with the classical two-state, concerted model for allosteric enzymes (*1*). The cooperative binding of both inhibitor and substrate is inconsistent with the model. Furthermore, because Trp inhibition of the hybrid complex is partially competitive with respect to Chr, whereas inhibition of the wt complex and the monomeric TrpE subunit is strictly competitive, it can be inferred that the conformational and kinetic consequences of Trp binding to a single TrpE subunit of the complex are not symmetrical. Instead, the effects appear to be more stringent for the active site of the liganded TrpE subunit than for the active site of the unliganded subunit. Finally, the results of dye-ligand chromatography of the wt and various mutant enzymes have indicated at least three conformational states for the complex, with the unliganded enzyme being conformationally distinct from that of the Trp-liganded and Chr-liganded enzymes (*16*). Thus the properties of the AS-PRT complex appear to be better described by a variation of the sequential model for allosteric enzymes (*2*) where multiple conformational states arise as a result of the tertiary and quaternary effects of the successive binding of ligands by the subunits of the enzyme.

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13. Mutant TrpE subunits are designated by the nature and location of the amino acid change. The single amino acid code (F, Phe; I, Ile; S, Ser; and T, Thr) is used. Thus, $TrpE^{S40F}$ and $TrpE^{T425I}$ represent mutant subunits with amino acid changes of Ser^{40} → Phe and Thr^{425} → Ile, respectively.
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15. The $(TrpE^{T425I})_2(TrpD^{+})_2$ complex lacks AS-Gln activity but has wt PRT activity with normal sensitivity to feedback inhibition (*16*). The $(TrpE^{S40F})_2(TrpD^{+})_2$ complex has wt AS-Gln and PRT activities, both of which are completely resistant to inhibition by Trp (*8*).
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21. Mutant *S. typhimurium* TrpE subunits were prepared from *E. coli* strains CB96/pSTG39 ($TrpE^{S40F}$) and CB96/pSTH16 ($TrpE^{T425I}$). Wild-type *S. typhimurium* TrpD subunit was prepared from *E. coli* strain CB25/pSTG119 ($TrpD^{+}$). Plasmids pSTG39 and pSTG119, derivatives of pBR327, and plasmid pSTH16, a derivative of pBR322, carry their respective *trp* genes under the control of the native *trp* promoter. Host strains CB96 (C600 *ΔtrpED24 leuB6 hsdR thi-1*) and CB25 (*W3110 ΔtrpE-A2 tna2 bglR*) were provided by C. Yanofsky. Overexpression of the mutant TrpE and wt $TrpD^{+}$ polypeptides was achieved by derepression of the genes in cultures grown under conditions of Trp limitation (*8*) in supplemented minimal medium [K_2HPO_4 (10.5 g/liter), KH_2PO_4 (4.5 g/liter), $(NH_4)_2SO_4$ (1.0 g/liter), $MgSO_4$ (0.1 g/liter), glucose (2.5 g/liter), thiamine (50 μM), L-Trp (3 mg/liter), Leu (25 mg/liter), each of the other 18 protein amino acids (50 mg/liter), and ampicillin (25 mg/liter)]. Crude extracts were prepared and partially purified by ammonium sulfate precipitation (38% saturation) as described (*4*). For the preparation of radiolabeled $TrpE^{S40F}$ and $TrpE^{T425I}$ subunits, cultures of CB96/pSTG39 and CB96/pSTH16 were grown as above except that either 100 μCi [U-^{14}C]L-leucine (184 mCi/mmol) or 600 μCi [3,4,5-^{3}H]L-leucine (108 mCi/mmol) were added at the time of inoculation, as indicated.
22. The $TrpD^{+}$ subunit dimerizes when present in vitro at high concentrations [M. Grieshaber and R. Bauerle, *Biochemistry* **13**, 373 (1974)]. Although the dimeric form has undiminished PRT activity, it is not competent for assembly with the TrpE subunit. We estimated the amount of the assembly-competent monomer in the $TrpD^{+}$ preparation immediately before its use in the assembly experiment by assaying both its PRT activity and its ability to complement TrpE in the AS-Gln reaction (*4*). Any increase in the PRT/AS-Gln activity ratio over that of the wt complex (1.4) was used to estimate the proportion of monomer present and to calculate the amount of the preparation needed for the experiment.
23. The sonication of mixtures of whole cells, instead of the mixing of partially purified subunits as used in the experiment of Table 1, minimized the problem of spontaneous dimerization of the $TrpD^{+}$ subunit, but prevented the use of a defined stoichiometry of subunits because of the different levels of expression of the three subunits.
24. The ^{3}H and ^{14}C radioactivities were determined in 0.50-ml samples of each fraction in the LKB model 1215 liquid scintillation counter (Pharmacia LKB) with Ready-Solv counting fluid (Beckman). In Fig. 1A, counts were corrected for spillover, which was <1% for ^{3}H and ~8% for ^{14}C. Offscale counts in fraction 1 of each experiment were as follows: ^{3}H, 45,800 cpm; ^{14}C, 36,200 cpm (Fig. 1A); ^{3}H, 42,500 cpm (Fig. 1B); and ^{14}C, 36,000 cpm (Fig. 1C). AS-Gln activity was assayed as described (*4*).

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A Corticosteroid Receptor in Neuronal Membranes

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Steroids may rapidly alter neuronal function and behavior through poorly characterized, direct actions on neuronal membranes. The membrane-bound receptors mediating these behavioral responses have not been identified. [^{3}H]Corticosterone labels a population of specific, high-affinity recognition sites (dissociation constant = 0.51 nanomolar) in synaptic membranes from an amphibian brain. These binding sites were localized by receptor autoradiography in the neuropil, outside the regions of perikarya. The affinities of corticoids for this [^{3}H]corticosterone binding site were linearly related to their potencies in rapidly suppressing male reproductive behavior. Thus, it appears that brain membranes contain a corticosteroid receptor that could participate in the regulation of behavior.

IN THE CLASSIC MODEL OF STEROID hormone action, steroids bind to intracellular receptors, which act as ligand-dependent transcription factors that regulate gene expression (*1*). In addition to these well-known actions of steroid receptors, steroids may alter brain function through nongenomic mechanisms (*2*). For example, in rats, short-term exposure to the gonadal steroid progesterone is associated with rapid changes in behavior, and this effect occurs in the absence of new protein synthesis (*3*). Gonadal and adrenal steroid hormones can alter neuronal firing activity within milliseconds to minutes of administration (*4*), and these responses can occur in brain regions lacking classic steroid receptors (*5*) or if steroid access to intracellular receptors is blocked (*6*). These events appear to be mediated by direct steroid action on neuronal membranes, but there is little information concerning steroid binding to membrane-bound recognition sites in the brain (*7, 8*).

To investigate the possibility that glucocorticoid receptors occur on neuronal membranes, we performed radioligand binding studies on synaptic (P2) membranes from brains of the amphibian *Taricha granulosa*, which is known to have rapid behavioral responses to corticosterone (CORT) (*9*). Equilibrium saturation binding experiments (*10, 11*) indicated that ^{3}H-labeled CORT binding to brain membranes was specific, saturable, and of high affinity [dissociation

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constant (K_d) = 0.51 nM] (Fig. 1A). Kinetic experiments indicated that specific binding of [^{3}H]CORT was relatively rapid and reversible (Fig. 1, B and C). The K_d calculated from kinetic data (0.16 nM) was close to the K_d estimated from equilibrium saturation data. The affinity of this site differs from the low affinity (120 nM) of the [^{3}H]CORT binding site described in the synaptosomal fraction from rats (*7*).

The specific binding of [^{3}H]CORT was temperature-sensitive—it was greatest at 30°C but eliminated when assays were performed at 60°C (*12*). Specific binding was also inhibited in a concentration-dependent manner by treatment of the membranes with the protease trypsin (0.001 to 1.0 mg/ml). Together, these data suggest that [^{3}H]-CORT binds to a proteinaceous recognition site in the P2 fraction (*13*).

The binding site was highly specific for CORT (Table 1) and did not have a pharmacological signature resembling that of mammalian (*14*) or amphibian (*15*) intracellular corticoid receptors. The mineralocorticoid aldosterone, the glucocorticoid dexamethasone, and other Type I and Type II corticoid receptor ligands we tested did not display high affinity for this membrane-associated binding site. Furthermore, the [^{3}H]CORT binding site did not appear to be associated with the γ-aminobutyric acid ($GABA_A$) receptor. The steroid metabolites 5α-pregnan-3α,21-diol-20-one (5α-THDOC) and 5α-pregnan-3α-ol-20-one (3α-OH-DHP) are potent modulators of mammalian (*16*) and *Taricha* (*17*) $GABA_A$ receptors, but 5α-THDOC inhibited [^{3}H]CORT binding with only modest affinity, whereas 3α-OH-DHP was devoid of activity (Table 1). In addition, nonsteroidal $GABA_A$ receptor ligands did not displace [^{3}H]CORT binding to membranes. The inability of $GABA_A$ receptor ligands to alter [^{3}H]CORT binding is consistent with the inability of CORT to alter $GABA_A$ receptor ligand binding or GABA-stimulated chloride flux in rodent (*18*) and *Taricha* brains (*17*).

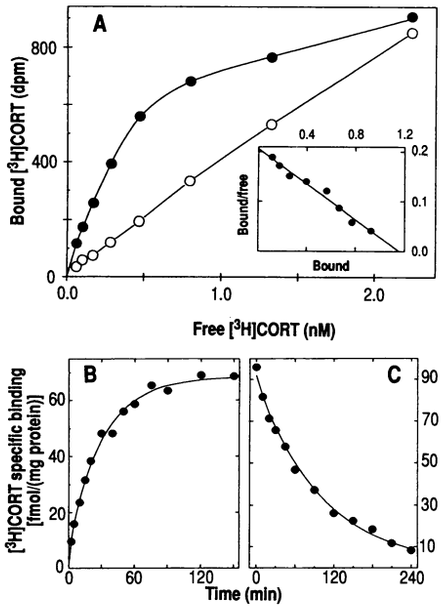

Fig. 1. Binding of [^{3}H]CORT to crude synaptosomal membranes. (**A**) Equilibrium saturation binding. P2 fraction resuspensions were incubated with increasing concentrations of [^{3}H]CORT (101.6 Ci/mmol; New England Nuclear, Boston). Specific binding (●) equals total binding (not shown) minus nonspecific binding (○). Estimates of K_d = 0.51 ± 0.04 nM and maximum binding capacity = 146 ± 4.4 fmol per milligram of protein were obtained by nonlinear regression analysis (Lundon 1). The data were best fit by a one-site model. (**Inset**) Scatchard replot of data was linear; Hill coefficient = 0.98 (LIGAND). (**B**) Association of [^{3}H]CORT-specific binding to brain membranes. P2 fraction resuspensions were incubated with [^{3}H]CORT (0.5 nM) for increasing intervals. Data were fit by a one-site model (LIGAND); pseudo–first-order rate constant, k_{obs}, is 0.054 ± 0.005 min^{-1}. (**C**) Dissociation of [^{3}H]CORT from neuronal membranes. P2 fraction resuspensions were incubated for 2 hours with [^{3}H]CORT (0.5 nM) before addition of unlabeled CORT (100 μM). Data were best described with a one-site model; dissociation rate constant, k_{-1}, is 0.013 ± 0.001 min^{-1}.

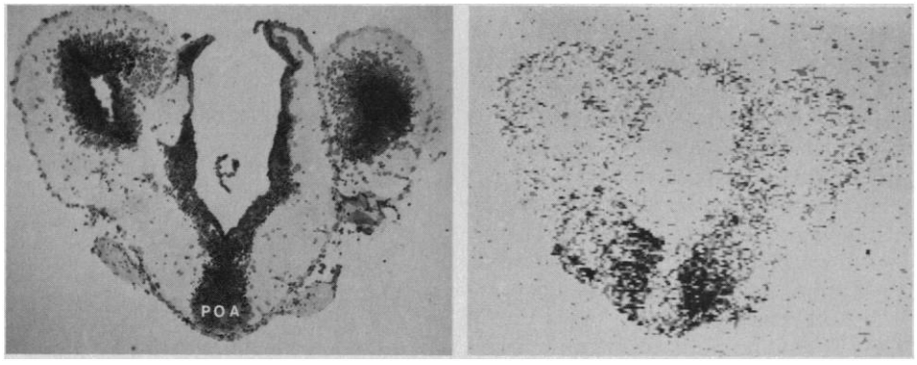

Fig. 2. Distribution of putative, membrane-bound [^{3}H]CORT binding sites in the POA. Section (**left**) showing darkly stained perikarya of POA neurons. Autoradiogram of identical section (**right**) shows localization of [^{3}H]CORT-specific binding sites in the neuropil surrounding perikarya of POA neurons.

Because the P2 fraction contains mitochondria as well as pre- and postsynaptic membranes (*19*) and [^{3}H]CORT binds to adrenal and liver (*20*) mitochondria, we examined [^{3}H]CORT binding to synaptosomal and mitochondrial fractions prepared by discontinuous sucrose gradient centrifugation (*21*). We also measured the binding of the muscarinic cholinergic antagonist, quinuclidinyl benzilate (QNB) (*22*), and succinate cytochrome c reductase activity (*23*) as markers for synaptic membranes and mitochondria, respectively. The succinate cytochrome c reductase assay indicated that mitochondria were well separated from synaptic membranes (1.2, 10.6, and 2.8 units per minute per milligram of protein for homogenate, mitochondrial fraction, and synaptosomal fraction, respectively).

Our data indicate that [^{3}H]CORT binds to synaptic membranes. The specific binding of [^{3}H]CORT was most enriched, more than 11-fold, in the synaptosomal fraction. (The ratios of [^{3}H]CORT-specific binding activity to binding activity in homogenate were as follows: crude cytosolic, 0.06; nuclear, 0.59; mitochondrial, 7.04; synaptosomal, 11.50.) Furthermore, [^{3}H]CORT binding activity paralleled the enrichment of muscarinic receptor–specific binding in the synaptosomal fraction. (The ratios of [^{3}H]QNB-specific binding activity to [^{3}H]QNB binding activity in homogenate were as follows: crude cytosolic, 0.06; nuclear, 0.68; mitochondrial, 3.06; synaptosomal, 11.20) (*24*). The minimal amount of [^{3}H]CORT binding in the nuclear and crude cytosolic fractions demonstrated that, under these conditions, [^{3}H]CORT binding to intracellular receptors is negligible, thus providing further evidence that [^{3}H]CORT binding activity in the synaptosomal fraction is not attributable to intracellular receptor contamination.

To further substantiate the presence of CORT binding sites in synaptic membranes, we performed in vitro receptor autoradiography. Sections of *Taricha* brain were incubated with [^{3}H]CORT in the presence of unlabeled ligands specific for intracellular receptors (*25*). Under these conditions, [^{3}H]CORT-specific binding sites were detectable, with the greatest density in distinct regions of neuropil in areas including the amygdala, preoptic area (POA), and hypothalamus (Fig. 2). These [^{3}H]CORT binding sites were located almost exclusively outside regions of perikarya. The localization of binding sites in neuropil, areas rich in synaptic terminals, provides corroborating evidence for the presence of CORT binding sites on synaptic membranes.

A series of behavior experiments were designed to determine if [^{3}H]CORT bind-

Table 1. Potency of steroids as inhibitors of the specific binding of [^{3}H]CORT to crude synaptosomal membranes. Inhibition constant (K_i) values and slopes were determined by nonlinear regression analysis (LIGAND); data are mean ± SEM. Maximal inhibition was reported for 1 μM competitor (Sigma, Steraloids, New England Nuclear; RU 38486 courtesy of Roussel-UCLAF, France) and 0.5 nM [^{3}H]CORT. The highest concentration of solvent used (0.3% ethanol–0.1% dimethyl sulfoxide) was added to control tubes.

Compound	K_i (nM)	Maximal inhibition (%)	Slope
Corticosterone	0.11 ± 0.006	100	0.96 ± 0.03
Cortisol	3.75 ± 0.56	100	0.92 ± 0.08
Aldosterone	293 ± 13	69	1.06 ± 0.06
5α-THDOC*	297 ± 14	70	0.96 ± 0.05
RU 28362	569 ± 40	36	1.14 ± 0.24
Progesterone	759 ± 113	37	0.99 ± 0.10
Testosterone	1138 ± 63	32	0.96 ± 0.03
ZK91587	>5000	13	
Dexamethasone	>5000	11	
RU 38486	>5000	8	
3α-OH-DHP*	>5000	5	

*Potent modulators of $GABA_A$ receptors.

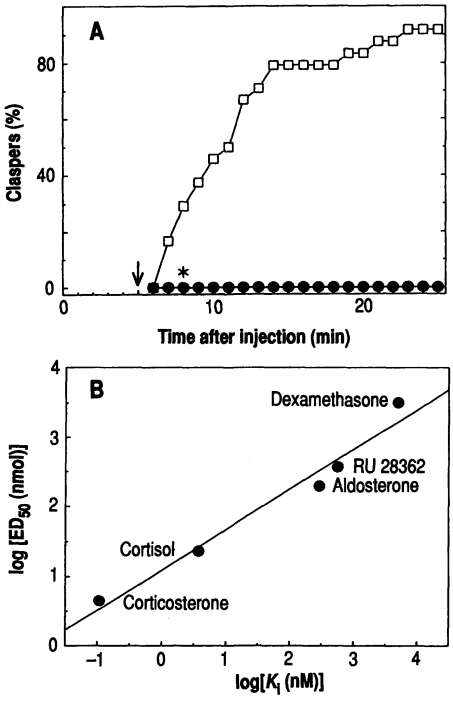

Fig. 3. Inhibition of male sexual behavior. (**A**) Latency of response to CORT. Males were injected with 32 nmol (11 μg) of CORT (●) or vehicle (□), n = 14. Arrow indicates time of addition of females to tanks. Data are reported as cumulative percentage of claspers at 1-min intervals. Males injected with CORT were significantly inhibited (*) within 3 min of testing (Fisher exact test, P = 0.025). (**B**) Linear relationship between potency of steroids in inhibition of [^{3}H]CORT binding (ordinate) and potency in inhibition of sexual behavior (abscissa). Males were injected with one of five to seven doses of steroid or of vehicle (n = 24 for each dose of each steroid, except n = 14 for RU 28362). Data were recorded as number of claspers in 20-min tests (except cortisol, for which 60-min tests were performed late in the breeding season). Half-maximal effective dose (ED_{50}) values for inhibition of male clasping behavior versus controls were obtained by probit analysis (*29*).

ing sites could be behaviorally relevant receptors (*26*). Stress can suppress the sexual behavior of male *Taricha*; this rapid response is dependent on the adrenal (interrenal) steroid CORT and is mimicked by CORT injection (*9*). Male sexual behavior was suppressed within 8 min of intraperitoneal injection, compared to behavior of vehicle-injected controls (Fig. 3A). A behavioral response this rapid is consistent with the presence of CORT receptors on synaptic membranes. Sexual behavior was inhibited by a low dose of CORT (2.5 nmol; Fisher exact test, $P < 0.005$), consistent with a high-affinity recognition site for CORT. We established dose responses for a series of steroids as inhibitors of male sexual behavior (Fig. 3B). The potencies of corticoids in rapid inhibition of sexual behavior were linearly related to their potencies in inhibition of [^{3}H]CORT binding (slope ± SE = 0.58 ± 0.06; $P < 0.003$). These data suggest that this binding site, or pharmacologically similar receptors in the spinal cord or peripheral tissue, could be involved in stress-induced suppression of reproductive behavior.

In conclusion, our binding studies reveal the presence of a specific, high-affinity, ligand-receptor interaction (*27*) in synaptic membranes. Autoradiographic studies provide further evidence for the localization of CORT binding sites in synaptic membranes (in brain regions known to regulate sexual behaviors). The linear relationship between the potencies of compounds in interaction with this CORT receptor and inhibition of behavior suggests that this receptor may mediate CORT regulation of sexual behavior (*28*).

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10. Whole brains from males were homogenized in cold 0.32 M sucrose containing 5 mM Hepes (pH = 7.45) and centrifuged at 1,000*g* for 15 min, and the resulting supernatant was centrifuged at 30,000*g* for 40 min. The P2 pellet was frozen, then resuspended in 150 volumes (w/v) cold buffer (Hepes, 25 mM; EDTA-free acid, 1 mM; bacitracin, 60 μg/ml; pH = 7.45) for 3 hours to dissociate endogenous ligand. After centrifugation at 30,000*g* for 30 min, the final pellet was frozen, resuspended in assay buffer (Hepes, 25 mM; EDTA salt, 0.5 mM; NaCl, 200 mM; pH = 7.45) to a protein concentration of 450 to 550 μg/ml. Protein concentration was determined by Bio-Rad (Rockford, IL) microassay with a bovine serum albumin standard.
11. Incubations (45 to 55 μg of protein in 300 μl) were terminated after 2 hours by vacuum filtration and a 9-ml wash with buffer over Whatman GF-C filters. Radioactivity bound to filters was measured by liquid scintillation spectroscopy. Nonspecific binding was determined by addition of 10 μM unlabeled CORT. Specific binding was typically 75 to 80% of total binding. All binding experiments were performed in triplicate and were repeated at least twice, with similar results.
12. Specific binding was low at 2°C, moderate at 15°C and 45°C, and negligible at 60°C. Binding studies were routinely performed at 15°C, which is in the physiological temperature range of *Taricha*.
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22. We incubated P2 fraction resuspensions with 0.5 nM [^{3}H]QNB ± 10 μM scopalamine to determine nonspecific binding.
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24. The greater enrichment of [^{3}H]CORT binding in the mitochondrial fraction relative to [^{3}H]QNB suggests there may be binding sites for CORT in neuronal mitochondria as well as in synaptic membranes.
25. Thaw-mounted 25-μm brain sections were incubated for 30 min at 22°C with assay buffer containing dexamethasone and ZK91587 (200 nM), then incubated for 2 hours with [^{3}H]CORT (2 nM) in buffer containing dexamethasone and ZK91587 (200 nM). Nonspecific binding was determined in alternate sections by the addition of CORT (10 μM). The reaction was terminated by two 3-min washes in ice-cold buffer. Sections were dried under cool air, apposed to ^{3}H-sensitive film for 2 months, and stained with toluidine blue for histology. The autoradiogram was analyzed by computer-assisted densitometry (DUMAS). Specific binding was determined by subtraction of nonspecific binding from the aligned total binding sections.
26. Adult male *Taricha* in breeding condition were collected locally and experiments were conducted in the field. Males (mean weight, 22 g) received intraperitoneal injections (0.1 ml) of steroid or vehicle (amphibian Ringers with 8% ethanol–2% dimethyl sulfoxide). Testing was initiated 5 min after injection, when stimulus females were added to tanks holding males. Males displaying sexual behavior (dorsal amplectic clasping of female) were removed from tanks. Females received 500 μg of progesterone by injection 24 hours before tests to enhance attractivity [F. L. Moore, *Copeia* **3**, 530 (1978)].
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An Analog of Myristic Acid with Selective Toxicity for African Trypanosomes

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***Trypanosoma brucei*, the protozoan parasite responsible for African sleeping sickness, evades the host immune response through the process of antigenic variation. The variant antigen, known as the variant surface glycoprotein (VSG), is anchored to the cell surface by a glycosyl phosphatidylinositol (GPI) structure that contains myristate (*n*-tetradecanoate) as its only fatty acid component. The utilization of heteroatom-containing analogs of myristate was studied both in a cell-free system and in vivo. Results indicated that the specificity of fatty acid incorporation depends on chain length rather than on hydrophobicity. One analog, 10-(propoxy)decanoic acid, was highly toxic to trypanosomes in culture although it is nontoxic to mammalian cells.**

THE EFFECTS OF AFRICAN TRYPANOSOMIASIS on human health and on livestock production are crippling (*1*). Of the 50 million people at risk for sleeping sickness, fewer than 20% have access to protection from, or treatment for, this frequently lethal illness. In addition, the only drug widely used to combat late-stage (meningoencephalitic) disease, melarsoprol, causes fatal side effects in up to 5% of those treated (*2*). There is thus a need for new approaches to chemotherapy and for the design of less toxic drugs. One general approach to drug development is to examine the pathogenic organism for features not shared with its host, in the hope of exploiting these differences to generate selectively toxic agents.

The attachment of surface proteins to eukaryotic cell membranes via GPI structures has been described only in the last few years (*3, 4*). The trypanosome VSG is the best characterized protein of this type, and it differs from mammalian GPI-anchored proteins in containing exclusively myristate (14:0, a fully saturated 14-carbon fatty acid) in its GPI moiety (Fig. 1A) (*5*). During VSG biosynthesis, the anchor is preformed as a GPI called glycolipid A (*6*) [or P2 (*7*)], to which the COOH-terminus of the polypeptide is later attached. The pathway of GPI biosynthesis in trypanosomes has been elucidated by means of a cell-free system (*8–10*) capable of forming glycolipid A. In the presence of uridine 5′-diphosphate–*N*-acetylglucosamine (UDP-GlcNAc) and guanosine 5′-diphosphate–mannose (GDP-Man), the glycan portion of this GPI is constructed de novo on endogenous phosphatidylinositol (PI), producing glycolipid A′ (*8*). This GPI contains two fatty acids more hydrophobic than myristate [one of which is stearate (18:0)], which are sequentially replaced by myristate via a process termed fatty acid remodeling (*10*). First, glycolipid A′ is deacylated to form glycolipid θ, a lyso intermediate. In the presence of myristoyl coenzyme A (CoA), myristate is added to glycolipid θ to form glycolipid A″, with one myristate and one stearate. The final steps in the process involve replacement of the stearate with a second myristate, to form glycolipid A. In the absence of UDP-GlcNAc and GDP-Man, there is no de novo GPI biosynthesis in the cell-free system; however, ^{3}H-labeled myristate is still incorporated into preexisting glycolipid A by a presumed acyl exchange process (*10*).

It is intriguing that the trypanosome anchor strictly requires myristate, particularly because this fatty acid is rare in the GPIs of mammalian proteins (*3*). The specificity is more striking because *T. brucei* lacks the ability to synthesize fatty acids de novo (*11*) and must import these compounds from the infected host's bloodstream. Because mammalian serum contains relatively little myristate [for example, 1 to 2% of fatty acids from total lipids in rat plasma (*12*)], the import process may be quite selective.

To investigate the biological function of the myristoyl moieties of the VSG anchor, we introduced analogs of myristate into the trypanosome system. These fatty acid ana-

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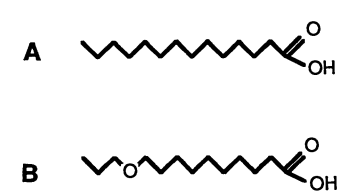

Fig. 1. Structures of myristate (**A**) and O-11 (**B**).