstrated the presence of specific, saturable membrane receptors for Ap_4A in brain, liver, kidney, cardiac, spleen, and adipose tissue, with the greatest density of receptors found in cardiac tissue (Hilderman et al., 1991). The distribution of the receptor in diverse tissue types suggests that circulating Ap_4A may have an extremely important modulatory or signaling function. We have identified the Ap_4A receptor in mouse heart membrane fractions as a 30-kDa polypeptide (Walker et al., 1993) and have isolated Mabs against the receptor. These Mabs inhibit Ap_4A binding to its membrane receptor and recognize four membrane proteins with molecular masses of 67, 55, 42, and 30 kDa (Walker & Hilderman, 1993). This suggests that the precursor for the Ap_4A receptor is a 67-kDa polypeptide which undergoes multiple cleavage events. This is consistent with our data showing that, prior to binding Ap_4A, the receptor is activated by a membrane-bound serine protease (Walker et al., 1993; Walker & Hilderman, 1993).

Since Ap_4A has been shown to bind to intracellular proteins (Kobayashi & Kuratomi, 1989), rat brain synaptosomes (Pintor et al., 1993), and intact cultured chromaffin cells (Pintor et al., 1991a,b), we feel it is important to determine the cellular distribution of the Ap_4A receptor. In this article, we demonstrate that Ap_4A binds to intact mouse heart cells and that at least 77% of the active Ap_4A receptor is associated with the plasma membrane.

MATERIALS AND METHODS

Cell Lines and Materials. Swiss mice were obtained from Charles River Laboratories. The animals were housed in an animal facility maintained with a photoperiod of 15L:9D and temperatures of 22–25 °C. [^{3}H]Ap_4A was purchased from Amersham International. Goat anti-rat IgG conjugated to alkaline phosphatase and streptavidin conjugated to alkaline phosphatase were purchased from Bio-Rad. Rat IgG was purchased from Zymed Laboratories. Goat anti-rat IgG–agarose, collagenase type I, and fraction V BSA were

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purchased from Sigma. The sulfo-*N*-hydroxysuccinimide ester of biotin was purchased from Pierce. Immobilon PVDF transfer membranes were purchased from Millipore. Nonidet P-40 was purchased from Fluka Chemie. Zwittergent 3-14 was purchased from Calbiochem. All reagents were of analytical reagent grade or better.

Isolation of Individual Heart Cells. Individual heart cells were isolated by a modification of the collagenase digestion procedure as described (West et al., 1986). The final cell suspension was resuspended in Tyrodes' solution (12 mM $NaHCO_3$ (pH 7.4), 0.4 mM NaH_2PO_4, 140 mM NaCl, 0.5 mM $MgCl_2$, 2.7 mM KCl, and 5.6 mM glucose) containing 1 mM $CaCl_2$ at a concentration of 7.5×10^7 cells/mL. The individual heart cells were judged to be intact by the following three criteria: (1) the cells were 85–90% viable as shown by trypan blue dye exclusion; (2) counting of the total number of cells in a hemacytometer demonstrated a loss of only 10% of the cells after Ap_4A binding for 20 min at 20 °C; (3) there was only a 15–19% release of lactate dehydrogenase after incubating the cells for 20 min at 20 °C.

Optimum Ap_4A Binding to Single Cells. Each reaction mixture contained 1.0×10^6 cells, 16 μL of 5× Tyrodes' solution, and 0.033 μM [^{3}H]Ap_4A (15000–20000 cpm/pmol) in a final volume of 0.1 mL. The samples were incubated for 15 min at 20 °C, filtered, and counted as described (Hilderman et al., 1991).

Lactate Dehydrogenase Assay. Individual heart cells (1.0×10^6) were resuspended in 0.1 mL of Tyrodes' solution, incubated at 20 °C for 15 min, and centrifuged at 1000*g* for 3 min. The supernatant was removed and diluted to 1.0 mL with Tyrodes' solution containing a final concentration of 140 μm NADH and 1.5 mM pyruvate. The disappearance of NADH was measured at 340 nm. The cell pellet was then resuspended in 0.1 mL of Tyrodes' solution, sonicated, and centrifuged at 16000*g* for 10 min, and the supernatant was also assayed for lactate dehydrogenase activity. As a control, 1.0×10^6 individual heart cells were resuspended in Tyrodes' solution, sonicated, and centrifuged at 16000*g* for 10 min, and the supernatant was assayed for lactate dehydrogenase activity.

Preparation of Monoclonal Antibodies against the 30-kDa Receptor Protein. These antibodies were isolated as described (Walker & Hilderman, 1993). The Mab clone that was used in all experiments described in this article is TL4. An unrelated Mab prepared against chicken brain *N*-cadherin (kindly supplied by Dr. Jack Lilien) was used as a control and is referred to as CBC.

Detection of TL4 Binding to Individual Heart Cells by ELISA. Poly-L-Lys (50 μg/mL) (100 μL) in Tyrodes' solution was added to microtiter wells and incubated overnight at 4 °C. The plates were washed two times with 100 μL of Tyrodes' solution prior to the addition of 1.5×10^6/well and assayed as described (Walker & Hilderman, 1993).

Biotinylation of Individual Heart Cells. Biotinylation was performed as described (Sargiacomo et al., 1989). Cells (6.0×10^6) were resuspended into 600 μL of Tyrodes' solution containing 1.0 mg/mL of the sulfo-*N*-hydroxysuccinimide ester of biotin (sulfo-NHS-biotin) and incubated for 60 min at 4 °C, washed three times with Tyrodes' solution, resuspended into 50 μL of lysis buffer (10 mM Hepes (pH 7.5), 140 mM NaCl, 4 mM EDTA, 2.5% Nonidet-P40, and 2.5% Zwittergent 3-14) and incubated with shaking, for at min at room temperature. Buffer (450 μL) containing 10 mM Hepes (pH 7.5), 140 mM NaCl, and 4 mM EDTA (nonlysis buffer) was added to reduce the detergent concentrations to 0.5%, and these samples were centrifuged at 16000*g* for 10 min. Under these conditions, the Ap_4A receptor remains in the pellet. The receptor was immunoprecipitated as described (Balsamo & Lilien, 1990). The immunoprecipitated receptor was resuspended in 25 μL of 83.3 mM Tris-HCl (pH 8.0), 6% (w/v) SDS, 1% Triton X-100, and 4.4 M urea followed by the addition of 75 μg of DTT and 57.5 μg of iodoacetamide and denatured by boiling. Samples were loaded onto an 8% discontinuous polyacrylamide gel and run as described (Laemmli, 1970). After SDS–PAGE, Western transfer was performed as described (Walker et al., 1993) and immunoblotted with streptavidin conjugated to alkaline phosphatase, also as described (Harlow & Lane, 1988).

Photolabeling and Analysis of Photolabeling Reactions by SDS–PAGE for Identification of the Ap_4A Receptor. All operations involving 8-azido nucleotides were performed under subdued light. The [α-^{32}P]-8-N_3Ap_4A was synthesized and purified as described (Walker et al., 1993). Each reaction mixture contained 1.5×10^6 cells in 400 μL of Tyrodes' solution plus 1×10^6 cpm of 0.4 nM [α-^{32}P]-8-N_3Ap_4A. These reaction mixtures were incubated for 20 min at 20 °C in the dark. The samples were irradiated at 4 °C using a short-wavelength UV lamp at 254 nm at a dose rate of 300 μW/cm^2 for 60 s (at a distance of 12 cm from the surface of the film). SDS–PAGE, Western transfer, and autoradiography were performed as described (Walker et al., 1993).

Subcellular Fractionation of the Ap_4A Receptor. Eight mouse hearts (1.0 gm) were suspended in 40 vol of Hepes-buffered saline with glucose and potassium (HBSGK), which contained 20 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM glucose, 3 mM KCl, and 1 mM $CaCl_2$ in addition to 0.1% Triton X-100. The suspended hearts were minced and homogenized with a Dounce homogenizer. After homogenization, the sample was filtered through cheesecloth (crude extract). A 0.2-mL sample of the crude extract was removed and the rest was centrifuged at 1000*g* for 10 min at 4 °C to isolate the nuclear fraction. The nuclear fraction was washed twice with HBSGK buffer containing 0.1% Triton and then resuspended in 22 mL of HBSGK buffer containing 0.1% Triton. A 0.2-mL sample was removed from the postnuclear supernatant prior to centrifugation at 12000*g* for 10 min at 4 °C to isolate the mitochondrial fraction. The mitochondrial pellet was washed twice with HBSGK buffer containing 0.1% Triton and resuspended in 20 mL of HBSGK buffer containing 0.1% Triton X-100. A 0.2-mL sample of the postmitochondrial supernatant was removed prior to centrifugation at 100000*g* for 90 min at 4 °C to isolate the membrane fraction. The membrane fraction was resuspended in 2.0 mL of HBSGK buffer containing 0.1% Triton X-100 and 8.5% sucrose.

Plasma membranes were separated from the sarcoplasmic reticulum by sucrose density gradient centrifugation as described (Kidwai et al., 1971). Two bands had Ap_4A binding activity. The upper band (F_1) was shown to be enriched for plasma membranes by the following three criteria: (1) F_1 is enriched with plasma membrane markers. 5′-Nucleotidase and K^+-activated phosphatase were assayed as described (Kidwai et al., 1971) and showed 6.2- and 2.4-fold enhancements, respectively. (2) Greater than 85% of the protein in F_1 bound to concanavalin A affinity resin, and this protein was eluted from the column with α-methyl mannoside. (3) The buoyant density of F_1 is 1.108 g/mL, which is consistent with the reported value of 1.105 for cardiac muscle plasma membranes (Misselwitz et al., 1979). In the absence of any accepted sarcoplasmic reticulum markers, we could not determine the extent of cross-contamination of plasma

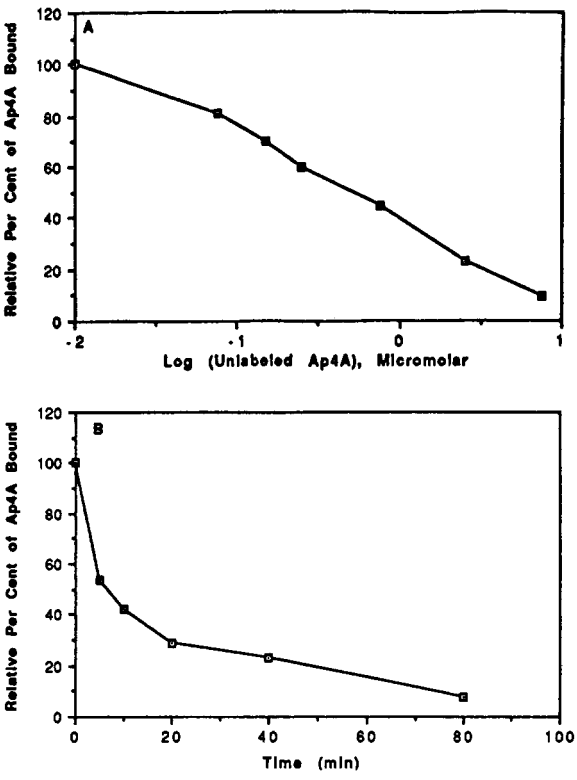

FIGURE 1: Inhibition of [^{3}H]Ap_4A binding to individual heart cells by unlabeled Ap_4A. The binding assay is described in the text. (A) Varying concentrations of unlabeled Ap_4A at the concentrations indicated and 0.033 μM [^{3}H]Ap_4A (specific activity, 9000–10000 cpm/pmol) were incubated with 1.0×10^6 heart cells for 15 min at 20 °C. (B) 0.033 μM [^{3}H]Ap_4A (specific activity, 9000–10000 cpm/pmol) was incubated with 1×10^6 heart cells for 15 min at 20 °C. The samples were then brought to 1.0 mM with nonradioactive Ap_4A (time zero in the figure), and binding was determined at the times indicated. All samples were collected on glass fiber disks and counted as described (Hilderman et al., 1991).

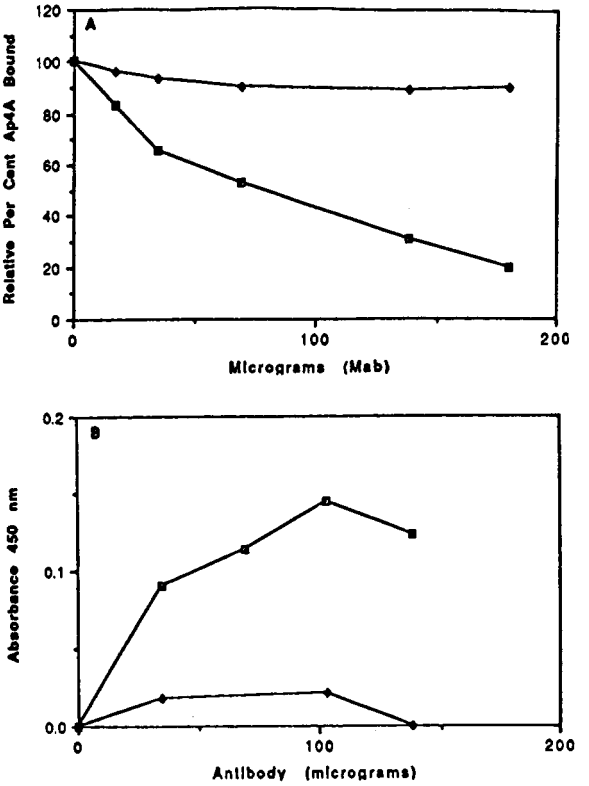

FIGURE 2: TL4 inhibition of Ap_4A binding to individual heart cells. (A) 1.0×10^6 heart cells were resuspended in Tyrodes' solution plus varying amounts of TL4 (⊡) or CBC (◆) and incubated for 60 min at 4 °C. The samples were then centrifuged for 3 min at 1000g. The pellets were resuspended in 0.1 mL of Tyrodes' solution plus 0.033 μM [^{3}H]Ap_4A (specific activity, 9000–10000 cpm/pmol) and incubated for 15 min at 20 °C. All samples were collected on glass fiber disks and counted as described (18). (B) 1.0×10^6 cells were bound to poly-L-Lys in microtiter plates as described in Materials and Methods. Varying amounts of TL4 (⊡) or CBC (◆) were added, and the samples were incubated for 60 min at 4 °C prior to the addition of the secondary antibody and developed as described in Materials and Methods.

membrane by this fraction. However, greater than 90% of the hazy lower band (F_2) did not bind to concanavalin A resin. Furthermore, the mitochondrial pellet contained 8 times more cytochrome c oxidase activity (mitochondrial marker) than F_1 or F_2. These data are consistent with F_1 being predominantly plasma membranes and F_2 most likely being sarcoplasmic reticulum.

Protein Determination. Protein concentrations were determined by dye binding (Bradford, 1976).

RESULTS

Ap_4A Binding to Individual Heart Cells. Ap_4A binding to individual heart cells was linear from 0.5×10^5 to 2×10^6 cells (data not shown). All subsequent experiments were performed using 1.0×10^6 cells. The optimum temperature was determined to be 20 °C; relative values obtained at saturation for binding with respect to temperature were 59, 100, 81, 59, 24%, respectively, for 12, 20, 30, 37, and 42 °C. To determine whether Ap_4A was being hydrolyzed during the incubation, [^{3}H]Ap_4A was incubated with 1.0×10^6 cells for 15 min at 20 °C and then subjected to thin-layer chromatography (Hilderman et al., 1991). There is no evidence of Ap_4A breakdown under the conditions of the assay (data not shown).

Using the optimum temperature of 20 °C, Ap_4A binding to individual heart cells was linear for 5 min and reached a plateau by 10 min. Longer incubation periods, up to 60 min, did not significantly alter the amount of Ap_4A bound (data not shown). All subsequent experiments were performed at 20 °C for 15 min.

The ability of nonradioactive Ap_4A to displace radioactive Ap_4A binding is presented in Figure 1A. Variable amounts of nonradioactive Ap_4A and a fixed amount of [^{3}H]Ap_4A were incubated with heart cells for 15 min at 20 °C. Nonradioactive Ap_4A effectively competes with [^{3}H]Ap_4A for binding sites (>90% inhibition of [^{3}H]Ap_4A for binding at 7.5 μM nonradioactive Ap_4A). ATP, ADP, AMP, and adenosine do not effectively compete at concentrations up to 7.5 μM, while Ap_5A was almost as effective as Ap_4A in displacing [^{3}H[Ap_4A (data not shown).

Unlabeled Ap_4A, when added following a 15-min incubation with [^{3}H]Ap_4A, also effectively competes for binding with prebound [^{3}H]Ap_4A. Under the conditions of our assay, nonradioactive Ap_4A displaces about 65% of the prebound [^{3}H]Ap_4A within 20 min and greater at 90% of the prebound [^{3}H]Ap_4A after 80 min (Figure 1B). This is consistent with Ap_4A binding being reversible, and it demonstrated that Ap_4A does not enter the cell by either endocytosis or pinotcytosis processes.

Monoclonal Antibodies against the Ap_4A Receptor Inhibit Binding. We have previously isolated Mabs (TL4) against the Ap_4A receptor that inhibit Ap_4A binding to its receptor in a mouse heart membrane fraction (Walker & Hilderman, 1993). TL4 preincubated with individual cells, followed by washing of the cells, inhibits [^{3}H]Ap_4A binding to its receptor, while an unrelated Mab against chicken brain N-cadherin (CBC) does not (Figure 2A). Furthermore, when individual

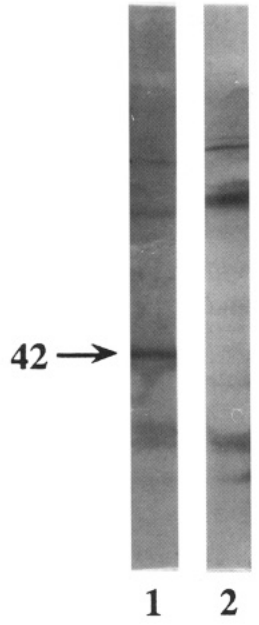

FIGURE 3: Labeling individual heart cells with sulfo-NHS-biotin. 6 × 10^6 individual heart cells were biotinylated, immunoprecipitated followed by SDS–PAGE, and Western transferred. The Western transfer strips were developed with streptavidin conjugated to alkaline phosphatase as described in Materials and Methods. Lane 1: Immunoprecpitation was performed with 40 μg of TL4. Lane 2: Immunoprecipitation was performed with 40 μg of rat IgG. Molecular mass value is denoted by the arrow.

heart cells are bound to ELISA plates, the Ap_4A receptor can be detected by binding to TL4 followed by reaction with goat anti-rat IgG conjugated with alkaline phosphatase (Figure 2B).

Labeling the Receptor on Individual Heart Cells with Sulfo-NHS-biotin and [α-^{32}P]-8-N_3Ap_4A. As a prelude to determining whether the Ap_4A receptor could be labeled with the cell-impermeable reagent sulfo-NHS-biotin, we determined whether the label entered cells, possibly through pores created during cell preparation. Individual heart cells were biotinylated, sonicated, and subjected to differential centrifugation. Up to 30 μg of cytosolic proteins and nuclear/mitochondria pellet fractions were subjected to SDS–PAGE and then Western transfers prepared and developed with streptavidin conjugated to alkaline phosphatase. There were only two streptavidin-positive polypeptides in the cytosolic fraction, with molecular masses of 125 and 90 kDa, and no streptavidin-positive polypeptides in the nuclear and mitochondrial pellet (data not shown). This is consistent with biotinylation occurring primarily on cell surface proteins.

To determine whether the Ap_4A receptor was labeled, individual heart cells were biotinylated, lysed, immunoprecipitated with either TL4 or rat IgG, subjected to SDS–PAGE, Western transferred, and developed with streptavidin conjugated to alkaline phosphatase. The only polypeptide immunoprecipitated by TL4 that is not also immunoprecipitated by rat IgG is a 42-kDa polypeptide (Figure 3). TL4 recognizes, by immunoblotting, three polypeptides from labeled individual heart cells with molecular masses of 67, 55, and 42 kDa (Figure 4, lane 1). These data are consistent with the 42-kDa polypeptide being present at the cell surface. Since the 67- and 55-kDa polypeptides were not immunoprecipitated, this suggests that the TL4 epitope on these two polypeptides is not accessible on intact heart cells.

To identify the actual receptor on intact cells, individual heart cells were incubated with [α-^{32}P]-8-N_3Ap_4A for 15 min

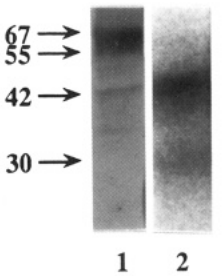

FIGURE 4: SDS–PAGE and autoradiography of the photolabeled receptor of individual heart cells. 1.5 × 10^6 cells were incubated in Tyrodes' solution containing 1.0 × 10^6 cpm of [α-^{32}P]-8-N_3Ap_4A. These reaction mixtures were incubated for 15 min at 20 °C in the dark, photolabeled, and then run on SDS–PAGE and Western transferred. Lane 1: The Western-transferred strip was immunoblotted with 30 μg of TL4 as described in Materials and Methods. Lane 2: Autoradiography was performed as described in Materials and Methods. Molecular mass values are denoted by the arrows.

at 20 °C prior to UV cross-linking. SDS–PAGE followed by Western transfer and autoradiography reveals labeling of the 42-kDa polypeptide and a small amount of a 30-kDa polypeptide (Figure 4, lane 2).

These data are consistent with our observations in heart membranes. However, in membrane preparations, TL4 also recognizes a 30-kDa polypeptide which is the predominant receptor type (Walker et al., 1993; Walker & Hilderman, 1993). Since proteolytic processing is essential to Ap_4A binding, the simplest explanation for this difference in the predominant receptor (42 kDa on cells vs 30 kDa on membranes) is that in intact cells, processing is limited. Since the studies with heart cells must be performed in a physiological buffer, and studies with membrane homogenates were performed with a buffer optimized for Ap_4A binding containing only 67 mM Tris-HCl (pH 7.7) and 100 μM $MgCl_2$ (Hilderman et al., 1991), it is possible that a component in the physiological buffer (Tyrodes' solution) limits conversion of the 42- to the 30-kDa receptor. This is indeed the case as [α-^{32}P]-8-N_3Ap_4A is cross-linked to both the 30-kDa and the 42-kDa polypeptides when membrane homogenates are incubated in Tyrodes' solution (data not shown). By running a matrix of Ap_4A binding experiments with membrane homogenates in which individual components of Tyrodes' solution were omitted, we have found that the component limiting the appearance of the 30-kDa polypeptide is the physiological concentration of NaCl (data not shown). This suggests that conversion of the 42-kDa and the 30-kDa polypeptide is artifactual.

Cellular Distribution of the Ap_4A Receptor. The data presented above indicate that the Ap_4A receptor is located on the cell surface. However, these data do not eliminate the possibility of an internal receptor. To determine what percentage of the Ap_4A receptor is associated with plasma membranes, mouse hearts were fractionated by differential centrifugation (Table 1). Membrane fractions were further fractionated by sucrose density gradient centrifugation (data not shown). Two membraneous components were shown to have Ap_4A binding activity. As described in Materials and Methods, the upper band is enriched for plasma membrane (F_1) while the lower band is most likely enriched for sarcoplasmic reticulum (F_2).

Ap_4A binding to F_1 and F_2 was linear from 2.5 to 25 μg of protein (data not shown). All subsequent experiments were performed using 11.6 μg. In F_1, Ap_4A binding was linear for 20 min and reached a plateau by 40 min. Longer incubations of up to 120 min did not significantly alter the amount of

Table 1: Subcellular Distribution of the Ap_4A Receptor[a]

fraction	total pmol of Ap_4A bound	relative percent	relative percent of Ap_4A binding inhibited by TL4
crude extract	300.1	100	76.1
nuclear fraction			
supernatant	254.1	84.5	75.6
pellet	11.4	3.8	75.6
mitochondrial fraction			
supernatant	182.5	60.8	79.6
pellet	<0.1		
membrane fraction			
supernatant	<0.1		
pellet	174.6	58.2	75.1

[a] Subcellular fractions were performed with four mouse hearts, as described in Materials and Methods. Ap_4A binding used the optimum conditions as described (Walker & Hilderman, 1993), except the membranes were resuspended in HSGBK with 0.1% Triton X-100. A 20-μL sample of each fraction was incubated with 0.033 μM [^{3}H]Ap_4A (specific activity, 9000–10000 cpm/pmol) for 20 min at 20 °C. TL4 inhibition studies were performed with 80 μg of antibody as described (Walker & Hilderman, 1993).

Table 2: Membrane Distribution of the Ap_4A Receptor

	nmol of Ap_4A bound/mg of protein[a]	nmol of Mab bound/mg of protein[b]
plasma membrane	0.35	0.13
sarcoplasmic reticulum	0.10	1.6

[a] The specific activities were calculated by extrapolation of a time course of Ap_4A binding curve to determine the minimum membrane at saturation of Ap_4A binding. [b] The specific activities were calculated by extrapolation of a membrane dosage of Mab curve to determine the minimum membrane concentration at saturation and using 150 kDa as the molecular mass of the Mab.

Ap_4A bound. F_2 also reached a plateau by 40 min, but the total amount of Ap_4A bound was significantly less (data not shown). Furthermore, F_1 bound 0.35 nmol of Ap_4A/mg of protein, while F_2 bound 0.1 nmol of Ap_4A/mg of protein (Table 2); this is consistent with at least 77% of the total active Ap_4A receptor in the membrane fraction being associated with plasma membranes.

TL4 inhibits the binding of Ap_4A to both F_1 and F_2 at essentially the same percentage (data not shown). Surprisingly, the total amount of TL4 bound to F_2 was significantly greater than the amount bound to F_1. F_2 bound 1.6 nmol of TL4/mg of membrane, while F_1 only bound 0.13 nmol of TL4/mg of membrane (Table 2). These data are consistent with the active form of the receptor being associated with the plasma membrane, while the sarcoplasmic reticulum contains significantly higher amounts of the precursor forms. Possibly, the forms associated with the sarcoplasmic reticulum are being processed for incorporation into the plasma membrane. We are in the process of determining the mechanism of receptor biosynthesis.

In order to determine specificity of Ap_4A binding to F_1 membranes, variable amounts of unlabeled nucleotides were incubated with a fixed concentration of [^{3}H]Ap_4A. Using the program Ligand (Munson & Rodbard, 1980), we were able to calculate K_D values for Ap_4A (Table 3). The K_D value is 10.8-, 12.0-, and 197.3-fold lower than the values for ATP, ADP, and adenosine, respectively. However, the K_D values for Ap_5A is only 1.9-fold lower (Table 3).

Table 3: K_D Values for Ap_4A and Other Nucleotides[a]

displacement nucleotide	K_D value (μM)
Ap_4A	0.074
Ap_4A	0.14
ATP	0.80
ADP	0.89
adenosine	14.60

[a] The displacement binding assays were performed as described (Hilderman et al., 1991). Unlabeled nucleotide concentration varied from 0.04 to 4.0 μM, and 0.033 μM [^{3}H]Ap_4A (specific activity, 9000–10000 cpm/pmol) was incubated with 11.6 μg of F_1 for 60 min at 20 °C.

DISCUSSION

In previous studies, we have demonstrated that [α-^{32}P]-8-N_3Ap_4A is cross-linked to a 30-kDa polypeptide (Walker et al., 1993) in mouse heart membranes. In addition, Mabs directed against the 30-kDa polypeptide recognize membrane polypeptides with molecular masses of 67, 55, 42, and 30 kDa (Walker & Hilderman, 1993). We have identified a serine protease essential for activation of the Ap_4A receptor (Walker et al., 1993; Walker & Hilderman, 1993). This suggests that the Ap_4A receptor exists as a precursor which is activated by proteolytic processing. In this article, we demonstrate that the Ap_4A receptor is localized on the cell surface of individual mouse heart cells. Ap_4A binding to individual cells is specific, saturable, and reversible with nonlabeled Ap_4A (Figure 1). Ap_4A binding to intact cells is also inhibited by the anti-30-kDa receptor Mab, TL4 (Figure 2), which immunoprecipitates a labeled 42-kDa polypeptide on cells labeled with the impermeable reagent, sulfo-NHS-biotin. On immunoblotting, the antibody recognizes three polypeptides with molecular masses of 67, 55, and 42 kDa (Figure 3). In addition, the 42-kDa polypeptide along with a small amount of a 30-kDa polypeptide is cross-linked with [α-^{32}P]-8-N_3Ap_4A (Figure 4). Thus, we conclude that the predominant receptor for Ap_4A on intact heart cells is a 42-kDa polypeptide.

The multiple polypeptides recognized by TL4 appear to be precursors generated by proteolytic processing on intact heart cells. In isolated membranes, there appears to be an additional processing step which predominantly generates the 30-kDa receptor. It is not known whether the protease involved in the conversion of the 42- to the 30-kDa polypeptide is part of the Ap_4A receptor system or whether it is a contaminating enzyme in the membrane homogenate. Since the physiological salt component of Tyrodes' solution inhibits the appearance of the 30-kDa receptor, it is reasonable to suggest that this conversion is artifactual. In order to further define the relationship of the polypeptides recognized by TL4, we are in the process of purifying each of these polypeptides and obtaining peptide maps and N-terminal sequences.

We have previously reported that Ap_4A binding to its membrane receptor is enhanced 3.3-fold during an activation step which requires both a serine protease and divalent cations (Walker et al., 1993). This activation affects Ap_4A binding to both intact cells and isolated plasma membranes. Isolation of intact cells requires incubations at 37 °C in the presence of both Mg^{2+} and Ca^{2+} ions. This undoubtedly results in activation of the receptor during cell preparation. This is born out by the fact that the total amount of Ap_4A bound to heart cells activated for 60 min is essentially the same as the amount bound to heart cells that were not activated. In the case of plasma membrane isolation, the serine protease is separated from the membrane receptor. Thus, optimal activation does not occur unless the activator containing fraction is added. These studies are presently being pursued.

Our K_D value of 0.074 μM for Ap_4A binding to plasma membranes is 9.60-fold lower than the value we obtained in partially purified membranes (Hilderman et al., 1991) and is

consistent with the range of Ap_4A K_D values reported for synaptosomes (Pintor et al., 1993).

Using the program Ligand and the data in Figure 1, we were able to calculate a value of 2.5×10^6 receptors per heart cell. This value is within the range of a physiologically significant receptor. However, using the nanomoles of Ap_4A bound per milligram of plasma membrane (Table 2), we calculated that the Ap_4A receptor is approximately 1.5% of the total membrane protein. Pintor and Miras-Portugal (1993) have also reported that the amount of Ap_4A receptor per milligram of synaptosomal membrane is high. These values appear to be high for most physiological receptor systems. The difference between the number of receptor sites on intact cells versus that presumably present on plasma membranes is an engima. Two explanations are possible: (1) cryptic receptors are present which become available on membrane isolation, and (2) following membrane isolation, more than 1 mol of Ap_4A is bound per mole of receptor. We favor the first explanation, as the amount of Ap_4A bound to plasma membrane receptor is equivalent to the amount of antibody bound (Table 2).

Localization of the Ap_4A receptor on the cell surface further supports the notion that circulating Ap_4A is a modulator of cellular function, presumably during physiological stress. We have demonstrated that Ap_4A causes a rapid and reversible Ca^{2+} flux (E. P. Pivorun and R. H. Hilderman, unpublished observations), which may be one mechanism through which circulating Ap_4A modulates cell function. Under physiological conditions, the circulating concentration of Ap_4A is too low for detection (E. P. Pivorun and R. H. Hilderman, unpublished observations), while the circulating concentration of ATP is 0.1 μM (Vassort et al., 1993). This indicates that there is not a significant amount of ATP occupying the Ap_4A receptor. Thus, a localized increase in Ap_4A due to platelet activation or chromaffin cell secretion, which also results in a transient release of ATP, will favor Ap_4A binding. We are now beginning to understand the biochemistry of Ap_4A binding and are starting to direct our attention to the cellular response to Ap_4A binding.

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