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ACCELERATED COMMUNICATION

Novel Amiloride Analog Allosterically Modulates the α_2-Adrenergic Receptor but Does Not Inhibit Na^+/H^+ Exchange

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SUMMARY

Two novel amiloride analogs have been synthesized during the course of efforts to develop a photoaffinity label for the amiloride allosteric domain on α_2-adrenergic receptors. One of these, 5-[*N*-2′-aminoethyl-*N*′-isopropyl]amiloride-*N*-[4″-azidosalicylamide] (A-EIA-AS), markedly accelerates the rate of dissociation of [^{3}H]yohimbine from affinity-purified α_2-adrenergic receptors, an assay for allosteric modulation of receptor-adrenergic ligand interactions. In contrast, this agent does not appreciably inhibit Na^+/H^+ exchange, measured as 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIA)-inhibitable $^{22}Na^+$ uptake into cultured renal epithelial cells. A second analog, 5-[*N*-2′-(4″-azidosalicylamidino)ethyl-*N*′-isopropyl]amiloride (ASA-EIA), does not foster an accelerated rate of dissociation of [^{3}H]yohimbine binding from the α_2 receptor but does block the ability of A-EIA-AS to do so, suggesting that ASA-EIA and A-EIA-AS interact at a common binding site. Interestingly, the ability of EIA to accelerate [^{3}H]yohimbine dissociation is not blocked by ASA-EIA, a finding that may indicate that EIA and A-EIA-AS allosterically modulate α_2 receptor-ligand interactions via distinct or nonoverlapping binding sites.

The α_2-adrenergic receptor is one of a family of seven membrane-spanning receptors that are linked to the inhibition of adenylate cyclase via an inhibitory GTP-binding protein. It has been demonstrated previously that adrenergic ligand binding to the α_{2A} and α_{2B} subtypes of this receptor, as well as the D_2 dopamine receptor, can be modulated allosterically by monovalent cations and amiloride analogs (1–5). Mutagenesis studies have shown that the allosteric regulatory site on the α_2 receptor for cations is distinct from that for amiloride analogs (6). Thus, mutation of Asp^{79} of the α_2 receptor to an asparagine results in a complete loss of allosteric regulation of the receptor by cations (6), without perturbing the ability of amiloride analogs to modulate allosterically receptor interactions with adrenergic agents.

In order to refine our understanding of allosteric modulation of the α_2 receptor by amiloride analogs, we wish to map this domain of the α_2-adrenergic receptor biochemically, as a prelude to rational mutagenesis studies intended to evaluate the functional relevance of allosteric modulation of receptor-ligand interactions by amiloride analogs.

Previous studies have demonstrated that analogs of amiloride that block Na^+/H^+ exchange, e.g., the 5-amino-substituted analogs EIA and MIA, also allosterically regulate adrenergic ligand binding to the α_2-adrenergic receptor, whereas analogs that do not block Na^+/H^+ exchange, e.g., the guanidino-substituted analog DCB and the guanidino- and 5-amino-substituted analog CBDMB, are less effective in modulating the α_2 receptor, at equimolar concentrations (1, 2, 7). Unfortunately, many of the available amiloride analogs that have been used to modify the epithelial Na^+ channel covalently (8) are modified on the guanidino moiety and lack substitution at the 5-amino position. In addition, 5-amino-substituted analogs that have been used to modify the renal Na^+/H^+ exchanger either are not radiolabeled or do not possess a photoactivatable moiety (9, 10). Consequently, we have attempted to develop a photoactivatable, radioiodinated, amiloride analog to label covalently the allosteric regulatory site on α_2-adrenergic receptors, with the specificity characteristic of the functional effects elicited by

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ABBREVIATIONS: EIA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; DCB, 3′,4′-dichlorobenzamil; CBDMB, 5-(*N*-4-chlorobenzyl)-2′,4′-dimethylbenzamil; A-EIA-AS, 5-[*N*-2′-aminoethyl-*N*′-isopropyl]amiloride-*N*-[4″-azidosalicylamide]; ASA-EIA, 5-[*N*-2′-(4″-azidosalicylamidino)ethyl-*N*′-isopropyl]amiloride; DPBS, Dulbecco's phosphate-buffered saline; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

amiloride analogs. In the course of developing such a covalent label, we have synthesized a variety of novel amiloride analogs. Examination of these analogs with respect to their ability to modulate adrenergic ligand binding to the α_2-adrenergic receptor, compared with modification of Na^+/H^+ exchange activity, has led to two interesting and unanticipated observations. First, an analog (dubbed A-EIA-AS) was developed that altered adrenergic ligand binding to the α_2-adrenergic receptor but did not block Na^+/H^+ exchange in renal epithelial cells in culture. This is the first indication that an amiloride analog can modulate adrenergic ligand binding to the α_2 receptor without having overlapping effects at the Na^+/H^+ exchanger. Second, an analog (ASA-EIA) was developed that did not allosterically modulate binding to the α_2 receptor but, nonetheless, was able to block the allosteric regulatory effects of A-EIA-AS. In contrast, ASA-EIA did not block the allosteric regulatory effects elicited by EIA. These latter observations suggest either that there is more than one amiloride analog binding site on the α_2 receptor or that nonoverlapping sites in one binding pocket exist for these modulatory agents.

Experimental Procedures

Materials

The synthesis of the novel amiloride analogs A-EIA-AS and ASA-EIA will be described elsewhere.[1] EIA, DCB, and CBDMB were synthesized as previously described (7, 11) and purchased from Dr. E. J. Cragoe, Jr. The yohimbine-agarose affinity resin was synthesized in our laboratory as previously described (12). [^{3}H]Yohimbine, *p*-[^{125}I] iodoclonidine, and $^{22}Na^+$ were purchased from Du Pont-New England Nuclear. Cell culture medium was obtained from GIBCO Laboratories. Fetal calf serum was from Sigma. The porcine kidney cell line $LLCPK_1/Cl_4$ was generously provided by Dr. Carolyn Slayman (Yale University). Transwell filter dishes (24 mm) were purchased from Costar.

Methods

Transfection and expression of the α_2-adrenergic receptor in $LLCPK_1/Cl_4$ cells. $LLCPK_1/Cl_4$ cells were co-transfected with an expression plasmid ($pCMV_4$) containing the α_2-adrenergic receptor gene (13) and a plasmid containing a neomycin resistance gene, using a standard calcium phosphate transfection protocol (14). Neomycin-resistant clones were screened for α_2-adrenergic receptor binding with [^{3}H]yohimbine and *p*-[^{125}I]iodoclonidine. A clone that overexpressed the α_2 receptor (referred to throughout the text as the $LLCPK_1$/O-clone) was selected as a source from which to purify the α_2-adrenergic receptor.

Culture of the $LLCPK_1/Cl_4$ parental cell line and $LLCPK_1$/O-clone. The parental $LLCPK_1/Cl_4$ and transfected $LLCPK_1$/O-clone cell lines were maintained in α-minimal essential medium with 10% fetal calf serum, 100 IU of penicillin, and 100 μg of streptomycin, in an atmosphere of 5% CO_2/95% air. The parental $LLCPK_1/Cl_4$ cell line was used for Na^+/H^+ exchange experiments in which the ability of amiloride analogs to block ^{22}Na uptake was assessed. For these experiments, $LLCPK_1/Cl_4$ cells were plated on 0.4-μm Transwell filter dishes, at a density of 2×10^5 cells/24-mm well (day 0). The medium was replaced with fresh medium on day 4 and every day thereafter. The cells were typically used on day 7, 8, or 9.

The $LLCPK_1$/O-clone cells were used as a source for purification of the porcine brain α_2-adrenergic receptor. Cells were plated on 150-mm dishes, at a density of 1.8×10^6 cells/dish. Cells were typically grown for 4 days and were intentionally allowed to grow on top of one another, to conserve plasticware and medium.

Solubilization and purification of the α_2-adrenergic receptor. $LLCPK_1$/O-clone cells were harvested by removal of the medium, placement of plates on ice, and pipetting of 10 ml of DPBS (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. The DPBS was then removed, and 10 ml of DPBS containing 1 mM EDTA were added. Cells were gently resuspended with a rubber policeman, pooled, and transferred to 50-ml Sorvall tubes. The tubes were centrifuged in an SS 34 rotor at 4° for 10 min at 18,000 rpm, in a Sorvall RC-5B centrifuge. The supernatant liquid was discarded, and each pellet was resuspended, using a transfer pipet, in 10 ml of 15 mM HEPES, 5 mM EGTA, 5 mM EDTA, pH 8, 10^{-5} M phenylmethylsulfonyl fluoride. Lysates were then pipetted up and down 20 times through a 20-gauge needle. The particulate preparations were then pooled into four 50-ml Sorvall tubes and centrifuged as described above. The supernatant liquid was discarded, and the pellets were resuspended in 5 ml of 25 mM glycylglycine, 20 mM HEPES, 40 mM NaCl, 5 mM EGTA, pH 8 (membrane buffer), using a transfer pipet, and were then transferred to a Teflon-glass homogenizer. The tube was then rinsed with 2 ml of membrane buffer, and the rinse was added to the homogenizer. Membranes were homogenized by 10 up and down passes on ice, in the homogenizer. The membranes were then pooled and assayed by incubation of 10 μl of the preparation with 7.5 nM [^{3}H]yohimbine for 30 min at 25°. Bound radioligand was separated from free by vacuum filtration over Whatman GF-B filters. The estimates of binding were determined in order to optimize the detergent/receptor ratio for solubilization.

Membranes were then placed in 50-ml Sorvall tubes and centrifuged at 4° for 30 min at 18,000 rpm. Each pellet (corresponding to approximately 400 pmol of α_2 receptor binding) was resuspended in 5 ml of solubilization buffer (1% digitonin, 25 mM glycylglycine, 20 mM HEPES, 100 mM NaCl, 5 mM EGTA, pH 8). The membranes were then transferred to a Teflon-glass homogenizer, and a 2-ml rinse of solubilization buffer was added to each tube. The receptor was extracted into the digitonin-containing buffer by homogenization with 10 up and down passes, followed by sonication for 30 min on ice in a bath sonicator. Before sonication, the solubilized preparation was diluted to an approximate receptor concentration of 6.25 pmol/ml. The digitonin extract was centrifuged at 29,000 rpm (100,000 × *g*) in a type 30 rotor for 1 hr at 4°, in a Sorvall OTD55B ultracentrifuge. The supernatants of this centrifugation were operationally defined as the "solubilized receptor preparations." Soluble α_2-adrenergic receptor binding was assayed as previously described (12).

Purification of α_2-adrenergic receptor. Digitonin-solubilized preparations of the α_2-adrenergic receptor (approximately 150×10^6 cpm of [^{3}H]yohimbine binding activity detected at 7.5 nM radioligand, i.e., approximately 2 nmol of binding activity) were adsorbed to 4 ml of yohimbine-agarose, at a flow rate of 10 ml/hr, in a 15- × 0.9-cm (inner diameter) Pharmacia column, at 4°. It typically took 4 days to adsorb the entire volume of the receptor preparation. The affinity resin was then washed with 0.3% digitonin, 50 mM HEPES, 500 mM NaCl, 5 mM EGTA, pH 7.6, for 2.5 days, at a flow rate of 10 ml/hr. The resin was then washed for 2 hr at 10 ml/hr in 0.2% digitonin, 50 mM HEPES, 5 mM EGTA, pH 7.6, without added sodium. The resin was then equilibrated with the α_2 antagonist phentolamine, under conditions in which elution in the presence of the adrenergic ligand does not readily occur (i.e., wash with 0.2% digitonin, 50 mM HEPES, 0 mM NaCl, 5 mM EGTA, 10^{-4} M phentolamine, pH 7.6, for 2 hr at 10 ml/hr). The α_2-adrenergic receptor was then eluted from the affinity resin with 0.2% digitonin, 50 mM HEPES, 50 mM NaCl, 5 mM EGTA, 10^{-4} M phentolamine, pH 7.6, at a flow rate of 4 ml/hr. Phentolamine was removed by exchange over 0.5- × 7-cm Sephadex G-50 columns before eluates were assayed for [^{3}H]yohimbine binding, to determine receptor binding activity in the various column eluates (12). For the study of allosteric modulation of α_2 receptor binding, 2-ml aliquots of the column eluates were exchanged into 0.2% digitonin, 25 mM glycylglycine, 40 mM HEPES, 100 mM *N*-methyl-D-glucamine, 5 mM EGTA, pH 8, using 1.0- × 30-cm Sephadex G50 columns (12).

[1] C. Prakash, S. W. Womble, A. L. Wilson, L. E. Limbird, and I. A. Blair, manuscript in preparation.

Monitoring of the rate of [^{3}H]yohimbine dissociation. Association of 7.5 nM [^{3}H]yohimbine with yohimbine-agarose-purified α_2-adrenergic receptor was performed in 0.2% digitonin, 25 mM glycylglycine, 40 mM HEPES, 100 mM *N*-methyl-D-glucamine, 5 mM EGTA, pH 8, for 1 hr at 15°. The incubation temperature was then lowered to 10° for 30 min before initiation of the dissociation phase of the reaction by the addition of 50 μM unlabeled yohimbine. It was previously determined that the addition of even more yohimbine did not further alter the rate of [^{3}H]yohimbine dissociation, indicating that 50 μM yohimbine was sufficient to occupy fully the adrenergic binding pocket during the dissociation phase. The rate of [^{3}H]yohimbine dissociation was then monitored in the absence and presence of the various amiloride analogs, at the indicated concentrations. Because the adrenergic binding pocket was occupied fully by either [^{3}H]yohimbine or unlabeled yohimbine during the dissociation phase of the experiment, any influence of amiloride analogs on the rate of [^{3}H]yohimbine dissociation must be due to interaction at another, termed allosteric, site.

Bound radioligand was separated from free [^{3}H]yohimbine by Sephadex G50 chromatography (12). Separation times ranged from 12 to 20 min, depending on the age of the column. Nonspecific binding was defined as that [^{3}H]yohimbine binding detected in the presence of 10 μM phentolamine.

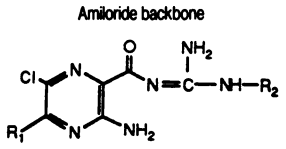
Monitoring of basolateral Na^+/H^+ exchange in $LLCPK_1/Cl_4$ cells. Tissue culture-treated, six-well, Transwell filter dishes (Costar, Cambridge, MA) were used as the substrate for $LLCPK_1/Cl_4$ cells. We examined basolateral rather than apical Na^+/H^+ exchange activity in $LLCPK_1/Cl_4$ cells because basolateral exchange is 300-fold more sensitive to inhibition by amiloride and its analogs than is apical Na^+/H^+ exchange and, thus, increases the sensitivity for detection of effects of novel amiloride analogs on this transport activity (15). The cells were plated at a density of 2×10^5 cells/filter, in α-minimal essential medium, under 5% CO_2/95% air and were grown in culture for 7 days, until confluence was obtained. The medium was changed on day 4 and then on every other day. A confluent monolayer was obtained around day 7 (15). For ^{22}Na uptake analysis, filter inserts were transferred to clean culture wells, and the remaining medium was removed by aspiration. The Transwell cultures were maintained at 37° during assay by placement of the plates on a Fisher plate warmer. To each compartment was added 1.5 ml of KMBS buffer (10 mM MOPS, 125 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM *N*-methyl-D-glucamine, pH 6.5) containing 5 μM nigericin. The nigericin was added to equilibrate extracellular and intracellular pH, and this set intracellular pH at pH 6.5. The plate was swirled gently for the first 20 sec of minutes 1, 2, and 3 and was kept at 37° for a total of 10 min. After this time, the KMBS-nigericin buffer was removed by aspiration and replaced with 1.5 ml of KMBS buffer containing 0.5% bovine serum albumin, to bind any residual nigericin, and 500 μM ouabain, to inhibit Na^+/K^+ ATPase activity before the $^{22}Na^+$ uptake phase of the experiment. The plate was again swirled gently for the first 20 sec of minutes 1, 2, and 3 and was kept at 37° for a total of 5 min. For measurement of $^{22}Na^+$ uptake on the basolateral side of the polarized $LLCPK_1/Cl_4$ cells, the Transwell membrane was gently lifted from the well and placed in another well containing 1.5 ml of the $^{22}Na^+$ uptake buffer. The composition of the $^{22}Na^+$ uptake buffer was 10 mM MOPS, 129 mM *N*-methyl-D-glucamine, 10 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4, 500 μM ouabain, and 1 μCi/ml $^{22}Na^+$. When evaluated, amiloride analogs were added directly to the $^{22}Na^+$ uptake buffer before transfer of the Transwell filter to the culture well. The plate was then swirled continuously for exactly 2 min. The buffer was then aspirated from each compartment, and the filter unit was gently washed three times in ice-cold 0.1 M $MgCl_2$. The cells were then solubilized with 1.5 ml of 0.5% sodium dodecyl sulfate that was placed in each compartment and swirled for 45 sec. The resulting liquid was removed and analyzed for radioactivity in a Beckman Gamma 4000 γ counter. Na^+/H^+ exchange activity was defined as amiloride-sensitive $^{22}Na^+$ uptake detected on the basolateral surface of polarized $LLCPK_1/Cl_4$ cells, which, based on the well characterized transport properties of the Cl_4 clonal cell line, is an appropriate operational definition for Na^+/H^+ exchange (15).

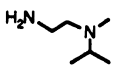
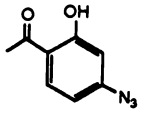
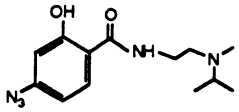
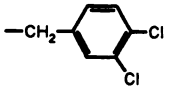
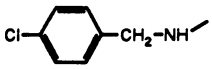
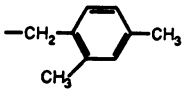
Results and Discussion

It has been shown previously that amiloride analogs that are substituted at the 5-amino position of the parent compound amiloride, such as EIA and MIA, accelerate the rate of [^{3}H]yohimbine dissociation from the α_2-adrenergic receptor. In contrast, DCB, a guanidino-substituted analog, and CBDMB, an analog substituted on both the 5-amino and guanidino moieties, do not appreciably accelerate dissociation when used at equimolar concentrations (1–3). The structures of the newly synthesized amiloride analogs (A-EIA-AS and ASA-EIA) are shown in Table 1 and are compared with the structures of the parent compound, amiloride, and of analogs previously characterized for their effects on the α_2-adrenergic receptor. The ability of amiloride analogs to alter the rate of [^{3}H]yohimbine dissociation from partially purified α_2-adrenergic receptors is utilized in our laboratory as a measure of the ability of regulatory agents to modulate allosterically adrenergic ligand binding to the α_2 receptor (see Experimental Procedures). Interestingly, A-EIA-AS, an analog that is substituted on both the 5-amino and guanidino moieties of the parent compound amiloride, accelerates the rate of dissociation much more dramatically than does EIA, which, to date, had been the most effective amiloride analog allosteric modulator of the α_2 receptor (Fig. 1A) (1–3). The effect of A-EIA-AS to accelerate the rate of [^{3}H]yohimbine dissociation from the α_2 receptor appears to be very rapid. Approximately 80% of the tritiated antagonist is dissociated from the receptor, in the experiments depicted in Fig. 1A, by the 20-min time point. Although it would be desirable to consider time points earlier than 20 min, it is technically unreasonable to do so, based on the limitation imposed by the time required to separate bound from free radioligand by G50 chromatography (see Experimental Procedures). In any case, the effect of A-EIA-AS appears to be qualitatively greater in rate and extent than previously observed for any amiloride analog modulation of the α_2 receptor. Concentration-response curves for the ability of A-EIA-AS, EIA, and ASA-EIA to accelerate the rate of [^{3}H]yohimbine dissociation are shown in Fig. 2. The EC_{50} values for A-EIA-AS and EIA appear to be very similar (approximately 40 μM and 50 μM, respectively), but A-EIA-AS appears to be more efficacious, because it elicits a greater maximal response. The concentration-response curve for A-EIA-AS also is particularly steep and may reflect a cooperative interaction of the amiloride analog-binding domain with the adrenergic ligand-binding pocket. In contrast to the effects of A-EIA-AS, ASA-EIA, an analog substituted solely at the 5-amino position of amiloride, does not detectably alter the rate of [^{3}H]yohimbine dissociation from the receptor. It should be noted that both A-EIA-AS and ASA-EIA possess the azidosalicylamide moiety. In ASA-EIA this moiety is attached at the 5-amino position, whereas in A-EIA-AS it is attached to the guanidino moiety. Thus, it probably is not only the azidosalicylamide attachment of A-EIA-AS that makes it an effective allosteric modulator of the α_2 receptor; the site of attachment of the azidosalicylamide moiety is also critical.

Given the previous finding that amiloride analogs that allosterically regulate the α_2-adrenergic receptor also block Na^+/H^+ exchange and that analogs that do not modulate the α_2 receptor are not effective blockers of Na^+/H^+ exchange (1–3, 7), it was

Fig. 1. Amiloride analog structures



Substituent		Abbreviation	Chemical name
5-Amino, R_1	Guanidino, R_2		
		A-EIA-AS	5-[*N*-2′-Aminoethyl-*N*′-isopropyl]amiloride-*N*-[4″-azidosalicylamide]
	H	ASA-EIA	5-[*N*-2′-(4″-Azidosalicylamidino)ethyl-*N*′-isopropyl]amiloride
	H	EIA	5-(*N*-Ethyl-*N*-isopropyl)amiloride
		DCB	3′,4′-Dichlorobenzamil
		CBDMB	5-(*N*-4-Chlorobenzyl)-2′,4′-dimethylbenzamil)

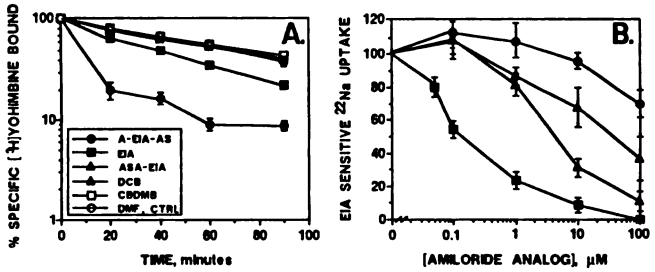


Fig. 1. A-EIA-SA allosterically modulates ligand binding to the α_2-adrenergic receptor but does not block Na^+/H^+ exchange. A, The rate of [^{3}H]yohimbine dissociation from the α_2-adrenergic receptor was evaluated as described in Experimental Procedures. The data shown represent the means ± standard errors from three experiments, each performed in triplicate, in which the ability of 100 μM A-EIA-AS, ASA-EIA, EIA, DCB, and CBDMB to alter the rate of [^{3}H]yohimbine dissociation from the α_2-adrenergic receptor was determined. B, The ability of A-EIA-AS (●), EIA (■), ASA-EIA (▲), and DCB (△) to block ^{22}Na uptake in $LLCPK_1/Cl_4$ cells at the indicated concentrations was evaluated (see Experimental Procedures). These data represent the means ± standard errors of three experiments. *DMF*, dimethylformamide.

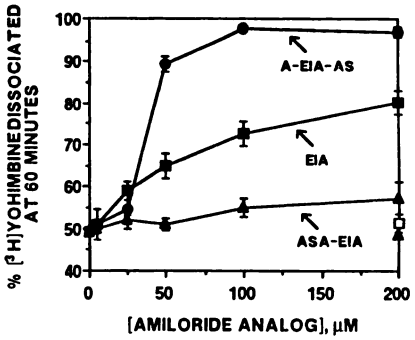


Fig. 2. A-EIA-AS and EIA have similar potencies, but A-EIA-AS is more efficacious with respect to its ability to modulate the α_2-adrenergic receptor allosterically. The ability of A-EIA-AS (●), EIA (■), ASA-EIA (▲), DCB (△), and CBDMB (□) to accelerate the rate of [^{3}H]yohimbine dissociation from the α_2-adrenergic receptor was evaluated, with the indicated concentrations of analog, 60 min after the initiation of the dissociation phase of the reaction (see Experimental Procedures). Data shown represent the means ± standard errors of three experiments performed in triplicate. The data are not normalized but, rather, are plotted as percentage of [^{3}H]yohimbine dissociated in the absence of amiloride analog (control). In this series of experiments, the extent to which [^{3}H]yohimbine dissociated from the receptor in the presence of 100 μM A-EIA-AS was slightly greater than that observed for the data shown in Fig. 1 (e.g., an average of 98% versus 91% dissociated at 60 min).

of interest to examine A-EIA-AS and ASA-EIA with respect to their ability to block Na^+/H^+ exchange. Surprisingly, A-EIA-AS does not significantly block Na^+/H^+ exchange, as monitored in the porcine kidney $LLCPK_1/Cl_4$ cell line, whereas ASA-EIA does, although not as potently as does EIA (Fig. 1B). The differential quantitative effects of A-EIA-AS for allosteric modulation of the α_2-adrenergic receptor versus blockade of Na^+/H^+ exchange are significant, because this is the first example of an amiloride analog that markedly modulates α_2 receptor-adrenergic ligand interactions but does not similarly inhibit Na^+/H^+ exchange. These data suggest that it is possible to design compounds that interact selectively with the amiloride analog binding site of the α_2 receptor while not having overlapping effects at the Na^+/H^+ exchanger, as a first step to evalu-

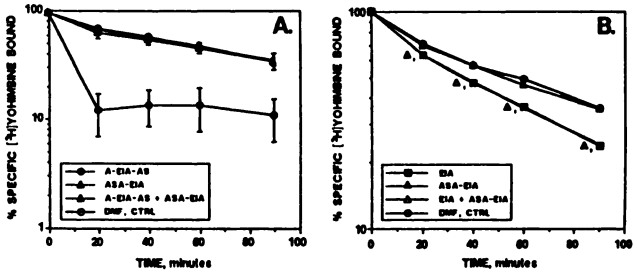


Fig. 3. ASA-EIA blocks the ability of A-EIA-AS but not EIA to modulate the α_2-adrenergic receptor allosterically. The rate of [^{3}H]yohimbine dissociation from the α_2-adrenergic receptor was evaluated (see Experimental Procedures) at the indicated times, in the presence of 50 μM A-EIA-AS, 200 μM ASA-EIA, or 50 μM A-EIA-AS and 200 μM ASA-EIA in combination (A) or 50 μM EIA, 200 μM ASA-EIA, or 50 μM EIA and 200 μM ASA-EIA in combination (B). Data shown are the means ± standard errors of three experiments performed in triplicate. Because the data in the presence of EIA alone and EIA plus ASA-EIA are superimposable, only one *line* can be drawn (△,■). A, Standard errors are shown. B, Standard error bars do not exceed the symbol size. Note that the ranges of the *ordinates* in A and B are different. *DMF*, dimethylformamide.

ating the effects of these compounds on α_2-adrenergic receptor-mediated functions.

The data in Fig. 3 suggest that the different effectiveness of A-EIA-AS and EIA for eliciting allosteric modulation of adrenergic ligand binding to the α_2 receptor may result from the ability of these two agents to interact with different allosteric sites on the receptor. Thus, although ASA-EIA does not accelerate the rate of [^{3}H]yohimbine dissociation, it can completely block A-EIA-AS-mediated acceleration of dissociation. ASA-EIA does not, however, alter the effect of EIA on α_2 receptor-ligand interactions. This differential protection by ASA-EIA from the effects of A-EIA-AS, but not of EIA, on allosteric modulation of α_2 receptor-ligand interactions suggests that A-EIA-AS and ASA-EIA interact with a site that is distinct from the EIA binding site. However, simultaneous addition of EIA and A-EIA-AS does not further enhance the effects of A-EIA-AS (data not shown). This apparent nonadditivity of EIA and A-EIA-AS may mean that nonoverlapping sites for differing amiloride analogs exist within a single binding pocket or that two allosteric modulatory sites do exist for amiloride analogs on the α_2 receptor but, once maximal conformational changes have been activated by A-EIA-AS, the independent but less dramatic effects of EIA cannot be detected.

Conclusions

The present findings demonstrate that a novel amiloride analog, dubbed A-EIA-AS, is able to accelerate markedly the dissociation of [^{3}H]yohimbine binding from the α_2-adrenergic receptor, an assay for allosteric modulation of adrenergic ligand-receptor interactions, without perturbing Na^+/H^+ exchange activity. These findings are important because the ability to modulate α_2-adrenergic receptor-ligand interactions without perturbing Na^+/H^+ exchange (Fig. 1B) or Na^+/Ca^{2+} exchange[2] provides an opportunity to examine the effects of amiloride analogs on α_2-adrenergic receptor-mediated functions without confounding effects due to blockade of Na^+ transport systems. In much the same way, the ability of ASA-EIA to block allosteric effects of A-EIA-AS provides a tool to examine the possible role of endogenous substances at this site as allosteric modifiers of α_2-adrenergic receptors, or of other receptors previously demonstrated to be allosterically modulated by amiloride or its analogs, such as D_2 dopamine receptors (4). Finally, the novel amiloride analogs described, A-EIA-SA and ASA-EIA, are both azido derivatives of amiloride and, thus, can be exploited to modify covalently the α_2-adrenergic receptor, for mapping of the amiloride regulatory domains and for exploration of whether, as suggested by ASA-EIA protection data, more than one modulatory binding pocket exists for amiloride analogs on the α_2-adrenergic receptor.

[2] The ability of A-EIA-AS to inhibit Na^+/Ca^{2+} exchange was evaluated by Dr. Fred Mandel, The Upjohn Co., in sarcolemmal preparations; A-EIA-AS was found to have no effect, when evaluated at concentrations up to 100 μM (unpublished observations).

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