Activation of the Mouse Heart Adenosine 5′,5‴-P_1-P_4-Tetraphosphate Receptor†

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ABSTRACT: We have previously demonstrated that mouse brain membrane fractions have a specific, saturable receptor for diadenylated nucleotides. Binding is specific for two adenosines, and the length of the phosphate bridge is critical, with four phosphates being optimal [Hilderman et al. (1991) *J. Biol. Chem. 266*, 6915–6918]. In this report, we demonstrate that adenosine 5′,5‴-P_1,P_4-tetraphosphate (Ap_4A) binding to its receptor is dependent upon an activation step that requires divalent cations and a serine protease. Monoclonal antibodies (Mabs) are identified that inhibit Ap_4A binding to its membrane receptor. These antibodies recognize a 212-kDa membrane protein. However, SDS–PAGE analysis of Ap_4A cross-linked to membrane fractions reveals that Ap_4A is not attached to the 212-kDa peptide but to a 30-kDa polypeptide. Appearance of the 30-kDa polypeptide is dependent on the activation step, and one of the inhibitory antibodies blocks its appearance. We suggest that the protease-dependent processing step involves cleavage of the 212-kDa component with the appearance of an active 30-kDa receptor.

It has been hypothesized that Ap_4A and other adenine dinucleotides may represent a new class of molecules that modulate physiological stress through binding to distinct receptors (Hoyle, 1990). Consistent with this hypothesis, there is accumulating evidence that Ap_4A is released from cells and acts as an extracellular signal. Ap_4A and Ap_3A have been shown to be stored in micromolar to millimolar concentrations in the dense granules of blood platelets (Flodgaard & Klenow, 1982; Luthje & Ogilive, 1983) and in adrenal medullary chromaffin granules (Rodriguez et al., 1988). Both dinucleotides are released from platelets into the extracellular milieu after activation, with local extracellular concentrations exceeding 100 μM (Flodgaard & Klenow, 1982). Ap_4A and Ap_3A also affect platelet function. Ap_4A inhibits ADP-induced platelet aggregation (Zamecnik et al., 1992; Busse et al., 1988; Harrison et al., 1975) while Ap_3A causes platelet aggregation (Busse et al., 1988). Extracellular Ap_4A has been shown to have antithrombotic potency (Louie et al., 1988) and to influence smooth muscle tone of isolated rabbit arteries (Pohl et al., 1987; Luthje & Ogilvie, 1987).

Beyond its effect within the bloodstream, Ap_4A has pronounced effects on hepatic parenchymal and nonparenchymal cell function (Busshardt et al., 1989). Furthermore, application of Ap_4A to perfused isolated chromaffin cells results in a significant inhibition of catecholamine release that is concentration dependent. Ap_4A modulation of catecholamine release is also consistent with the presence of a cell surface receptor on adrenal chromaffin cells (Casto et al., 1990).

Our laboratory has demonstrated the presence of specific, saturable membrane binding sites or receptors for Ap_4A in brain, cardiac, liver, kidney, spleen, and adipose tissue (Hilderman et al., 1991). The distribution of this site in a diversity of tissue types suggests that Ap_4A may have an extremely important general modulatory function, possibly alerting tissues to the presence and magnitude of specific or generalized stress conditions. Thus, this site might act as a classical receptor altering cell function. The activation of Ap_4A receptors along with the activation of catecholamine or glucocorticoid receptors may amplify the response of the tissue to the classical stress hormones.

It is our goal to identify the molecular nature of the Ap_4A receptor in order to define its physiological role and mechanism of action. As a step in this direction, we report here that a protease-dependent activation step is essential for Ap_4A binding and characterize two classes of Mabs that block Ap_4A binding to its receptor.

MATERIALS AND METHODS

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 room temperatures of 22–25 °C. [^{3}H]Ap_4A was purchased from Amersham International and [α-^{32}P]ATP was purchased from NEN Nuclear. Goat anti-rat IgG conjugated with alkaline phosphatase was purchased from Bio-Rad. Immobilon PVDF transfer membranes were purchased from Millipore. All tissue culture reagents were of tissue culture grade. All nucleotides, 8-N_3AMP, and carbodiimide were purchased from Sigma Chemical Co. All other reagents were of analytical reagent grade or better.

Synthesis and Purification of [α'-^{32}P]8-N_3Ap_4A. All operations involving 8-azidonucleotides were performed under subdued light. [α'-^{32}P]8-N_3A_4A was synthesized as described (Prescott & McClennan, 1990). This analog is synthesized by using 8-N_3AMP and [^{32}P]ATP and thus contains a single photoreactive 8-azidoadenosine group. The advantage of using the analog synthesized from these substrates is that the position of the labeled phosphorus in relation to the 8-azidoadenosine moiety will minimize nonspecific labeling resulting from analog breakdown. The [α'-^{32}P]8-N_3A_4A was purified using borate chromatography (Barnes et al., 1985). The purity of the preparation was determined by thin-layer chromatography using PEI–cellulose plates (Hilderman & Ortwerth, 1987) and by HPLC using a Whatman Partisil 10 SAX column and an isocratic elution with 0.27 M ammonium phosphate, pH 5.5. The preparation contained greater than 95% [α'-^{32}P]8-N_3Ap_4A with a yield of 10–15%. The specific activity was 5.5×10^{-4} bequerels/mol.

Isolation of Membrane Extracts. All membrane fractions were prepared as previously described (Hilderman et al., 1991).

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These membranes were isolated by centrifugation at 40000*g* for 10 min and contain a mixture of plasma membranes, nuclear membranes, mitochondrial membranes, and other membranes.

Filter Assay for Receptor Activation of [^3H]Ap_4A. Activation is defined as the enhanced binding of Ap_4A to its receptor. Membrane fractions of 0.5-mL aliquots were centrifuged at 18000*g* for 10 min at 4 °C and resuspended in 0.5 mL of binding buffer (67 mM Tris HCl, pH 7.7, and 100 μM $MgCl_2$). These samples were activated by incubating for 60 min at 29 °C. The optimum temperature for activation was determined to be 29 °C; the relative values of Ap_4A binding obtained with respect to activation temperature were 82.4%, 100%, 79.1%, respectively, for 20, 29, and 37 °C. After activation, the samples were centrifuged at 18000*g* for 10 min at 4 °C and resuspended in 0.5 mL of triple-distilled water and the protein concentrations were determined; 22 μg of membrane extracts was assayed in binding buffer as described (Hilderman et al., 1991), except the samples were incubated for 30 min at 20 °C.

Photolabeling and Analysis of Photolabeling Reactions by SDS–PAGE for Identification of the Ap_4A Receptor. Each reaction mixture contained 67 mM Tris-HCl, pH 7.7, 100 μM $MgCl_2$, 1 × 10^6 cpm of 0.4 nM [α'-^{32}P]8-N_3A_4A, and 25 μg of membrane protein in a final volume of 0.4 mL. These reaction mixtures were incubated for 15 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 $\mu W/cm^2$ for 60 s (at a distance of 12 cm from the surface of the film). The samples were then centrifuged at 40000*g* for 10 min at 4 °C, and the pellet was resuspended in 0.5 mL of 1 M NaCl and recentrifuged at 40000*g* for 10 min. This pellet was then washed twice with 0.5 mL of 67 mM Tris-HCl, pH 7.7, and 100 μM $MgCl_2$. After washing, the pellet was resuspended into 40 μL of 83.3 mM Tris-HCl, pH 8.0, 6% (w/v) SDS, 1% (v/v) Triton 100, 4.4 M urea, 75 μg of DTT, 57.5 μg of iodoacetamide (IAA), and 0.01% (w/v) bromophenol blue, and aliquots of 30 μL were loaded onto 4–20% continuous polyacrylamide gels and run as described (Laemmli, 1970). After SDS–PAGE the photolabeled samples were transferred to Immobilon PVDF transfer membranes using a TE series transphor electrophoresis unit. The transfer was carried out at 100 mA for 18 h at 4 °C. After Western transfer, the strips were washed twice with TBS and dried and autoradiography was carried out at −80 °C for 2–4 days using Kodak X-OMATAR X-ray film and Cronex Lighting Plus intensifying screens.

Preparation of Monoclonal Antibodies. Mouse heart membrane fractions were prepared as described (Hilderman et al., 1991) and emulsified with 0.25 mL of phosphate-buffered saline, pH 7.4 (PBS), and 0.25 mL of complete Freund's adjuvant. This emulsion was injected subcutaneously into female Wistar rats (Charles River). After two weeks a booster injection was administered intraperitoneally. This booster antigen was emulsified in 0.25 mL of PBS and 0.25 mL of incomplete Freund's adjuvant. A third booster was injected intraperitoneally two weeks later. Thirty days after the third injection, the final booster was administered and the animal was bled by cardiac puncture for isolation polyclonal antibodies. The spleen cells were also isolated and fused with the myeloma cell line P3-X63-AgU1. These cells were cultured, and hybrids were selected using standard procedures (Kohler & Milstein, 1975). Culture fluids from hybridomas obtained from the fusion were first screened by ELISA as described (Harlow & Lane, 1988) using goat anti-rat IgG conjugated with alkaline phosphatase, with the following modifications: the assay was performed for 1 h at 37 °C in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, and 0.1 mg of *p*-nitrophenyl phosphate. ELISA-positive cultures were then screened for their ability to inhibit Ap_4A binding to membranes. These positive cultures were plated at limiting dilution, and the resulting positive clones were rescreened by ELISA and for inhibition of Ap_4A binding. Two clones were ultimately chosen for detailed analysis; these are referred to as JW17 and JJ1. The Mabs were concentrated from the culture fluids by ammonium sulfate precipitation, dialyzed against TBS, and stored at −70 °C at a concentration of 3–5 mg/mL. An unrelated Mab prepared against chicken brain *N*-cadherin was used as a control (kindly supplied by Dr. Jack Lilien and referred to as CBC).

Western Transfer and Immunoblotting of Mouse Heart Membranes. After SDS–PAGE the samples were transferred to Immobilon PVDF transfer membranes using a TE series transphor electrophoresis unit. The transfer was carried out at 100 mA for 18 h at 4 °C using a Bio-Rad Model 422 electro-eluter. After Western transfer, the strips were washed twice with TBS, blocked with Blotto (5% milk and 0.4% Tween 20 in TBS) for 60 min at 37 °C, and then washed three times with a blocking solution (5% goat serum and 0.4% Tween 20 in TBS). Mabs (in blocking solution) were incubated with the Western transfer strips for 60 min at room temperature and washed three times with blocking solution, and then a 1:1000 dilution of goat anti-rat IgG conjugated with alkaline phosphatase (in blocking solution) was incubated with the strips for 60 min at room temperature and these strips were washed three times with blocking solution prior to development as described (Harlow & Lane, 1988).

Protein Determination. Protein concentrations were determined by the Bradford method (Bradford, 1976).

RESULTS

Heart membranes have the highest density of Ap_4A binding sites (Hilderman et al., 1991); therefore all analyses were done on heart membrane fractions. Optimization of binding conditions and displacement assays were carried out as previously described for brain membrane fractions (Hilderman et al., 1991). Results with heart membranes were essentially the same as found with brain membranes (Hilderman et al., 1991).

Receptor Activation Is Required for Ap_4A Binding. As reported previously, saturable Ap_4A binding with brain or heart membrane fractions is not obtained for at least 60 min at an optimum temperature of 29 °C (Hilderman et al., 1991). This prolonged time of incubation suggests that there may be a receptor activation step required for Ap_4A binding. To determine if receptor activation is required, membrane fractions were preincubated for 60 min at 29 °C, prior to the addition of [^{3}H]Ap_4A. Under these preincubation conditions, [^{3}H]Ap_4A binding to membrane fractions reaches a plateau after 20 min (Figure 1A) at an incubation temperature of 20 °C. Twenty degrees was choosen because it was the lowest temperature to give maximal Ap_4A binding (Figure 1B). Thus, the kinetics of Ap_4A binding following a preincubation at 29 °C (activated membranes) more closely resembles typical ligand/receptor binding kinetics.

Receptor activation is also dependent on divalent cations (Table I). If membranes are preincubated in the absence of added magnesium ions or in the presence of 1 mM EDTA, the amount of Ap_4A bound is reduced to 62.3% and 42.4%, respectively, of activated control membranes. After activation,

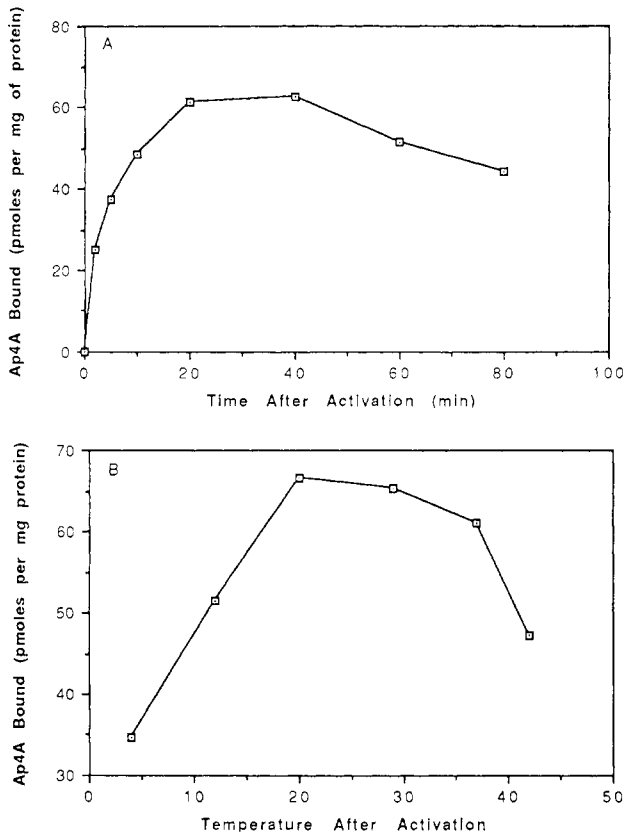

FIGURE 1: Time course and temperature curve of Ap_4A binding to activated mouse heart membrane fractions. All membrane samples were prepared, activated as described in Materials and Methods. (A) Time course; (B) temperature curve.

Table I: Effect of EDTA on Receptor Activation[a]

sample	Ap_4A bound (pmol/mg of protein)	relative percent
activated	61.9	100
no $MgCl_2$ during activation	38.6	62.3
1 mM EDTA during activation	26.2	42.4
no $MgCl_2$ plus 1 mM EDTA during activation	7.6	12.3

[a] All membrane samples were prepared, activated, and assayed for [^{3}H]Ap_4A binding as described in Materials and Methods. The concentration of $MgCl_2$ in the reference solution was 100 μM.

EDTA must be removed from the membrane fractions because magnesium ions are also required for Ap_4A binding (data not shown). EDTA was removed from the activated membrane fractions by washing three times with binding buffer. To demonstrate that EDTA was effectively removed by these washings, 1 mM EDTA was added to preactivated membrane fractions and the samples were immediately washed three times, resuspended in binding buffer, and assayed for Ap_4A binding. These fractions had greater than 95% Ap_4A binding activity compared to activated control membrane fractions (data not shown).

The effect of other divalent cations on receptor activation was determined since other divalent cations have been shown to stimulate Ap_4A binding (Hilderman et al., 1991; Prescott & McClennan, 1990; Kobayashi & Kuratomi, 1989). Preincubation with cobalt ions results in about 50% more binding than with magnesium ions while preincubation with zinc or manganese ions results in binding equivalent to that seen with magnesium ions (data not shown). Even though cobalt ions are more effective than magnesium ions in activating Ap_4A receptors, magnesium ions were used because of their presumed physiological importance.

Table II: Effect of PMSF and DIPF on Receptor Activation[a]

sample	Ap_4A bound (pmol/mg of protein)	relative percent
control[b]	61.1	100
2 mM PMSF[c]	30.1	49.3
2 mM DIPF[d]	29.1	47.6
1% 2-propanol[e]	59.0	96.3

[a] All membrane samples were prepared, activated and assayed for [^{3}H]Ap_4A binding as described in Materials and Methods. [a] Activated membrane samples were assayed for 20 min at 20 °C. [b] Membrane samples were activated in the presence of 2 mM PMSF, centrifuged to remove unbound PMSF, and then assayed for 20 min at 20 °C in the absence of PMSF. [c] Membrane samples were activated in the presence of 2 mM DIPF, centrifuged to remove unbound DIPF, and then assayed for 20 min at 20 °C in the absence of DIPF. [d] Membrane samples were activated in the presence of 1% 2-propanol, centrifuged to remove 2-propanol, and then assayed for 20 min at 20 °C in absence of 2-propanol.

Proteolysis Is Required for Receptor Activation. To determine if proteolysis was required for activation, several protease inhibitors were added to the binding buffer during the preincubation at 29 °C for 60 min. Only phenylmethanesulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DIPF) reduced the amount of Ap_4A bound (49.3% and 47.6%, respectively) (Table II). The decrease in Ap_4A binding was not due to 2-propanol, the solvent in which both PMSF and DIPF were added (Table II). These data are consistent with a serine protease being involved in receptor activation.

Identification of the Ap_4A Receptor. To identify the receptor, membrane fractions were preincubated for various lengths of time at 29 °C prior to binding and UV cross-linking with [α'-^{32}P]8-N_3Ap_4A. SDS–PAGE, followed by autoradiography, reveals labeling of a 30-kDa polypeptide (Figure 2A). No other polypeptides were labeled under these conditions. A densitometer tracing of the autoradiography reveals that labeling of the 30-kDa polypeptide is time dependent reaching a plateau by 40 min (Figure 2B).

To demonstrate that the covalent binding to the 30-kDa polypeptide is specific for Ap_4A, activated membrane fractions were UV cross-linked with [α'-^{32}P]8-N_3Ap_4A in the presence of varying amounts of unlabeled Ap_4A. A 1000-fold excess of unlabeled Ap_4A competes with [α'-^{32}P]8-N_3Ap_4A for binding to the 30-kDa polypeptide while a 10000-fold excess of ATP does not compete. The Ap_4A receptor has also been identified as a 30-kDa polypeptide in brain, thigh, kidney, and liver membranes in the same manner (data not shown). The data thus far are consistent with the hypothesis that the Ap_4A receptor is a 30-kDa polypeptide that binds Ap_4A following an activation step.

If a serine protease is indeed involved in processing of a precursor polypeptide to the active 30-kDa form, PMSF added during the activation step should inhibit formation of the 30-kDa receptor. SDS–PAGE followed by autoradiography reveals that membrane fractions preincubated in the presence of PMSF do not have [α'-^{32}P]8-N_3Ap_4A cross-linked to a 30-kDa polypeptide (Figure 3). Also, membrane fractions activated in the presence of 1 mM EDTA do not have [α'-^{32}P]8-N_3Ap_4A cross-linked to a 30-kDa polypeptide (data not shown). This suggests that the 30-kDa polypeptide may be a proteolytic fragment of a larger polypeptide.

Monoclonal Antibodies Identify a Potential Receptor Precursor. In an attempt to understand the relationship between the 30-kDa receptor and a putative precursor molecule, membrane fractions were used to raise monoclonal

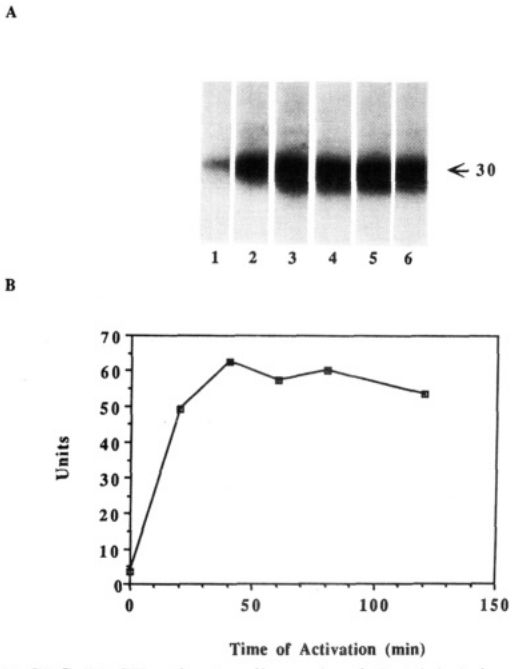

FIGURE 2: SDS–PAGE and autoradiography of photolabeled receptor after mouse heart membrane preincubations. Membrane samples (24 μg) were preincubated for various lengths of time at 29 °C. After preincubation, the samples were centrifuged at 40000g for 10 min at 4 °C and resuspended in 0.4 mL of binding buffer containing 1 × 10^6 cpm of [α'-^{32}P]8-N_3Ap$_4$A (0.4 nM). These reaction mixtures were incubated for 15 min at 20 °C in the dark and photolabeled and analyzed by SDS–PAGE as described in Materials and Methods. (A) Autoradiography of SDS–PAGE preincubated for (lane 1) 0 min, (lane 2) 20 min, (lane 3) 40 min, (lane 4) 60 min, (lane 5) 80 min, and (lane 6) 120 min. The molecular weight value is denoted by the arrow. (B) Densitometer tracing of the autoradiograph using a Pharamcia LKB UltroScan XL enhanced densitometer connected to an IBM PS2 Model 55 computer with LKB GelScan XL (version 2.1) software. Units on the ordinate are equal to A_{533} × mm^2.

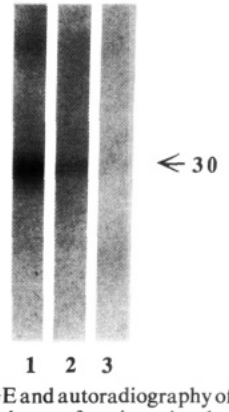

FIGURE 3: SDS–PAGE and autoradiography of photolabeled receptor of mouse heart membrane fractions in the presence of phenylmethanesulfonyl fluoride. Membrane samples (24 μg) were processed as (lane 1) membrane fractions activated as described in Materials and Methods, (lane 2) unactivated membrane fractions, and (lane 3) membrane fractions activated in the presence of 2 mM PMSF. After activation, all samples were centrifuged at 40000g for 10 min and resuspended in binding buffer containing 2 mM PMSF prior to photolabeling as described in Figure 2. SDS–PAGE and autoradiography were performed as described in Materials and Methods. The molecular weight value is denoted by the arrow.

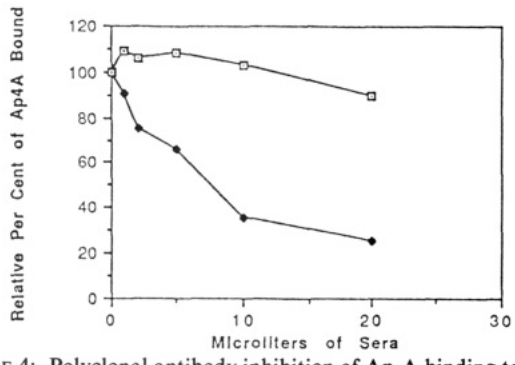

FIGURE 4: Polyclonal antibody inhibition of Ap$_4$A binding to heart membrane homogenates. Ap$_4$A binding experiments were performed as described (Hilderman et al., 1991) except the samples were first preincubated with sera for 10 min at 4 °C prior to the addition of 0.033 μM [^{3}H]Ap$_4$A (15 000 to 20 000 cpm/pmol). After the addition of [^{3}H]Ap$_4$A, the samples were incubated for an additional 80 min at 4 °C. Symbols: (◆) rat polyclonal sera and (⊡) preimmune sera.

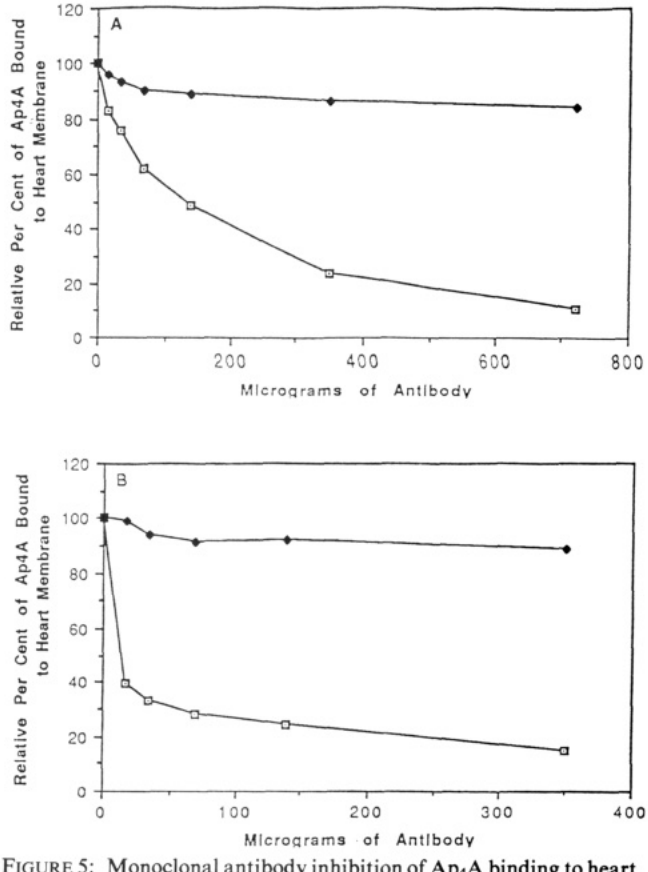

FIGURE 5: Monoclonal antibody inhibition of Ap$_4$A binding to heart membrane homogenates. (A) Membrane samples (10 μg) were preincubated with either JW17 (⊡) or CBC (◆) in assay buffer for 60 min at 4 °C with varying amounts of antibody and then centrifuged for 10 min at 16000g. The pellets were resuspended in assay buffer plus 0.033 μM [^{3}H]Ap$_4$A (15 000–20 000 cpm/pmol), and the samples were assayed after incubation for an additional 80 min at 29 °C. (B) Membrane samples (10 μg) were preincubated with either JJ1 (⊡) or CBC (◆) as described above in (A). All membrane samples were collected on glass fiber discs and counted as described (Hilderman et al., 1991).

antibodies which were screened for their ability to block Ap$_4$A binding to its membrane receptor.

Myeloma cells were fused with spleen cells isolated from a rat whose sera was able to block Ap$_4$A binding (Figure 4). The resulting hybridomas were screened and subcloned by assaying for inhibition of Ap$_4$A binding to heart membrane fractions. Two different classes of Mabs that inhibit Ap$_4$A binding (JW17 and JJ1) were isolated.

Both classes of Mabs, JW17 and JJ1, inhibit Ap$_4$A binding to membranes (Figure 5). JW17 inhibits Ap$_4$A binding only in membranes isolated from heart, thigh muscles, and aorta while JJ1 inhibits Ap$_4$A binding to membranes isolated from heart, brain, liver, kidney, and skeletal muscle (data not shown). An unrelated Mab (CBC) does not inhibit Ap$_4$A

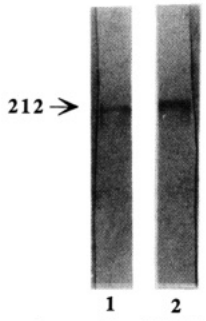

FIGURE 6: Antibody capture using JW17 and JJ1 of Western transferred mouse heart membrane proteins. Membrane samples (20 μg) were subjected to SDS–PAGE, transferred, blotted, and developed as described in Materials and Methods. Lane A: JW17 was used at a concentration of 30 μg/mL. Lane B: JJ1 was used at a concentration of 20 μg/mL. The molecular weight value is denoted by the arrow.

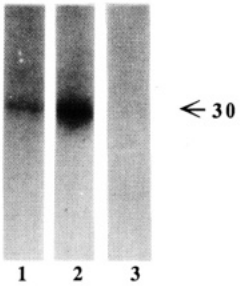

FIGURE 7: SDS–PAGE and autoradiography of photolabeled receptor of mouse heart membrane fractions in the presence of Mabs. Membrane samples (24 μg) were processed as (Lane 1) unactivated membrane fractions, (lane 2) membranes activated and then incubated with 160 μg of JW17 for 60 min at 4 °C prior to photolabeling, (lane 3) membrane fractions incubated with 160 μg of JW17 for 80 min at 29 °C prior to photolabeling. After activation, all samples were washed and photolabeled as described in Figure 2. SDS–PAGE and autoradiography were performed as described in Materials and Methods. The molecular weight value is denoted by the arrow.

binding (Figure 5). JJ1 also inhibits Ap_4A binding at much lower antibody concentrations than does JW17 (Figure 5).

The above data indicate that JW17 and JJ1 recognize different epitopes. However, both Mabs recognize a 212-kDa polypeptide following Western transfer of SDS–PAGE nonactivated membranes (Figure 6) and activated membranes (data not shown). Since [α'-^{32}P]8-N_3Ap_4A is cross-linked only to a 30-kDa polypeptide and the Mabs block Ap_4A binding, this suggests that the Mabs inhibit Ap_4A binding by blocking the activation step. Consistent with this notion, if JW17 is added to membrane fractions during the activation step, [α'-^{32}P]8-N_3Ap_4A is not cross-linked to the 30-kDa polypeptide (Figure 7, lane 3), but if the membranes are activated and then incubated with JW17 prior to the addition of [α'-^{32}P]8-N_3Ap_4A, the 30-kDa polypeptide is photo-cross-linked (Figure 7, lane 2).

DISCUSSION

We have identified the Ap_4A receptor as a 30-kDa polypeptide using a covalent radiolabeled derivative of Ap_4A. Prior to binding Ap_4A the receptor must be activated and this activation is dependent upon both divalent cations and a serine protease.

Mabs that inhibit the photolabeling of the 30-kDa polypeptide with [α'-^{32}P]8-N_3A_4A when added during activation recognize a second polypeptide of 212 kDa. These same Mabs do not inhibit cross-linking when added following the activation step. This suggests that the 212-kDa polypeptide is either (1) a serine protease that activates the Ap_4A receptor, (2) a precursor to the Ap_4A receptor, or (3) a precursor molecule that contains both the serine protease and the receptor that undergoes activation by autocatalytic processing. It is also possible that the 212-kDa polypeptide is not related to the serine protease or the 30-kDa receptor but is an unknown component of the Ap_4A receptor system. Recently, other investigators have demonstrated that a highly specific interleukin-1β-converting enzyme cleaves an IL-1β precursor to produce an active IL-1β (Thronberry et al., 1992; Cerretti et al., 1992). This enzyme is a cysteine protease that is composed of two nonidentical subunits that are derived from a single proenzyme, possibly by autoproteolysis. The activation of the Ap_4A receptor system by a serine protease may represent a similar control system. In order to ascertain the relationship of the 30-kDa Ap_4A receptor to the 212-kDa polypeptide, we are in the process of raising antibodies against the 30-kDa receptor.

Receptor activation also requires divalent cations. To our knowledge, the only serine proteases that require magnesium ions are C3 convertase, C5 convertase, and complement factor D (Barrett & McDonald, 1980). Thus, the Ap_4A receptor activation step may be the result of a unique serine protease or other enzymes labile to PMSF and/or require more than one step.

From the data presented in this communication, we cannot determine if the Ap_4A receptor is associated with internal membranes or on the cell surface. Other investigators have demonstrated that there are soluble intracellular Ap_4A binding proteins (Kobayashi & Kuratomi, 1989; Grau et al., 1982). It has also been reported that there are Ap_4A binding sites on intact cultured chromaffin cells (Pintor et al., 1991). We have recently obtained evidence that individual heart cells have the same saturable and specific Ap_4A binding as heart membranes. Furthermore, our Mabs also inhibit Ap_4A binding to the individual heart cells. This indicates that the Ap_4A receptor is localized on the cell surface. It will be important to establish whether the previously described soluble and membrane-bound receptors are related to the membrane-bound receptor characterized here.

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