Estradiol Selectively Regulates α_{1B} -Noradrenergic Receptors in the Hypothalamus and Preoptic Area

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We previously demonstrated that estradiol administered *in vivo* elevates the number of α_1 -adrenoceptors in preoptic area (POA) and hypothalamic membranes from ovariectomized female rats and potentiates α_1 receptor augmentation of β -adrenoceptor-stimulated cAMP formation in slices from these brain regions. Present studies examined (1) if estradiol selectively regulates any α_1 -adrenoceptor subtype, and (2) which α_1 receptor subtype mediates the augmentation of cAMP synthesis. Hypothalamic and POA membranes from estradiol-treated rats, when compared to ovariectomized rats, had modestly (30–50%) but significantly elevated numbers of ^3H -prazosin (α_1) binding sites. Estradiol affected neither the number of α_1 receptor sites in frontal cortex nor the affinity of ^3H -prazosin binding in any brain region examined. Results of binding studies conducted in the presence of chlorethylclonidine, a selective, irreversible inactivator of the α_{1B} receptor subtype, indicated that the estrogen-dependent increase in total α_1 binding sites in POA and hypothalamic membranes was attributable to a selective, five- to sixfold increase in α_{1B} receptor number. Progesterone had no measurable effects on α_1 receptor binding. Blockade of α_{1B} receptors with chlorethylclonidine eliminated phenylephrine augmentation of isoproterenol-stimulated cAMP formation in slices, whereas the α_{1A} antagonist 5-methyl-urapidil did not. This suggests that the α_{1B} receptor subtype potentiates cAMP formation. Thus, the increased α_1 receptor augmentation of cAMP formation seen in slices from estradiol-treated rats is correlated with increased α_{1B} receptor number.

In some tissues, including brain slices, activation of α_1 -adren-
 ergic receptors potentiates agonist- and forskolin-stimulated
 cAMP accumulation (Perkins and Moore, 1973; Daly et al.,
 1980; Duman et al., 1985; Sugden et al., 1985; Etgen and Petitti,
 1987; Petitti and Etgen, 1990, 1991). Ovarian steroids modulate
 α_1 -adrenoceptor augmentation of cAMP formation in brain ar-
 eas that regulate female reproductive function. When compared
 to hypothalamic and preoptic area (POA) slices from ovariec-
 tomized (OVX) rats, slices from estradiol (E_2)-treated females
 exhibit enhanced α_1 receptor augmentation of cAMP formation
 (Petitti and Etgen, 1990). This change in α_1 receptor facilitation

of cAMP synthesis is correlated with a modest (20–30%) ele-
 vation in the total number of specific ^3H -prazosin binding sites
 in hypothalamic and POA membranes (Etgen and Karkanas,
 1990). Interestingly, progesterone (P) abolishes α_1 receptor po-
 tentiation of cAMP synthesis in slices from E_2 -primed female
 rats (Petitti and Etgen, 1989, 1990, 1992) without affecting the
 number or affinity of ^3H -prazosin binding sites (Etgen and Kar-
 kanas, 1990).

Norepinephrine (NE) receptors have long been divided into
 β , α_1 , and α_2 subtypes. However, it is now clear that these three
 classes of adrenergic receptors each can be subdivided further
 based upon both pharmacological and molecular biological cri-
 teria (for recent reviews, see Lomasney et al., 1991; Szabadi and
 Bradshaw, 1991). The β receptors can be subdivided into β_1 and
 β_2 receptor subtypes (Minneman and Molinoff, 1980), the α_1
 receptors can be subdivided into α_{1A} and α_{1B} (and perhaps α_{1C})
 receptor subtypes (McGrath, 1982; Morrow and Creese, 1986;
 Minneman et al., 1988; Terman et al., 1990; Harrison et al.,
 1991), and the α_2 receptors can be subdivided into at least α_{2A} ,
 α_{2B} , and α_{2C} receptor subtypes (Bylund, 1988; Bylund et al., 1988;
 Harrison et al., 1991). Morrow and Creese (1986) originally
 reported that two classes of α_1 receptor binding sites in rat ce-
 rebral cortex could be differentiated by their affinities for the
 competitive antagonists WB4101 and phentolamine, and desig-
 nated them α_{1A} and α_{1B} . Han et al. (1987a,b) subsequently
 demonstrated that these binding sites could be distinguished by
 their different sensitivities to inactivation by the site-directed
 alkylating agent chlorethylclonidine (CEC). They demonstrated
 that the α_{1A} site was relatively CEC insensitive whereas the α_{1B}
 site was sensitive to irreversible inactivation by CEC.

The identity of the α_1 receptor subtype mediating cAMP po-
 tentiation remains unresolved. For example, Johnson and
 Minneman (1986, 1987) suggest that the α_1 receptor subtype
 that augments cAMP accumulation is similar to the α_1 receptor
 that activates inositol phospholipid hydrolysis, most likely the
 α_{1B} subtype, whereas Robinson and Kendall (1989) suggest that
 it is not. Recent studies employing both CEC and antagonists
 with high selectivity for the α_{1A} subtype concluded that the
 receptors mediating cAMP potentiation and phosphoinositol
 hydrolysis in brain slices fit neither the current α_{1A} nor the α_{1B}
 classification (Minneman and Atkinson, 1991). Therefore, the
 purposes of the present studies were (1) to evaluate the possi-
 bility that the small E_2 -induced increase in total α_1 receptor
 binding in hypothalamus and POA is attributable to selective
 alterations in a specific α_1 receptor subtype, and (2) to identify
 which α_1 -adrenoceptor subtype mediates the augmentation of
 cAMP formation in hypothalamic and POA slices. The selective
 α_{1A} receptor antagonist 5-methyl-urapidil (5-MU; Gross et al.,

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1988), WB4101, an antagonist with higher affinity for the α_{1A} than for the α_{1B} subtype, and CEC, an irreversible, α_{1B} -selective alkylating agent, were used to distinguish the α_1 receptor subtypes.

Some of these findings have appeared in preliminary form (Etgen et al., 1992).

Materials and Methods

Animals and hormone treatments. Female Sprague-Dawley rats obtained from Taconic Farm (Germantown, NY) and weighing 150–175 gm were ovariectomized bilaterally under Metofane anesthesia 4–7 d prior to use. Estrogen treatment consisted of two subcutaneous injections of 2 μ g of E_2 benzoate (EB) given 24 and 48 hr before death. P treatment consisted of an injection of 500 μ g of P given subcutaneously 3.5 hr before sacrifice. EB and P were dissolved in peanut oil and injected in a volume of 0.1 ml.

Tissue preparation. Animals were killed by decapitation, and their brains were rapidly removed and placed on ice. The entire hypothalamus and POA were removed, and 350 μ m slices were cut on a McIlwain tissue chopper beginning approximately 2 mm anterior to the optic chiasm and ending 1 mm anterior to the mammillary bodies. Based on anatomical landmarks observed in comparable slices from fixed tissue, four slices of POA and three slices of middle hypothalamus (MH) were obtained as described earlier (Etgen and Petitti, 1986, 1987) and used for cAMP assays and for preparation of membranes for some radioligand binding assays. The MH slices include the arcuate nucleus, the ventromedial nucleus, the dorsomedial nucleus, and much of the lateral hypothalamus. For binding competition studies, membranes from the entire hypothalamus plus POA were used (see below). A sample of frontal cortex (CTX; 250–350 mg) was also removed from each brain for radioligand binding assays.

Radioligand binding assay. To provide sufficient material for Scatchard analysis, tissue from two rats given identical hormone injections was combined for the POA and MH regions. For competition studies, combined hypothalamus plus POA from three rats were used. Tissue samples were homogenized separately in 5 ml of ice-cold Tris-MgCl₂ buffer (50 mM Tris HCl; 10 mM MgCl₂; pH 7.4) with a Polytron at speed 5–6 for 20 sec. The homogenates were centrifuged for 10 min at 20,000 $\times$ g, the supernatant discarded, and the pellet containing the membrane fraction frozen at -70°C until assay. Freezing of the crude membrane fraction does not result in a measurable loss of any NE receptor subtype (Etgen and Karkanias, 1990).

For analysis of total α_1 binding, frozen membranes were resuspended in 6 ml buffer (Tris-MgCl₂) and preincubated for 10 min at 37°C . The preincubation was terminated by addition of 6 ml of ice-cold Tris-MgCl₂ buffer and centrifugation for 10 min at 20,000 $\times$ g. The supernatant was discarded and the pellet resuspended in 6 ml Tris-MgCl₂ buffer. For Scatchard analysis, triplicate 200 μ l aliquots were incubated for 20 min at 37°C with 0.05–5 nM ^3H -prazosin with and without a 2000-fold excess of phentolamine to assess nonspecific binding. Competition studies were conducted by incubating triplicate aliquots of membrane suspensions with 2 nM ^3H -prazosin in the presence of radioinert antagonists (WB4101 or 5-MU). Antagonist concentrations were varied from 10^{-4} to 10^{-11} M in $1/2$ -log increments. Bound and free ^3H -prazosin were separated by rapid filtration through glass fiber filters (FPB-148 Whatman GF/B) on a Brandel (Gaithersburg, MD) cell harvester. Filters were washed with 5 $\times$ 2 ml of ice cold buffer, and radioactivity was determined. To reduce nonspecific adsorption of ligands, filters were soaked in 1% polyethylenimine for 1 hr prior to use (Bruns et al., 1983). Ligand affinities (K_D and K_i), apparent receptor numbers (B_{max}), and Hill coefficients were calculated using the LIGAND program of Munson and Rodbard (1980).

To distinguish α_{1A} and α_{1B} receptor subtypes, the effects of CEC inactivation on ^3H -prazosin binding in brain membranes were studied. The water solubility of CEC may prevent it from gaining access to binding sites enclosed in vesicles that may form following tissue homogenization in a Tris-MgCl₂ buffer (Minneman et al., 1988). In agreement with these observations, pilot experiments indicated that Tris-MgCl₂ buffer is unsuitable for assessing CEC inactivation of α_{1B} receptors (N. Petitti and A. M. Etgen, unpublished observations). Therefore, the binding assays were performed in hypotonic, 10 mM Na-HEPES buffer (pH 7.6) to promote vesicle lysis. Frozen membranes were resuspended in 6 ml Na-HEPES buffer and split into two equal fractions. Each frac-

tion was preincubated for 10 min at 37°C with vehicle or with 10 μM CEC. Reactions were stopped by addition of 6 ml of ice-cold Na-HEPES buffer and centrifugation for 10 min at 20,000 $\times$ g. The supernatant was discarded and the pellet resuspended in 6 ml Tris-MgCl₂ buffer. Specific ^3H -prazosin binding after CEC inactivation reflects the α_{1A} -adrenergic receptor population. The α_{1B} receptor population was determined by subtracting the binding of ^3H -prazosin after CEC inactivation from total specific ^3H -prazosin binding.

Determination of slice cAMP accumulation. Each POA and MH slice was maintained at 34 – 35°C in a shaking water bath (80 oscillations/min) in an individual tissue culture well containing 300 μ l of a modified Yamamoto's medium (Yamamoto, 1972) in an O_2/CO_2 (95:5)-saturated environment. The incubation conditions were identical to those used in our previous work except that NaHCO_3 was replaced by 10 mM HEPES in the Yamamoto's medium. Slices were preincubated for 75 min prior to the addition of the NE agonists. CEC and 5-MU were added directly to the incubation wells 30 min and 10 min, respectively, prior to phenylephrine (PHE) and isoproterenol (ISO). At the end of the 20 min incubation period with the NE agonists, the slices were transferred rapidly to 400 μ l of ice cold 5% (w/v) trichloroacetic acid. The slices were disrupted by sonication, and the supernatant (containing cAMP) and pellet (containing tissue protein) were separated by centrifugation. The pellet was dissolved in 2.0 M NaOH for determination of protein content. The supernatant was acidified with 1.0 M HCl, and trichloroacetic acid was removed with 4 vol of washed ether. The resulting aqueous extracts were concentrated by lyophilization and analyzed for cAMP content using a modified Gilman protein binding assay (Brostrom and Kon, 1974).

Chemicals. EB and P were purchased from Steraloids, Inc. (Wilton, NH). Metofane was obtained from Pitman-Moore, Inc. (Atlanta, GA). Radiolabeled ^3H -prazosin (87 Ci/mmol) was obtained from New England Nuclear (Boston, MA). PHE, ISO, and phentolamine were purchased from Sigma (St. Louis, MO). CEC and WB4101 were purchased from Research Biochemicals, Inc. (Natick, MA), and 5-MU was generously provided by Byk Gulden (Konstanz, Germany).

Analysis of data. Aliquots of each slice and membrane fraction were analyzed for protein content by the method of Larson et al. (1986). For binding assays, receptor number (i.e., B_{max}) was expressed as fmol/mg protein. For cAMP experiments, data were converted to pmol cAMP/mg protein. Values for the four POA or three MH slices were averaged to give a single value for each brain region for each rat. Significant differences between means were determined using analysis of variance. Planned post hoc comparisons were made using a Newman-Keuls multiple range test and/or *t* tests. Differences were considered statistically significant if $p < 0.05$.

Results

Effects of steroids on α_1 receptor number and binding affinity

Prior studies indicated that EB enhanced α_1 receptor potentiation of cAMP formation in MH and POA slices (Etgen and Petitti, 1987; Petitti and Etgen, 1990) and that this was correlated with modest increases in ^3H -prazosin binding (Etgen and Karkanias, 1990). Thus, initial binding studies replicated the effects of ovarian steroids on total α_1 receptor binding. Figure 1 and Table 1 show that EB significantly increased total α_1 receptor number in POA (33%) and MH (49%) membranes ($p < 0.05$) with no change in ^3H -prazosin binding affinity. Approximately 75% of the total ^3H -prazosin binding represented specific binding. As reported previously, the administration of P to EB-primed rats did not alter the EB-induced increase in ^3H -prazosin binding in POA and MH membranes, nor were the binding parameters of α_1 sites in CTX membranes influenced by hormone treatment (Fig. 1, Table 1).

To determine whether α_{1A} or α_{1B} receptors are selectively altered by estrogen, we evaluated the effects of CEC pretreatment on ^3H -prazosin binding in POA, MH, and CTX membranes. Figure 2 shows representative protein-standardized Scatchard plots of α_1 receptor binding in MH membranes from OVX and estrogen-treated rats preincubated in the absence or presence of CEC to inactivate α_{1B} receptors. In all tissues from both OVX

Figure 1. Effects of *in vivo* treatments with ovarian steroids on ^3H -prazosin binding. OVX female rats were primed with vehicle (OVX), with EB only (2 μg , 24 and 48 hr before death), or with EB + 500 μg of P 3.5 hr before death. B_{max} values were obtained by Scatchard analysis of ^3H -prazosin binding in the presence or absence of excess phentolamine to assess nonspecific binding. Each value represents the mean of four to six independent replications and is expressed as fmol/mg protein ($\pm$ SEM). *, significantly greater than OVX ($p < 0.05$); **, significantly greater than OVX ($p < 0.01$).

and E_2 -treated females, ^3H -prazosin appeared to bind to a single class of saturable, high-affinity binding sites, and Hill coefficients were not significantly different from 1.0 (data not shown). E_2 administration had little influence on the number of CEC-insensitive binding sites despite a substantial increase in total specific ^3H -prazosin binding sites. This is illustrated for group data by the finding that administration of neither EB nor EB+P significantly influenced CEC-insensitive sites, that is, the number of α_{1A} receptors, in any brain region (Fig. 3B). In agreement with Figure 1, EB did increase the total number of ^3H -prazosin binding sites in both POA and MH membranes but not in CTX membranes (Fig. 3A). Moreover, POA ($p < 0.05$) and MH ($p < 0.01$) membranes from E_2 -treated females demonstrated a five- to sixfold increase in CEC-sensitive sites (i.e., α_{1B} receptor number) when compared to OVX controls (Fig. 3C). This effect of estrogen was not observed in CTX membranes. The administration of P to EB-primed rats did not modify the EB-induced increase in ^3H -prazosin binding in the presence or absence of CEC in POA or MH membranes. Thus, the failure of P to modify total prazosin binding cannot be attributed to reciprocal changes in the concentration of the α_{1A} and α_{1B} subtypes.

In vivo hormone treatment did not influence ^3H -prazosin

Figure 2. Representative Scatchard plots of total and CEC-insensitive ^3H -prazosin binding in hypothalamic membranes from OVX and EB-treated female rats. OVX female rats were primed with EB only (2 μg , 24 and 48 hr before death) or with vehicle before death (OVX). Hypothalamic membranes were preincubated in Na-HEPES buffer for 10 min at 37°C with (solid circles) or without 10 μM CEC (open circles). Values were obtained by Scatchard analysis of ^3H -prazosin binding in the presence or absence of excess phentolamine to assess nonspecific binding. Similar binding results were found in several independent pools of hypothalamic and POA tissue from OVX and EB-treated animals (see Fig. 3).

binding affinity (data not shown). However, CEC caused an approximately twofold increase in K_D for ^3H -prazosin binding in all three tissues ($p < 0.0001$). A similar CEC-dependent change in K_D for ^{125}I -BE-2254 binding has been observed in the hypothalamus (Wilson and Minneman, 1989) and liver (Han et al., 1987a; Minneman et al., 1988). This would suggest that α_{1B} receptors have a slightly higher affinity for prazosin than α_{1A} receptors. We were unable to resolve total ^3H -prazosin binding into two components by subjecting saturation isotherms to a two-site model, presumably because the affinities of the α_{1A} and α_{1B} sites for prazosin differ by only twofold.

Additional binding studies were carried out to confirm the selectivity of CEC for α_{1B} -adrenoceptors and the specificity of estrogen action on that receptor subtype. Figure 4 shows that the displacement of ^3H -prazosin by WB4101, an antagonist with relatively higher affinity for α_{1A} than for α_{1B} receptors, was similar in hypothalamic-POA membranes from OVX rats preincubated in the presence or absence of CEC. In both cases, the slope coefficient of the competition curve was close to 1.0 (Table 2), indicative of a single receptor population. Hence, these observations are consistent with the interpretation that in membranes from OVX females, most α_1 receptors are of the α_{1A} subtype (i.e., high affinity for WB4101). By contrast, the slope coefficient of the competition curve for WB4101 displacement

Table 1. Effects of *in vivo* hormone treatments on ^3H -prazosin binding affinity

Region	K_D (nm $\pm$ SEM)		
	OVX	EB	EB + P
POA	0.35 $\pm$ 0.1	0.43 $\pm$ 0.8	0.43 $\pm$ 0.3
MH	0.41 $\pm$ 0.3	0.45 $\pm$ 0.7	0.49 $\pm$ 0.6
CTX	0.60 $\pm$ 0.3	0.87 $\pm$ 0.1	0.69 $\pm$ 0.2

OVX female rats were primed with vehicle (OVX), with EB only (2 μg , 24 and 48 hr before death), or with EB + 500 μg of P 3.5 hr before death. K_D values were obtained from Scatchard analysis of ^3H -prazosin binding. Each value represents the mean of four or five independent replications. B_{max} values are shown in Figure 1.

Figure 3. Effects of *in vivo* treatments with ovarian steroids on putative α_{1A} - and α_{1B} -adrenergic receptors. OVX female rats were primed with vehicle (OVX), with EB only (2 μ g, 24 and 48 hr before death), or with EB + 500 μ g of P 3.5 hr before death. To distinguish α_{1A} and α_{1B} receptors, POA, MH, and CTX tissues were preincubated in Na-HEPES buffer for 10 min at 37°C with or without 10 μ M CEC. Total 3 H-prazosin binding (A), corrected for nonspecific binding, reflects both α_1 receptor subtypes. B shows the α_{1A} -adrenergic receptor population that is measurable after CEC inactivation. The α_{1B} receptor population (C) was determined by subtracting the binding of 3 H-prazosin after CEC inactivation from total 3 H-prazosin binding. B_{max} values were obtained by Scatchard analysis. Each value represents the mean of three to five independent replications and is expressed in fmol/mg protein ($\pm$ SEM). *, significantly greater than OVX ($p < 0.05$); **, significantly greater than OVX ($p < 0.01$).

of 3 H-prazosin binding in membranes from EB-treated females was less than 1.0 in the absence of CEC (Table 2), indicating the presence of a heterogeneous receptor population. In addition, the WB4101 competition curve was shifted to left in the presence of CEC (Fig. 4), an observation consistent with the removal of the lower-affinity α_{1B} receptor subtype. Moreover, CEC significantly reduces the K_i of WB4101 ($p < 0.05$) in both OVX and E_2 -treated tissue (Table 2). Taken together, these ob-

Table 2. WB4101 and 5-MU displacement of 3 H-prazosin binding sites in hypothalamic-POA membranes

Competitor		Slope coefficient	K_i (nM)
WB4101	OVX		
	–CEC	0.90 ± 0.18	3.9 ± 0.85
	+CEC	0.95 ± 0.09	1.3 ± 0.06
	EB		
5-MU	OVX		
	–CEC	0.70 ± 0.26	2.5 ± 0.99
	+CEC	1.0 ± 0.15	0.48 ± 0.11
	OVX		
	–CEC	0.39 ± 0.10	407 ± 108
	+CEC	1.1 ± 0.05	85 ± 36

OVX female rats were primed with vehicle (OVX) or with 2 μ g of EB 24 and 48 hr before death. Membranes were preincubated in Na-HEPES buffer in the presence (+CEC) or absence (–CEC) of 10 μ M CEC. Aliquots of the membranes were then incubated with 2 nM 3 H-prazosin and increasing concentrations of WB4101 or 5-MU. Slope coefficients and K_i values were obtained by computer modeling using the LIGAND program of Munson and Rodbard (1980). Representative competition curves are shown in Figures 4 and 5. Values represent the mean $\pm$ SEM of three (WB4101) or two (5-MU) independent replications. CEC and EB significantly reduced the apparent K_i of WB4101 ($p < 0.001$ and $p < 0.02$, respectively).

servations confirm other reports that CEC selectively inactivated the α_{1B} receptor subtype. They are also consistent with the interpretation that E_2 selectively increased the proportion of 3 H-prazosin binding attributable to α_{1B} receptors.

Displacement of 3 H-prazosin by the α_{1A} -selective antagonist 5-MU was also evaluated in hypothalamic-POA membranes from OVX rats (Fig. 5). Approximately 80% of 3 H-prazosin was displaced by 5-MU in the absence of CEC preincubation, indicating that the predominant receptor is of the α_{1A} subtype. When membranes were preincubated in the presence of CEC, the 5-MU competition curve shifted to the left, the slope coefficient was closer to 1.0, and the K_i decreased (Table 3). Because there is some evidence that 5-MU is the antagonist of choice for blocking α_{1A} -adrenoceptors (Han and Minneman, 1991), and because it clearly competed for prazosin binding in hypothalamic-POA membranes, we chose 5-MU for use in our slice studies.

Effects of CEC and 5-MU on α_1 receptor augmentation of cAMP formation

In our previous studies (Etgen and Petitti, 1987; Petitti and Etgen, 1990), α_1 receptor potentiation of the cAMP response to β -adrenergic receptor stimulation was more robust in POA and MH slices from E_2 -treated than from OVX females. Thus, experiments to determine whether α_{1A} - or α_{1B} -adrenoceptors mediate augmentation of ISO-stimulated cAMP formation were conducted in slices from EB-treated animals (Table 3). The combination of α_1 and β receptor activation (PHE+ISO) increased cAMP accumulation significantly more than ISO alone ($p < 0.01$). PHE alone had no effect on cAMP synthesis in these brain slices (Petitti and Etgen, 1990, 1991). When slices were preincubated with CEC to inactivate α_{1B} receptors, PHE potentiation of ISO-stimulated cAMP accumulation was eliminated. In contrast, the α_{1A} receptor antagonist 5-MU at a concentration that should block all or most α_{1A} sites (Fig. 5) did not alter PHE augmentation of cAMP synthesis in either POA or MH slices. Neither CEC nor 5-MU significantly altered basal cAMP levels in POA or MH slices. Although CEC appeared to have a modest suppressive effect on ISO-stimulated cAMP accumulation, this effect was not statistically significant.

Figure 4. Representative competition curves of WB4101 displacement of ^3H -prazosin binding in control and CEC-pretreated membranes from combined hypothalamus and POA of OVX and EB-treated female rats. OVX female rats were primed with vehicle (OVX) or with EB (2 μg) 24 and 48 hr before death. Membranes from combined hypothalamus and POA were preincubated in Na-HEPES buffer for 10 min at 37°C with (open circles) or without (solid circles) 10 μM CEC prior to incubation with 2 nM ^3H -prazosin and increasing concentrations of WB4101. Similar results were observed in several independent pools of hypothalamic and POA membranes from OVX and EB-treated animals. Binding parameters (see Table 2) were obtained by computer modeling using the LIGAND program of Munson and Rodbard (1980).

Discussion

This is the first demonstration that an endogenous factor, that is, the steroid hormone E_2 , regulates a specific α_1 -adrenoceptor subtype in the CNS. The conclusion that E_2 selectively increases the number of α_{1B} -adrenergic binding sites in female rat hypothalamus and POA is supported by two findings. First, *in vivo* administration of E_2 increases the number of CEC-sensitive, but not CEC-insensitive ^3H -prazosin binding sites. Second, the competition curve for displacement of prazosin by the α_{1A} -preferring antagonist WB4101 is influenced more markedly by preincubation with the α_{1B} -preferring alkylating agent CEC in mem-

Table 3. Effects of CEC and 5-MU on PHE augmentation of ISO-stimulated cAMP formation in slices from EB-primed rats

	cAMP (pmol/mg protein $\pm$ SEM)	
	POA	MH
Basal	5.88 $\pm$ 0.56	6.36 $\pm$ 0.74
ISO (1 μM)	11.8 $\pm$ 0.95*	12.4 $\pm$ 1.35*
ISO + PHE (10 μM)	27.7 $\pm$ 2.20**	26.0 $\pm$ 2.82**
CEC (100 μM)	5.79 $\pm$ 0.56	6.85 $\pm$ 0.74
+ ISO	8.53 $\pm$ 0.96*	9.92 $\pm$ 0.41*
+ ISO + PHE	8.28 $\pm$ 0.30*	12.1 $\pm$ 0.63*
5-MU (1 μM)	6.87 $\pm$ 0.56	6.54 $\pm$ 0.15
+ ISO	13.9 $\pm$ 0.78*	15.0 $\pm$ 0.93*
+ ISO + PHE	25.3 $\pm$ 1.51**	25.5 $\pm$ 1.46**

Each value represents the mean of four to five independent replications.

* Significantly greater than basal ($p < 0.01$).

** Significantly greater than ISO alone ($p < 0.05$).

Figure 5. Representative competition curves of 5-MU displacement of ^3H -prazosin binding in control and CEC-pretreated hypothalamic-POA membranes from OVX female rats. Combined hypothalamic-POA membranes from OVX rats were preincubated in Na-HEPES buffer for 10 min at 37°C with (open circles) or without (solid circles) 10 μM CEC prior to incubation with 2 nM ^3H -prazosin and increasing concentrations of 5-MU. Similar results were observed in a second preparation of hypothalamic-POA membranes from OVX female rats. Binding parameters (see Table 2) were obtained by computer modeling using the LIGAND program of Munson and Rodbard (1980).

branes from estrogen-treated than from OVX females. Taken together, these observations suggest that in OVX females, α_{1B} receptors comprise only a small fraction of the total α_1 binding sites. An earlier biochemical study (Johnson and Minneman, 1987) reported that in male rat hypothalamus, only one-third to one-half of α_1 binding sites are CEC sensitive. A more recent report that utilized receptor autoradiography to distinguish between α_1 receptor subtypes (Blendy et al., 1990) likewise found a 3:1 ratio of α_{1A} : α_{1B} sites in male rat hypothalamus. We now find that estrogen priming increases the ratio of α_{1B} : α_{1A} sites in female rat hypothalamus and POA, although the proportion of α_{1B} receptors is never more than 50% of the total ^3H -prazosin binding in either brain region.

In agreement with other reports (Wilson and Minneman, 1989; Blendy et al., 1990; Han and Minneman, 1991), frontal CTX membranes have relatively more α_{1B} than α_{1A} receptors. Estrogen administration does not significantly affect prazosin binding to either α_1 receptor subtype in this relatively estrogen receptor-poor brain region (Pfaff and Keiner, 1973). This finding is consistent with the interpretation that E_2 -induced increases in hypothalamic-POA α_{1B} receptors may be mediated by genomic actions of intracellular estrogen receptors. The long time lag between hormone administration and increases in prazosin binding observed in our earlier study (Etgen and Karkanas, 1990) also supports a genomic mechanism of E_2 action.

Although previous reports have shown that estrogen influences α_1 -adrenoceptor function and number (Weiland and Wise, 1987; Condon et al., 1989; Roberts et al., 1989; Etgen and Karkanas, 1990; Petitti and Etgen, 1990; Favit et al., 1991), the methods used in those studies did not distinguish between the two α_1 receptor subtypes. The finding that E_2 selectively upregulates α_{1B} receptors, which account for the minority of total ^3H -prazosin binding in the POA and hypothalamus of OVX females, may explain some of the inconsistencies in the literature on estrogen regulation of hypothalamic α_1 -adrenoceptors. Likewise, the data suggest that the modest (30–50%) estrogen-dependent increase in the total number of hypothalamic and POA α_1 binding sites (Etgen and Karkanas, 1990; Fig. 1) actually represents a nearly 600% increase in α_{1B} receptors. A large increase in α_{1B} receptor number is difficult to detect when total ^3H -prazosin binding is measured. However, it must also be noted that the present data do not rule out the possibility that increases in α_{1B} receptors are also limited to specific hypothalamic nuclei, further compounding the difficulty of detecting hormone-dependent changes when large tissue blocks are analyzed.

Present data also suggest that α_1 -adrenoceptor augmentation of cAMP generation in hypothalamic and POA slices is mediated by the α_{1B} subtype. This conclusion is supported by the observation that inactivating α_{1B} receptors by preincubation with CEC abolishes PHE potentiation of ISO-stimulated cAMP synthesis in slices from estrogen-primed females whereas blocking α_{1A} receptors with 5-MU does not. Because we have also shown that α_1 -adrenoceptor potentiation of cAMP accumulation in hypothalamic slices is blocked by inhibitors of protein kinase C and phospholipase C (Petitti and Etgen, 1991), it is reasonable to propose that α_{1B} -adrenoceptor modulation of adenylyl cyclase may be mediated via activation of phosphoinositol hydrolysis. This hypothesis is consistent with data from other laboratories demonstrating that α_{1B} receptor activation stimulates the formation of inositol phosphates (Han et al., 1987b; Michel et al., 1990; Terman et al., 1990). Furthermore, since E_2 priming en-

hances PHE potentiation of ISO-stimulated cAMP formation, our results suggest that estrogen enhances signaling via phospholipase C. This interpretation is congruent with the recent proposal by Mobbs et al. (1991) that phospholipase C- α may be a widespread mediator of E_2 action in many tissues. Moreover, estrogen has been reported to increase α_1 -adrenoceptor-mediated phosphoinositol hydrolysis in rabbit myometrium (Riemer et al., 1987) and in rat cortical slices (Favit et al., 1991). The finding that α_{1B} receptors mediate adenylyl cyclase potentiation in hypothalamus and POA may also explain the inability of the potent α_1 receptor agonist methoxamine to augment ISO stimulation of cAMP formation in slices from these brain areas (Petitti and Etgen, unpublished observations). A recent study in rat hepatocytes reported that methoxamine preferentially stimulates the α_{1A} receptor subtype to increase calcium influx (Tsujimoto et al., 1989).

However, there is still limited information regarding which α_1 receptor subtypes are linked to specific intracellular signaling mechanisms. Moreover, the intracellular mediators of α_1 -adrenoceptor potentiation of adenylyl cyclase activity may be cell type specific. For example, in pinealocytes, the arachidonic acid cascade rather than phosphoinositol hydrolysis is likely to underlie α_1 -adrenergic enhancement of agonist-stimulated cyclic nucleotide accumulation (Chik et al., 1991). There is some evidence that α_{1A} -mediated responses require calcium influx whereas responses to α_{1B} receptors involve mobilization of intracellular calcium stores via phosphoinositol hydrolysis (see Han et al., 1987b, 1990; Tsujimoto et al., 1989; Wilson and Minneman, 1990). However, stimulation of either α_{1A} or α_{1B} receptors can lead to phosphoinositol hydrolysis (Robinson and Kendall, 1990; Wilson and Minneman, 1990). Recent studies utilizing 5-MU, CEC, and nifedipine, another α_{1A} -preferring antagonist, to examine the signal transduction pathways linked to the two receptor subtypes in brain slices and primary glial cultures failed to resolve this issue (Minneman and Atkinson, 1991). Moreover, in contrast with our results, those investigators found that α_1 -adrenoceptor potentiation of cAMP responses in cortical and hippocampal slices was insensitive to CEC and was inhibited in a noncompetitive fashion by 5-MU, leading them to conclude that the α_1 receptors mediating cAMP accumulation do not easily fall into the traditional α_{1A} / α_{1B} subclassification and may not be linked to phosphoinositol hydrolysis. The discrepancies between our findings and the work of other laboratories may reflect tissue and/or sex differences in the coupling of α_1 receptor subtypes to specific membrane effectors. Alternatively, as postulated by Minneman and Atkinson (1991), α_1 receptor subtypes in addition to those fitting in with the current α_{1A} / α_{1B} scheme may exist. In support of this possibility is the finding that the properties of cloned, putative α_{1A} and α_{1B} receptors, when expressed in mammalian cells, are not entirely consistent with the α_{1A} / α_{1B} classification (see Harrison et al., 1991).

The present analysis of ^3H -prazosin binding also demonstrates that administration of P to estrogen-primed female rats does not modify the number of either α_{1A} or α_{1B} binding sites in hypothalamus or POA. This is an interesting finding in light of our consistent observation that when hypothalamic and POA tissue from estrogen-primed rats is exposed to P either *in vivo* (Petitti and Etgen, 1990) or *in vitro* (Petitti and Etgen, 1992), α_1 receptor augmentation of β -receptor-stimulated cAMP formation is abolished. Thus, the P-induced reduction in α_1 receptor coupling to the cAMP second messenger system does not

correlate with the density or affinity of α_{1B} receptors, the subtype that apparently mediates cAMP potentiation in these tissues. This suggests that P may act on factors controlling α_{1B} receptor coupling to its effector system (e.g., G-protein coupling, availability of substrates, enzymes, or G-proteins).

In summary, these results demonstrate (1) that activation of the α_{1B} -adrenergic receptor subtype mediates potentiation of ISO-stimulated cAMP formation in hypothalamic and POA slices and (2) that estrogen selectively increases α_{1B} -adrenoceptor number in these brain regions. Furthermore, P does not influence ^3H -prazosin binding in estrogen-primed rats. Thus, the loss of α_1 receptor potentiation of cAMP synthesis in EB+P-treated rats is not attributable to downregulation of α_1 or α_{1B} receptors but may reflect a modification of the coupling of the α_{1B} receptor to various components of its signal transduction system. Because behavioral data from this laboratory suggest that activation of hypothalamic α_1 -adrenoceptors by endogenously released NE may participate in the hormonal facilitation of female reproductive behavior (see Etgen et al., 1992), an important direction for future study will be to determine which α_1 receptor subtypes and signal transduction mechanisms underlie the modulatory actions of NE on female reproductive function.

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