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Benzodiazepines and peptides stimulate pregnenolone synthesis in brain mitochondria

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Abstract

Mitochondria isolated from rat brain were found to cleave cholesterol to produce pregnenolone, the precursor for hormonal steroids, at a mean rate of 21.0 pmol pregnenolone · mg protein^{-1} · min^{-1}. This rate-limiting step in steroidogenesis was significantly stimulated by PK 11195 (1-(2-chlorophenyl)-*N*-methyl-(1-methylpropyl)-3-isoquinoline carboxamide) and Ro5 4864 (4′-chlorodiazepam), ligands which bind to peripheral benzodiazepine receptors with high affinity. Low-affinity ligands for the peripheral benzodiazepine receptor such as Ro15 1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzo-3-carboxylate) and clonazepam had no significant effect on the rate of pregnenolone synthesis. Furthermore, the rank order of potency of these compounds as inhibitors of [^{3}H]Ro5 4864 binding was identical to the rank order for steroid production. Since the 86-amino acid peptide diazepam binding inhibitor is also thought to bind to the peripheral benzodiazepine receptor, four fragments of this peptide, a random sequence and steroidogenesis activator peptide were also evaluated for their ability to interact with peripheral benzodiazepine receptors and to stimulate steroidogenesis in rat brain mitochondria. Steroidogenesis activator peptide and two fragments of diazepam binding inhibitor significantly stimulated pregnenolone biosynthesis. In contrast to the peripheral benzodiazepine receptor ligands, no correlation between peptide potency in displacing [^{3}H]Ro5 4864 binding and steroidogenesis was observed.

Keywords: Steroidogenesis; Brain, rat; Benzodiazepine receptor, peripheral; Mitochondrion

1. Introduction

At least two distinct types of benzodiazepine receptors have been identified in the central nervous system. The central benzodiazepine receptor that is allosterically coupled to the γ-aminobutyric acid$_A$ (GABA$_A$) receptor-chloride ionophore has been well described (Stephenson, 1988). A pharmacologically distinct but less well characterized benzodiazepine recognition site is the peripheral-type benzodiazepine receptor. Fractionation studies have localized the peripheral benzodiazepine receptor to the outer mitochondrial membrane (Anholt et al., 1986). These putative receptors are abundant in both neural and peripheral tissues where they have recently been implicated to play a regulatory role in the synthesis of steroids (Barnea et al., 1989; Mukhin et al., 1989; Yanagibashi et al., 1989; Besman et al., 1989; Papadopoulos et al., 1990, 1991). These findings are significant when one considers compelling evidence demonstrating that steroids are potent modulators of GABAergic neurotransmission (Gee, 1988; Olsen and Tobin, 1990; Paul and Purdy, 1992).

Peripheral benzodiazepine receptors are characterized by their high affinity for Ro5 4864 (4′-chlorodiazepam), an atypical benzodiazepine, and low affinity for clonazepam, a benzodiazepine with high affinity for the central benzodiazepine receptor (Braestrup and Nielsen, 1980; Schoemaker et al., 1982). Recent studies

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in adrenocortical cells have demonstrated that the peripheral benzodiazepine receptor may specifically promote the delivery of cholesterol from intracellular stores across the outer mitochondrial membrane into the inner mitochondrial membrane in response to certain benzodiazepines (Yanagibashi et al., 1988, 1989; Krueger and Papadopoulos, 1990). The transport of cholesterol to the C_{27} side-chain-cleavage cytochrome P_{450} enzyme system associated with the inner membrane is the rate-limiting step in steroidogenesis. The initial and committed step in steroid biosynthesis is the cleavage of cholesterol to form pregnenolone, the common precursor for steroid hormones. Thus, elevating the intra-mitochondrial cholesterol concentration can increase the rate of steroidogenesis.

In the last several years, some interesting pharmacological properties of a group of putative benzodiazepine receptor ligands termed endozepines, endogenous peptide ligands, has been revealed (Barbaccia et al., 1988). One member of this class that has been isolated to homogeneity in rat, bovine and human brain is diazepam binding inhibitor (Guidotti et al., 1983; Marquadt et al., 1986), an 86-amino acid peptide so named for its ability to inhibit diazepam binding in synaptosomes. Diazepam binding inhibitor can be proteolytically cleaved into shorter fragments that retain differential activities for the central and peripheral benzodiazepine receptors. Another 30-amino acid peptide that has been identified in peripheral tissues and demonstrated to have a similar functional effect at the peripheral benzodiazepine receptor is steroidogenesis activator peptide (Pedersen and Brownie, 1987). Steroidogenesis activator peptide has no primary sequence homology to diazepam binding inhibitor, but it has been suggested that it is derived from the 78000 Da glucose-regulated protein (GRP78) previously described (Li et al., 1989). Together, these peptides may represent a pool of endogenous ligands for the peripheral benzodiazepine receptor that are capable of mediating an effect through the peripheral benzodiazepine receptor in response to cellular signals.

The concept that steroid biosynthesis may occur in tissue other than the classical steroidogenic tissues has been suggested recently; de novo synthesis of pregnenolone in central nervous system-derived tissues such as oligodendrocytes and cultured brain cells has been reported (Le Goascogne et al., 1987; Yi Hu et al., 1987; Jung-Testas et al., 1989). In light of mounting evidence supporting a mechanism mediated through the peripheral benzodiazepine receptor by in vivo studies in the brain and in adrenocortical and Leydig cell mitochondria (Yanagibashi et al., 1988, 1989; Mukhin et al., 1989; Besman et al., 1989; Papadopoulos et al., 1990, 1991; Krueger and Papadopoulos, 1990; Korneyev et al., 1993), it was of interest to determine whether benzodiazepine receptor ligands and these peptides could also stimulate steroidogenesis in brain mitochondria and if they share a common site of action.

2. Materials and methods

2.1. Mitochondrial preparation

The method described by Clark and Nicklas (1970) was used to isolate a relatively pure mitochondrial population from brain tissues. Briefly, male Sprague-Dawley rats were decapitated and forebrains rapidly removed in cold isolation medium (0.25 M sucrose, 10 mM Tris and 0.5 mM K^+-EDTA, pH 7.4). The tissue was finely chopped with scissors and placed in a glass homogenizer. Using 5 ml of isolation medium per forebrain, the tissue was homogenized manually with eight strokes. An additional 10 ml of isolation medium was added and the homogenate centrifuged at $2000 \times g$ for 3 min. The supernatant was then collected and centrifuged at $12500 \times g$ for 8 min. This yielded a crude mitochondrial pellet which was resuspended in 3% Ficoll medium (3% Ficoll, 0.12 M mannitol, 0.03 M sucrose, 25 mM K^+-EDTA, pH 7.4) to a volume of 10 ml and layered onto 20 ml of 6% Ficoll medium (6% Ficoll, 0.24 M mannitol, 0.06 M sucrose, 50 mM K^+-EDTA, pH 7.4). The gradient was centrifuged for 30 min at $11500 \times g$. The supernatant was then decanted and the pure mitochondrial pellet was washed in 10 ml isolation medium and centrifuged for 10 min at $12500 \times g$. Mitochondria were resuspended in 0.32 M sucrose for steroidogenesis or phosphate buffer saline (50 mM Na/K phosphate pH = 7.4 containing 200 mM NaCl) for binding assays.

2.2. Peptide synthesis

All peptides were synthesized using *t*-butyloxycarbonyl-aminoacyl-OCH_z-PAM resins (Fluka, Ronkonkoma, NY, USA) and an automated solid-phase protocol, based on the principles outlined by Merrifield (1963, 1965) on an Applied Biosystems Model 430 peptide synthesizer. To ensure a high degree of coupling efficiency all amino acids were double coupled. After addition of the final amino acid the resin was dried in vacuo prior to hydrofluoric acid cleavage. For those sequences containing dinitrophenyl-histidine, thiolysis was performed. The peptide resin (0.25 g) was treated for 2×30 min at 25°C with 15 ml of 10% diisopropylethylamine-20% β-mercaptoethanol in dimethylformamide to remove the dinitrophenol protecting group. The N^{α}-*t*-butyloxycarbonyl group was subsequently removed with 65% trifluoroacetic acid for 10 min at 25°C. Peptides not containing histidine were treated with trifluoroacetic acid only. The peptide-res-

in was dried and treated immediately with anhydrous hydrofluoric acid.

2.3. Stimulation of pregnenolone synthesis

Mitochondrial preparations were resuspended in 200 μl of 0.32 M sucrose for each brain used. Steroidogenesis was measured in 3.3 ml of 0.015 M triethanolamine (pH = 7.3) using 200 μl aliquots of mitochondrial homogenate. Cyanoketone (Sterling-Winthrop Labs, New York, USA) was added to a final concentration of 5 μM to prevent further metabolism of pregnenolone and the mixture pre-incubated at 37°C for 5 min. A 200 μl aliquot was removed to determine basal pregnenolone synthesis. Isocitric acid (Sigma Chemical Co., St. Louis, MO, USA) was then added as a source of reducing equivalents (5 mM), the compound being evaluated for stimulatory effects on steroidogenesis (1 μM) and 100 μM cholesterol to ensure that cholesterol was not a limiting factor. All conditions contained equal amounts of dimethyl sulfoxide (DMSO, Sigma Chemical Co.) which was used to dissolve non-peptide compounds. Additional 200 μl aliquots were taken at the desired time points and transferred to 2 ml chloroform with 0.5 ml water and mixed immediately for 10 s. The organic phase was removed to a separate container and the aqueous phase extracted with 2 ml chloroform two more times. The chloroform was then evaporated under nitrogen and samples were redissolved in 100 μl of 95% ethanol for radioimmunoassay of pregnenolone or 50 μl of methylene chloride for analysis by high-pressure liquid chromatography (HPLC). Recovery of a tritiated standard averaged about 90%.

2.4. Quantitation of pregnenolone synthesis

The radioimmunoassay protocol developed by Bergon and coworkers (Bergon et al., 1974) was followed to quantitate pregnenolone. A gelatin phosphate buffer was prepared from 50 mM Na/K PO_4 stock by adding 0.15 M NaCl, 0.015 M NaN_3 and dissolving gelatin (Knox, unflavored) to make a 0.1% solution. All assay tubes contained the following: 0.2 ml gelatin phosphate buffer, 0.1 ml (25 000 dpm) of [^{3}H]pregnenolone (New England Nuclear, 25.0 Ci/mmol), 0.1 ml pregnenolone antibody preparation (Pantex, Santa Monica, CA, USA), and 0.1 ml pregnenolone sample diluted 1 : 10 with assay buffer. The tubes were mixed and incubated at room temperature for 20 min. Following 3 more hours of incubation at 0–4°C, 1 ml of dextran-coated charcoal solution was added to each tube. The dextran-coated charcoal was prepared by adding 2.5 g charcoal (Norit A, Serva, New York, USA) and 0.25 g dextran (Serva) to 1 liter of gelatin phosphate buffer. The tubes were mixed and centrifuged at 2000 rpm for 15 min at 4°C. The supernatant was transferred to scintillation vials and radioactivity quantified by liquid scintillation spectrometry. Individual standard curves were plotted using a least-squares, non-linear regression program (GraphPad Inplot, San Diego, CA, USA) to obtain values for the concentration at which half-maximal [^{3}H]pregnenolone binding occurs and B_{max}. These values were then used to derive the equation defining the standard curve for each assay. The amount of pregnenolone in each sample was determined from its respective standard curve. Under these conditions, 0.05 pg of pregnenolone was detectable and used as the lower limit of the standard curve. All of the [^{3}H]pregnenolone bound to the antibody was displaced by 4 pg of cold pregnenolone (the upper limit of the standard curve). No cross-reactivity was observed with up to 500 ng of progesterone, cholesterol or 5α-pregnan-3α-ol-20-one.

2.5. Chromatography

Pregnenolone levels were determined on a Gilson HPLC using a Microsorb 5 μm silica column (25 cm) with an 85 : 15 hexane : acetone mixture at a flow rate of 1 ml/min. The retention time for pregnenolone was between 8.5 and 10 min using an Evaporative Light Scattering Detector.

2.6. [^{3}H]Ro5 4864 binding assays

Mitochondrial pellets were resuspended in 6 ml per brain of phosphate-buffered saline. Aliquots (100 μl) of mitochondria were incubated with 1 nM [^{3}H]Ro5 4864 (New England Nuclear, 86.9 Ci/mmol) and various concentrations of the compound or peptide being tested in buffer for 2 h at 0–4°C. For the saturation experiments, various concentrations of [^{3}H]Ro5 4864 were incubated with mitochondria in the presence or absence of 10 μM steroidogenesis activator peptide. The total incubation volume was 1 ml. Non-specific binding was defined as binding in the presence of 3 μM Ro5 4864. Incubations were terminated by rapid filtration through No. 32 glass fiber filters (Schleicher and Schuell, Keene, NH, USA), followed immediately by three washes with ice-cold buffer. Filter bound radioactivity was quantified by liquid scintillation spectrometry. The K_d values were calculated as the negative reciprocal of the slope of the regression line (GraphPad Inplot, San Diego, CA, USA) from the saturation binding data. Significant differences were determined by Student's paired t-test ($P < 0.001$).

2.7. [^{3}H]Ro5 4864 dissociation and association kinetics

Dissociation kinetics of [^{3}H]Ro5 4864 under control conditions were determined by incubating mito-

chondria with 2 nM [^{3}H]Ro5 4864 for 2 h at 0–4°C (steady-state conditions). The effect of a peptide on the dissociation of [^{3}H]Ro5 4864 was determined by including 10 μM steroidogenesis activator peptide or 15 μM octadecaneuropeptide during the 2 h preincubation period. Binding in the presence of 3 μM cold Ro5 4864 was defined as nonspecific. Dissociation of [^{3}H]Ro5 4864 was initiated by the addition of 3 μM Ro5 4864 at various time points. The natural log of the amount of [^{3}H]Ro5 4864 bound at a given time t as a percentage of the total bound at time $t = 0$ was plotted versus time. The k_{-1} was derived from the slope of the best fit of the data by computerized linear regression (GraphPad Inplot, San Diego, CA, USA). Association kinetics under control conditions were performed by incubating mitochondria with 2 nM [^{3}H]Ro5 4864 at 0–4°C for various periods of time up to 1 h. The effect of a peptide on the association of [^{3}H]Ro5 4864 was determined by including 10 μM steroidogenesis activator peptide or 15 μM octadecaneuropeptide during the incubation period. Nonspecific binding was defined as the binding in the presence of 3 μM cold Ro5 4864. The natural log of the ratio of the amount bound at steady state divided by the difference between the amount bound at steady state and at time t was plotted versus time. The k_{obs} was derived from the slope of the best fit of the data by computerized linear regression (GraphPad Inplot, San Diego, CA, USA). The k_{+1} was calculated from the equation $k_{+1} = (k_{obs} - k_{-1})/F$ where F equals the free radioligand concentration.

3. Results

3.1. Activity of benzodiazepine receptor ligands

Five benzodiazepine receptor ligands were evaluated for their ability to inhibit [^{3}H]Ro5 4864 binding in brain mitochondrial preparations. The compounds exhibited various potencies that ranged over four orders of magnitude. The rank order (PK 11195 > Ro5 4864 > diazepam > Ro15 1788 > clonazepam) in brain was consistent with previously reported data (Schoemaker et al., 1982). Each compound was tested for its ability to stimulate the synthesis of pregnenolone. The rank order of IC_{50} values correlated one-to-one with their effect on steroidogenesis as measured by the change in the rate of pregnenolone synthesis in the presence of 1 μM compound (Table 1). Diazepam, Ro5 4864 and PK 11195 (1-(2-chlorophenyl)-*N*-methyl-(1-methylpropyl)-3-isoquinoline carboxamide) significantly increased pregnenolone biosynthesis at least twice that of control conditions, 21.0 ± 4.14 pmol pregnenolone · mg protein^{-1} · min^{-1} (n = 11), whereas clonazepam and Ro15 1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzo-3-carboxylate) had no significant effect. HPLC analysis of clonazepam-, Ro5 4864- and PK 11195-stimulated extracts detected increasing levels of pregnenolone qualitatively consistent with the results obtained by radioimmunoassay. Therefore the radioimmunoassay was used routinely to determine pregnenolone levels. Clonazepam did not produce detectable levels of pregnenolone whereas Ro5 4864 and PK 11195 stimulated a significant increase in the amount of pregnenolone. Scatchard analysis of saturation binding data indicated the presence

Table 1
The potency of various benzodiazepine receptor ligands in displacing [^{3}H]Ro5 4864 binding and their effect on the rate of pregnenolone biosynthesis in brain mitochondria

Ligand	IC_{50} (nM)	$\Delta \pm$ S.E.M.
Clonazepam	3650	8 ± 11.2
Ro15 1788	825	12 ± 8.0
Diazepam	92	36 ± 7.9 [a]
Ro5 4864	3	63 ± 5.1 [b]
PK 11195	1	154 ± 116 [b]

The IC_{50} is defined as the concentration at which half-maximal inhibition of 1 nM [^{3}H]Ro5 4864 binding occurs. Mean difference (Δ) between control (21.0 ± 4.14 pmol pregnenolone · mg protein^{-1} · min^{-1}) and stimulated (+1 μM ligand) conditions expressed as mean ± S.E.M. pmol pregnenolone · min^{-1} · mg protein^{-1} of at least three independent experiments. Significantly different from control at [a] $P < 0.025$ and [b] $P < 0.005$ by Student's paired t-test.

Table 2
Kinetic parameters for the association and dissociation of 2 nM [^{3}H]Ro5 4864 in the presence or absence of 10 μM steroidogenesis activator peptide or 15 μM octadecaneuropeptide

	k_{-1} (min^{-1})	k_{obs} (min^{-1})	k_{+1} (min^{-1} nM^{-1})	K_d (nM)
Control	0.028 ± 0.0036	0.061 ± 0.0062	0.017	1.6
+ 10 μM steroidogenesis activator peptide	0.047 ± 0.0015 [a]	0.064 ± 0.0040	0.009	5.2
+ 15 μM octadecaneuropeptide	0.025 ± 0.0079	0.073 ± 0.0079	0.024	1.0

Mitochondria were incubated with 2 nM [^{3}H]Ro5 4864 at 0–4°C in the absence and presence of peptide using 3 μM cold Ro5 4864 to define nonspecific binding. Dissociation was initiated by the addition of 3 μM Ro5 4864. Rate constants, k_{-1} and k_{obs}, were derived from computerized linear regression of log of specific bound versus time and the natural log of the amount bound at a given time t as a percentage of the total bound at time $t = 0$ versus time, respectively. The k_{+1} was calculated from the equation $k_{+1} = (k_{obs} - k_{-1})/F$ where F equals the free radioligand concentration. The K_d values were calculated from the equation $K_d = k_{-1}/k_{+1}$. [a] Significantly different from control at $P < 0.001$ by Student's paired t-test.

Table 3
Amino acid sequences of peptides evaluated for activity in displacing [^{3}H]Ro5 4864 and steroidogenesis

Name	Sequence
Diazepam binding inhibitor (1–86)	SQADFDKAAEEVKRLKTQPTDEEMLFIYSHFK QATVGDVNTDRPGLLDLKGKAKWDSWNKLK GTSKENAMKTYVEKVEELKKKYGI
Triakontatetraneuropeptide (17–50)	TQPTDEEMLFIYSHFKQATVGDVNTDRPGLLKLK
Octadecaneuropeptide (33–50)	QATVGDVNTDRPGLLKLK
Diazepam binding inhibitor (42–50)	DRPGLLDLK
Diazepam binding inhibitor (42–50) NH_2	DRPGLLDLK-NH_2
Steroidogenesis activator peptide (1–30)	IVQPIISKLYGSGGPPPTGEEDTDEKKDEL
Random sequence	QQHIEKKMLRLEGHGDPLHLEEVKRHK

of a single high-affinity binding site for [^{3}H]Ro5 4864 with a dissociation constant (K_d) of 2.9 ± 0.36 nM and a B_{max} of 770 ± 29 fmol/mg protein. This equilibrium dissociation constant is consistent with the IC_{50} derived from [^{3}H]Ro5 4864 displacement data.

3.2. Activity of peptides

Given the ability of steroidogenesis activator peptide to stimulate steroidogenesis, it was of interest to determine if this effect was mediated by the peripheral benzodiazepine receptor in brain mitochondria. In the presence of 10 μM steroidogenesis activator peptide, the apparent equilibrium dissociation constant for [^{3}H]Ro5 4864 was significantly different from that under control conditons while the B_{max} was unchanged. Further analysis of the effect of steroidogenesis activator peptide on the binding of [^{3}H]Ro5 4864 to the peripheral benzodiazepine receptor was performed by measuring the effect of the peptide on the rate of dissociation. The k_{-1} values were significantly different between control conditions and in the presence of 10 μM steroidogenesis activator peptide resulting in rate constants of 0.028 min^{-1} and 0.047 min^{-1} respectively. There was no significant change in the k_{+1} in the presence of 10 μM steroidogenesis activator peptide (Table 2). Thus, in the presence of 10 μM steroidogenesis activator peptide the kinetically derived K_d was 5.2 nM, consistent with the K_d determined by equilibrium saturation experiments. In contrast, the presence of 15 μM octadecaneuropeptide had no effect on kinetically derived parameters (Table 2) and thus, the kinetically derived K_d was not significantly different from control.

Four fragments of diazepam binding inhibitor with reported contrasting binding activities at the central and peripheral benzodiazepine receptors were evaluated (Table 3). Octadecaneuropeptide (amino acids 33–50) has been reported to have low potency at the peripheral benzodiazepine receptor and greater potency at the central benzodiazepine receptor whereas triakontatetraneuropeptide (amino acids 17–50) is just the reverse (Barbaccia et al., 1988; Berkovich et al., 1990). Additionally, two shorter diazepam binding inhibitor fragments containing amino acids 42–50 (diazepam binding inhibitor 42–50), one having an amide group at the carboxy terminus (diazepam binding inhibitor 42–50 NH_2), and steroidogenesis activator peptide were evaluated. A random sequence of amino acids was included as a control. In contrast to that observed for the benzodiazepine ligands, the IC_{50} of these peptides against [^{3}H]Ro5 4864 did not correspond with their functional potency in stimulating steroidogenesis (Table 4). Indeed, a random sequence of amino acids displayed ability to inhibit peripheral benzodiazepine receptor binding under conditions

Table 4
The potency of various peptides in displacing [^{3}H]Ro5 4864 and their effect on the rate of pregnenolone biosynthesis in brain mitochondria

Peptide	IC_{50} (μM)	Δ ± S.E.M.
Octadecaneuropeptide + triakontatetraneuropeptide		−6 ± 2.2
Diazepam binding inhibitor (17–50), triakontatetraneuropeptide	5	−2 ± 6.4
Random sequence	25	7 ± 8.4
Diazepam binding inhibitor (42–50) NH_2	31	8 ± 7.1
Steroidogenesis activator peptide	46	8 ± 1.0 [a]
Diazepam binding inhibitor (33–50), octadecaneuropeptide	35	17 ± 5.7 [b]
Diazepam binding inhibitor (42–50)	43	19 ± 5.6 [b]

The IC_{50} is defined as the concentration at which half-maximal inhibition of 1 nM [^{3}H]Ro5 4864 binding occurs. Mean difference (Δ) between control (21.0 ± 4.14 pmol pregnenolone · mg protein^{-1} · min^{-1}) and stimulated (+1 μM peptide) rates expressed as mean ± S.E.M. pmol pregnenolone · min^{-1} · mg protein^{-1} of at least four independent experiments. Significantly different from control at [a] $P < 0.005$ and [b] $P < 0.05$ by Student's paired t-test.

identical to those used to measure benzodiazepine ligand activity relatively equal to that of the proposed endogenous modulators. Due to the low potency of these peptides as inhibitors of [^{3}H]Ro5 4864 binding to the peripheral benzodiazepine receptor, it was of interest to determine if this observation was simply a result of the peptides sequestering radioligand. None of the peptides evaluated sequestered [^{3}H]Ro5 4864 at concentrations up to 100 μM except triakontatetraneuropeptide which affected [^{3}H]Ro5 4864 binding in the absence of mitochondria at concentrations greater than 10 μM (data not shown). Furthermore, bovine serum albumin, a completely unrelated protein, was unable to inhibit [^{3}H]Ro5 4864 binding to the peripheral benzodiazepine receptor at concentrations up to 17 μM (data not shown). It is noteworthy that triakontatetraneuropeptide, which has a 10-fold lower IC_{50} than the others, was unable to stimulate steroidogenesis under the same experimental conditions. Steroidogenesis activator peptide, octadecaneuropeptide and diazepam binding inhibitor (42–50) all significantly increased steroidogenesis. Interestingly, triakontatetraneuropeptide (1 μM) blocked the ability of octadecaneuropeptide to stimulate pregnenolone synthesis.

4. Discussion

Neurosteroids (LeGoascogne et al., 1987) are steroids synthesized by nervous tissue that may include naturally occurring metabolites of progesterone and deoxycorticosterone. These steroids have been postulated to be endogenous ligands of a steroid recognition site coupled to the $GABA_A$-benzodiazepine receptor complex. In light of the well-documented observations that certain neurosteroids and other synthetic neuroactive steroids (for review, see Paul and Purdy, 1992) are potent modulators of GABAergic transmission, the physiological importance of evaluating the steroidogenic capabilities of neural tissue becomes apparent. Evidence has accumulated over the past decade demonstrating the presence of pregnane steroids in the brain (LeGoascogne et al., 1987). Recent studies by Purdy and coworkers (Purdy et al., 1991) provided some evidence that these steroids may be synthesized de novo since the levels of the neurosteroids increase under stress even in adrenalectomized rats. The data presented here add further support to this notion. The ability of oligodendrocytes and brain cell cultures to convert cholesterol to pregnenolone has been shown previously (Yi Hu et al., 1987; Jung-Testas et al., 1989). We have demonstrated here that the basal rate of pregnenolone synthesis can be stimulated by peripheral benzodiazepine receptor ligands in brain mitochondria. The rank order potencies of these ligands at the peripheral benzodiazepine receptor coincide with the rank order of these ligands in stimulating pregnenolone synthesis, suggesting the possible involvement of the peripheral benzodiazepine receptor in this important metabolic pathway.

The initial and rate-limiting step in steroidogenesis is the transport of cholesterol across the outer mitochondrial membrane. Due to the strategic location of peripheral benzodiazepine receptors on the outer membrane (Anholt et al., 1986), it is conceivable that the peripheral benzodiazepine receptor can be a mediator in this process. Recent reports have presented data strongly suggestive of a coupling between the peripheral benzodiazepine receptor and steroid biosynthesis in brain using in vivo protocols, adrenocortical and Leydig cells (Mukhin et al., 1989; Yanagibashi et al., 1989; Besman et al., 1989; Papadopoulos et al., 1990, 1991; Korneyev et al., 1993). The interpretation of these data has extended this association to a specific role for peripheral benzodiazepine receptors in facilitating the translocation of cholesterol into the mitochondria. Our results with benzodiazepine receptor ligands suggest that as in peripheral tissues, brain-derived mitochondria also have peripheral benzodiazepine receptor sites capable of mediating the stimulation of steroidogenesis. The relative potency of these ligands in displacing [^{3}H]Ro5 4864 correlates well with their ability to stimulate the synthesis of pregnenolone.

The effect of steroidogenesis activator peptide on steroidogenesis and its interaction with the peripheral benzodiazepine receptor is complex. The observed change in the dissociation rate constant of [^{3}H]-Ro5 4864 in the presence of steroidogenesis activator peptide and no change in the association rate constant is suggestive of an allosteric effect of steroidogenesis activator peptide on the binding of [^{3}H]Ro5 4864. The dissociation of [^{3}H]Ro5 4864 from the remaining sites would not be affected if steroidogenesis activator peptide and Ro5 4864 share the same site. Using Michaelis-Menten kinetics as a model, it is possible to predict the effect of steroidogenesis activator peptide on the apparent K_d (K_{dapp}) of Ro5 4864 for the peripheral benzodiazepine receptor if the interaction is competitive. In the presence of 10 μM steroidogenesis activator peptide and assuming a K_i of 46 μM for steroidogenesis activator peptide, one would predict a K_{dapp} for [^{3}H]Ro5 4864 of 3.5 nM. This value is inconsistent with the experimentally derived K_{dapp} of 5.7 nM. Conversely, when using 5.7 nM as the K_{dapp} an IC_{50} of 10 μM (we obtained an IC_{50} of 46 μM experimentally) for steroidogenesis activator peptide in the [^{3}H]Ro5 4864 binding assay is calculated. Thus, both kinetically- and equilibrium-derived K_{dapp} values suggest the interaction between steroidogenesis activator peptide and peripheral benzodiazepine receptor site is inconsistent with simple competitive interactions. Although we have presented intriguing data demonstrat-

ing that steroidogenesis activator peptide has an effect on the peripheral benzodiazepine receptor in brain mitochondria, its significance is unknown since steroidogenesis activator peptide has not thus far been measured in brain. However, precedence for the possibility of peptide involvement in steroidogenesis was established several years ago when steroidogenesis activator peptide was isolated from both Leydig cells and adrenocortical tissues (Pedersen and Brownie, 1983, 1987; Frustaci et al., 1989). Although a mechanism of action of steroidogenesis activator peptide was not clearly defined, these studies suggested a role for steroidogenesis activator peptide in enhancing cholesterol's availability to cytochrome P_{450}. Assuming that peripheral benzodiazepine receptors in brain mitochondria function similiar to those in peripheral tissues, steroidogenesis activator peptide would be expected to act in brain-derived mitochondria. Although steroidogenesis activator peptide may not be an endogenous modulator of central nervous system steroidogenesis, its effect in the studies described here is consistent with those reported for this peptide in other steroidogenic tissues.

Of perhaps greater physiological relevance is the isolation and identification of diazepam binding inhibitor as an endogenous peptide in the brain with the ability to bind peripheral benzodiazepine receptors. Diazepam binding inhibitor and some of its processing products (e.g., triakontatetraneuropeptide) have been found to bind with low micromolar potencies at the peripheral benzodiazepine receptor (Barbaccia et al., 1988; Berkovich et al., 1990). Interestingly, octadecaneuropeptide has been reported to preferentially bind central benzodiazepine receptors (Ferrero et al., 1986; Berkovich et al., 1990). Accordingly, it had been proposed that these peptides are endogenous modulators of steroidogenesis in the central nervous system. Using kinetic experiments to evaluate the interaction of octadecaneuropeptide and Ro5 4864 at the peripheral benzodiazepine receptor, no significant effect on either the k_{-1} or the k_{+1} was observed. Thus, the kinetically derived K_d values in control conditions and in the presence of octadecaneuropeptide were not different. These data argue against allosteric modulation of the peripheral benzodiazepine receptor by octadecaneuropeptide in brain mitochondria and imply octadecaneuropeptide displaces [^{3}H]Ro5 4864 binding competitively. Interestingly stimulation of steroidogenesis in brain mitochondria by two of the diazepam binding inhibitor fragments was observed. Under our conditions, triakontatetraneuropeptide had a 10-fold greater potency in displacing [^{3}H]Ro5 4864 binding than the other peptides in the group; however, this did not translate into steroidogenic efficacy. In fact, triakontatetraneuropeptide had no measurable effect on the rate of pregnenolone synthesis. In contrast, both octadecaneuropeptide and diazepam binding inhibitor (42–50) significantly stimulated steroidogenesis. Using a glioma cell line, Papadopoulos et al. (1992) reported an increase in pregnenolone formation by diazepam binding inhibitor and triakontatetraneuropeptide. However, both octadecaneuropeptide and clonazepam were observed to have no effect on pregnenolone synthesis. Additionally, in vivo studies have shown that PK 11195 antagonizes steroidogenesis (Korneyev et al., 1993). Subsequent studies in Leydig cells showed the action of diazepam binding inhibitor on steroid biosynthesis was not blocked by PK 11195, but the effect of diazepam binding inhibitor was mimicked by octadecaneuropeptide (Garnier et al., 1993). Interestingly, it was reported that diazepam binding inhibitor had no effect on pregnenolone synthesis in adrenocortical cells. On the basis of those experiments, the authors postulated that in Leydig cells the effect of diazepam binding inhibitor on steroidogenesis is mediated through a peripheral benzodiazepine receptor-independent mechanism. In the present experiments, triakontatetraneuropeptide was found to partially inhibit the stimulatory effect of octadecaneuropeptide. This is suggestive of an antagonist-like action of triakontatetraneuropeptide at the site at which octadecaneuropeptide acts to stimulate steroidogenesis. Whether octadecaneuropeptide and triakontatetraneuropeptide share the same site of action remains to be determined.

The weak potency of any of these peptides in displacing [^{3}H]Ro5 4864 from the peripheral benzodiazepine receptor cannot be explained by the sequestering of [^{3}H]Ro5 4864. Based on the contrasting effects of steroidogenesis activator peptide and octadecaneuropeptide on the kinetics of [^{3}H]Ro5 4864 binding, it is unlikely that the peptides all share a common site of action at the peripheral benzodiazepine receptor with the nonpeptide peripheral benzodiazepine receptor ligands. This is further supported by the lack of correlation between binding potency at the peripheral benzodiazepine receptor and efficacy in steroidogenesis. In a typical competitive interaction, the K_d changes in a predictable fashion while the B_{max} remains the same in the presence of the competitor. Among the peptides we evaluated, steroidogenesis activator peptide appears to act independent of the peripheral benzodiazepine receptor whereas octadecaneuropeptide may act on the peripheral benzodiazepine receptor. The inconsistency of the actions of diazepam binding inhibitor and its processing products on pregnenolone synthesis in various tissues as reported in the literature suggests that their actions may be complex and somewhat dependent on the specific experimental protocol in which they are evaluated. It also highlights the need for careful evaluation of the individual characteristics of these peptides as modulators of binding to the peripheral benzodiazepine receptor. Together with the

kinetic data described in this report, the possibility of a site and mechanism of action for some of these peptides that is independent of the peripheral benzodiazepine receptor in brain mitochondria cannot be ruled out. In view of the high micromolar concentrations needed to stimulate steroidogenesis, their physiological role if any is dependent upon the local concentrations of these peptides in the brain.

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