0026-895X/94/061071-11$03.00/0

MOLECULAR PHARMACOLOGY, 45:1071-1081, 1994.

Introduction of Purified α_{2A}-Adrenergic Receptors into Uniformly Oriented, Unilamellar, Phospholipid Vesicles: Productive Coupling to G Proteins but Lack of Receptor-Dependent Ion Transport

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Received December 14, 1993; Accepted March 21, 1994

SUMMARY

Introduction of highly purified α_{2A}-adrenergic receptors (α_{2A}AR) into lipid vesicles resulted in vesicle preparations that were unilamellar in structure, nonleaky to monovalent cations, and uniformly oriented such that the cytoplasmic domains of the α_{2A}AR faced the vesicle exterior. In this orientation, addition of G_i/G_o G proteins yielded a 4–5-fold stimulation of agonist-dependent guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding to the G protein α subunit. These nonleaky, uniformly oriented, α_{2A}AR-containing vesicle preparations allowed us to explore the hypothesis that the α_{2A}AR itself, or in combination with G_i/G_o proteins, is able to effect ion translocation. Measurements of $^{22}Na^+$ uptake, $^{22}Na^+$ efflux, and H^+ movement revealed no detectable agonist-stimulated, receptor-dependent, ion translocation, even in the presence of G proteins, suggesting that allosteric regulation of α_{2A}AR by cations and amiloride analogs is not an indication that the α_{2A}AR itself is an ion transporter. Nonetheless, the methodology developed in the present studies for preparation of nonleaky vesicles containing receptor and G proteins should be well suited for evaluating the stoichiometry and selectivity of receptor-G protein interactions and, in particular, G protein specificity in mediating receptor-dependent regulation of voltage-gated or receptor-operated ion channels.

A wide array of hormones and neurotransmitters mediate their diverse physiological effects by first activating cell surface receptors coupled to G proteins. Much of our understanding of the interactions that occur between receptors and G proteins has come from reconstitution studies in which purified protein components have been evaluated for their ability to interact in a functionally relevant manner, such as accelerating GTPase activity or GTPγS binding. Similar experimental strategies also have been utilized to explore the specificity of interaction of receptors with various G proteins. These studies have provided a great deal of information about the probable mechanism of interaction between receptors and G proteins. Most studies to date, however, have lacked information regarding the uniformity, or not, of receptor orientation in the reconstitution mixture, and thus relative stoichiometries of receptors accessible for interaction with G proteins cannot be definitively compared when evaluating, for example, the specificity of interactions between a given receptor population and various G protein populations. In addition, the known ability of G protein-coupled receptors to modulate a variety of ion channels suggests that the study of receptor-activated ion transport in a reconstitution system requires well sealed unilamellar vesicles that do not allow leak of ions across the phospholipid membrane.

In this manuscript, we describe the development of methodology to yield unilamellar phospholipid vesicles containing functional α_2AR of the α_{2A} subtype that are appropriately nonleaky to ions. The α_{2A}AR molecules appear to be uniformly oriented in this preparation such that the extracellular domain of the α_{2A}AR faces the vesicle interior. Using these vesicles, we have demonstrated that the α_{2A}AR couples to G proteins and accelerates GTPγS binding in a manner that is apparently quantitatively superior to reconstitution of these same molecular species in receptor/lipid/detergent mixtures. Our data also demonstrate that receptor coupling to G proteins is retained after hydrolysis of the cytoplasmic domains of the α_{2A}AR, analogously to previous studies with the βAR (1). Binding to

This work was supported by Grants HL25182 and GM31954 from the National Institutes of Health. J.R.K. was supported by a Medical Scientist Training Program Training Grant (GM07347) from the National Institutes of Health.
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ABBREVIATIONS: GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; NMDG, *N*-methyl-D-glucamine; AR, adrenergic receptor(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PIPES, 1,4-piperazinediethanesulfonic acid; WGA, wheat germ agglutinin.

the α_{2A}AR (2–5), like that to other receptors coupled to inhibition of adenylyl cyclase (6–9), is allosterically regulated by cations and analogs of amiloride. Because our findings have demonstrated that these allosteric effects are due to interactions at the level of the α_{2A}AR molecule itself (5), we sought to determine whether these allosteric effects might reflect the ability of the α_{2A}AR to translocate ions in a manner analogous to that of bacteriorhodopsin (10, 11), which is topographically similar to (12), albeit structurally distinct from (13, 14), vertebrate rhodopsin and, by inference, G protein-coupled receptors. Despite the ability of our vesicle preparations to maintain substantial ion gradients, we detected no evidence of α_{2A}AR-mediated ion translocation, even in the presence of G proteins. Nonetheless, the present studies establish a methodology for preparation of unilamellar nonleaky phospholipid vesicles of known receptor orientation that will be of value not only in analyzing the ability of all receptors in the seven-transmembrane domain family to interact with distinct subunit combinations of G proteins but also in evaluating the components necessary for coupling to a variety of effector systems, including receptor-operated or voltage-gated ion channels.

Experimental Procedures

Materials

[^{3}H]Yohimbine (80 Ci/mmol), $^{22}Na^+$ (carrier free), and ^{125}I (carrier free) were from DuPont-New England Nuclear. Chloramine T was from Kodak. Crude soybean phosphatidylcholine (asolectin) was from Sigma. High purity glycerol and Sephadex G-50 (fine) were purchased from Pharmacia. CHAPS was from Pierce Biochemical and digitonin was from Gallard-Schlessinger. Bio-Rad was the source for Dowex AG 50W-X8 resin (20–50 mesh). WGA-agarose was purchased from Vector Laboratories. AR ligands were kind gifts from the following sources: phentolamine from Ciba-Geigy, (−)-epinephrine from Sterling-Winthrop, and UK14304 from Reckitt-Colman.

Methods

Purification of α_{2A}AR for insertion into phospholipid vesicles. Homogeneous preparations of α_{2A}AR were prepared from digitonin-solubilized porcine brain extracts using two sequential yohimbine-agarose chromatography steps, as described by Repaske *et al.* (15). Partially purified α_{2A}AR preparations were produced by a single yohimbine-agarose affinity purification followed by WGA-agarose chromatography, essentially as described (16). The sequential yohimbine-agarose/WGA-agarose chromatography was performed as follows. Eluates from a single yohimbine-agarose chromatography step, containing approximately 500 pmol of α_{2A}AR, were pooled and concentrated to a volume of 50 ml using an Amicon 30YM membrane. NaCl and $MgCl_2$ were added to the concentrated preparation to final concentrations of 500 mM and 10 mM, respectively. This material was then added to 2 ml of WGA-agarose resin that had been washed sequentially with 10 resin volumes of 0.3 M NaCl, 10 volumes of 0.5 M NaCl, and 10 volumes of buffer containing 0.5% digitonin, 50 mM HEPES, pH 8.0, 500 mM NaCl, and 2 mM EGTA and was rotated for 2 hr in 10 volumes of 0.25% digitonin, 50 mM HEPES, pH 8.0, 500 mM NaCl, 2 mM EGTA, 10 mM $MgCl_2$, before addition of the receptor preparation. The receptor preparation was added and rotated overnight with the WGA-agarose resin at 4°. The resin was slowly transferred to a siliconized 2-ml Bio-Rad column and the resin was packed at a flow rate of 25 ml/hr in the presence of the original receptor-containing solution. The resin was then washed with 6 ml of 0.25% digitonin, 50 mM HEPES, pH 8.0, 500 mM NaCl, 2 mM EGTA, 10 mM $MgCl_2$; 4 ml of 0.25% digitonin, 50 mM HEPES, pH 8.0, 250 mM NaCl, 2 mM EGTA, 10 mM $MgCl_2$; 20 ml of 0.25% digitonin, 50 mM HEPES, pH 8.0, 100 mM NaCl, 2 mM EGTA, 10 mM $MgCl_2$; and 6 ml of 0.25% digitonin, 50 mM HEPES, pH 8.0, 100 mM NaCl, 2 mM EGTA. $MgCl_2$ was deleted from the final wash and elution buffers to foster destabilization of WGA-α_2AR interactions. The α_{2A}AR was eluted from the WGA-agarose column at 4 ml/hr with 0.25% digitonin, 50 mM HEPES, pH 8.0, 100 mM NaCl, 2 mM EGTA, 500 mM *N*-acetylglucosamine. One-milliliter fractions were collected and 10-μl aliquots were assayed for [^{3}H]yohimbine binding (15).

Eluates from either the sequential yohimbine-agarose/WGA-agarose purification or two sequential yohimbine-agarose chromatography steps were pooled, concentrated, and exchanged (on a Sephadex G-50 column) into buffer containing 0.12% digitonin, 25 mM glycylglycine, and "intravesicular components" as needed to create varying ultimate "intravesicular conditions" in individual experiments (see Results and figure legends). The final α_{2A}AR concentration in these preparations was approximately 150 pmol/ml. Homogeneous α_{2A}AR preparations have a specific binding activity of ~45,000 pmol of [^{3}H]yohimbine bound/mg of protein (15). However, many of the preparations used were not homogeneous preparations derived from two rounds of yohimbine agarose but were derived by a single round of yohimbine-agarose chromatography followed by WGA chromatography. These preparations had impurities, as assessed by either silver stain or radioiodination analysis of the preparations.

Insertion of the α_{2A}AR into phospholipid vesicles. For each reconstitution experiment, 1.2 ml of partially purified or homogeneous α_{2A}AR (150–200 pmol of α_{2A}AR), prepared as described above and suspended in intravesicular buffer, was incubated briefly with 20 μM phentolamine, an αAR antagonist intended to stabilize α_{2A}AR function during subsequent steps, and mixed with 400 μl of 100% glycerol for an additional 10–30-min incubation at 15°. Crude soybean lipids (40% phosphatidylcholine, asolectin; Sigma P3644) were solubilized at a concentration of 6.25 mg/ml in intravesicular buffer containing 1% CHAPS. A 400-μl aliquot of this solubilized lipid was added to the receptor/glycerol mixture and the entire mixture (2 ml total) was then incubated for 30 min at 15–20°.

After this incubation, a 1- × 60-cm Sephadex G-50 (fine) column was used to facilitate removal of the detergents digitonin and CHAPS and to catalyze the formation of the proteoliposomes. Immediately before each use, the Sephadex column (at 4°) was precoated at a flow rate of 25 ml/hr with approximately 70 ml of 1% crude soybean lipids that had been dissolved in intravesicular buffer by vigorous vortexing. This step was followed by washing the column with 20 ml of intravesicular buffer at the same flow rate. After the column was precoated with lipids, the 2-ml α_{2A}AR/lipid/glycerol mixture was applied to the column and eluted with intravesicular buffer at a flow rate of 9 ml/hr. One-milliliter fractions were collected. Those fractions that contained vesicles, as identified by their turbidity, were pooled. Vesicles either were used directly from the column or, to concentrate the vesicle preparation, were pelleted at 39,000 rpm in a 40Ti rotor (100,000 × *g*) for 2.5 hr and resuspended by pipeting in 200 μl of intravesicular buffer. In some experiments, aggregated non-receptor-containing lipid was removed by centrifugation at 2000 rpm in a Sorvall GLC centrifuge (4°) before the high-speed concentration of unilamellar vesicles described above. For each series of experiments reported, control studies were performed using protein-free vesicular preparations or proteoliposomes that had been incubated at 65° for 30 min to inactivate α_{2A}AR function (4).

Determination of protein and lipid recovery in reconstitution. Recovery of phospholipid after detergent removal and vesicle formation was determined by comparing the inorganic phosphate content of the reconstitution mixture and the resulting vesicles, using the method described by Ames (17).

Because available radioligands for α_2AR identification are hydrophobic and achieve high levels of nonspecific binding in the vesicle preparations, steady state binding of [^{3}H]yohimbine, for example, could not be used to quantify recovery of α_{2A}AR in varying vesicular preparations. Therefore, to determine recovery of vesicle-associated α_{2A}AR, a 10-μl aliquot of the reconstitution mixture (2 ml total) and a 1-μl aliquot of

the pelleted α_{2A}AR-containing vesicles (200 μl total) were radioiodinated by incubation with 10 μl of ^{125}I (20 μCi in 150 mM sodium phosphate buffer, pH 7.4) and 5 μl of chloramine T (2 mg/ml in 50 mM sodium phosphate buffer, pH 7.4), in a final volume of 25 μl. Radioiodinated samples were analyzed on a 12% polyacrylamide gel (18) and subjected to autoradiography. The α_{2A}AR in each sample was quantitated by cutting the area corresponding to the α_{2A}AR from the gel and counting the gel slices in a γ counter. The recovery of α_{2A}AR in reconstituted preparations, based on a ratio of ^{125}I cpm in eluted vesicle preparations to ^{125}I cpm in starting material, was 75%. In our experience, radioiodination is superior to silver stain or other means for identification of trace contaminants in the α_{2A}AR preparations.

$^{22}Na^+$ flux experiments. Direct $^{22}Na^+$ uptake. Uptake of Na^+ into vesicles was monitored using $^{22}Na^+$. The final concentration of Na^+ in the extravesicular buffer was 10 mM, whereas the intravesicular buffer was prepared with no Na^+ added, thus creating an initial 10 mM driving force for $^{22}Na^+$ uptake. By varying the pH of the extravesicular buffer, this method also can assess Na^+/H^+ exchange (intravesicular pH = 6.5). For these analyses, the intravesicular buffer was composed of 25 mM glycylglycine, 25 mM HEPES, 25 mM PIPES, and 100 mM NMDG-HCl (a Na^+ substitute), pH 6.5, and the extravesicular uptake solution was composed of 10 mM NaCl, 25 mM glycylglycine, 25 mM HEPES, 25 mM PIPES, 100 mM NMDG-HCl, pH 6.5 (to measure Na^+ transport) or pH 8.0 (to measure Na^+/H^+ exchange). $^{22}Na^+$ uptake was measured essentially as described by Gasko *et al.* (19). A 10-μl aliquot of vesicles was added to 40 μl of extravesicular uptake solution containing 20 μCi/ml $^{22}Na^+$ and was incubated at 25° for various times. This reaction mixture was then applied to Dowex columns to remove all extravesicular Na^+ and thus terminate the uptake reaction. The vesicles were eluted with 2.0 ml of 204 mM Tris, pH 8.3, at 4° and the radioactivity in the eluates (corresponding to the amount of vesicle-associated $^{22}Na^+$) was quantified using a γ counter. The choice of 204 mM Tris-HCl was intended to retain iso-osmolality of the solutions; osmolality was measured directly by using instrumentation based on freezing-point determinations. The columns used for Na^+ removal were prepared as follows. Dowex AG 50W-X8 (20–50 mesh) resin was converted to the Tris form by repeated washes with 1 M Tris, pH 10. Two milliliters of resin were poured into Evergreen polycarbonate columns plugged with siliconized Whatman no. 1 filter paper. The columns were precoated with 2 column volumes of 1% bovine serum albumin in 204 mM Tris, pH 8.1, followed by 2 column volumes of 204 mM Tris, pH 8.1.

$^{22}Na^+$ uptake driven by electrical diffusion potential. In this assay, developed by Garty *et al.* (20), vesicles are prepared with high interior Na^+ or K^+ concentrations, whereas external Na^+ or K^+ is removed and replaced with Tris buffer. In Na^+-loaded vesicles containing Na^+ transport proteins or, alternatively, in K^+-loaded vesicles upon the addition of the K^+ ionophore valinomycin, Na^+ or K^+ moves outward, thus creating an interior negative diffusion potential. Despite the large outward concentration gradient, an isotope ($^{22}Na^+$) added to the outside of these vesicles moves into the vesicles that contain Na^+ transport machinery, driven by this interior negative membrane potential. This method is extremely sensitive for detection of small Na^+ uptake signals. In addition, the opposing nature of the chemical and electrical gradients results in tracer equilibration that occurs over several minutes, rather than within seconds as in most standard Na^+ uptake procedures. Furthermore, if the presence of an electrical potential across the membrane were crucial for the α_2AR to assure an appropriate conformation for ion transport, this method would provide the needed electrical potential. For these studies, vesicles were prepared as described above, with an intravesicular buffer of either 100 mM NaCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA, or 75 mM NaCl, 75 mM KCl, 25 mM HEPES, pH 8.0, 1 mM EDTA (for experiments where the K^+ ionophore valinomycin was used to create the negative diffusion potential). To remove external cations from the vesicle preparations before the incubation, aliquots of nonconcentrated (150–200 μl) vesicles were loaded on 2-ml Dowex columns (AG 50W-X8 Tris form; prepared as described above) that had been prewashed with 3 ml of 225 mM sucrose, 10% bovine serum albumin, before use. Vesicles were eluted with 1.5 ml of 225 mM sucrose (uptake buffer) and divided into two or three different reactions containing various agents (e.g., the α_2AR agonist UK14304, the non-subtype-selective αAR antagonist phentolamine, or the ionophore valinomycin). After a 1–3-min incubation with these agents, the uptake assay was initiated by addition of 1–2 μCi of $^{22}Na^+$ to each reaction. At various time points, the uptake reaction was terminated by passing 100–200-μl aliquots of vesicles over Dowex AG 50W-X8 columns made as described above. The vesicles were eluted in 1.5 ml of ice-cold 225 mM sucrose and counted in a γ counter. A 5-μl aliquot of the initial uptake reaction also was counted and was used to standardize each reaction for the amount of $^{22}Na^+$ present in the assay.

Na^+ efflux assays. The Na^+ efflux assay used in this study measures movement of Na^+ out of vesicles with or without a Na^+ driving force. By varying the pH of the extravesicular buffer, this method also can assess Na^+/H^+ exchange. Vesicles were prepared with intravesicular buffer of 100 mM NaCl, 25 mM glycylglycine, 1 mM EDTA, concentrated by centrifugation at 100,000 × *g* for 2.5 hr, and resuspended in 500 μl of intravesicular buffer containing 100 mM NaCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA, and 20 μCi of $^{22}Na^+$. Vesicles were incubated for 20 hr at 4° to load vesicles with $^{22}Na^+$. Note that, although the vesicles are very impermeant to Na^+, enough $^{22}Na^+$ enters the vesicles after 20 hr (approximately 2% of total $^{22}Na^+$ present) to allow detection of efflux. To begin the assay, Na^+ was removed by passing a 100-μl aliquot of vesicles over a 2-ml Dowex column and eluting the vesicles with 3 ml of 100 mM NMDG-HCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA. The eluted vesicles were then diluted 10–20-fold in different buffers with different final pH values, ion gradients as driving forces, and/or drugs, as described in the figure legends, and were incubated at 4° or 25°. At the times indicated, aliquots were withdrawn and filtered under vacuum through GF/F filters. The filters were washed with 8 ml of ice-cold buffer (100 mM NaCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA) and counted in a γ counter.

Assessment of proton movements using acridine orange. Changes in acridine orange absorbance were measured using a dual-wavelength Amino-Bowman spectrophotometer or a SPEX microfluorometer, both of which were equipped with a stirring motor. The excitation wavelength was 493 nm and the emission wavelength was 530 nm. Vesicles were formed in the presence of intravesicular buffer (25 mM glycylglycine, 100 mM NMDG-HCl, 10 mM KCl, pH 6.5), concentrated by centrifugation, and resuspended in 200 μl of intravesicular buffer. One milliliter of extravesicular buffer (25 mM glycylglycine, 100 mM NMDG-HCl, 10 mM KCl, pH 7–8) containing 20 μM acridine orange was allowed to equilibrate for 5 min, with stirring, in a quartz cuvette at 25°. After equilibration, a 15–30-μl aliquot of the vesicles was added. Upon vesicle addition, the acridine orange (a weak base) became concentrated in the interior of the vesicle because of the lower intravesicular pH, and its fluorescence emission was quenched as a result of this concentrating process. Once this emission signal stabilized and a new baseline was achieved, Na^+ or other ions were added to the extravesicular solution along with α_2AR agonists and/or antagonists and amiloride analogs. Any acceleration of Na^+/H^+ exchange would result in the movement of H^+ out of the vesicle, a parallel efflux of acridine orange, and a subsequent increase in acridine orange fluorescence. Inhibition of Na^+/H^+ exchange, e.g., by amiloride analogs, would be predicted to have the opposite effects. In all experiments, the Na^+/H^+ ionophore monensin was used as a positive control to assess the extent of the H^+ gradient across the vesicle at the termination of the experiment. The response to monensin was not attenuated even after long incubations of the vesicles in extravesicular buffer (up to 3 hr), indicating that the vesicles were indeed nonleaky and the H^+ gradient was stable.

Determination of receptor orientation in vesicle preparations. Receptor phosphorylation by cAMP-dependent protein kinase. Aliquots of intact α_{2A}AR-containing vesicles or CHAPS-permeabilized vesicles (CHAPS was added to the vesicle preparation to a final concentration of 0.4%) were incubated at room temperature for 1 hr

with 0.02 mg/ml concentrations of the catalytic subunit of cAMP-dependent protein kinase (prepared in 175 mM KH_2PO_4, pH 6.8, 0.05 mM DTT), in the presence of 0.025 mM ATP, 5 mM $MgCl_2$, and [γ-^{32}P]ATP (3–5 μCi/40-μl reaction). The reaction was quenched by the addition of a 500-fold excess concentration of ATP and the samples were subjected to analysis by SDS-PAGE and autoradiography. The amount of phosphorylated α_{2A}AR was quantitated by cutting out the gel slices corresponding to the migration of the α_{2A}AR and counting them in a Packard scintillation counter.

Sensitivity of vesicle-associated receptor to trypsin. For these studies, reconstitution of α_{2A}AR was performed as usual, except that 20% of the total receptor used for reconstitution was subjected to a covalent photoaffinity labeling procedure (21) using 17α-hydroxy-20α-yohimban-16β-[*N*-(4-azido-3-[^{125}I]iodo)-phenethyl]carboxamide (22) before vesicle formation. Thus, after detergent removal and vesicle formation, a fraction of the vesicle-associated α_2AR population was radiolabeled with the covalently bound α_2AR antagonist. Aliquots of intact vesicles versus CHAPS-solubilized vesicles were then incubated overnight at 15° with varying amounts of trypsin, indicated as percentage (w/w) of trypsin. Therefore, 100% trypsin is equivalent to equal weights of trypsin and receptor, calculating the receptor as 45,000 kDa, based on the mass of polypeptide predicted from the sum of amino acids (21). After this incubation, a 5-fold excess of soybean trypsin inhibitor was added to the samples and they were analyzed by SDS-PAGE and autoradiography.

Reconstitution of vesicle-associated α_{2A}AR with G proteins and assay of agonist-stimulated [^{35}S]GTPγS binding. A 25-μl aliquot of α_{2A}AR-containing vesicles (~2.5 pmol of α_{2A}AR) made as described previously was mixed with 100 pmol of a purified G_i/G_o G protein mixture (2.5 μl) suspended in 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1–0.3 mM NaCl, 0.5–2% sodium cholate, prepared as described previously (23). The mixture was incubated on ice for 1 hr and then diluted dropwise to 1 ml, with vortexing, with buffer containing 20 mM HEPES, 100 mM NaCl, and 2 mM EDTA, pH 8.0. For comparison of the effectiveness of α_{2A}AR-G protein interactions in nonleaky vesicle-based reconstitution assays versus more typical protein/lipid/detergent reconstitution assays, detergent-solubilized purified α_{2A}AR (2.0–2.5 pmol) were mixed with cholate-purified G_i/G_o proteins (100 pmol) (24) in the presence of 0.6 mg/ml soybean phosphatidylcholine plus 0.06 mg/ml cholesterol. The reaction mixture was incubated on ice for 1 hr, diluted to 300 μl, passed over a 5-ml (0.7- × 16-cm) G-50 column to facilitate detergent removal and protein-protein interactions, equilibrated, and eluted with 1.2 ml of 20 mM HEPES, 1 mM EDTA, 2 mM $MgCl_2$, 100 mM NaCl, as described previously (24). DTT was added to 5 mM and the samples were kept on ice.

An important difference between the reconstitution protocol using a 1- × 60-cm column to generate nonleaky unilamellar vesicles and that using the 0.7- × 16-cm column employed here and described previously (24) is that detergent appears to be less effectively removed over this 0.7- × 16-cm column, yielding nonturbid preparations, compared with eluates of the 1.0- × 60-cm column used for preparation of ion-impermeant unilamellar vesicles. Apparently the 60-cm height of the G-50 column used for preparation of vesicles is sufficient to resolve the vesicle preparations from detergent micelles that elute just after the void volume in G-50 fine Sephadex columns. Thus, the reconstituted preparations obtained using the smaller 0.7- × 16-cm column are referred to throughout the text as protein/lipid/detergent mixtures, for brevity in distinguishing these preparations from the unilamellar receptor-containing lipid vesicle preparations obtained after chromatography on a 1- × 60-cm column.

The yield of G proteins in the final reconstituted preparations was evaluated by assay of an aliquot of the starting material versus the final reconstituted preparations in the presence of 0.1% Lubrol PX and 25 mM $MgCl_2$, which identifies all [^{35}S]GTPγS binding sites independently of receptor activation. For protein/lipid/detergent mixtures prepared on 0.7- × 16-cm columns, recovery of G proteins was 89–97%, compared with the starting material. The orientation of G proteins in these reconstituted vesicles was not directly determined. For reconstitution of G proteins into sealed vesicles prepared on 1- × 60-cm G-50 columns, recovery of G proteins in the reconstituted material was 85–100%, compared with the starting material.

[^{35}S]GTPγS binding was assayed as described by Northup *et al.* (25). An aliquot of a receptor-G protein reconstitution mixture (25–50 μl) was incubated with receptor agonists or antagonists, in the presence of 5 mM $MgCl_2$, for 15 min at 20°. GDP was then added to a final concentration of 5 μM and the reactions were incubated for an additional 5 min. GDP was critical for minimizing receptor- or agonist-independent GTPγS binding (24). The GTPγS binding reaction was then initiated by the addition of 10× reaction buffer consisting of 200 mM Tris, pH 8.0, 1 μM GTPγS, 10 mM EDTA, 10 mM DTT, 1 M NaCl, and [^{35}S]GTPγS (3 × 10^6 cpm/ml). The reactions were incubated at 20°; aliquots were removed at various times and placed in 3 ml of ice-cold wash buffer (20 mM Tris·HCl, 100 mM NaCl, 25 mM $MgCl_2$). The terminated reactions were filtered onto BA85 nitrocellulose filters, dried, dissolved in 2 ml of ethylene glycol monoethyl ether, and counted in a Packard scintillation counter (in 10 ml of DuPont NEF 963 scintillation fluor).

Preliminary studies intended to enhance the fraction of G proteins activated by α_{2A}AR were undertaken using the protocol to create protein/lipid/detergent mixtures. The final protocol selected utilizes α_{2A}AR/G proteins in a 1:40 molar ratio. Increasing the receptor concentration 2-fold (to create a 1:20 α_{2A}AR/G protein molar ratio) or increasing the G protein concentration 5-fold (to create a 1:200 α_{2A}AR/G protein molar ratio) did not increase the amount of agonist-stimulated [^{35}S]GTPγS binding detected per G protein added, suggesting that the 2.5 pmol of α_{2A}AR/100 pmol of G protein proportion used represented the optimal experimental conditions for our reconstitution protocol. In the sealed vesicle preparations it can be calculated that approximately five G proteins are activated per α_{2A}AR in the 10-min incubation. This estimate is based on the following calculation. Recovery of the initial 2.5 pmol of α_{2A}AR was 75%, yielding 1.88 pmol of α_{2A}AR in the reconstituted preparation. Recovery of G proteins (100 pmol) was 90%, yielding 90 pmol of G_i/G_o in the reconstituted preparation. Approximately 10% of the G proteins were activated in a 10-min incubation, indicating that 1.88 pmol of α_{2A}AR activated 9 pmol of G protein or that 1 receptor activates ~5 G proteins in these preparations.

Results and Discussion

α_{2A}AR can be introduced into unilamellar lipid vesicles. Fig. 1 demonstrates the properties of the α_{2A}AR-containing phospholipid vesicles formed after detergent removal over the Sephadex G-50 column, as described in Experimental Procedures. As shown in Fig. 1A, electron microscopic analysis revealed that the vesicles are unilamellar in structure and possess a mean diameter of 900 Å (range, approximately 650–1050 Å). Fig. 1B compares the protein composition of partially purified α_{2A}AR (sequential yohimbine-agarose/WGA-agarose chromatography) and homogeneous α_{2A}AR (two sequential yohimbine-agarose chromatography steps) introduced into the lipid vesicles. Properties of vesicles containing these two α_{2A}AR preparations were structurally indistinguishable, including nonleakiness (see below). Recovery of receptor protein in vesicles was assessed in multiple experiments using radioiodination and comparison (by cutting out and counting radiolabeled bands from SDS-PAGE) of starting material versus receptor in the final vesicles and was at least 75% of receptor added to the preparation before chromatography over the 1- × 60-cm Sephadex G-50 column (Fig. 1B, *lane 2* versus *lane 3*).

The vesicles obtained are appropriately nonleaky for ion translocation studies. An important component of these

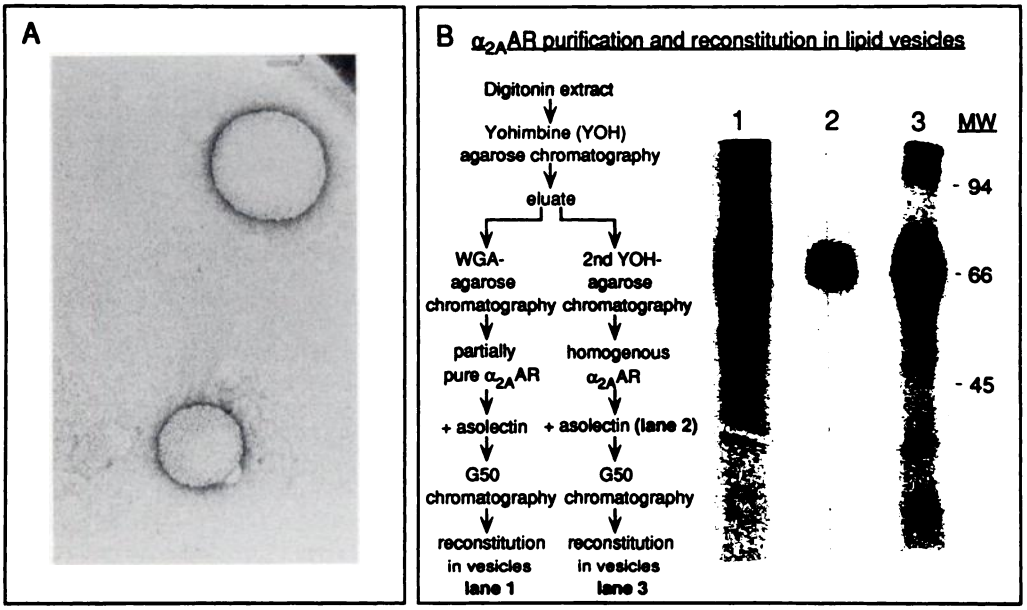

Fig. 1. Characterization of α_{2A}AR-containing phospholipid vesicles. A, Electron micrograph of negatively stained α_{2A}AR-containing vesicles. Receptor-containing vesicles were prepared by chromatography over a 1- × 60-cm Sephadex G-50 column, as described in Experimental Procedures. Based on the analysis of multiple micrographs, the calculated mean diameter of the vesicle preparations was 900 Å. B, Characterization of the α_{2A}AR preparations used in vesicle studies. Aliquots of receptor-containing vesicles prepared with partially purified (*lane 1*) or homogeneous (*lane 3*) α_{2A}AR were radioiodinated as described in Experimental Procedures and were analyzed by SDS-PAGE. Each *lane* represents 1/200th of a single vesicle preparation. An aliquot of the initial reconstitution mixture of homogeneous α_{2A}AR (after CHAPS and asolectin addition and before G-50 chromatography) was radioiodinated as described in Experimental Procedures and analyzed by SDS-PAGE (*lane 2*). This aliquot represents 1/200th of the total reconstitution mixture. Recovery of purified α_{2A}AR after vesicle formation (material shown in *lane 3*) was determined by comparing *lanes 3* (final G-50 eluate) and *2* (receptor plus lipid mixture starting material before G-50 chromatography and vesicle formation). The lower molecular weight bands migrating faster than the α_{2A}AR are due to contaminants in the asolectin preparation that coelute with the receptor/vesicle preparations after Sephadex G-50 chromatography.

experiments was to generate vesicles that were nonleaky in nature, so that they could be used in subsequent experiments for analyses of receptor-mediated ion translocation. Several methods were used to assess the permeation properties of the vesicles.

Data regarding Na^+ transport across these vesicles are shown in Fig. 2. As shown in Fig. 2A, the time-dependent influx of $^{22}Na^+$ into the vesicles was minimal, compared with the instantaneous and significant entry of $^{22}Na^+$ observed when the Na^+ ionophore monensin was added. Similarly, as shown in Fig. 2B, when the lipid vesicles were first loaded with $^{22}Na^+$, as described in Experimental Procedures, and efflux of $^{22}Na^+$ from the vesicles was determined, a similar impermeance to Na^+ was observed. However, immediate and marked efflux of $^{22}Na^+$ efflux detected upon the addition of monensin indicated that $^{22}Na^+$ was present inside the vesicles and could have diffused outward were the vesicles leaky in nature. H^+ movement across these vesicles was similarly minimal, based upon the observation that a H^+ gradient across these vesicles, as measured by acridine orange fluorescence, was stable for ≥3 hr (see Fig. 7 and related methods, below), again confirming the nonleaky nature of the α_{2A}AR-containing vesicle preparations.

The α_{2A}AR is uniformly oriented in the vesicles. For evaluation of the α_{2A}AR-containing lipid vesicles in reconstitution experiments with G proteins and, in the future, for reconstitution with possible effector proteins, it was important to establish the orientation of the receptor in the vesicles. Only by quantitating receptor orientation in the vesicles would it be possible to establish the stoichiometry of receptor, G proteins, and other molecules in terms of their accessibility for interaction with one another in reconstitution studies. Orientation of the vesicles was established using two independent methods. First, as shown in Fig. 3A, we used cAMP-dependent protein kinase to phosphorylate the α_{2A}AR. Based on the predicted amino acid sequence of the porcine α_{2A}AR (21), a single cAMP-dependent protein kinase substrate site is predicted in the α_{2A}AR at residue Thr^{373}, which lies at the termination of the predicted third cytoplasmic loop of the receptor. As shown in Fig. 3A, the accessibility of the receptor to cAMP-dependent protein kinase A was indistinguishable whether the receptor used as substrate was provided in nonleaky unilamellar vesicles, as described in Figs. 1 and 2, or was provided as substrate in vesicles solubilized with the detergent CHAPS. The data are consistent with the interpretation that the α_{2A}AR is largely if not exclusively in an orientation in which the cytoplasmic portion of α_{2A}AR faces the exterior of the vesicle.

Similar interpretations regarding α_{2A}AR orientation in the unilamellar vesicles derive from data such as those described in Fig. 3B. Based on previous studies with the α_{2A}AR (26), exposure of the detergent-solubilized α_{2A}AR to trypsin leads to the cleavage of the third cytoplasmic loop and carboxyl terminus of the α_{2A}AR to yield a glycosylated hydrophobic tryptic core that contains a major fragment, consisting of the amino terminus and the first five membrane-spanning domains, that migrates in SDS-PAGE at ~45,000 kDa (see schematic diagram above Fig. 3B and see Ref. 26). Thus, tryptic cleavage sites predicted to reside in intracellular loop 1 or 2 or extracellular loop 2 must be protected from proteolysis due to the tertiary structure of the α_{2A}AR. If the receptor were oriented "right side out" in the vesicles, so that the extracellular domain of the

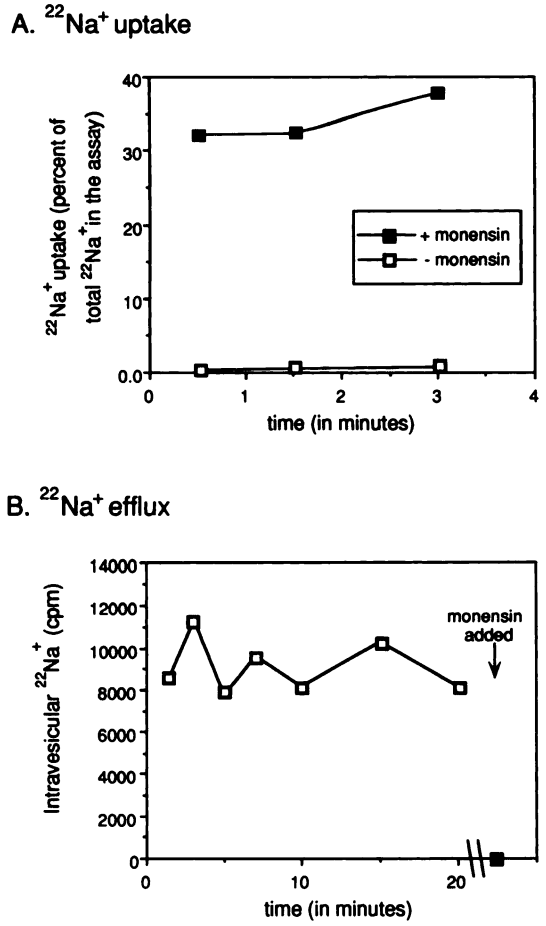

Fig. 2. Analysis of the permeability of α_{2A}AR-containing vesicles to $^{22}Na^+$. A, ^{22}Na uptake. Phospholipid vesicles were prepared with an intravesicular buffer of 100 mM NaCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA, using partially purified α_{2A}AR that had been heated to 65° for 15 min to destroy receptor binding and function (4). $^{22}Na^+$ uptake driven by electrical diffusion potential was assayed for 0.5, 1.5, or 3.0 min in the absence or presence of the Na^+ ionophore monensin, as described in Experimental Procedures. Maximal uptake of $^{22}Na^+$ in the absence of monensin was 0.7% of the total $^{22}Na^+$ in the assay. Uptake of $^{22}Na^+$ in the presence of monensin was 38% of total $^{22}Na^+$ in the assay. B, $^{22}Na^+$ efflux. To remove extravesicular $^{22}Na^+$, a 100-μl aliquot of $^{22}Na^+$-loaded phospholipid vesicles, prepared with partially purified α_{2A}AR as described in Experimental Procedures, was loaded onto Dowex columns and eluted with 3 ml of 100 mM NMDG-HCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA. A 1.5-ml aliquot of these vesicles was diluted to 20 ml in buffer (50 mM NaCl, 25 mM glycylglycine, 1 mM EDTA, pH 8.0) and incubated at 4°, thus creating an outward driving force for $^{22}Na^+$ efflux (intravesicular buffer contained 100 mM NaCl). At the times indicated, 2-ml aliquots were withdrawn from the reaction and filtered onto GF/F filters as described in Experimental Procedures. Monensin (10 μM) was added to an aliquot of vesicles at the end of the assay, to determine the signal expected if the vesicles were "leaky" and freely permeable to $^{22}Na^+$.

α_{2A}AR were uniformly exposed to the extravesicular space, then digestion with trypsin would not yield the M_r 45,000 fragment but, rather, would result in an undigested α_{2A}AR, because the third extracellular loop connecting transmembrane spans 6 and 7 contains no tryptic cleavage sites. In contrast, if the α_{2A}AR is oriented "inside out" in the sealed vesicles, as suggested by receptor accessibility to cAMP-dependent protein kinase (Fig. 3A), then tryptic digestion should result in the formation of a M_r 45,000 cleavage product (composed of the glycosylated amino terminus and transmembrane domains 1–5) and smaller fragments derived from transmembrane spans 6 and 7 (26). Thus, tryptic cleavage of the α_{2A}AR-containing vesicle preparations should provide diagnostic information regarding α_{2A}AR orientation in the vesicles. As shown in Fig. 3B, exposure of the nonleaky, α_{2A}AR-containing, unilamellar vesicle preparation to 10% (w/w) trypsin resulted in the degradation of the majority of the α_{2A}AR to the predicted M_r 45,000 fragment, and digestion with 100% (w/w) trypsin resulted in complete degradation of intact receptor to this M_r 45,000 tryptic core and smaller peptide fragments. These data are consistent with the interpretation that the α_{2A}AR in these vesicular preparations is uniformly oriented inside out, relative to its orientation on the surface of target cells, such that the cytoplasmic domains of the α_{2A}AR face the vesicle exterior. Because we were not certain of the α_{2A}AR orientation before undertaking these studies, we also permeabilized vesicles with CHAPS to provide access of trypsin to the intravesicular space and to residues in the third cytoplasmic loop of the α_{2A}AR, were it oriented right side out. The α_{2A}AR appeared sensitive to trypsin at lower percentages (w/w) in the presence of CHAPS, likely due to the ability of CHAPS to deform receptor structure (ligand binding to the α_{2A}AR is inactivated in the presence of CHAPS) and facilitate access to tryptic cleavage sites in the unfolded receptor. Taken together, the data in Fig. 3 suggest that the α_{2A}AR in these lipid vesicle preparations is uniformly oriented inside out, an orientation that must be energetically preferred by serpentine receptors, because introduction of structurally similar βAR into lipid vesicles also appears to achieve a similar, uniformly oriented, inside-out orientation of the receptor (27).

The vesicle-associated α_{2A}AR productively couples to G proteins. Having determined that the vesicle preparation was unilamellar and well sealed, with the α_{2A}AR in an inside-out orientation, we were interested in determining the ability of this receptor preparation to interact with G proteins. In native target cells, the α_{2A}AR mediates diverse physiological effects via interaction with pertussis toxin substrates, namely proteins of the G_i/G_o protein family. Thus, we incubated vesicle preparations containing the native α_{2A}AR or trypsin-proteolyzed α_{2A}AR with a G_i/G_o protein mixture purified from bovine brain (23). After incubation of the receptor-containing vesicles with the G protein in a small volume, which maximized receptor-G protein encounters, the vesicle preparation was diluted 40-fold before assessment of functional interaction of receptor and G proteins, using agonist-stimulated [^{35}S]GTPγS binding as a measure of productive α_{2A}AR-G protein coupling. Despite the inside-out nature of the vesicles, the hydrophobic agonist and antagonist ligands readily accessed the AR binding pocket in the transmembrane (transvesicle) domain of the α_{2A}AR. As shown in Fig. 4, A and B, interaction of the receptor and G protein yielded a 4–5-fold increase in [^{35}S]GTPγS binding in the presence of the α_2 agonist UK14304, compared with the [^{35}S]GTPγS binding achieved by the G protein when the αAR antagonist phentolamine was added to the incubation. This demonstrates that the α_{2A}AR reconstituted in these vesicles is functional. Receptor-dependent activation of G proteins also was achieved in vesicular preparations where the α_{2A}AR was first incubated with trypsin. The attenuation of the fold stimulation by α_{2A}AR agonists after receptor hydrolysis with trypsin, particularly at concentrations of trypsin of >10% (w/w), may result from complete degradation of a fraction of the α_{2A}AR due to the lack of AR antagonist as stabilizing ligand during the proteolysis (in contrast to the incubation conditions used

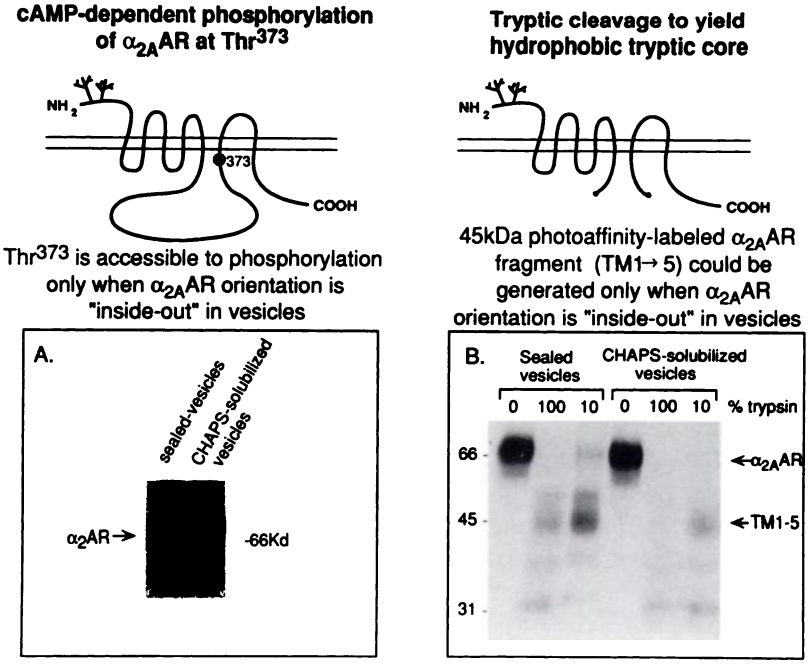

Fig. 3. Assessment of the orientation of the α_{2A}AR in phospholipid vesicles. A, Receptor phosphorylation. A 25-μl aliquot of intact α_{2A}AR-containing vesicles, or a 25-μl aliquot of vesicles to which CHAPS was added at 0.4% to render the receptor accessible to cAMP-dependent protein kinase-catalyzed phosphorylation regardless of receptor orientation in the vesicles, was subjected to phosphorylation by cAMP-dependent protein kinase, as described in Experimental Procedures, and analyzed by SDS-PAGE (12% polyacrylamide gel) and autoradiography. The area corresponding to phosphorylated α_{2A}AR was excised from the gel and counted in a scintillation counter. The receptor band from intact vesicles incorporated 6368 cpm of [^{32}P]PO$_4$, compared with 6428 cpm for the CHAPS-solubilized vesicles. The vesicles studied in the absence of added CHAPS also were used for transport studies and were shown to be nonleaky, based on $^{22}Na^+$ influx. B, Trypsin sensitivity. A 45-μl aliquot of intact 17α-hydroxy-20α-yohimban-16β[*N*-(4-azido-3-[^{125}I]iodo)-phenethyl]carboxamide–labeled α_{2A}AR-containing vesicles prepared as described in Experimental Procedures and a 45-μl aliquot of vesicles to which CHAPS was added at 0.4% to solubilize the vesicles were incubated overnight at 15° with 0 (control), 10%, or 100% (w/w) trypsin. A 5-fold excess of soybean trypsin inhibitor was added at the end of the incubation and the samples were analyzed by SDS-PAGE and autoradiography. The experiments were performed twice with separate vesicle preparations, with similar results.

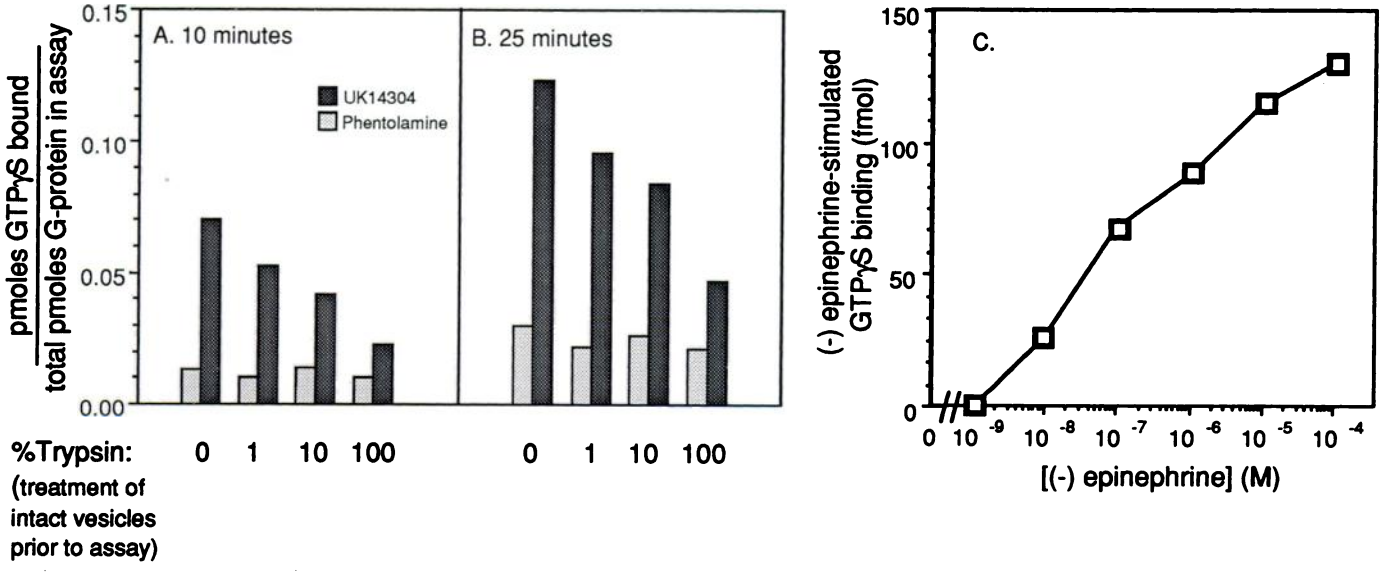

Fig. 4. Agonist-stimulated [^{35}S]GTPγS binding in α_{2A}AR-containing vesicles reconstituted with G proteins. A and B, Individual 25-μl aliquots of α_{2A}AR-containing phospholipid vesicles (containing approximately 2.5 pmol of α_{2A}AR) were incubated overnight at 15° with 0 (control), 1, 10, or 100% (w/w) trypsin. After the incubation, excess soybean trypsin inhibitor was added to the samples and G proteins were reconstituted with the α_{2A}AR-containing vesicles, as described in Experimental Procedures. [^{35}S]GTPγS binding was assessed at 10 min (A) or 25 min (B), in the presence of the agonist UK14304 or the antagonist phentolamine. C, [^{35}S]GTPγS binding was assessed in the presence of varying concentrations of (−)-epinephrine.

in Fig. 3B); antagonist could not be added in these experiments, where we chose to subsequently assess agonist-activated α_{2A}AR-G protein interactions. Regardless of the attenuation of the fold stimulation for agonist activation of G proteins by trypsin-digested α_{2A}AR in the vesicles, the observation that the tryptic core of the α_{2A}AR can activate G proteins is in agreement with previous work by Ross *et al.* (1) on the βAR, where a trypsin-generated hydrophobic core was able to couple effectively to G_s. Fig. 4C demonstrates that [^{35}S]GTPγS binding in these α_{2A}AR-G protein reconstitution assays also could be activated by the endogenous catecholamine agonist epinephrine, in a concentration-dependent manner. Interestingly, as shown in Fig. 5, the stimulation of [^{35}S]GTPγS binding by vesicle-associated α_{2A}AR obtained on 1- × 60-cm columns, as shown in this and several comparable experiments, was at least 2-fold greater than agonist-dependent stimulation of [^{35}S]GTPγS binding by these same receptor and G protein components evaluated in a protein/detergent/lipid reconstitution mixture obtained by detergent removal on a 0.7- × 16-cm G-50 column (see Experimental Procedures). Thus, the presentation of the α_{2A}AR to G proteins in the uniformly oriented, unilamellar, sealed vesicles apparently enhances the efficiency of receptor-G protein coupling.

The α_{2A}AR does not translocate ions in vesicles. As indicated earlier, a number of lines of evidence have caused us to postulate that the α_{2A}AR might itself be able to effect ion translocation. Thus, homogeneous preparations of the receptor are allosterically modulated by monovalent cations as well as by analogs of amiloride that inhibit Na^+/H^+ exchange activity. In addition, topographically similar receptors, such as bacteriorhodopsin and halorhodopsin, mediate light-activated ion transport. Bacteriorhodopsin-mediated light-activated Na^+/H^+ exchange in bacteria results from light-activated proton efflux effected by the rhodopsin molecule itself, electrically compensated by an equivalent Na^+ influx via an independent molecule to yield a net Na^+/H^+ exchange activity (28–35). However, recent reports comparing the structure of G protein-coupled bovine rhodopsin with bacteriorhodopsin suggest significant differences in the bilayer structure of G protein-coupled receptors, compared with bacteriorphodopsin, including the absence of a readily detectable "pore" (14). Despite the anticipated difference in bacteriorhodopsin versus α_{2A}AR structure, the vesicle preparations obtained in the present studies were nonetheless uniquely appropriate for evaluating whether α_{2A}AR-mediated ion translocation occurs. Ion translocation mediated by the α_{2A}AR was evaluated using three independent experimental strategies, i.e., $^{22}Na^+$ uptake, $^{22}Na^+$ efflux, and H^+ movement.

As shown in Fig. 6A, receptor-containing vesicle preparations did not effect $^{22}Na^+$ uptake driven by an electrochemical gradient at 4°, either in the absence or in the presence of receptor agonists. As summarized in Table 1, a similar lack of $^{22}Na^+$ uptake was detected in vesicle preparations incubated at 25°, in vesicles incubated with intravesicular K^+ plus valinomycin to create an electrical gradient, or in vesicles reconstituted with a G_i/G_o mixture. In all experiments, we are confident that receptor-mediated $^{22}Na^+$ transport would have been detected if it had occurred, because the ionophore monensin resulted in robust uptake in this assay (Fig. 6A). Direct Na^+ uptake experiments performed in the absence of an opposing chemical gradient (see Experimental Procedures) also failed to demonstrate any receptor-dependent $^{22}Na^+$ translocation. Similarly, agonist stimulation had no effect on $^{22}Na^+$ efflux from α_{2A}AR-containing vesicles (Fig. 6B). Thus, it appears that the α_{2A}AR is not capable of effecting an agonist-dependent movement of sodium across the vesicle surface that is experimentally detectable in this biological preparation. Creating a H^+ gradient in opposing directions to the Na^+ gradient in direct $^{22}Na^+$ uptake studies (data not shown) or in $^{22}Na^+$ efflux studies (Fig. 6B) also did not permit detection of receptor-dependent or agonist-activated ion translocation.

As shown in Fig. 7, there also was no detectable agonist-mediated H^+ movement across α_{2A}AR-containing lipid vesicles. For these studies, H^+ movement was measured using the dye acridine orange. Although the precise mechanism by which this dye works is not known, it has been observed that concentration of the dye into lipid vesicles leads to a quenching of the fluorescence signal of the dye. Proton movements out of the vesicle, in parallel with redistribution of the pH-sensitive acridine orange dye, would lead to an increase in the fluorescence signal due to a decrease in local acridine orange concentration and thus a decrease in quenching of the fluorescence. As shown

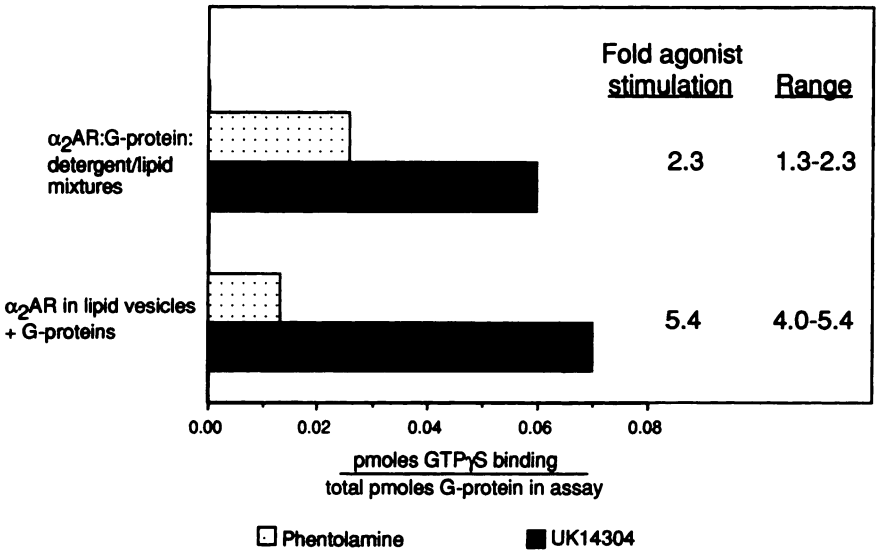

Fig. 5. Comparison of agonist-stimulated [^{35}S]GTPγS binding in sealed α_{2A}AR-containing lipid vesicles versus nonvesicular preparations. The α_{2A}AR was reconstituted with a crude G_i/G_o preparation using two different methods, 1) mixing 2.5 pmol of digitonin-solubilized α_{2A}AR with 100 pmol of purified G_i/G_o, 0.6 mg/ml asolectin, and 0.06 mg/ml cholesterol, incubating the mixture on ice for 1 hr, and fostering vesicle formation by passing the mixture over a 5-ml G-50 column (*α_{2A}AR:G-protein:detergent/lipid mixtures*) or 2) mixing α_{2A}AR-containing vesicles (2.5 pmol of receptor) with 100 pmol of G_i/G_o as described in Experimental Procedures (*α_{2A}AR in lipid vesicles + G-proteins*). GTP$^{35}\gamma$S binding assays were performed as described in Experimental Procedures. The data shown represent the maximal fold stimulation obtained for each method over a variety of time points in different experiments (range for α_{2A}AR G protein/detergent/lipid mixture preparations, 1.3–2.3-fold stimulation, five experiments; range for sealed vesicle preparations, 4.0–5.4 fold stimulation, two experiments).

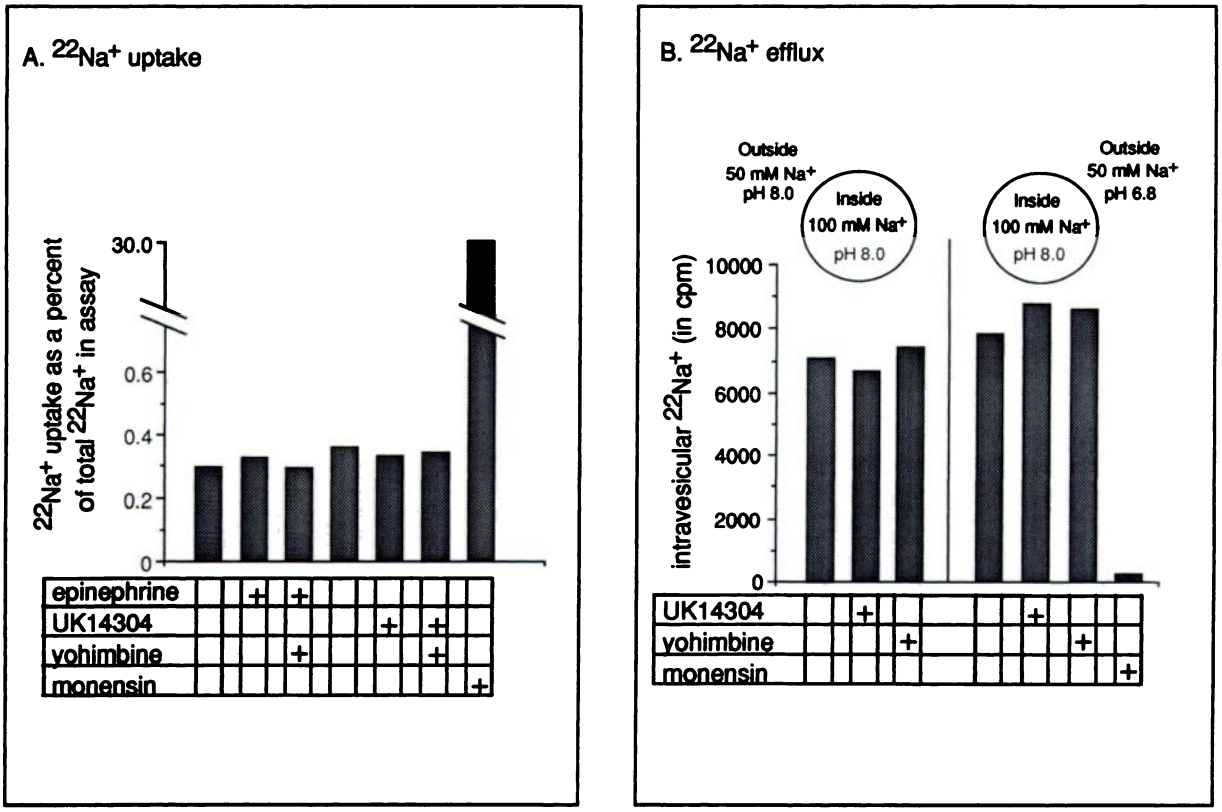

Fig. 6. Ion transport studies. A, ^{22}Na uptake. Phospholipid vesicles were prepared with an intravesicular buffer of 100 mM NaCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA, using partially purified α_{2A}AR. $^{22}Na^+$ uptake driven by electrical diffusion potential was assayed for 2 min in the presence of epinephrine (100 μM), UK14304 (1 μM), or yohimbine (1 μM), as described in Experimental Procedures. The ionophore monensin (10 μM) was added to control tubes. Each *point* was assayed in duplicate and standardized to the amount of $^{22}Na^+$ in each assay. B, ^{22}Na efflux. A 100-μl aliquot of $^{22}Na^+$-loaded phospholipid vesicles prepared with partially purified α_{2A}AR as described in Experimental Procedures (intravesicular buffer, 100 mM NaCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA) was loaded onto Dowex columns and eluted with 3 ml of 100 mM NMDG-HCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA, to remove extravesicular $^{22}Na^+$. Vesicles (1.5 ml) were diluted to 30 ml in buffer containing either 50 mM NaCl, 25 mM glycylglycine, and 1 mM EDTA, pH 8.0 (assay for Na^+ flux), or 50 mM NaCl, 25 mM glycylglycine, and 1 mM EDTA, pH 6.8 (assay for Na^+/H^+ exchange). After dilution, the vesicles were aliquoted into three 10-ml reactions, to which no drug, UK14304 (10 μM), or yohimbine (1 μM) was added, and were incubated at 20° for 4 min. Two fractions (2 ml each) were withdrawn at this time, filtered onto GF/F filters, and counted as described in Experimental Procedures. Monensin (10 μM) was added to vesicles as a positive control for $^{22}Na^+$ efflux. The experiments were performed at least two times, with comparable results.

TABLE 1

Lack of agonist-stimulated $^{22}Na^+$ transport in α_{2A}AR-containing lipid vesicles under various experimental conditions

$^{22}Na^+$ uptake driven by electrical diffusion potential was assayed for 2 min in the presence of the α_2AR agonist UK14304 (10 μM) or the antagonist yohimbine (10 μM), essentially as described in Experimental Procedures, under the following conditions. All data were standardized to the amount of $^{22}Na^+$ in the assay. I) The entire assay was performed at 4°. II) The entire assay was performed at 25°. III) Vesicles were prepared with 75 mM KCl, 75 mM NaCl, 25 mM glycylglycine, pH 8.0, 1 mM EDTA. At the initiation of the uptake assay (at 25°), 10 μM valinomycin (a K^+ ionophore) was added to create a strong electrical diffusion potential. IV) Receptor-containing vesicles were reconstituted with G_i/G_o G protein mixture, as described in Experimental Procedures, and then used for uptake assays.

Assay conditions	Uptake of $^{22}Na^+$		
	+Yohimbine	+UK14304	+Monensin
	% of total $^{22}Na^+$		
I. 4°	0.34	0.33	22
II. 25°	0.38	0.43	ND[a]
III. 25° + valinomycin	1.0	1.0	30
IV. 25° + G proteins[b]	0.06	0.06	6.0

[a] ND, not done.

[b] Because of the dilution of the vesicles that occurred during reconstitution with G proteins (assay condition IV), fewer vesicles were used for each assay than in the reactions under conditions I, II, and III.

in Fig. 7, addition of the lipid vesicles containing α_{2A}AR led to a significant quenching of the acridine orange fluorescence signal. However, the addition of extravesicular Na^+ did not change the rate at which the signal re-equilibrated, nor did the addition of the α_2AR agonist UK14304. Thus, it appears that the receptor is not able to alter H^+ movement either upon the introduction of Na^+ or, importantly, upon the introduction of the α_2AR agonist. However, the driving force for proton movement across the vesicles was demonstrated by the addition of monensin, which caused an immediate and dramatic increase in the fluorescence signal and returned the signal to that measured in the absence of the lipid vesicles, indicating that the intra- and extravesicular pH values had equilibrated due to the introduction of monensin. The monensin signal confirmed that an ion gradient for H^+ movement did exist across the nonleaky vesicle preparation but this movement was not modulated by the addition of either extravesicular Na^+ or the α_2AR agonist UK14304 in the α_{2A}AR-containing lipid vesicle preparations. Furthermore, in preliminary studies undertaken to establish the feasibility of detecting H^+ movement in biological preparations that contained H^+ transporters at a density less than or equal to the α_{2A}AR density in our vesicle preparation,

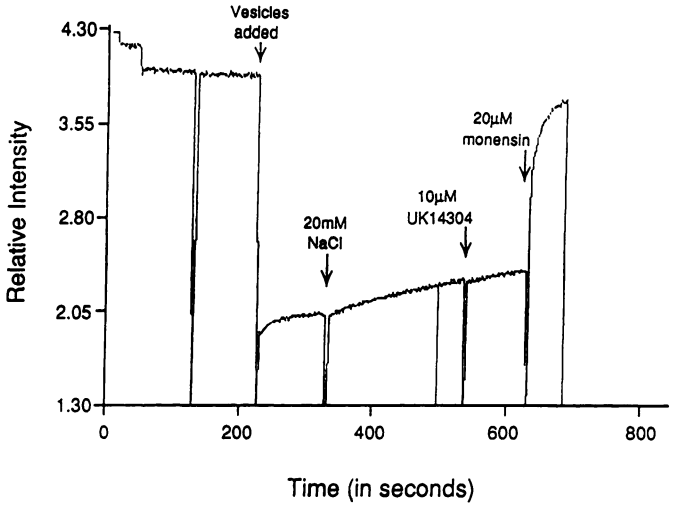

Fig. 7. Measurement of Na^+/H^+ exchange using acridine orange. Measurement of Na^+/H^+ exchange was performed using a Spex microfluorometer essentially described in Experimental Procedures, except that in this experiment α_{2A}AR-containing vesicles were concentrated, after formation, by centrifugation at 18,000 rpm (39,000 × *g*) in a Sorvall SS-34 rotor for 30 min at 4°, rather than at 39,000 rpm (100,000 × *g*) for 2.5 hr. We observed that, although this lower speed/shorter duration concentration step resulted in a 30% lower yield of receptor protein, compared with the higher speed centrifugation (presumably due to incomplete pelleting of smaller vesicles with the lower speed centrifugation), the resulting vesicles were found to be even less leaky to protons than vesicles prepared with the standard protocol. However, similar results were obtained regardless of the vesicle concentration method.

we readily detected Na^+-stimulated H^+ efflux from brush border vesicles that were isolated from rat ileum and contained Na^+/H^+ transporters at a density calculated to be 20% of the density of α_{2A}AR in our reconstituted vesicles (36). These findings indicate that Na^+/H^+ exchange activity, if it occurred in our α_{2A}AR-containing vesicles, should have been detectable using the experimental procedures used. Thus, the findings in Figs. 6 and 7 provide no evidence for detectable ion translocation mediated by the α_{2A}AR, implying that the receptor molecule itself, or in association with G proteins, is not capable of moving ions.

Conclusions

The present data demonstrate that the α_{2A}AR, either in homogeneous form or in highly enriched preparations, can be introduced into lipid vesicle preparations such that the resulting lipid vesicles are unilamellar in structure, nonleaky to monovalent cations, and uniformly oriented in terms of receptor orientation. In this orientation, introduction of G proteins yields a 4–5-fold stimulation of agonist-dependent [^{35}S]GTPγS binding to the G protein α subunit. The uniform orientation of the receptors in these preparations facilitates appropriate receptor-G protein interactions and suggests that the methodology developed should be well suited for evaluating the stoichiometry of receptor-G protein coupling as well as the selectivity of receptor-G protein interactions. Finally, these nonleaky, uniformly oriented, receptor-containing vesicle preparations allowed us to explore the hypothesis that the α_{2A}AR itself, or in combination with G_i/G_o proteins, was able to effect ion translocation. However, measurements of $^{22}Na^+$ uptake, $^{22}Na^+$ efflux, and H^+ movement revealed no detectable receptor-dependent ion translocation. Nonetheless, the nonleaky nature of these vesicles to monovalent cations in the absence and in the presence of G proteins suggests that such preparations will be particularly valuable for introduction of other effectors, such as voltage-gated or receptor-activated ion channels, to examine G protein subunit selectivity and stoichiometry in mediating receptor-dependent regulation of these ion channels, in an experimental situation where the protein component stoichiometry and transvesicular ion content can be rigorously and independently varied.

Acknowledgments

We wish to heartily thank Dr. Paul Sternweis of the University of Texas, Southwestern Medical Center (Dallas, TX), for providing the purified G_i/G_o mixtures and for advice and stimulating discussion, offered with his distinctive blend of scientific insight and humor, regarding receptor-G protein reconstitution during the course of these studies. We also thank Dr. Sternweis for critical reading of the manuscript. Purified cAMP-dependent protein kinase catalytic subunit was kindly provided by Dr. Jackie Corbin of the Department of Molecular Physiology and Biophysics at Vanderbilt University Medical Center. We are grateful to Dr. Joachim Sommer of the Veterans Administration Medical Center at Duke University for electron microscopic analysis of our vesicle preparations. Finally, we gratefully acknowledge the assistance of Fayez Ghishan and his laboratory colleagues in the Division of Pediatric Gastroenterology at Vanderbilt University Medical Center for their help with the preparation of ileal brush border vesicles.

References

1. Rubenstein, R. C., S. K. Wong, and E. M. Ross. The hydrophobic tryptic core of the β-adrenergic receptor retains G_s regulatory activity in response to agonists and thiols. *J. Biol. Chem.* **262**:16655–16662 (1987).
2. Tsai, B. S., and R. J. Lefkowitz. Agonist-specific effects of monovalent and divalent cations on adenylate cyclase-coupled α_2-adrenergic receptors in rabbit platelets. *Mol. Pharmacol.* **14**:540–548 (1978).
3. Motulsky, H., and P. A Insel. Influence of sodium on the α_2-adrenergic receptor system of human platelets. *J. Biol. Chem.* **258**:3913–3919 (1983).
4. Limbird, L. E., and J. L. Speck. *N*-Ethylmaleimide, elevated temperature, and digitonin solubilization eliminate guanine nucleotide but not sodium effects on human platelet α_2-adrenergic receptor-agonist interactions. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **72**:164–170 (1983).
5. Nunnari, J. M., M. G. Repaske, S. Brandon, and L. E. Limbird. Modulation of α_2-adrenergic receptor-ligand interactions by Na^+, H^+ and inhibitors of Na^+/H^+ exchange. *J. Biol. Chem.* **262**: 12387–12312 (1987).
6. Pert, C. B., and S. H. Snyder. Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Mol. Pharmacol.* **10**:868–879 (1974).
7. Blume, A. J., D. Lichtshtein, and G. Boone. Coupling of opiate receptors to adenylate cyclase: requirement for Na^+ and GTP. *Proc. Natl. Acad. Sci. USA* **76**:5626–5630 (1978).
8. Jakobs, K.-H. Inhibition of adenylate cyclase by hormones and neurotransmitters. *Mol. Cell. Endocrinol.* **16**:147–156 (1979).
9. Neve, K. A., R. A. Hennigsen, J. M. Kinzie, T. DePaulis, D. C. Schmidt, R. M. Kessler, and A. Janowsky. Sodium-dependent isomerization of dopamine D-2 receptors characterized using [^{125}]epidepride, a high affinity substituted benzamide ligand. *J. Pharmacol. Exp. Ther.* **252**:1108–1116 (1990).
10. Osterhelt, D., and W. Stoekenius. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nature (Lond.)* **233**:149–152 (1971).
11. Drachev, L. A., A. D. Koulen, S. A. Ostroumov, and V. P. Skulacher. Electrogenesis by bacteriohrodopsin incorporated in a planar phospholipid membrane. *FEBS Lett.* **39**:43–45 (1974).
12. Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing. Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.* **213**:899–929 (1990).
13. Schertler, G. F., C. Villa, and R. Henderson. Projection structure of rhodopsin. *Nature (Lond.)* **362**:770–772 (1993).
14. Baldwin, J. M. The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.* **12**:1693–1703 (1993).
15. Repaske, M. G., J. M. Nunnari, and L. E. Limbird. Purification of the *alpha*$_2$-adrenergic receptor from porcine brain using a yohimbine-agarose affinity matrix. *J. Biol. Chem.* **262**:12381–12386 (1987).
16. Domino, S. E., M. G. Repaske, C. A. Bonner, M. E. Kennedy, A. L. Wilson, S. Brandon, and L. E. Limbird. Synthesis of a yohimbine-agarose matrix useful for large-scale and micropurification of multiple α_2-receptor subtypes. *Methods Enzymol.* **215**:181–200 (1992).
17. Ames, B. N. Assay of inorganic phosphate, total phosphate, and phosphatases. *Methods Enzymol.* **8**:115–117 (1966).
18. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lo...)* **227**:680–685 (1970).
19. Gasko, O. D., A. F. Knowles, H. G. Sherter, E. M. Suoinna, and E. Racker. The use of ion-exchange resins for studying ion transport in biological systems. *Anal. Biochem.* **72**:57–65 (1976).
20. Garty, H., B. Rudy, and S. J. D. Karlish. A simple and sensitive procedure

for measuring isotope fluxes through ion-specific channels in heterogeneous populations of membrane vesicles. *J. Biol. Chem.* **258**:13094–13099 (1983).
21. Guyer, C. A., D. A. Horstman, A. L. Wilson, J. D. Clark, E. J. Cragoe, Jr., and L. E. Limbird. The hydrophobic tryptic core of the porcine α_2-adrenergic receptor retains allosteric modulation of binding by Na^+, H^+ and 5-amino substituted analogs of amiloride. *J. Biol. Chem.* **265**:17318–17322 (1990).
22. Lanier, S. M., H. J. Hess, A. Grodski, and R. M. Graham. Synthesis and characterization of a high affinity radioiodinated probe for the α_2-adrenergic receptor. *Mol. Pharmacol.* **29**:219–227 (1986).
23. Sternweis, P., and J. D. Robishaw. Isolation of two proteins with high affinity for guanine nucleotides from extracts of bovine brain. *J. Biol. Chem.* **259**:13806–13813 (1984).
24. Florio, V. A., and P. C. Sternweis. Mechanisms of muscarinic receptor action on G_o in reconstituted phospholipid vesicles. *J. Biol. Chem.* **264**:3909–3915 (1989).
25. Northup, J. K., M. D. Smigel, and A. G. Gilman. The guanine nucleotide activating site of the regulatory component of adenylate cyclase: identification by ligand binding. *J. Biol. Chem.* **257**:11416–11423 (1982).
26. Wilson, A. L., C. A. Guyer, E. J. Cragoe, Jr., and L. E. Limbird. The hydrophobic tryptic core of the porcine α_2-adrenergic receptor retains allosteric modulation of binding by Na^+, H^+ and 5-amino substituted analogs of amiloride. *J. Biol. Chem.* **265**:17328–17322 (1990).
27. Kurstjens, N. P., M. Frölich, C. Dees, R. C. Cantrill, M. Hekman, and E. J. Helmreich. Binding of α- and γ-subunits of G_s to β_1-adrenoceptor in sealed unilamellar lipid vesicles. *Eur. J. Biochem.* **197**:167–176 (1991).
28. Lanyi, J. K., and R. E. MacDonald. Existence of electrogenic hydrogen ion/sodium ion antiport in *Halobacterium halobrium* cell envelope vesicles. *Biochemistry* **15**:4608–4614 (1976).
29. Lanyi, J. K., and M. P. Silverman. Gating effect in *Halobacterium halobium* membrane transport. *J. Biol. Chem.* **254**:4750–4755 (1979).
30. Garcia, M. L., A. A. Guffanti, and T. A. Krulwich. Characterization of the Na^+/H^+ antiporter of alkalophilic bacilli *in vivo*: $\Delta\psi$-dependent $^{22}Na^+$ efflux from whole cells. *J. Bacteriol.* **156**:1151–1157 (1983).
31. Bassilana, M., E. Damiano, and G. LeBlanc. Relationships between the Na^+-H^+ antiport activity and the components of the electrochemical proton gradient in *Escherichia coli* membrane vesicles. *Biochemistry* **23**:1015–1022 (1984).
32. Bassilana, M., E. Damiano, and G. LeBlanc. Kinetic properties of Na^+-H^+ antiport in *Escherichia coli* membrane vesicles: effects of imposed electrical potential, proton gradient and internal pH. *Biochemistry* **23**:5288–5298 (1984).
33. Seto-Young, D., M. L. Garcia, and T. A. Krulwich. Reconstitution of resolved muscarinic cholinergic receptors with purified GTP-binding proteins. *J. Biol. Chem.* **260**:11393–11395 (1985).
34. Murakami, N., and T. Konishi. DCCD-sensitive Na^+ transport in the membrane vesicles of *Halobacterium halobium*. *J. Biol. Chem.* **103**:231–236 (1988).
35. Koniski, T., and N. Murakami. $\Delta\psi$-dependent gating of Na^+/H^+ exchange in *Halobacterium halobrium*: a $\Delta\mu$ H^+ driven Na^+ pump. *J. Biochem. (Tokyo)* **103**:231–236 (1988).
36. Weinman, E. J., S. Shenolikar, E. J. Cragoe, Jr., and W. P. Dubinsky. Solubilization and reconstitution of renal brush border Na^+/H^+ exchanger. *J. Membr. Biol.* **101**:1–9 (1988).

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