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Solubilization of a Guanine Nucleotide-Sensitive Form of the P_{2Y}-Purinergic Receptor

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SUMMARY

P_{2Y}-Purinergic receptors were solubilized from turkey erythrocyte plasma membranes with the nonionic detergent digitonin. Adenosine 5′-*O*-(2-[^{35}S]thiodiphosphate) ([^{35}S]ADPβS) labeled a single population of soluble high affinity sites (K_d = 12.9 nM; B_{max} = 4.5 pmol/mg of protein) in an equilibrium binding assay; adenine nucleotide analogs competitively inhibited [^{35}S]ADPβS binding with a rank order of potency consistent with that for P_{2Y}-purinergic receptors. Radioligand binding to solubilized P_{2Y}-purinergic receptors was noncompetitively inhibited by guanine nucleotides with a rank order of potency that was in agreement with the potency order observed for guanine nucleotide-mediated inhibition of [^{35}S]ADPβS binding in purified turkey erythrocyte plasma membranes. The rate constant for dissociation of [^{35}S]ADPβS from solubilized receptors was increased 2.3-fold by guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS). Plasma membrane P_{2Y}-purinergic receptors were labeled with [^{35}S]ADPβS or covalently labeled with the photoaffinity probe 3′-*O*-(4-benzoyl)benzoyl adenosine 5′-[α-^{32}P]triphosphate ([α-^{32}P]BzATP) before solubilization and gel filtration chromatography on Superose 12. [^{35}S]ADPβS- or [α-^{32}P]BzATP-labeled species eluted as a single peak of radioactivity of apparent M_r ≥300,000. Incubation of the M_r ≥300,000 protein species with GTPγS before rechromatography resulted in loss of labeling of proteins by [^{35}S]ADPβS and a shift in apparent size of the covalently [α-^{32}P]BzATP-labeled species to a single peak of radioactivity of approximate M_r 70,000. These results suggest that a P_{2Y}-purinergic receptor-guanine nucleotide regulatory protein complex is stable to membrane solubilization with digitonin, even in the absence of prebound agonist.

Extracellular adenine nucleotides apparently regulate a number of physiological processes through receptors for adenosine (P_1-purinergic receptors), which have been subclassified into A_1- and A_2-purinergic receptors (1), and receptors for ATP and ADP (P_2-purinergic receptors), which have been subdivided into P_{2X}- and P_{2Y}-purinergic receptors (2). The pharmacological properties, structure-activity relationships, and biochemical mechanisms of P_1-purinergic receptors have been widely studied in the last 10 years. In contrast, full acceptance of the existence of P_2-purinergic receptors is only a recent occurrence, and the molecular events that accompany P_2-purinergic receptor occupancy remain essentially undefined. Very little information exists for P_{2X}-purinergic receptors, although their mechanism of signaling may involve, in part, activation of a Ca^{2+}/cation channel (3, 4). Activation of receptors exhibiting pharmacological properties similar to those initially proposed for a P_{2Y}-purinergic receptor has been shown to promote phospholipase C-catalyzed inositol lipid hydrolysis and/or Ca^{2+} mobilization (5–11). This effect may not be limited to P_{2Y}-purinergic receptors, because another receptor for extracellular ATP with pharmacological properties distinct from those of P_{2X}- and P_{2Y}-purinergic receptors also may activate phospholipase C and mobilize Ca^{2+} (12–16).

Turkey erythrocyte membranes have served as a useful model to study the guanine nucleotide-dependent regulation of phospholipase C (6, 17–21). They also have proven useful for the study of a receptor expressing pharmacological properties analogous to those originally described by Burnstock and Kennedy (2) for P_{2Y}-purinergic receptors. This receptor regulates the turkey erythrocyte phospholipase C in a guanine nucleotide-dependent fashion (6), and its abundance in a homogeneous cell preparation has permitted its direct radiolabeling with the agonist [^{35}S]ADPβS (22). Further, [^{32}P]BzATP has been developed as a photoprobe to covalently radiolabel a 53,000-Da protein in turkey erythrocyte plasma membranes that expresses the pharmacological specificity of a P_{2Y}-purinergic receptor (23).

We have extended these direct studies of a P_2-purinergic receptor and report here that a turkey erythrocyte plasma

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ABBREVIATIONS: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); 2-MeSATP, 2-methylthio-adenosine 5′-triphosphate; ATPγS, adenosine 5′-thiotriphosphate; App(NH)p, 5′-adenylylimidodiphosphate; [α-^{32}P]BzATP, 3′-*O*-(4-benzoyl)benzoyl adenosine 5′-[α-^{32}P]triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Gpp(NH)p, 5′-guanylylimidodiphosphate; GDPβS, guanosine 5′-*O*-(thiodiphosphate); G protein, guanine nucleotide-binding protein; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

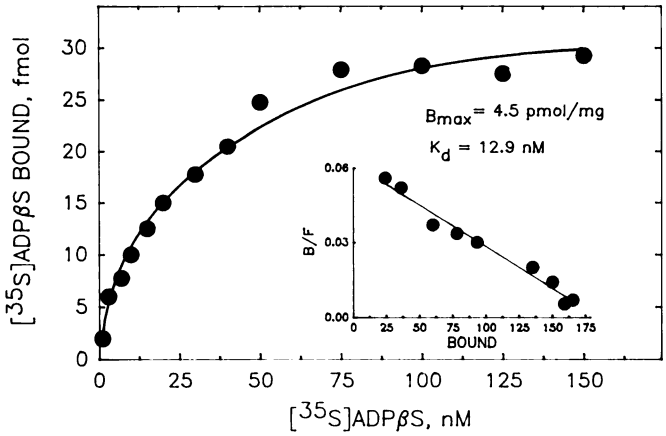

Fig. 1. Saturation binding isotherm. A soluble fraction was obtained after digitonin treatment of erythrocyte membranes, as described in Experimental Procedures. One to 10 micrograms of protein were incubated with increasing concentrations of [^{35}S]ADPβS (1–150 nM), as described in Experimental Procedures. Nonspecific binding was defined with 10 μM ADPβS or 100 μM ATPγS. The data shown are representative of results obtained in nine separate experiments. *Inset*, Scatchard transformation. [^{35}S]ADPβS specifically bound (fmol) (*abscissa*) is plotted versus the amount of radioligand bound, divided by free radioligand concentration (*ordinate*); K_d = 12.9 ± 2.7 nM and B_{max} = 4.5 ± 0.9 pmol/mg.

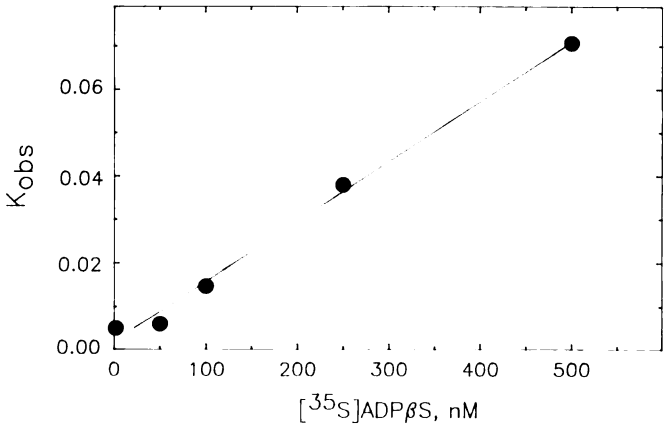

Fig. 2. Determination of rate constants. The figure shown is a transformation of association rate plots carried out at five different concentrations of [^{35}S]ADPβS (2, 50, 100, 250, and 500 nM). The k_{obs} was calculated, assuming pseudo-first-order conditions, from a plot of $\ln[DR_{eq}]/([DR_{eq}] - [DR])$ versus time, for each radioligand concentration. Linear regression analysis of a replot of k_{obs} (*abscissa*) versus radioligand concentration (*ordinate*) resulted in a slope or association rate constant (k_1) of 3.22 ± 1.85 × 10^6 min^{-1} M^{-1} and an interpolated intercept or dissociation rate constant (k_2) of 0.011 ± 0.009 min^{-1}. The data shown are representative of results obtained in two separate experiments.

membrane protein can be solubilized by digitonin in an active form that retains retaining the pharmacological characteristics of membrane-bound P_{2Y} receptors. Size-exclusion chromatography provides physical evidence to support the idea that a multimolecular complex of P_{2Y}-purinergic receptor and its associated G protein is stable to detergent solubilization.

Experimental Procedures

Materials. All of the adenine and guanine nucleotides were obtained from Boehringer Mannheim, with the exception of GMP, UTP, and App(NH)p, which were purchased from Sigma, and 2-MeSATP, which was from Research Biochemicals (Natick, MA). Whatman cellulose nitrate filters (0.45 μm) were obtained from American Scientific Supply,

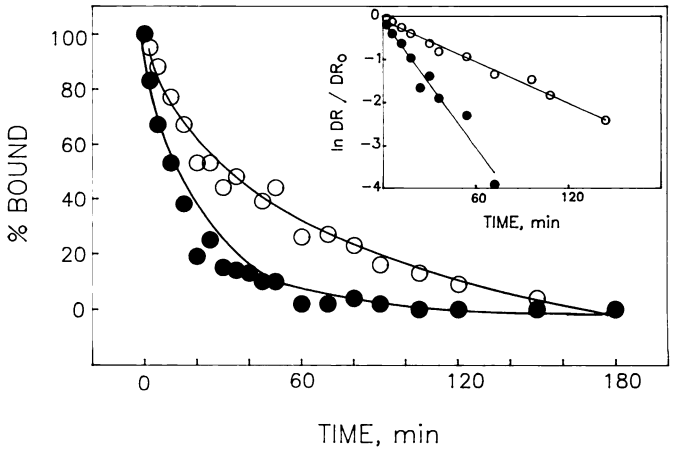

Fig. 3. Effect of guanine nucleotide on the rate of dissociation of [^{35}S]ADPβS. Incubations were allowed to proceed to equilibrium (180 min), at which point 100 μM ATPγS (○) or 100 μM ATPγS plus 100 μM GTPγS (●) were added to initiate dissociation, and aliquots were filtered at the indicated times. The data are plotted as the amount of [^{35}S]ADPβS bound at each respective time, divided by the amount of [^{35}S]ADPβS bound immediately before addition of excess nucleotide (percentage bound), versus time. *Inset*, semilogarithmic plot of $\ln DR/DR_o$ versus time, where *DR* is the amount of [^{35}S]ADPβS specifically bound at the indicated time and DR_o is the amount of [^{35}S]ADPβS specifically bound at equilibrium, yields a slope or dissociation rate constant ($-k_2$) of 0.021 ± 0.001 min^{-1} for addition of ATPγS alone and 0.048 ± 0.007 min^{-1} for concurrent addition of ATPγS and GTPγS. The data shown are representative of results obtained in two separate experiments.

and polypropylene test tubes were obtained from Sarstedt (Princeton, NJ). [^{35}S]ADPβS was custom synthesized by Dr. Roger Shaw (Du Pont-New England Nuclear Research Products), as previously described (22). [α-^{32}P]BzATP was synthesized as we have described previously in detail (23). Digitonin was obtained from Gallard-Schlesinger. The Superose 12 column was purchased from Pharmacia.

Purification of plasma membranes. Purified plasma membranes were prepared as previously described (22), with minor modifications. Washed packed turkey erythrocytes were resuspended in an equal volume of wash buffer (2.5 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 mM benzamidine, 0.1% dimethyl sulfoxide), placed in a Parr cell disruption bomb under nitrogen pressure of 1100 psi for 30 min, and slowly discharged into an equal volume of ice-cold lysis buffer containing 5 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 0.1 mM PMSF, and 0.1% dimethyl sulfoxide. The resultant suspension was centrifuged at 900 × *g* for 10 min at 4°, and the supernatant was carefully removed. The supernatant was centrifuged at 10,000 × *g* for 20 min, and the pellet was resuspended in ice-cold lysis buffer. Care was taken to resuspend only the easily dislodged, outer portion of the 10,000 × *g* pellet. The wash procedure was repeated four or five times. The pellet was resuspended in 20 mM Tris, pH 7.4, 5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 0.1 mM benzamidine, and centrifuged at 900 × *g*. Purified turkey erythrocyte membranes were recovered from the supernatant by centrifugation at 10,000 × *g* for 10 min at 4°. The process of centrifugation at 900 × *g* followed by centrifugation at 10,000 × *g* was repeated two to four times, until almost all contaminating nuclear material was removed. Aliquots were frozen at −80° in 1-ml volumes (approximately 1–2 mg/ml) until use.

Solubilization of P_{2Y}-purinergic receptors. Purified plasma membranes were thawed and centrifuged at 10,000 × *g*. The resultant pellet was resuspended in ice-cold solubilization buffer containing 50 mM NaCl, 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, and 0.6% digitonin (w/v), in a 10:1 digitonin to protein ratio, and was kept on ice for 45 min, with occasional mixing. The suspension was centrifuged in an Eppendorf microfuge at full speed for 30 min to remove insoluble material. Notably, ultracentrifugation of the resultant microfuge supernatant at 100,000 × *g* for 60 min resulted in no further

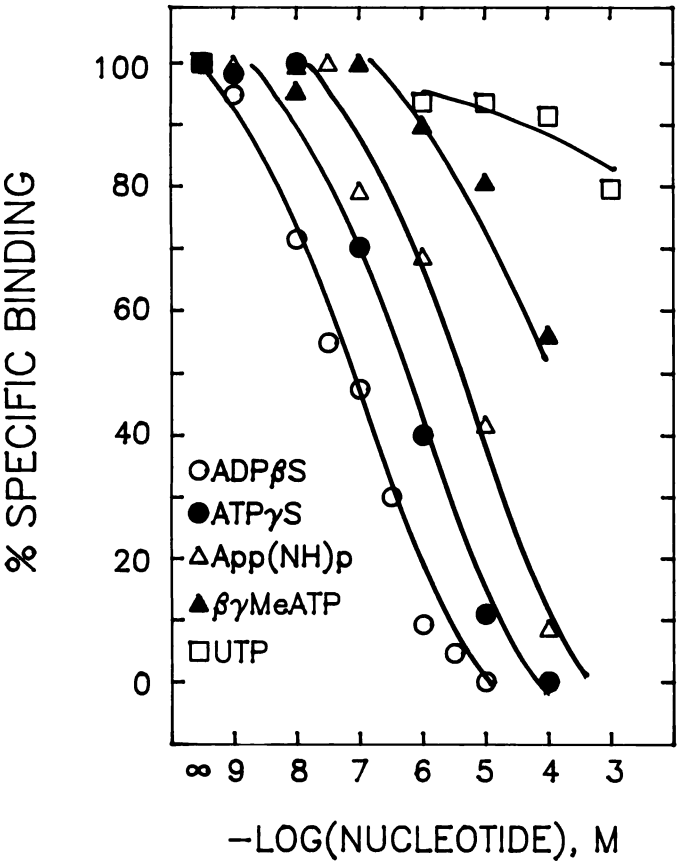

Fig. 4. Concentration-dependent inhibition by adenine nucleotide analogs of [^{35}S]ADPβS binding to soluble P_{2Y}-purinergic receptors. Approximately 0.5 nM [^{35}S]ADPβS was incubated with various concentrations of adenine nucleotides, as described in Experimental Procedures. The amount of [^{35}S]ADPβS specifically bound, expressed as a percentage of specific binding in the absence of nucleotide, was plotted versus the concentration of competing nucleotide. Nonspecific binding was defined with 10 μM ADPβS. The data shown are representative of results obtained in three separate experiments. *β,γMeATP*, β,γ-methylene-ATP.

sedimentation of [^{35}S]ADPβS-labeled protein. Under these conditions, approximately 50% of specific binding of [^{35}S]ADPβS and approximately 30% of total protein were recovered in the soluble fraction. Similar results were obtained using the covalent photolabel [α-^{32}P]BzATP, to preclude underestimation of the percentage of solubilization due to dissociation of bound [^{35}S]ADPβS from specific binding sites during solubilization.

P_{2Y}-Purinergic receptor binding assay. The receptor binding assay was initiated by addition of 1–10 μg of solubilized protein to polypropylene tubes containing [^{35}S]ADPβS (0.1–500 nM), in 10 mM HEPES, 1 mM EDTA, 0.3% digitonin, with various concentrations of ATP and ADP analogs, in a final volume of 0.2 ml. After a 3-hr incubation on ice, 10 ml of wash buffer (50 mM Tris, pH 7.4) at 4° were added to each tube. The samples were immediately filtered through cellulose nitrate filters, followed by another 10 ml of cold wash buffer. Nonspecific binding was defined as the amount of [^{35}S]ADPβS bound in the presence of 100 μM ATPγS or 10 μM ADPβS. Specific binding of [^{35}S]ADPβS was linear over the soluble protein range employed. Nonspecific binding of radioligand accounted for 5–10% of total radioactivity retained by the filter. In initial experiments, protein-associated radioligand was also separated from free radioligand using Sephadex G-25 columns. Results identical to those obtained with cellulose nitrate filters were obtained. Due to the convenience and reproducibility of the filtration assay, it was used in all [^{35}S]ADPβS binding assays reported here. Photoaffinity labeling of purified turkey erythrocyte plasma membranes with [α-^{32}P]BzATP was performed as previously described (22).

Gel filtration chromatography. FPLC was carried out at 4°, using a Superose 12 column (HR 10/30) equilibrated in 20 mM HEPES, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% digitonin (w/v). A sample volume of 0.2 ml was applied at a flow rate of 0.33 ml/min, and 1.5-min fractions were collected. When necessary, concentration of samples before injection was accomplished using a Centricon-30 microconcentrator (Amicon). Standard proteins prepared in solubilization buffer were chromatographed under conditions identical to those described above, and their elution volumes (V_e) were determined by monitoring of optical absorbance at 280 nm. The apparent size of the radiolabeled species was estimated based on interpolation using a standard curve (correlation coefficient = −0.86) constructed with the following proteins: amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa). Blue dextran and acetone were used to determine the excluded volume (V_o) and total liquid volume (V_t) of the Superose 12 column, respectively.

Other methods. Analysis of saturation and competition binding was performed using the EBDA/LIGAND program (24). Gel electrophoresis and autoradiography were carried out as previously described (23). Protein was determined by the method of Bradford (25) or Lowry *et al.* (26), using bovine serum albumin as standard.

Results

[^{35}S]ADPβS binding to solubilized membranes. [^{35}S]ADPβS binding to solubilized plasma membranes was performed at 4°, as described in Experimental Procedures. Binding of [^{35}S]ADPβS (0.5 nM) reached steady state within approximately 150 min at 4° (data not shown). The binding of [^{35}S]ADPβS was saturable and apparently occurred to a single class of sites (Fig. 1). A K_d value of 12.9 ± 2.7 nM and a B_{max} of 4.5

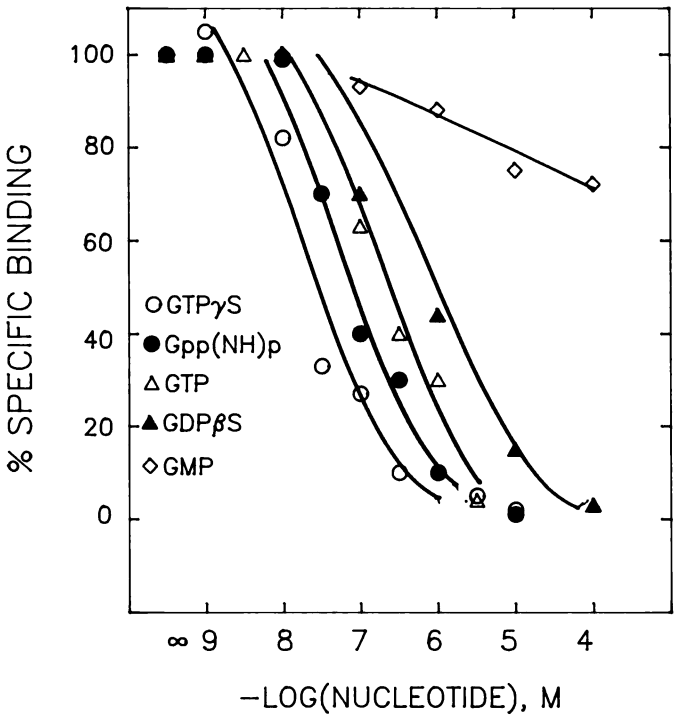

Fig. 5. Concentration-dependent inhibition by guanine nucleotide analogs of [^{35}S]ADPβS binding to soluble P_{2Y}-purinergic receptors. Approximately 0.5 nM [^{35}S]ADPβS was incubated with various concentrations of guanine nucleotides, as described in Experimental Procedures. The amount of [^{35}S]ADPβS specifically bound, expressed as a percentage of specific binding in the absence of nucleotide, was plotted versus the concentration of competing nucleotide. Nonspecific binding was defined with 10 μM ADPβS. The data shown are representative of results obtained in two separate experiments.

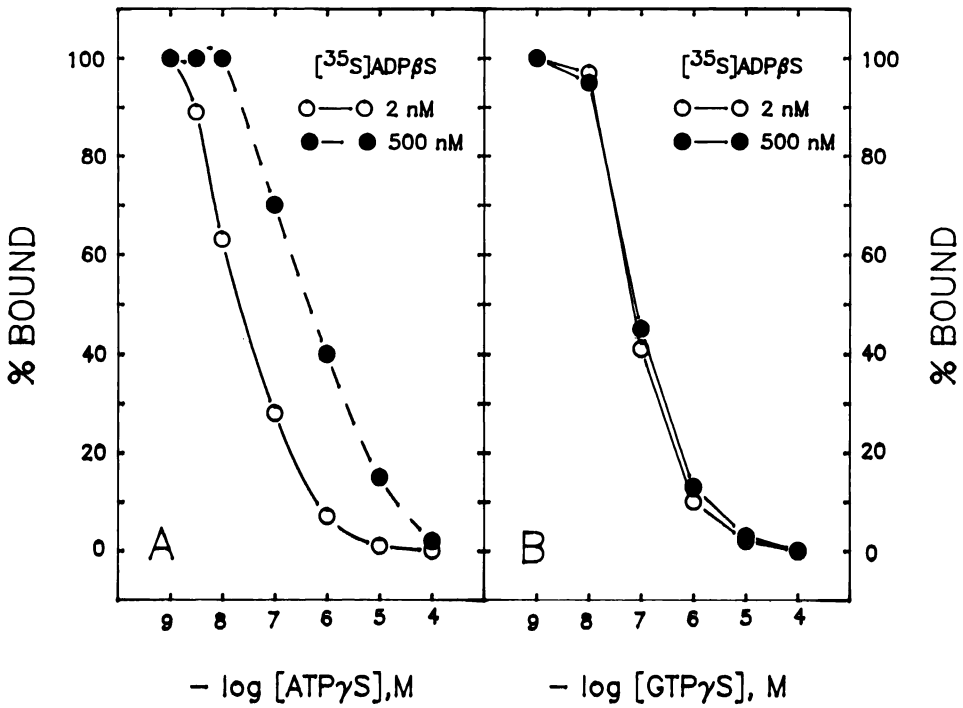

Fig. 6. Nucleotide inhibition curves obtained in the presence of two different concentrations of [^{35}S]ADPβS. The binding of [^{35}S]ADPβS was determined at two radioligand concentrations (2 and 500 nM) in the presence of various concentrations of nucleotides, as described in Experimental Procedures. The data shown are representative of results obtained in three separate experiments. A, ATPγS inhibition curves; B, GTPγS inhibition curves.

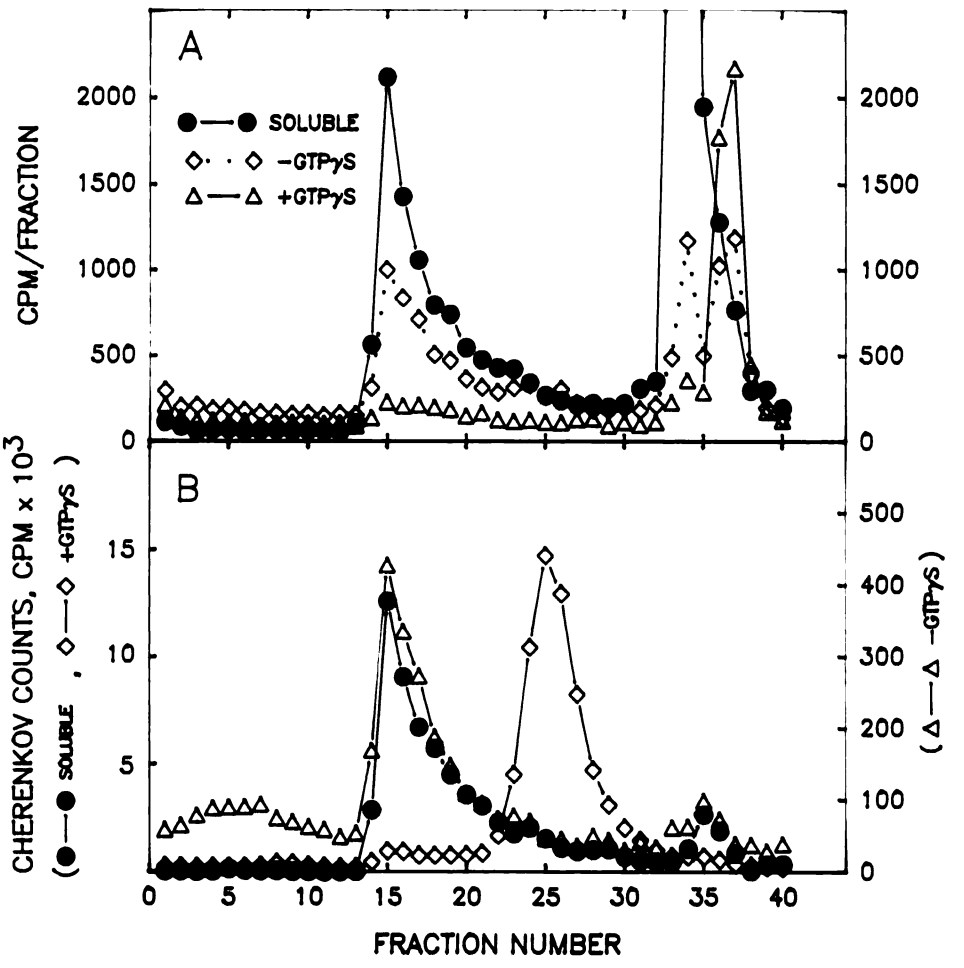

Fig. 7. FPLC Superose 12 size-exclusion chromatography of soluble P_{2Y}-purinergic receptors. A membrane aliquot was incubated with either [^{35}S]ADPβS or [α-^{32}P]BzATP and applied to a Superose 12 column, as described in Experimental Procedures. The peak fractions (fractions 15–18) were pooled, incubated with or without 100 μM GTPγS for 20 min at 30°, and separately chromatographed, as described in Experimental Procedures. A, Chromatography of receptors labeled with [^{35}S]ADPβS. B, Chromatography of receptors irreversibly labeled with [α-^{32}P]BzATP.

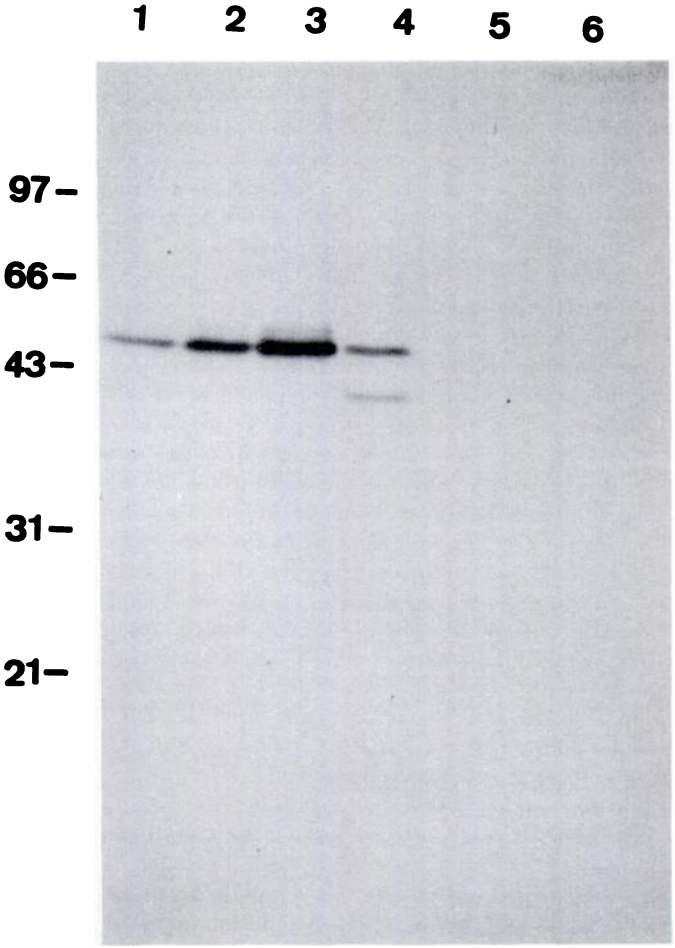

Fig. 8. Autoradiogram of SDS-PAGE of FPLC fractions. [α-^{32}P]BzATP-labeled membranes (*lane 1*), digitonin-solubilized [α-^{32}P]BzATP-labeled membranes (*lane 2*), the M_r ≥300,000 [α-^{32}P]BzATP-labeled protein species (*lane 3*) (see Fig. 7B, fractions 15–18), the GTPγS-shifted [α-^{32}P]BzATP-labeled M_r 70,000 species (*lane 4*) (see Fig. 7B, fractions 24–26 of +GTPγS plot), the M_r 70,000 region before treatment of the M_r ≥300,000 species with GTPγS (*lane 5*) (see Fig. 7B, fractions 24–26 of −GTPγS plot), and the fraction corresponding to unbound [α-^{32}P]BzATP (*lane 6*) (see Fig. 7B, fraction 35 of soluble plot) were electrophoresed on an 11% SDS-polyacrylamide gel, and an autoradiogram was obtained.

± 0.9 pmol/mg were obtained by Scatchard transformation of saturation binding isotherms (nine experiments; Fig. 1, *inset*). Association rate plots were also carried out at five different concentrations of [^{35}S]ADPβS (2, 50, 100, 250, and 500 nM). A plot of k_{obs} versus concentration of [^{35}S]ADPβS yielded an association rate constant of 3.2 ± 1.9 × 10^5 M^{-1} min^{-1} and a dissociation rate constant of 0.011 ± 0.009 min^{-1}, resulting in a kinetically derived K_d of 27.2 ± 12 nM, based on two separate experiments (Fig. 2). These results are in good agreement with the affinity and B_{max} for [^{35}S]ADPβS observed in turkey erythrocyte membranes. Separate determination of the dissociation rate constant by addition of a large excess of unlabeled ATPγS resulted in dissociation of radioligand with a single component and a calculated rate constant for dissociation of 0.021 ± 0.001 min^{-1} (Fig. 3). Combined addition of 100 μM GTPγS and 100 μM ATPγS resulted in a 2.3-fold increase in the rate of dissociation of [^{35}S]ADPβS, to 0.048 ± 0.007 min^{-1} (Fig. 3).

Concentration-dependent inhibition of [^{35}S]ADPβS binding by adenine and guanine nucleotides. The binding of [^{35}S]ADPβS to soluble binding sites was inhibited in a concentration-dependent manner by ADP and ATP analogs (Fig. 4). Inhibition followed law of mass action kinetics for interaction at a single site, i.e., Hill coefficients were close to 1 (≥0.98), and the rank order of potency of the ADP/ATP analogs was consistent with that previously reported for a P_{2Y}-purinergic receptor. This competitive inhibition of [^{35}S]ADPβS binding was selective for adenine nucleotides, because UTP had no effect. The hydrolyzable compounds 2MeSATP and ATP also competitively displaced [^{35}S]ADPβS binding in an equilibrium binding assay, with appropriate IC_{50} values for inhibition of [^{35}S]ADPβS binding to a P_{2Y}-purinergic receptor. However, the maximal inhibition of [^{35}S]ADPβS binding by 2MeSATP and ATP was variable and generally was less than that observed with ADPβS, ATPγS, App(NH)p, and β,γ-methylene-ATP (data not shown). This was apparently due to differential hydrolysis of the compounds by 5′-ectonucleotidases, because shorter (7-min) competition binding studies with ATP or 2MeSATP in the presence of the ATPase inhibitor oubain (100 μM) resulted in inhibition of [^{35}S]ADPβS binding to the same maximum limit as that obtained with nonhydrolyzable analogs (data not shown).

Guanine nucleotides inhibited the binding of [^{35}S]ADPβS (Fig. 5) with a rank order of potency similar to that observed for other receptors coupled to G proteins and for inhibition of [^{35}S]ADPβS binding to turkey erythrocyte membranes, i.e., GTPγS > Gpp(NH)p > GTP > GDPβS > GMP. [^{35}S]ADPβS binding assays were carried out (Fig. 6) using two different [^{35}S]ADPβS concentrations (2 and 500 nM), to rule out the possibility that guanine nucleotides may inhibit binding through direct competition for a receptor binding site rather than through a negative heterotropic interaction with a guanine nucleotide binding site, e.g., via a G protein. The inhibition curve for ATPγS was shifted approximately 20-fold to the right by increasing the radioligand concentration from 2 to 500 nM, which is consistent with competitive interaction between ATPγS and [^{35}S]ADPβS. The K_i values calculated using the Cheng-Prusoff relationship (27) resulted in an estimated K_i value of 15 and 13 nM for inhibition using 2 and 500 nM [^{35}S] ADPβS, respectively. Conversely, the inhibition curve for GTPγS was unaffected by a 250-fold increase in the concentration of radioligand, which is consistent with noncompetitive interaction between GTPγS and [^{35}S]ADPβS.

Estimation of the molecular size of the soluble ligand-receptor complex. The sensitivity of the P_{2Y}-receptor-ligand interaction to guanine nucleotides indicated that the soluble receptor may be physically associated with a G protein. To test this hypothesis, membranes were radiolabeled with [^{35}S] ADPβS before solubilization, and the molecular size of the prelabeled soluble receptor complex was estimated by gel filtration chromatography on a Superose 12 column, as described in Experimental Procedures. The reversibly [^{35}S]ADPβS-labeled species eluted after the column void volume and as a major peak of radioactivity at a volume corresponding to M_r ≥300,000 (Fig. 7A). We also used [α-^{32}P]BzATP to irreversibly label the P_{2Y}-purinergic receptor binding site (see Experimental Procedures and Ref. 22). Receptors covalently labeled with [α-^{32}P] BzATP also eluted from the gel filtration column as a major peak of radioactivity at a volume corresponding to M_r ≥300,000 (Fig. 7B). A peak of radioactivity eluted near the total volume of the column, with each radioligand corresponding to dissociated and/or free radioligand.

The effect of guanine nucleotides on the elution properties of the M_r ≥300,000 species was determined. After gel filtration chromatography of the [^{35}S]ADPβS- or [α-^{32}P]BzATP-labeled receptor complex, fractions containing the major M_r ≥300,000 species were pooled and concentrated. This material was incubated with 100 μM GTPγS for 20 min at 30° and subjected to gel filtration chromatography as described above. After treatment with GTPγS, protein labeling by the reversible radioligand [^{35}S]ADPβS was lost (Fig. 7A). In contrast, the covalently [α-^{32}P]BzATP-labeled protein species was shifted to a single species that eluted with a much smaller apparent size, M_r ~70,000 (Fig. 7B). Similar treatment in the absence of GTPγS did not produce a change in the elution properties of protein labeled with either radioligand (Fig. 7). This effect was specific to guanine nucleotides, because treatment of the M_r ≥300,000 species with 100 μM ATPγS did not result in a change in the observed elution pattern (data not shown). Autoradiograms obtained after SDS-PAGE of [α-^{32}P]BzATP-labeled membranes (Fig. 8, *lane 1*), [α-^{32}P]BzATP-labeled solubilized membranes (Fig. 8, *lane 2*), the FPLC [α-^{32}P]BzATP-labeled M_r ≥300,000 species (Fig. 8, *lane 3*), and the FPLC [α-^{32}P]BzATP-labeled GTPγS-shifted M_r 70,000 species (Fig. 8, *lane 4*) revealed predominantly a labeled protein of approximately 53,000 Da. Boyer *et al.* (23) have previously reported that this 53,000-Da species corresponds to a covalently labeled protein that displays the pharmacological characteristics of a P_{2Y}-purinergic receptor. The presence of this species in column fractions corresponding approximately to M_r 70,000 only occurred after treatment of the M_r ≥300,000 species with GTPγS (Fig. 8; compare *lane 4* and *lane 5*). The radioactivity eluting near the total column volume was not covalently associated with protein (Fig. 8, *lane 6*). Treatment of the M_r ≥300,000 species with 100 μM GTPγS for 20 min at 30° also resulted in the appearance of a [α-^{32}P]BzATP-labeled species of less than 53,000 Da on SDS-PAGE (Fig. 8, *lane 4*). This labeled protein species has been observed previously (23) and most likely represents a degradation of the 53,000-Da species as a result of treatment at 30° for 20 min.

Discussion

The existence of extracellular receptors for ATP has been controversial. Although there is now general acceptance of the idea that effects of extracellular adenine nucleotides include those mediated by ATP and ADP at P_2-purinergic receptors, as well as those more completely characterized for adenosine at P_1-purinergic receptors, the P_2-purinergic receptors remain difficult to study. The natural agonists are rapidly metabolized by extracellular ectonucleotidases, P_2-purinergic receptor subtype-selective agonists are scarce, and specific P_2-purinergic receptor antagonists are not available. Most of the knowledge accrued to date on P_2-purinergic receptors has derived from pharmacological studies of intact smooth muscle tissues and, to our knowledge, transformed cell lines expressing P_{2X}- and P_{2Y}-purinergic receptors have not been identified.

In light of the issues described above, it was fortuitous that we observed that ATP and ADP activate phospholipase C in turkey erythrocytes (6, 28) and that a receptor pharmacologically analogous to the P_{2Y}-purinergic receptor described originally by Burnstock and Kennedy (2) in studies of smooth muscle responses apparently is responsible for the action of adenine nucleotides in the turkey cell. Availability of this homogeneous cell preparation has allowed reversible radiolabeling of a binding site that fits the pharmacological specificity of a P_{2Y}-purinergic receptor (22), and a covalently binding radiolabel has been developed (23). Thus, at least in an avian cell, we are beginning to develop some understanding of the biochemical mechanisms associated with the action of a P_2-purinergic receptor, and we have begun to describe drug/receptor interactions using direct means. Work described here was undertaken with the goals of establishing that a protein retaining the pharmacological specificity of a P_2-purinergic receptor could be solubilized from plasma membranes by nonionic detergent and that, as with other G protein-regulated receptors, a P_2-purinergic receptor could be shown to be associated with a G protein in a macromolecular complex.

The properties of the [^{35}S]ADPβS binding site solubilized from turkey erythrocyte plasma membranes with digitonin are closely analogous to the binding parameters established for [^{35}S]ADPβS binding to purified turkey erythrocyte membranes. Thus, the K_d and B_{max} for [^{35}S]ADPβS binding to the digitonin-solubilized site are similar to the values obtained with plasma membranes, and the rank order of potency of adenine nucleotides for competitive inhibition of [^{35}S]ADPβS binding are identical for soluble and membrane-associated binding sites. The observation of high affinity binding of radiolabeled agonist to soluble receptors is in itself consistent with the idea that functional interaction between the P_{2Y}-purinergic receptor and its associated G protein is retained in the soluble preparation. It can be assumed that, without maintenance of a receptor-G protein complex, high affinity binding of agonist would not be observed, as has been reported for myriad other G protein-linked receptors (29). The relative stability of a supposed P_{2Y}-purinergic receptor-G protein complex upon solubilization in the absence of prebound ligand is not a unique observation, because similar results have been obtained for other receptors (30–34). These results are suggestive of close coupling between the receptor and G protein in their native membrane milieu but do not represent an inviolable property of G protein-associated receptors, because solubilization of a number of receptors in functional association with their G proteins requires agonist occupancy before solubilization (35–39). Why this would be the case with some receptors and not others is unclear. No unambiguous pattern based on the type of receptor or G protein is apparent, and data on many other potentially relevant factors, e.g., ratio of receptor to G protein, are not available. Whether this issue is of functional relevance, with "precoupled" receptor-G protein complexes playing a role that is kinetically or consequentially different from that of "agonist-promoted" receptor-G protein complexes, remains to be determined.

The apparent molecular size of the digitonin-solubilized P_{2Y}-purinergic receptor from turkey erythrocyte membranes was estimated to be M_r ≥300,000 by size-exclusion chromatography. The fact that chromatography of this high molecular weight species after incubation with guanine nucleotide resulted in a decrease in apparent size to a lower molecular weight species is suggestive of solubilization of the P_{2Y}-purinergic receptor and its associated G protein as a macromolecular complex. Critical to this interpretation, SDS-PAGE of the [^{32}P]BzATP-labeled species eluting from the size-exclusion matrix revealed that a 53,000-Da protein chromatographed with the high molecular weight species identified by [^{35}S]ADPβS labeling and eluted in

fractions of much smaller apparent size after treatment of fractions containing the $M_r \geq 300{,}000$ labeled species with guanine nucleotide. Furthermore, the hypothesis that the P_{2Y}-purinergic receptor and its associated G protein are solubilized as an interactive macromolecular complex is strengthened collectively by the sensitivity of [^{35}S]ADPβS binding to guanine nucleotides in a soluble equilibrium binding assay, the noncompetitive nature of this inhibition, and the increase in the rate constant for dissociation of [^{35}S]ADPβS caused by the addition of guanine nucleotide. It is of note that the apparent size of the micellar macromolecular complex, as estimated by FPLC size-exclusion chromatography, is larger than would be predicted for a 53,000-Da receptor and its associated G protein. This could reflect receptor association with other membrane proteins, complex aggregates, or problems inherent in estimation of the molecular size of detergent-solubilized membrane proteins.

In summary, the data presented here indicate that P_2-purinergic receptors are amenable to detergent solubilization in an active form and that, at least with an avian protein that expresses pharmacological properties of a P_{2Y}-purinergic receptor, solubilization is apparently in the form of a macromolecular complex of the receptor with another protein(s). The guanine nucleotide sensitivity of agonist binding to this complex and the elution of the covalently labeled receptor as a protein of much smaller apparent size after treatment of the solubilized receptor preparation with guanine nucleotides suggest that the receptor-associated protein is a G protein. A long term goal of these studies will be to purify the turkey P_{2Y}-purinergic receptor and reconstruct, in model phospholipid membranes, an active receptor-regulated phospholipase C from purified components.

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