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Decrease in Apparent *Alpha*$_1$-Adrenoceptor-G Protein Coupling During Maturation in Rat Aorta

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We have previously shown that the adrenoceptor agonist norepinephrine (NE) is more potent in eliciting contraction in aortas from 1-month-old Fischer 344 rats than it is in older animals. In the present study, we examined alpha$_1$*-adrenoceptor-guanine nucleotide regulatory binding protein (G protein) coupling in aortic membranes in order to investigate the mechanism for the age-dependent reduced responsiveness of aorta to NE. We used the guanosine 5′(βγ-imido)triphosphate (Gpp[NH]p)-induced shift in agonist binding affinity as a measure of the efficiency of* alpha$_1$*-adrenoceptor-G protein coupling. The binding of NE was assessed by measuring the displacement of 2-[β-(4-hydroxy-3-[*^{125}*I]iodophenyl)ethylaminomethyl] tetralone ([*^{125}*]-HEAT) by NE in aortic membranes. In 1-, 6-, and 24-month-old rat aortas, two apparent binding sites were detected in the competition isotherms for NE. This heterogeneous binding pattern was independent of Gpp(NH)p at all ages, and is likely to be due to a heterogeneous receptor population (*alpha$_{1a}$, alpha$_{1b}$, *and* alpha$_{1d}$ *subtypes). In 1-month-old rats, the high affinity binding of NE to* alpha$_1$*-adrenoceptors was sensitive to Gpp(NH)p, indicating a significant interaction between the receptor and G protein. This Gpp(NH)p-sensitive high affinity binding was not observed in aortas from 6- or 24-month-old animals. Despite the lack of Gpp(NH)p-sensitive high affinity binding of agonist in 6- or 24-month-old aortas, NE was still able to induce maximal contraction in these aortas, albeit, with a relatively low potency. A partial reduction in* alpha$_1$*-adrenoceptor-G protein coupling between 1 and 6 months of age can explain the observed decrease in agonist potency and the loss of Gpp(NH)p-sensitive high affinity binding of NE. This phenomenon can be explained as a reduction of allosteric coupling between the bindings of ligand and G protein to the receptor, that has been formulated in the ternary complex model. Computer simulation using the simple ternary complex model shows that manipulating the reciprocal coupling factor alone can lead to a loss of Gpp(NH)p-sensitive high affinity agonist binding, along with a reduction in agonist potency for contraction without altering the maximal response. Thus, a change in the relative expression of different* alpha$_1$*-adrenoceptor subtypes, which we have previously observed in the aorta, and which possess diverse intrinsic allosteric couplings, may be speculated to be the mechanism for the apparent reduction of* alpha$_1$*-adrenoceptor-G protein coupling during maturation.*

AGE-RELATED alteration in responsiveness to adrenergic neurotransmitter has been widely reported in various tissues (1,2). It has been shown that there is a decrease in the potency of norepinephrine (NE) to elicit vasocontraction in rat aorta during maturation (i.e., the period between 1 and 6 months (3–5). This does not seem to be due to a nonspecific alteration in contractility of the aorta, because the contractile responses to KCl or serotonin (5HT) are invariant with respect to age (Cai et al., 1994).

Three *alpha*$_1$-adrenoceptor subtypes have been cloned: *alpha*$_{1a}$, *alpha*$_{1b}$, *alpha*$_{1d}$, and mRNA for each has been detected in the rat aorta (6,7). The expression of *alpha*$_{1a}$, *alpha*$_{1b}$, and *alpha*$_{1d}$-adrenoceptors, increases, decreases and stays constant, respectively, during maturation in rat aorta, while the total number of *alpha*$_1$-adrenoceptors remains constant (8). Therefore, it is not possible to attribute the decrease in responsiveness to NE to an altered number of membrane receptors. Furthermore, decreased expression of aortic quanine nucleotide regulatory binding proteins (G proteins) cannot account for the decreased response (9,10). We therefore investigated receptor-G protein coupling, reduction of which can explain the reduced responsiveness to NE, by evaluating Gpp(NH)p sensitivity of agonist binding to *alpha*$_1$-adrenoceptors in 1-, 6-, and 24-month-old rat aortas. The possibility that an altered composition of *alpha*$_1$-adrenoceptor population during aging may lead to an apparent decrease in receptor-G protein coupling is discussed within the framework of the ternary complex model.

Methods

Animals.—Male Fischer 344 rats, 1, 6, and 24 months of age, were obtained from the National Center for Toxicological Research (Jefferson, AR) where they are bred and maintained under the auspices of the National Institute on Aging. Upon receipt at this institution animals were maintained for 1–2 weeks under barrier conditions comparable to those under which they were raised.

Contraction.—Rats were decapitated and the aorta was removed and placed in an ice-cold physiological salt solution (PSS) of the following composition: 120 mM NaCl; 4.7 mM KCl; 1.2 mM $MgCl_2$; 1.0 mM NaH_2PO_4; 2.5 mM $NaHCO_3$; 1.8 mM $CaCl_2$; 11 mM glucose; .024 mM EDTA. Vessels were cleaned of fat and connective tissue and cut into rings 3 mm wide. Aortic ring segments were mounted at 37°C in 15 ml organ baths using stainless steel hooks connected by fine gold chain at the bottom to a stationary glass rod attached to the bath and at the top to a Grass model

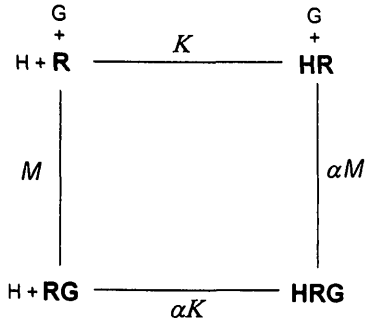
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FT.03 force-displacement transducer and bubbled continuously with 95% O_2/5% CO_2. Responses were recorded on a Grass Model 7 polygraph. Rings were equilibrated for 1 hour at a resting tension of 1.5 g. Concentration response curves were determined by cumulatively increasing the concentration of agonist. Rings were then washed extensively by several changes of PSS over 1–2 hours until tension stabilized at the precontraction level. The response to 60 mM KCl was obtained and the rings were subsequently blotted dry and weighed to normalize the NE concentration response curve.

Binding studies.—Rats were decapitated and the aorta was removed, placed in ice-cold 20 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 7.6) containing 154 mM NaCl (PBS), cleaned of fat and connective tissue, homogenized using a glass to glass homogenizer, and centrifuged at 500 × *g* for 10 min at 4°C. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C. The resulting pellet was resuspended, rehomogenized and then recentrifuged under the same conditions. The final pellet was suspended in PBS and used for binding assays. Protein content was measured according to the method of Bradford (11). Membranes were incubated-with 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl] tetralone ([^{125}I]-HEAT) (50–60 pM) in the presence or absence of 14–15 different concentrations of NE for 60 min at 37°C. Incubation volume was 0.25 ml of PBS containing 0.01 mg/ml of bovine serum albumin (20–30 μg membrane protein per tube). Displacement experiments with NE were performed in the absence or presence of Gpp(NH)p (0.1 mM). Reactions were terminated by rapid filtration using a Brandel cell harvester (Gaithersburg, MD) and Whatman, Inc. (Clifton, NJ) GF/B filters. Filters were washed four times with 4 ml of ice-cold PBS. The filter-bound radioactivity was determined in a Beckman gamma counter. Nonspecific binding was defined as binding in the presence of 0.1 mM prazosin or 1 mM NE with identical results. Assays were conducted in duplicate.

Data analysis and computer simulation.—Radioligand binding data were analyzed with the LIGAND program (12). A model for two binding sites was applied only when the LIGAND program fitted the data significantly better ($p < .05$) than the model for one binding site. Statistical significance was determined by an analysis of variance (ANOVA) followed by the post hoc Tukey test. A probability of .05 was considered significant.

The ternary complex model (13) shown in the figure was used to simulate the experimental results:



H, R, and G signify hormone, receptor and G protein, respectively. *K* and *M* represent the unconditional affinity for the formation HR and RG complexes, respectively, while αK and αM stand for their conditional counterparts (14). In this scheme, α is a measure of the reciprocal effect that hormone and G protein impart on the binding of each other (the allosteric coupling factor). Given the total concentrations of the components (H, R, and G), we calculated the equilibrium distribution of the free and complexed species numerically as described (15). We simulated the ligand binding as the total concentration of ligand-bound species (HR + HRG) and G protein activation as the total concentration of receptor-bound G protein (RG + HRG). In order to simulate the nucleotide effect on agonist binding, we calculated the binding in the presence and absence ($G_{total} = 2.10^{-11}$ and $G_{total} = 0$) of the G protein.

In the case of multiple receptors, we assumed that each receptor has its own G protein pool, which is equivalent to assuming a shared-pool of G protein where the density of G protein is in large excess with respect to receptor density.

Results

NE-induced contractions, in 1-, 6-, and 24-month-old aortas are shown in Figure 1*C*. Normalized with respect to responses induced by 60 mM KCl, NE-induced maximal responses were not significantly different among aortas from different ages. The EC_{50} of NE-stimulated contraction in 1-month-old aortas was 9 ± 3 nM, and this value increased significantly in older animals (40 ± 7 nM and 41 ± 6 in 6- and 24-month-old aortas, respectively, $p < .05$).

Figure 1*A* and 1*B* show competition binding isotherms of NE for [^{125}I]HEAT in the presence and absence of Gpp(NH)p. Since the binding curves of NE in 6- and 24-month-old aortas were indistinguishable (Figure 1), the data from 6-month-old aortas are only presented. A Gpp(NH)p-sensitive high affinity NE binding site [K_h = 25 nM) was observed only in 1-month-old aorta (Figure 1*A*). In the presence of Gpp(NH)p, however, a heterogeneous binding pattern for NE was detected in all ages, with high and low affinities (1 mo: k_h, 300 nM and k_l, 6,000 nM; 6 mo: k_h, 200 nM and k_l, 5,700 nM; 24-mo: k_h, 200 nM and k_l, 7,800 nM), respectively.

Computer simulations using the ternary complex model are shown in Figure 2. Manipulating the allosteric coupling factor (α) alone fits the phenomena observed in tissues of 1- and 6-month-old animals. The potency of agonist to stimulate G protein activation decreases while the maximal activation is unchanged (Figure 2*C*) and Gpp(NH)p-sensitive high affinity binding of agonist disappears with decreased coupling (compare Figure 2*A* and 2*B*). Inclusion of multiple receptors in the simulations (as imposed by the experimental data), did not change the general conclusion that can be drawn by using the simple model (data not shown).

Discussion

Previous studies showed that the potency of norepinephrine in eliciting aortic contraction is reduced during maturation (3–5). The reduced responsiveness may be due to a number of possible alterations in the excitation-

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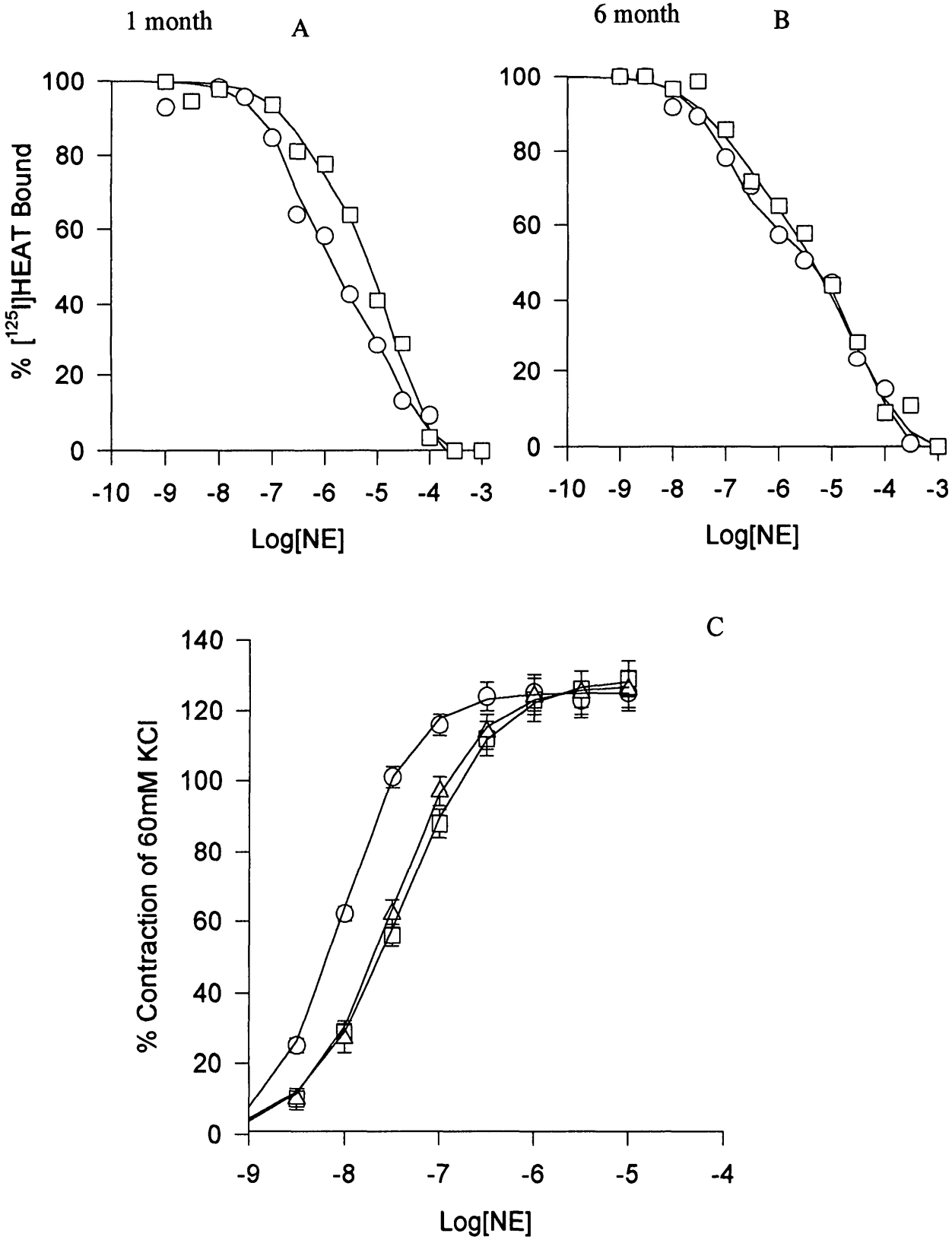


Figure 1. Inhibition of [^{125}I]-HEAT binding by NE in aortic membranes from (*A*) 1- and (*B*) 6-month-old animals. Aortic membranes were incubated with [^{125}I]-HEAT and the indicated concentrations of NE in the absence (○) or presence (□) of 0.1 mM Gpp(NH)p. Results are representative of three to four independent experiments. NE-stimulated contraction of aortic rings from 1- (○), 6- (□) and 24-mo (△)-old rats are shown in *C*. Data were normalized with respect to the contraction induced by 60 mM KCl for each experiment. Data are mean ± SEM of four to five separate experiments. GppNH(p) induced shifts in IC_{50} (i.e., ΔIC_{50}) values obtained in 1-mo-old aorta was significantly different from 6- or 24-mo-old aortas as assessed by Tukey's test ($p < .05$). Also, IC_{50} shifts in 6- or 24-mo-old aortas were not significantly different from 0, whereas the shift in 1-mo-old aorta was significant (Student's *t* test was used to test the hypothesis $\Delta IC_{50} = 0$).

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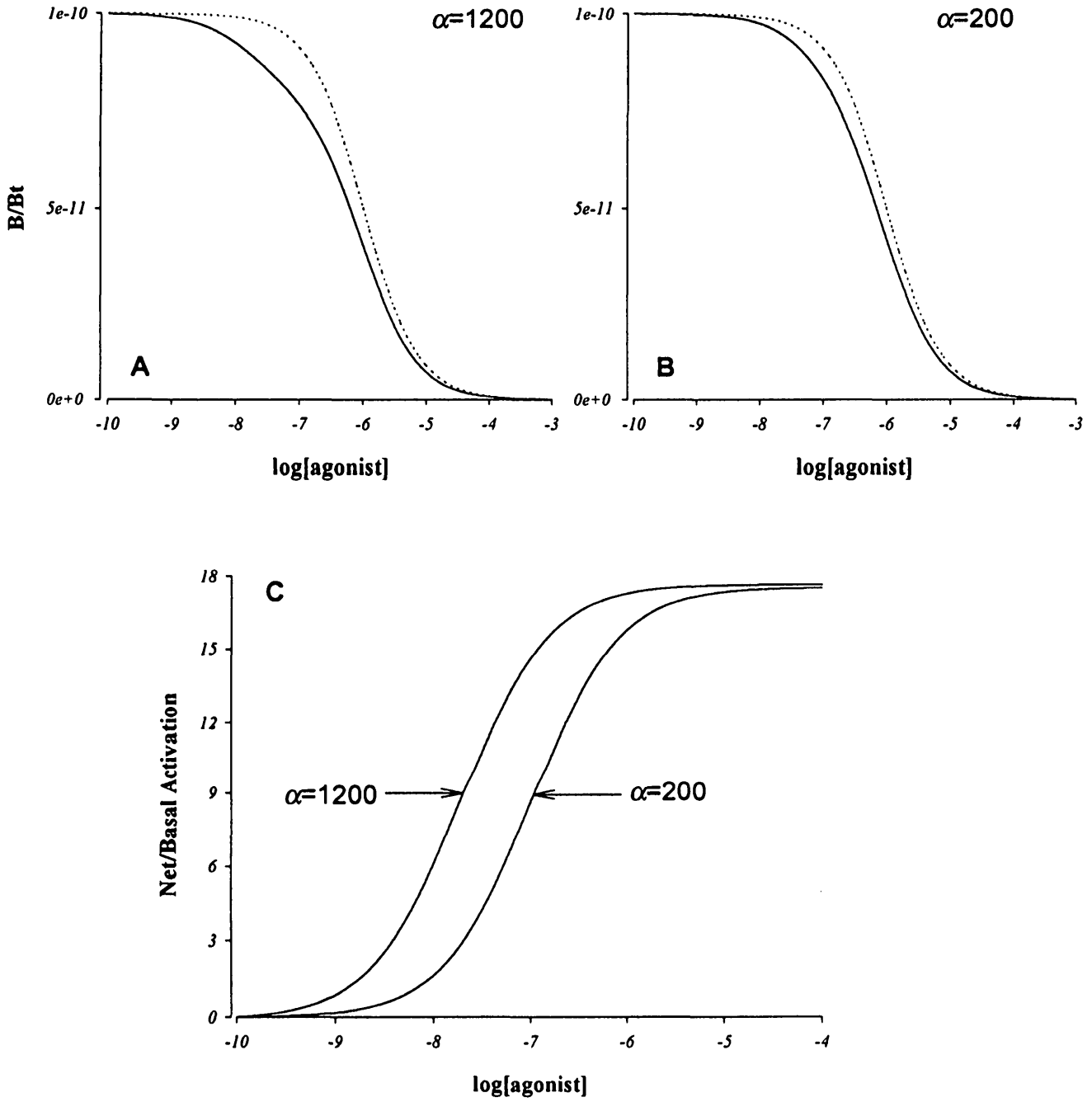


Figure 2. Simulation of the experimental data given in Figure 1, using the ternary complex model. (*A*) Shows the competition binding of a cold ligand with a tracer in the absence (solid curve) or presence (dotted curve) of guanine nucleotide. Binding of ligands was calculated as the total concentration of bound ligand (HR + HRG). Following parameter values were used: Total receptor = 10^{-10}, total G protein = 2.1^{-11}, and 0 for the absence and the presence of guanine nucleotide, respectively, K = 10^8, M = 5.10^8, and α = 1,200. (*B*) As in *A*, except α = 200. (*C*) Simulation of the G protein activation as the total concentration of receptor-bound G protein (RG + HRG), using the parameter values given in *A* or *B* (the curves on the left and right, respectively).

contraction coupling and these could occur at any level between the agonist-receptor interaction and the contractile machinery. Our previous results, however, suggested that the age-dependent alteration in rat aorta is rather specific to the adrenoceptor agonists and that it occurs at a proximal site in the pharmacomechanical coupling since KCl or 5-HT-induced contractions were not sensitive to age (16). It is also unlikely that the observed reduction in *alpha*$_1$-adrenoceptor responsiveness is due to a decrease in expression of *alpha*$_1$-adrenoceptors or the relevant G proteins in the membrane as we showed experimentally that their densities in the aorta remain relatively constant in 1-, 6-, and 24-month-old rats (4,8,10). In order to provide an explanation for the observed reduction of norepinephrine-induced contraction in rat aorta between 1 and 6 months, we investigated the Gpp(NH)p-sensitivity of norepinephrine binding

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to $alpha_1$-adrenoceptors as a measure of the strength of receptor-G protein coupling. The results show that the reduced responsiveness to norepinephrine is accompanied by a loss of Gpp(NH)p-sensitive high affinity agonist binding to $alpha_1$-adrenoceptors between 1 and 6 months. This phenomenon is consistent with the idea that the reduced responsiveness to norepinephrine is likely to be confined to the receptor-G protein interaction and that it can be discussed within the framework of the ternary complex model.

According to the ternary complex model, agonist has relatively high affinity for the G protein-bound receptor (RG) compared to the free receptor (R). The high affinity agonist binding is observable, however, only in the absence of added guanine nucleotide, which is assumed to abolish the binding affinity of receptor and G protein (M in model). Thus, the origin of the nucleotide-induced shift in agonist binding is interpreted as the diversity between the agonist affinities for free and G protein-bound receptors. The measure of this diversity in agonist affinity is expressed as a coupling factor (α) in the model. This coupling is strictly reciprocal in the sense that the difference between the affinities of agonist for free and G protein-bound receptors (i.e., αK/K) is equivalent to the difference between affinities of G protein for agonist-bound and unbound receptors (i.e., αM/M) (see model). Therefore, this allosteric coupling, which is reflected in the nucleotide-shift, can provide a measure of the strength of the agonist-induced activation of G proteins or the molecular efficacy of the agonist, considering that the crucial step in G protein-mediated signal transduction is the receptor-G protein interaction. Manipulating this parameter in the model simulates the experimental data obtained in the present study, so a reduction in the allosteric coupling between agonist and G protein during maturation may explain the observed disappearance of the nucleotide-shift without an observable change in the maximal response.

Manipulating the unconditional affinity for the formation of receptor-G protein complex (M) did not result in such a change. Reducing M, abolished the nucleotide-shift, but simultaneously reduced the maximal response (which is simulated as the maximal agonist-induced net binding of R to G). This result, together with the experimental data that shows no change in receptor or G protein expression in the aortic membrane (8–10) and, no nonspecific changes in the effector systems that are shared by $alpha_1$-adrenoceptor (as assessed by evaluating the 5HT-induced contraction), suggests a reduction in allosteric coupling between agonist and G protein during maturation.

The value for the allosteric coupling factor (α) depends on the identities of ligand, receptor and G protein, and also on the properties of the microenvironment in which these partners reside. Therefore, any change in membrane composition may underlie the observed change in allosteric coupling. In fact, age-related decrease in membrane fluidity was found to be correlated with decreased high-affinity agonist binding (which may be seen as a measure of α for a given ligand-receptor system) in rat aorta and in several other tissues (9,17–20).

A different mechanism for the present results can be considered as a maturation dependent alteration in composition of an heterogeneous receptor population, members of which possess diverse coupling values for the same ligand and G protein. In fact, we observed that the expression of aortic $alpha_{1a}$, $alpha_{1b}$, and $alpha_{1d}$-adrenoceptors increases, decreases, and stays constant, respectively, between the ages of 1 to 6 mo in the rat (8). A decrease in one receptor subtype is compensated by an increase in another subtype, so that the total receptor density stays constant. Such heterogeneity is also consistent with the two-site binding pattern observed in the presence of Gpp(NH)p at all ages. Therefore, the theoretical assumption that the allosteric coupling of the $alpha_{1a}$-adrenoceptor is less efficient than the $alpha_{1b}$-adrenoceptor, will produce an apparent reduction in allosteric coupling, and is consistent with the present observations. Theoretically replacing one receptor gradually with another one that possesses a lower α gives indistinguishable results from those obtained by reducing the α value of a single type of receptor (data not shown). At present it is not clear how to distinguish between the two possibilities that the apparent reduction in allosteric coupling between norepinephrine and G proteins on $alpha_1$-adrenoceptors during maturation is due to (*i*) a decrease in α for a particular receptor, or (*ii*) an increase in abundance of a receptor subtype having a lower α. The latter hypothesis, however, requires that the allosteric coupling factor for $alpha_{1a}$-adrenoceptor is lower than that for $alpha_{1b}$-adrenoceptor, which can be tested in an expression system under suitable conditions.

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