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Monovalent Cation and Amiloride Analog Modulation of Adrenergic Ligand Binding to the Unglycosylated α_{2B}-Adrenergic Receptor Subtype

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SUMMARY

The unglycosylated α_{2B} subtype of the α_2-adrenergic receptor found in NG-108-15 cells possesses allosteric regulation of adrenergic ligand binding by monovalent cations and 5-amino-substituted amiloride analogs. These findings demonstrate that allosteric modulation of adrenergic ligand binding is not a property unique to the α_{2A} subtype. The observation that amiloride analogs as well as monovalent cations can modulate adrenergic ligand binding to the nonglycosylated α_{2B} subtype indicates that charge shielding due to carbohydrate moieties does not play a role in this allosteric modulation but, rather, these regulatory effects result from interactions of cations and amiloride analogs with the protein moiety of the receptor. Furthermore, the observation that both α_{2A} and α_{2B} receptor subtypes are modulated by amiloride analogs suggests that structural domains that are conserved between the two are likely to be involved in this allosteric modulation.

The α_2-adrenergic receptor is one of a large population of receptors that are linked to inhibition of adenylate cyclase via a GTP-binding protein. The α_2-adrenergic receptor was defined originally by its sensitivity to blockade by the alkaloid yohimbine (1). Recently, it has been recognized that this yohimbine-sensitive receptor population exists as multiple subtypes that are distinguishable by pharmacological (2–4), biochemical (5), and molecular biological (6) differences. Human platelet and porcine brain cortex α_2-adrenergic receptors are well studied examples of the archetypical α_{2A} subtype, which is pharmacologically characterized by much greater affinity for yohimbine (K_i = nM) than for prazosin (K_i = μM) (3). In contrast, the α_{2B} subtype is more sensitive to blockade by the previously presumed α_1-adrenergic receptor-selective antagonist prazosin (3). Other properties distinguish the α_{2A} and α_{2B} receptor subtypes as well. Recent biochemical (5) and molecular biological (6) characterization of the α_{2B}-adrenergic receptor subtype has shown that it, unlike the α_{2A} subtype, is not glycosylated, at least via *N*-linked glycosylation.

Of particular interest in our laboratory is the allosteric modulation of adrenergic ligand binding at α_2 receptors by monovalent cations and 5-amino-substituted analogs of amiloride. This allosteric modulation has been characterized in detail for the α_{2A} subtype expressed in human platelets (7) and porcine brain cortex (8). The present studies were undertaken to determine whether allosteric modulation of adrenergic ligand binding also occurs at the α_{2B} receptor subtype. For these experiments, we exploited biological preparations previously demonstrated to contain only the α_{2A}- or α_{2B}-adrenergic receptor subtype (3, 4, 9). Thus, the structural and functional properties of the porcine brain α_{2A} receptor were compared with those of the α_{2B} receptor subtype derived from neuroblastoma × glioma NG-108-15 cells. If allosteric modulation is shared by these two α_2-adrenergic receptor subtypes, then these modulatory phenomena do not require *N*-linked glysosylation of the receptor, which is missing from the α_{2B} receptor subtype (5, 6). Furthermore, these studies would provide predictive insights into the receptor domains involved in allosteric modulation, because these would be structurally conserved between the α_{2A} and α_{2B} subtypes.

Experimental Procedures

Materials

The yohimbine-agarose affinity resin was synthesized in our laboratory, as previously described (9). The α_2-adrenergic receptor-selective

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ABBREVIATIONS: ^{125}I-Rau-AzPEC, 17α-hydroxy-20α-yohimban-16β-[*N*-(4-azido-3-[^{125}I]iodo)-phenethyl] carboxamide; EIA, ethylisopropyl amiloride; CBDMB, chlorobenzyldimethylbenzamil; WGA, wheat germ agglutinin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid; NMDG, *N*-methyl-D-glucamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

photoaffinity label ^{125}I-Rau-AzPEC was synthesized in our laboratory from the precursor 17α-hydroxy-20α-yohimban-16β-(*N*-4-aminophenethyl)carboxamide (provided by Dr. Stephen Lanier, Massachusetts General Hospital, Boston, MA), by the method of Lanier *et al.* (10). Amiloride analogs EIA, methylisobutyl amiloride, CBDMB, and dichlorobenzamil were synthesized as previously described (11, 12) and purchased from E.J.C. [^{3}H]Yohimbine and [^{3}H]rauwolscine were purchased from New England Nuclear. Na^{125}I was obtained from Amersham, *N*-glycanase from Genzyme, and WGA-agarose from Vector Laboratories. All cell culture reagents were obtained from GIBCO Laboratories. Fetal calf serum was from Sigma, and each lot of serum was selected based on catecholamine levels (≤400 pg/ml) and growth curves of NG-108-15 cells. Acrylamide, bisacrylamide, and SDS, all specially purified for electrophoresis, were from BDH Chemicals. Molecular weight markers were from Bio-Rad. All other chemicals were reagent grade.

Methods

Cell culture. The mouse neuroblastoma × rat glioma hybrid cell line (NG-108-15) was generously provided by the laboratories of Drs. Werner Klee and Marshall Nirenberg, National Institutes of Health, and was maintained in Dulbecco's modified Eagle's medium, pH 7.4, supplemented with 10% heat-inactivated fetal calf serum, 25 mM glucose, 0.1 mM hypoxanthine, 16 μM thymidine, 1 μM aminopterin, 2 mM glutamine, and penicillin/streptomycin (100 IU/100 μg), in a humidified atmosphere of 10% CO_2/90% air at 37°. Stock cells were plated at a density of 2×10^6 cells/75-cm^2 flask (Falcon) and maintained until confluent, typically 1 week. For individual experiments, confluent cells were harvested by gentle up and down resuspension in a sterile pipette and plated at a density of 2×10^6 cells/100-mm dish. Cells were then grown to confluency (typically 5 days) and serum deprived for 15 hr before being harvested. In our experience, apparent α_2 receptor density in NG-108-15 cells is increased 25–100% by overnight serum deprivation.

Solubilization and purification of the α_2-adrenergic receptor. NG-108-15 cells (approximately 4×10^8) plated on 40- × 100-mm dishes were harvested by removal of the medium and pipetting of 4 ml of Dulbecco's phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 6.89) into each dish. Cells were gently resuspended by mixing with the buffered saline in a polyethylene transfer pipette and pooled into 50-ml plastic conical tubes (10 dishes/50-ml tube) at room temperature. After cell counts were obtained (typically, 4×10^8 total cells were obtained from 40 dishes), the cells were pelleted by centrifugation for 5 min at room temperature at 1000 rpm, in a Sorvall GL-C tabletop centrifuge. The supernate was discarded, and the pelleted cells were allowed to sit on ice for 10 min before each pellet was resuspended in 2.5 ml of ice-cold lysing buffer containing protease inhibitors (15 mM HEPES, 5 mM EGTA, 5 mM EDTA, 10 mM benzamidine, 10 units/ml Trasylol, 0.16 mM phenylmethylsulfonyl fluoride, pH 7.4). The lysates obtained (approximately 3 ml) were homogenized by 10 up and down strokes in a Teflon-glass homogenizer held in ice, to which 5 ml of lysing buffer without protease inhibitors were added. Each 8 ml of homogenized lysate was transferred to a 40-ml Sorvall centrifuge tube and diluted to approximately 40 ml with additional lysing buffer, before centrifugation at 39,000 × *g* for 10 min at 4° in an SS34 rotor of a Sorvall RC-5B centrifuge. The supernate was discarded, and each pellet was resuspended in 8 ml of 50 mM HEPES, 100 mM NaCl, 5 mM EGTA, pH 8, and homogenized with 10 up and down strokes, as described above. The four homogenates, each derived from ten 100-mm dishes, were pooled and then transferred to two 40-ml Sorvall centrifuge tubes. The homogenates in each tube were then diluted to 40 ml with 50 mM HEPES, 100 mM NaCl, 5 mM EGTA, pH 8.0, and pelleted as described above. The resulting pellets were resuspended in 4 ml of freshly prepared extraction buffer (1% digitonin, 50 mM HEPES, 100 mM NaCl, 5 mM EGTA, pH 8.0) and resuspended using 10 up and down strokes in a Teflon-glass homogenizer. These detergent extracts were pooled, and additional extraction buffer was added to a final volume of 7 ml/1.5×10^8 cells (as determined from the original cell count before lysis and solubilization). This preparation was then sonicated in a bath sonifier (Heat Systems-Ultrasonics, Inc.) for 30 min in ice-cold water before centrifugation at 100,000 × *g* for 60 min at 4°. The supernate, operationally defined as our "solubilized preparation," was removed and saved. Occasionally, the pellets obtained after ultracentrifugation were reextracted in half the original volume of digitonin-containing buffer. However, this second extraction typically yielded less than 10% of the binding obtained from the first extraction.

Identification of α_2-adrenergic receptor in detergent-solubilized preparations. Characteristics of binding to detergent extracts of NG-108-15 cells were assayed as previously described for porcine brain α_2-adrenergic receptor (9). Either [^{3}H]yohimbine or [^{3}H]rauwolscine served as the radioligand to identify the α_2-adrenergic receptor, and nonspecific binding was defined as the amount of binding detected in the presence of 10 μM phentolamine. Most of the data presented

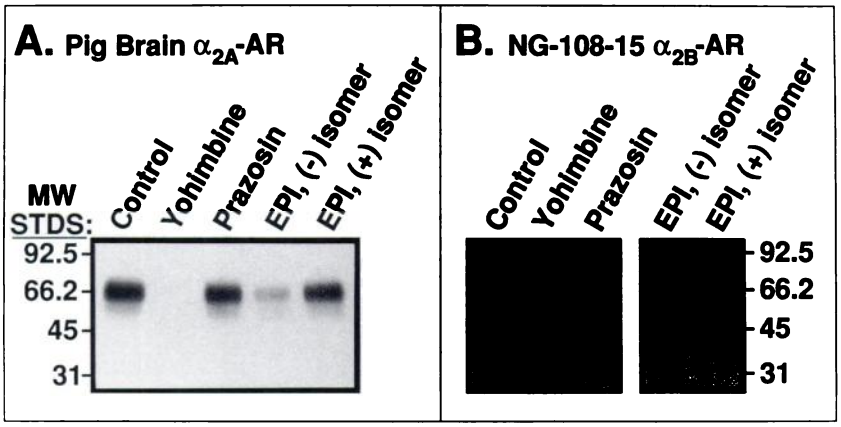

Fig. 1. α_2-Adrenergic receptor subtypes possess different structural and pharmacological properties. Partially purified α_2-adrenergic receptor (0.1–0.3 pmol) obtained from porcine brain cortex (α_{2A} subtype) (A) or NG-108-15 cells (α_{2B} subtype) (B) was incubated in a final volume of 150 μl with 2 nM of the α_2-adrenergic receptor-selective photoaffinity label ^{125}I-Rau-AzPEC, in 0.1% digitonin, 25 mM glycylglycine, 100 mM NaCl, 5 mM EGTA, pH 7.6, for 3 hr at 15° in the dark, in the absence or presence of 1 μM yohimbine, 1 μM prazosin, 50 μM (−)-epinephrine (*EPI*), or 50 μM (+)-epinephrine. Just before the photolysis reaction, 1 mM reduced glutathione was added to scavenge any reactive intermediates that might dissociate from the ligand binding site during subsequent photolysis, to minimize nonspecific labeling outside of the adrenergic ligand binding pocket. Each sample was photolyzed in a quartz tube by irradiation at 300 nm for 3 min at 4°, in a Rayonet photochemical mini-reactor. The samples were then subjected to SDS-PAGE and subsequent autoradiography.

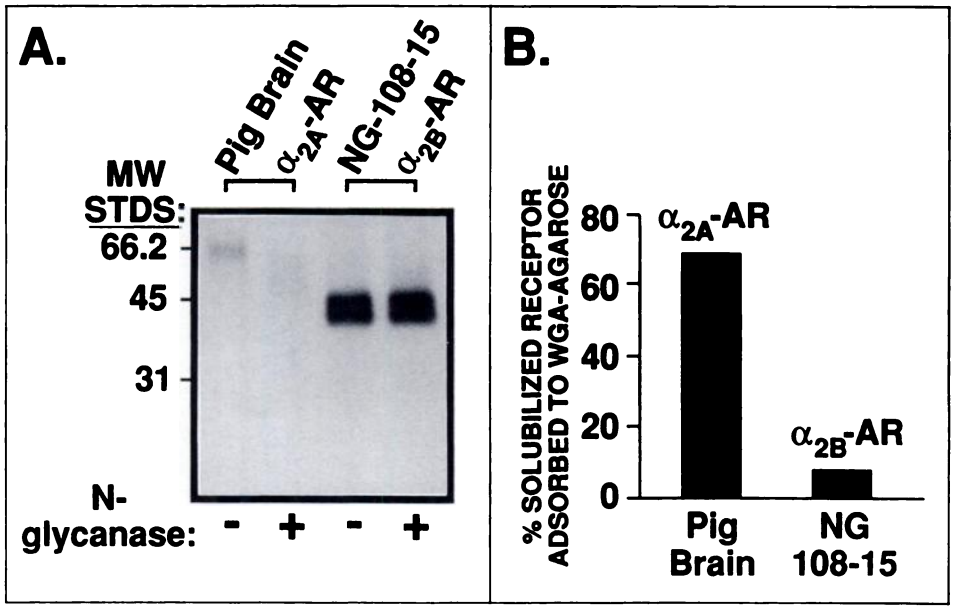

Fig. 2. α_2-Adrenergic receptor (α_2-*AR*) subtypes possess different glycosylation properties. (A) Preparations of porcine α_2-adrenergic receptor (approximately 280 fmol) that were partially purified by yohimbine-agarose affinity chromatography (9) and subsequent WGA-agarose chromatography (17), as well as NG-108-15 α_2-adrenergic receptor (approximately 95 fmol) that was purified by yohimbine-agarose affinity chromatography, were photolabeled as described in the legend to Fig. 1 and treated with *N*-glycanase, an enzyme that cleaves *N*-linked carbohydrate moieties from proteins (A). Receptor preparations were incubated with 1 unit of *N*-glycanase for 14 hr (overnight) at 23°, in a buffer consisting of 0.1% digitonin, 200 mM $NaPO_4$, 10 μM phentolamine, and 10 ng/μl soybean trypsin inhibitor, pH 8.6. An additional 1 unit of *N*-glycanase was then added to the incubation the following morning, and the deglycosylation reaction was allowed to continue for an additional 10 hr at 23°. The samples were then subjected to SDS-PAGE and subsequent autoradiography. (B) Partially purified porcine α_2-adrenergic receptor and digitonin-solubilized NG-108-15 α_2-adrenergic receptor preparations were added to WGA-agarose (10 volumes of receptor preparation/1 volume of resin) and rotated at 4° for 2 hr, in the presence of 0.2% digitonin, 50 mM HEPES, 500 mM NaCl, 5 mM EGTA, and 100 μM phentolamine, pH 7.6. The resin was then separated from the supernatant receptor preparation by centrifugation. Samples of the starting materials loaded onto each lectin, as well as samples of the supernates (i.e., nonadsorbed receptor), were assayed for [^{3}H]yohimbine binding as described in Experimental Procedures.

were obtained using [^{3}H]rauwolscine, because we learned during the course of our studies that its 3-fold higher affinity at α_{2B} receptors [0.68 nM (3)], compared with that of [^{3}H]yohimbine [1.76 nM (3)], resulted in greater ease of detection of ligand binding, using Sephadex G50 chromatography to resolve bound from free radioligand (9). The slightly lower affinity for [^{3}H]yohimbine observed in our studies (6.9 nM), compared with that in previous reports by Bylund *et al.* (3) (0.68 nM), is due to the pH of the incubations (pH 8.0 rather than 7.4) used in our assays for detection of ligand binding to detergent-solubilized receptors.

Yohimbine-agarose chromatography. Solubilized preparations of NG-108-15 α_2-adrenergic receptor, containing approximately 1×10^6 cpm of [^{3}H]rauwolscine binding activity (detected at 6.25 nM radioligand), were adsorbed to 2 ml of yohimbine-agarose, at a flow rate of 10 ml/hr, in a 4- × 0.7-cm siliconized column (Bio-Rad catalog no. 7370704). The affinity resin was then washed with 0.1% digitonin, 50 mM HEPES, 25 mM glycylglycine, 100 mM NaCl, 5 mM EGTA, pH 8, for 1 hr at a flow rate of 10 ml/hr, in order to remove loosely associated proteins. This wash was followed by a 1-hr wash (10 ml/hr) with the same buffer but without NaCl. The resin was then washed with 0.1% digitonin, 50 mM HEPES, 25 mM glycylglycine, 0 mM NaCl, 5 mM EGTA, 10^{-4} M phentolamine, pH 8, for 1 hr at 10 ml/hr, in order to equilibrate the resin with the α-adrenergic antagonist phentolamine under conditions (no NaCl) where elution in the presence of the adrenergic ligand does not occur. The α_2-adrenergic receptor was then eluted from the affinity resin with 0.1% digitonin, 50 mM HEPES, 25 mM glycylglycine, 600 mM NaCl, 5 mM EGTA, 10^{-4} M phentolamine, pH 8, at a flow rate of 3 ml/hr.

Phentolamine was removed from 50-μl aliquots of the column eluates, by exchange over 0.5 × 7-cm Sephadex G50 columns (9), before assay of [^{3}H]yohimbine binding to determine receptor binding activity in column fractions (9). Before the detailed study of the properties of ligand binding to the partially purified α_2 receptor preparation, 2-ml aliquots of the column eluates were exchanged into 0.1% digitonin-containing buffers, using 1.0- × 30-cm Sephadex G50 columns (9).

SDS-PAGE. All SDS-polyacrylamide gels were 7.5–20% gradient gels prepared by the method of Scherer *et al.* (13), with the exception that urea was omitted. Samples were prepared for electrophoresis by the addition of SDS-PAGE sample buffer to achieve final concentrations of 1.7% SDS, 33% glycerol, 0.17 M Tris·HCl, and 2 mM dithiothreitol. Gels were stained with Coomassie blue R250 to identify the molecular weight markers, dried, and subjected to autoradiography.

Results and Discussion

Comparison of the properties of photoaffinity labeling of α_2-adrenergic receptors derived from porcine brain cortex (α_{2A} subtype) and NG-108-15 cells (α_{2B} subtype) confirms their differences in antagonist selectivity, as well as revealing prominent structural differences in these two receptor subtypes. Whereas both receptors are protected from photolabeling stereoselectively by the (−) versus the (+)-stereoisomer of epinephrine and by the archetypical α_2-adrenergic antagonist yohimbine, the antagonist prazosin selectively decreases labeling of the α_{2B} subtype expressed in NG-108-15 cells (Fig. 1B), with little or no effect on the α_{2A} subtype purified from porcine brain cortex (Fig. 1A). Competition binding data with a variety of other adrenergic ligands confirm previous reports (3) of the α_{2B} selectivity of the α_2 receptor expressed in NG-108-15 cells, compared with the pharmacological selectivity of the α_{2A} receptor purified from porcine brain, notably the greater potency of the agonist oxymetazoline in competing for [^{3}H]yohimbine

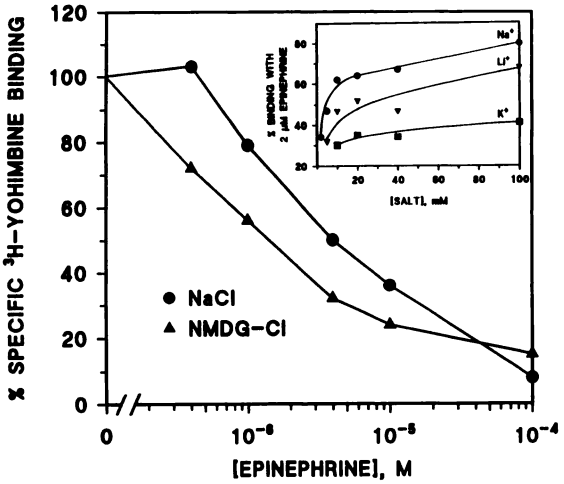

Fig. 3. Monovalent cations modulate epinephrine competition for radiolabeled antagonist binding. Digitonin-solubilized α_2-adrenergic receptors (approximately 30 fmol/incubation) from NG-108-15 cells were incubated in the presence of 10 nM [^{3}H]yohimbine and the indicated concentrations of epinephrine, in buffer containing 0.1% digitonin, 25 mM HEPES, 25 mM glycylglycine, 100 mM NaCl or 100 mM NMDG, 1 mM EGTA, and 10 mM $MgCl_2$, pH 8.0. Incubations were for 90 min at 15° and were terminated by G-50 column chromatography to separate bound from free radioligand. Nonspecific binding was taken to be that amount of [^{3}H]yohimbine bound in the presence of 10 μM phentolamine. Binding is expressed as percentage of control specific binding, which was typically 2400 cpm. The specificity of monovalent cations (*inset*) in influencing epinephrine competition for [^{3}H]yohimbine binding was evaluated by performing these experiments in the presence of a single final epinephrine concentration of 2 μM, with 0, 10, 20, 40, and 100 mM NaCl, LiCl, or KCl and the appropriate concentration of NMDG-Cl to maintain a constant salt concentration. These data are from one experiment, which is representative of three similar experiments performed in triplicate. In all cases, addition of Na^+ caused an apparent decrease in agonist potency, which varied from 2.5-fold (the data shown) to 5-fold.

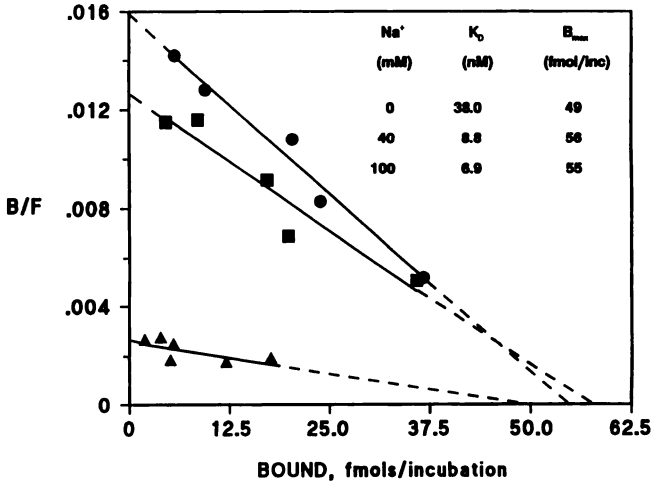

Fig. 4. Na^+ increases α_{2B}-adrenergic receptor affinity for the antagonist [^{3}H]rauwolscine. Digitonin-solubilized preparations of NG-108-15 α_2-adrenergic receptor were exchanged into Na^+-free buffer as described in Experimental Procedures and incubated with increasing concentrations of [^{3}H]rauwolscine for 90 min at 15°, in the presence of 0 (▲), 40 (■), or 100 (●) mM NaCl and appropriate concentrations of NMDG-Cl to maintain the added salt concentrations constant. The K_D and B_{max} values shown were obtained by linear regression. The data are from one experiment, which is representative of three separate experiments performed in triplicate. Similar effects of Na^+ were observed for [^{3}H]yohimbine binding to the NG-108-15 α_{2B} receptor. Thus, the K_D for [^{3}H]yohimbine was 15.7 nM in 0 NaCl (100 mM NMDG-Cl) and 6.1 nM in 100 mM NaCl. *B/F*, bound/free.

binding to porcine brain α_{2A} receptors (K_i = 4.0 nM), compared with NG-108-15 α_{2B} receptors (K_i = 0.6 μM) (data not shown).

Fig. 1 also reveals marked structural differences between the α_{2A} and α_{2B} subtypes. The relative migration of the α_{2A} receptor purified from porcine brain corresponds to an estimated M_r of approximately 68,000, whereas the α_{2B} receptor derived from NG-108-15 cells has an M_r of approximately 45,000. For the α_{2A} subtype, it is known that the apparent molecular size of this receptor on SDS-PAGE is influenced by glycosylation, because the gene coding for the α_{2A} subtype codes for a protein with a molecular weight of 48,981 (14, 15). This receptor also possesses two asparagine concensus sites for *N*-linked glycosylation in the amino-terminal stretch that is predicted to be expressed on the exofacial domain of target cells. The interpretation that the α_{2A} receptor is glycosylated is corroborated by the findings that the α_{2A} subtype can adsorb to WGA-agarose (Fig. 2B) and that the apparent molecular size of the receptor is reduced by treatment with deglycosylating enzymes, such as *N*-glycanase (Fig. 2A). In contrast, as shown in Fig. 2A, similar treatment of the NG-108-15 receptor with *N*-glycanase does not alter the migration of this receptor on SDS-PAGE. Similarly, the α_{2B}-adrenergic receptor of NG-108-15 cells does not adsorb to WGA-agarose, whereas the α_{2A}-adrenergic receptor subtype does (Fig. 2B). These findings with *N*-glycanase are consistent with previous reports for α_{2B} receptors obtained from fetal rat lung (5) and with the lack of concensus sites for *N*-linked glycosylation in the predicted amino acid sequence obtained for the α_{2B} receptor (6), suggesting that this subtype is not glycosylated *in situ*.

A primary goal of the present studies was to establish whether the allosteric regulation of α_2-adrenergic receptor binding by monovalent cations and 5-amino-substituted amiloride analogs, which is characteristic of the α_{2A} subtype (8), is a property also of the α_{2B} subtype. Regulation of adrenergic binding to the α_{2A} subtype of α_2-adrenergic receptors by monovalent cations is manifest in a variety of ways, including an apparent decrease in potency of agonists in competing for [^{3}H]yohimbine binding and an apparent increase in the receptor affinity for antagonists. As noted in Figs. 3 and 4, apparent changes in receptor binding affinity for adrenergic ligand due to monovalent cations also are detected in preparations of α_{2B} receptors derived from NG-108-15 cells. Because quantitatively similar changes in apparent receptor affinity occur for agonists and antagonists, our data cannot discriminate between a monovalent cation-induced decrease in receptor affinity for agonist and an increase in receptor affinity for antagonist, or both. The monovalent cation selectivity for detected changes in agonist potency ($Na^+ > Li^+ > K^+$) resembles that described previously for the α_2-adrenergic receptor of the α_{2A} subtype (8). The observation that monovalent cations influence ligand binding to adrenergic receptors of the α_{2A} and α_{2B} subtypes with a similar selectivity ($Na^+ > Li^+ > K^+$) and potency (EC_{50} = 2–5 mM Na^+) is of particular interest, because the highly glycosylated α_{2A} receptor and the nonglycosylated α_{2B} receptor offer very different charge-shielding potentials to monovalent cations. The observation that monovalent cations influence α_2-adrenergic receptor interactions at both subtypes indicates that the allosteric effect elicited by monovalent cations relies on interactions with the protein, rather than the carbohydrate, moiety of the α_2-adrenergic receptor. This interpretation is further substanti-

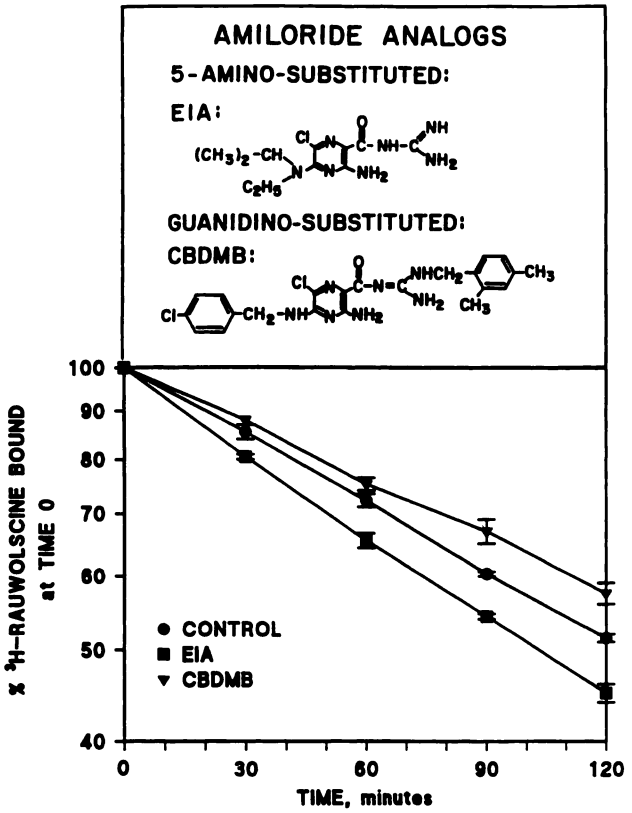

Fig. 5. Dissociation of [^{3}H]rauwolscine from α_{2B}-adrenergic receptors is accelerated by 5-amino-substituted analogs of amiloride. Association of digitonin-solubilized NG-108-15 α_2 receptors with 6.25 nM [^{3}H]rauwolscine was performed in buffer containing 0.2% digitonin, 25 mM glycylglycine, 40 mM HEPES, 100 mM NMDG-Cl, and 5 mM EGTA, pH 8.0, for 1 hr at 15°. The reaction was then transferred to a 10° water bath for 30 min to reequilibrate the incubation temperature prior to monitoring the rate of [^{3}H]rauwoiscine dissociation which occurred at 10°. The dissociation phase of the reaction was then initiated by the addition of unlabeled yohimbine to a final concentration of 50 μM. Amiloride analogs were then added, for determination of their effectiveness in influencing the rate of [^{3}H]rauwolscine dissociation. Control incubations were treated with the analog diluent, dimethylformamide. EIA and CBDMB were present at 100 μM. Data are presented as percentage of [^{3}H]rauwolscine bound at time 0, where 100% was 4500 cpm of specific binding. The data are expressed as the means ± standard errors of three separate experiments performed in triplicate. Statistical analysis, performed on the 90-min time points, indicated that the extent of dissociation in the presence of both EIA and CBDMB was significantly different from the control at a confidence level of >95%, as analyzed by the Student's *t* test.

ated by our own recent findings, which suggest that the aspartate residue that is predicted to lie in the inner lamella of the second transmembrane helix [corresponding to Asp-79 of the human (14) and porcine (15) α_{2A} receptor and to Asp-63 of the rat α_{2B} receptor (16)] plays a crucial role in monovalent cation regulation of adrenergic binding. This conclusion is based on the observation that substitution of Asp-79 with an asparagine residue by site-directed mutagenesis of the porcine α_{2A} receptor gene results in the loss of all manifestations of cation regulation of α_2-adrenergic receptor binding, including decreased potency of epinephrine in competing for [^{3}H]yohimbine binding, increased affinity for [^{3}H]yohimbine as revealed in saturation binding analysis, and facilitation of [^{3}H]yohimbine dissociation by monovalent cations (16).

Another way in which allosteric regulation of adrenergic ligand binding by monovalent cations has been monitored in the past (8) is by the ability of Na^+ to facilitate [^{3}H]yohimbine dissociation from the α_2-adrenergic receptor. This facilitated dissociation possesses an EC_{50} of approximately 2 mM NaCl and a selectivity of $Na^+ > Li^+ > K^+$, resembling the properties that are characteristic of cation-mediated decreases in epinephrine potency in competing for radiolabeled antagonist binding. In a sense, this acceleration of radiolabeled antagonist dissociation from the receptor is anomalous, because Na^+ increases α_2-adrenergic receptor affinity for antagonists (Fig. 4), which might be predicted to be manifested as a decrease in the rate of dissociation of these antagonists from the receptor. However, for the α_{2A}-adrenergic receptor of human platelet (7) and porcine brain cortex (8), Na^+ facilitates the rate of radioligand association as well as the rate of dissociation, and the greater effect on the association rate results in a net increased affinity for steady state binding of these antagonists. In contrast to these findings for the α_{2A} receptor, we detected no effect of Na^+ on the rate of [^{3}H]yohimbine or [^{3}H]rauwolscine dissociation from α_{2B}-adrenergic receptor derived from NG-108-15 cells. At present, we have no explanation for why Na^+ facilitates the rate of dissociation in the α_{2A}- but not the α_{2B}-adrenergic receptor subtype.

To evaluate the allosteric effect of amiloride analogs on adrenergic binding to the α_{2B}-adrenergic receptor of NG-108-15 cells, we examined whether these agents could alter the rate of [^{3}H]rauwolscine dissociation from the receptor. For these studies, receptor is incubated with [^{3}H]rauwolscine until steady state binding is achieved. Dissociation of the radioligand is monitored by addition of a large molar excess of unlabeled adrenergic agent, in this case 50 μM yohimbine, to the incubation to compete for radioligand rebinding to the receptor, once the radioligand has dissociated. Because 50 μM yohimbine will fully occupy all adrenergic sites (K_D for yohimbine = 7 nM), any effect of amiloride analog is interpreted as being due to interaction of these analogs at another, or "allosteric," site, which influences receptor affinity for antagonists. Fig. 5 demonstrates that the 5-amino-substituted analog of amiloride, EIA, accelerates the rate of dissociation of [^{3}H]rauwolscine from the receptor. Similar findings were observed for methylisobutyl amiloride, another 5-amino-substituted analog (data not shown). In contrast, the guanidino-substituted analogs of amiloride, CBDMB and dichlorobenzamil (not shown), do not accelerate the rate of dissociation of [^{3}H]rauwolscine from the α_{2B}-adrenergic receptor of NG-108-15 cells. In fact, CBDMB appears to slow the rate of dissociation. This preferential ability of 5-amino-substituted analogs, when compared with guanidino-substituted analogs of amiloride, to accelerate dissociation of radiolabeled antagonists from the α_2 receptor also is observed for allosteric modulation of the α_{2A} subtype, although CBDMB does not statistically significantly retard the rate of dissociation at this subtype (8). The finding that allosteric modulation of adrenergic ligand binding by amiloride analogs also occurs at α_2-adrenergic receptors of the α_{2B} subtype suggests that the domain that forms the allosteric binding pocket for amiloride analogs is defined by sequences or, more likely, tertiary structures that are conserved between the α_{2A}- and α_{2B}-adrenergic receptor subtypes.

Conclusions

The present studies demonstrate that the α_{2B}-adrenergic receptor subtype, at least as expressed in NG-108-15 cells,

possesses allosteric regulation of adrenergic binding by monovalent cations and 5-amino-substituted analogs of amiloride. Recent mutagenesis studies have implicated an aspartate residue [Asp-79 in the α_{2A} subtype (16)] as playing a crucial role in monovalent cation regulation of α_2 receptor-adrenergic ligand interactions, and an aspartate predicted to lie in a topographically identical position [Asp-63 (6)] has been detected in the derived amino acid sequence from a rat cDNA encoding an α_{2B} subtype receptor. Because modulation of α_2-adrenergic receptor binding by 5-amino-substituted amiloride analogs is unaltered in the mutant in which Asp-79 is substituted with an asparagine residue, it is clear that the domain involved in allosteric modulation of monovalent cations is distinct from that which mediates the effects of amiloride analogs on adrenergic binding. Although a comparison of the primary sequences of α_{2A} and α_{2B} subtypes can indicate those sequences common to both receptors and thus reveal "candidate sequences" for an allosteric regulatory domain, it is likely that the allosteric binding pocket for amiloride analogs is created by multiple noncontiguous stretches of amino acid sequence and that more direct biochemical studies will be necessary to refine our understanding of the residues involved in creating this allosteric binding pocket in α_2-adrenergic receptors.

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