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Ca^{2+}/Calmodulin System: Participation in the Progesterone-Induced Facilitation of Lordosis Behavior in the Ovariectomized Estrogen-Primed Rat

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Lordosis behavior Sexual behavior Rat

LORDOSIS is an estrogen-dependent essential reflex for reproduction in rats and many other mammals. This characteristic sexual behavior is produced as a response to copulatory stimulation by the male (2). It has been shown experimentally that in ovariectomized (Ovx) females, adequate sexual behavior depends on the sequential action of estradiol (E_2) and progesterone (P) (4). However, despite numerous experiments (36), the intimate brain mechanisms of these steroids on the modulation of sexual behavior are still insufficiently understood. A generalized mechanism of action for estrogens involves an association between estrogen and a cytoplasmic receptor, translocation of this estrogen complex to the nucleus, initiation of messenger RNA synthesis, and eventual production of new proteins. However, it seems that this increase in protein synthesis is insufficient to induce female sexual behavior (1).

There is more uncertainty concerning the mechanism of action of P (8). As in the case of estrogens, P is generally thought to pass through the plasma membrane and act at the level of the nucleus. However, some experimental results seem to be better explained by the participation of nongenomic mechanisms (8). Recent reports have clearly shown the presence of specific membrane receptors for P in some cells, including hypothalamic (20), oocytes (42), and spermatozoa

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(44). P binding to these cells induced immediate modifications on the intracellular concentration of free active ions, mainly Cl^- and Ca^{2+} (8,44).

Beginning with the pioneering work of Meyerson (28), an increasing number of research papers have approached the study of neural regulation of rodent sexual behavior from the pharmacologic point of view. In effect, our work and that of other groups indicates that the participation of P in lordosis behavior can be mimetized, in E_2-primed rats, by P-E_2 (39), luteinizing hormone-releasing hormone (LHRH) (31), forskolin, cholera toxin, cyclic nucleotides, both cyclic AMP and cyclic GMP (5), norepinephrine (12), α- and β-receptor agonists (11), and cholinergic agonists such as carbachol (19), and also presumably through muscarinic receptors (37). On the other hand, the sexuality-inducing behavior of P can be inhibited by some antagonist of these reagents (8,36). Reviewing these works, Whalen and Lauber (48) came to the conclusion that because serotonergic, dopaminergic, and noradrenergic agonists and antagonists, as well as cholinergic agonists, all facilitate sexual receptivity, it is possible that drugs from a variety of neurotransmitter categories act through a common nonspecific mechanism, possibly one involving increasing neural levels of cyclic guanosine monophosphate (cGMP) (48). On the other hand, most of these active substances share the property of acting, at least partially, through mechanisms modulated by the calcium/CaM system (7).

Calmodulin, a widely distributed Ca^{2+}-binding protein, participates in the regulation of many of the functions previously ascribed to intracellular Ca^{2+} (22,27). Among other important biochemical mechanisms related to the induction of sexual behavior, CaM is required in the calcium-dependent phosphorylation of receptors and autoreceptors (36). CaM is also required for the activation of a great number of enzymes, including the rate-limiting step in catecholamine synthesis, tyrosine hydroxylase (TS) (13), adenylate and guanylate cyclases, the Ca^{2+}-dependent protein kinases, and so forth. CaM inhibitors behave as important antagonist of neurotransmitter-receptor interactions, including dopaminergic, adrenergic, serotonineregic, and muscarininc cholinergic receptors (37,40, 47). In addition, Ca^{2+}-CaM complexes participate in nerve conduction and/or transport (14,33,34) and also as promoters of hormonal and neurotransmitter secretion in a wide variety of cells (7).

On basis of these data, we considered it interesting to study the effects of some inhibitors of the Ca^{2+}-CaM system on the P-facilitated sexual receptivity of ovariectomized estrogen-primed rats.

METHOD

Chemicals

Analytic-grade haloperidol, trifluoperazine (TFP), and pimozide were graciously donated by Janssen Pharmaceuticals (Beerse, Belgium). Verapamil was obtained from Knoll Pharmaceutical (Mexico City, Mexico), on its therapeutic parenteral presentation. Estradiol benzoate (EB) and P (P_4) were purchased from Sigma Chemical Co. (St. Louis, MO).

Young, sexually inexperienced Wistar female rats (250 ± 20 g) were used in this study. They were maintained at 23°C under a controlled reverse 14 L : 10 D and fed with Purina chow (BioServ, Frenchtown, NJ) and water ad lib. Ovariectomies were performed under ether anesthesia. Then, 2–3 weeks after ovariectomy, rats were primed with 2 μg EB (time 0), and 44 h later, they were injected with 2 mg P and divided into groups of 10 animals each. Immediately after the application of P, each group received, contralaterally, one of the following treatments (Table 1): saline (controls), sodium pentobarbital (25 mg/kg body wt.), TFP, promethazine (PMZ), chlorpromazine (CPZ), haloperidol (HAL), pimozide (PIM), and verapamil (VER). All injections were given subcutaneously. EB and P were dissolved in sesame oil and injected in a volume of 0.2 ml. The active agents were used at concentration of 1, 2, 4, and 8 mg/kg body wt. and were dissolved and applied in the manner previously described (38).

Animals were tested for sexual behavior with experienced males 4 h after the applications of P plus the neuroleptics. Observations were performed under subdued light, always during the dark phase of the life cycle, in circular Plexiglas arenas (53 cm in diam. and 45 cm in height). We allowed 10 vigorous mounts for each female; their receptivity was expressed by their lordosis quotient (LQ), or the number of lordosis per number of mounts. The observer was blinded to the pharmacologic treatment of individual females.

To discriminate between sedative and behavioral effects, each female was carefully checked immediately before the study of sexual behavior to detect any significant impairment of locomotor and/or postural reflexes. Animals whose reflexes were not considered normal were discarded from all subsequent observations. To further detect the presence of undesired sedation, the lordosis quality (15) was scored on a scale from 0 to 3, representing no lordosis, and light, moderate, and full dorsiflexion, respectively. Only lordosis corresponding to grades 2 and 3 were considered positive. In addition, we also observed the occurrence of precopulatory patterns of behavior on the part of the female: presenting posture, hopping, and darting, in the way proposed by Madlafousek and Hlinák (26).

We performed statistical analysis using a 486DX2 IBM-compatible PC and the Microstat II, Ecosoft Inc. (Indianapolis, IN, USA) statistical Package. Data were processed by multitrial analysis of variance (ANOVA) for two factors: compound administration and dose. We used 95% confidence for the mean test for post hoc comparison between groups. Fisher's exact tables were used to assess the significance of differences in the proportion of rats showing sexual behavior. Group comparisons were carried out by the Student's *t*-test.

RESULTS

Careful observation of the treated animals indicated that, as was previously observed (17), drugs with a low antipsychotic effect, such as prometazine, showed the highest levels of sedation of the tested drugs in the period immediately following the injection. This drug was also the least effective in modifying P-induced lordosis behavior. In addition, pentobarbital had a sedative effect equal to, and frequently more than, any of the anticalmodulin agents tested. Also, the hypnotic effect of pentobarbital was more prolonged, and some of the animals treated with this substance showed mild alterations of postural reflexes at the time of the sexual behavior test. The obtained results show that this mild impairment was not enough to interfere with the occurrence of the lordosis reflex. These findings allowed us to eliminate the role played by the sedative effect of the tested drugs as the main cause for the modifications observed in the sexual behavior. In addition, a balanced pattern of precopulatory activity (26) was generally observed in all animals that showed lordosis behavior.

Table 1 summarizes the results obtained in these experiments. All drugs tested at doses of 4 mg/kg or higher inhibited

TABLE 1

EFFECT OF SOME COMPOUNDS THAT INHIBIT THE Ca^{2+}-CaM SYSTEM ON THE HOMOTYPIC SEXUAL BEHAVIOR OF OVARIECTOMIZED RATS SEQUENTIALLY TREATED WITH EB AND P

Compound	Control	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg
Prometazine	0.95 ± 0.08	0.92 ± 0.07[a]	0.91 ± 0.06[a]	0.77 ± 0.22*[a]	0.55 ± 0.28*[a]
Chlorpromazine	1.00 ± 0.00	0.96 ± 0.03[a]	0.91 ± 0.05[a]	0.41 ± 0.26*[b]	0.26 ± 0.10*[b]
Haloperidol	0.92 ± 0.08	0.83 ± 0.06*[b]	0.66 ± 0.14*[b]	0.28 ± 0.16*[b]	0.12 ± 0.05*[c]
Trifluoperazine	1.00 ± 0.00	0.93 ± 0.06[a]	0.54 ± 0.25*[b]	0.21 ± 0.07*[c]	0.10 ± 0.03*[c]
Pimozide	0.95 ± 0.05	0.38 ± 0.07*[c]	0.26 ± 0.11*[c]	0.19 ± 0.06[c]	0.08 ± 0.02*[c]
Verapamil	0.97 ± 0.02	0.71 ± 0.13*[b]	0.48 ± 0.09*[b]	0.34 ± 0.13[b]	0.22 ± 0.05*[b]
Pentobarbital (25 mg/kg)	0.89 ± 0.10				

Lordosis quotient (mean ± SD).

*$p < .05$ compared with the group immediately to the left. Different superscript letters indicate the existence of a statistically significant difference ($\leq p < .05$) between the values on the same column.

lordosis. Only HAL, PIM, and VER were active with the lowest dose used (1 mg/kg). The maximum level of activity was shown by pimozide, although at a dose of 8 mg/kg, no statistical difference was found between this compound and TPZ or HAL. Results obtained by the application of pentobarbital (25 mg/kg) showed no statistically significant difference from the animals treated with saline only (controls).

Table 2 shows a comparison between our data on the effectiveness of the pharmacologic agents tested as inhibitors of P-facilitated sexual behavior and the data previously published by Levin and Weiss (24) on their activity as inhibitors of Ca^{2+}-CaM-dependent phosphodiesterase activity, and as inhibitors of the CaM-specific binding of calcium ions. The activity of the tested drugs on the facilitation of sexual behavior (as demonstrated by the effects on lordosis) induced by P in Ovx estrogen-primed rats, with the possible exception of chlorpromazine, appeared to be strictly related to their efficiency as inhibitors of CaM-dependent phosphodiesterase and as ligands for the Ca^{2+}-CaM complex: pimozide was the most active, followed by trifluoperazine, whereas prometazine was only marginally effective. In addition, verapamil, an inhibitor of slow calcium channels, induced effects that were comparable to those elicited by the more effective anticalmodulin drugs.

DISCUSSION

A good correlation exists between the effectiveness of the antipsychotics drugs tested as inhibitors of P-facilitated sexual behavior and the activity of these same drugs as inhibitors of Ca^{2+}-CaM-dependent phosphodiesterase activity, and as inhibitors of the CaM-specific binding of calcium ions. This correlation validates the possible participation of the Ca^{2+}-CaM system on the mechanism of action of P as a facilitator of the sexual copulatory responsiveness of the Ovx estrogen-primed rat. This possibility is further sustained by the similar inhibitory activity shown by verapamil, a blocker of the slow Ca^{2+} channels. In effect, nuclear magnetic resonance spectroscopy has recently shwon that Ca^{2+}-channel blockers (felodipine, nifedipine, diltiazem, prenylamine, etc.) interact with CaM (6,18). These results lead to the possibility that CaM may act as an intracellular site of action for this kind of pharmacologic agent, perhaps even accounting for its major pharmacologic effects (41).

It has been suggested that the central adrenergic transmission system is involved in the steroid induction of lordosis behavior in rodents (36); for example, the administration of norepinephrine, epinephrine, or adrenergic agonists may substitute for P in inducing lordosis in estrogen-primed rodents (12). Many CaM antagonists have great potency as receptor antagonists, including dopaminergic, adrenergic, serotoninergic, and muscarininc cholinergic receptors (37,40,47). However, many of the seemingly indirect CaM activities of the drugs tested may be interpreted as their action upon other parameters of the calcium metabolism (40,41).

Protein phosphorylation is known to be involved in the regulation of neuronal function (32,34). At least three distinct classes of well-characterized protein kinases are involved in the phosphorylation of a large number of neuronal proteins: kinase A, kinase C, and CaM-dependent kinase II. The first,

TABLE 2

EFFECTIVENESS OF PHARMACOLOGIC AGENTS TESTED AS INHIBITORS OF P-FACILITATED SEXUAL BEHAVIOR COMPARED WITH THEIR ACTIVITY AS INHIBITORS OF THE Ca^{2+}-/CaM BIOCHEMICAL SYSTEM

Agent Tested	ID_{50} Inhibition: Activation of Phosphodiesterase (μM)*	Specific Calcium Binding (nMol/mg Protein)*	ID_{50} Inhibition: Lordosis Quotient (mg/kg)
Pimozid	7.5	68.7	0.82
Trifluoperazin	10.4	58.3	1.8
Chlorpromazin	42.2	22.7	3.8
Haloperidol	63.0	12.8	2.4
Prometazin	334.0	3.8	10.3

*Data are calculated from the work of Levin and Weiss (24).

rate-limiting step in the biosynthesis of catecholamines is catalyzed by tyrosine hydroxylase (TH) (47). TH is a mixed-function oxidase whose activity is modulated by reversible phosphorylation (13), and is a substrate for all three kinases mentioned earlier (13,34). However, it is known that Ca^{2+}-CaM-dependent events are involved in maintaining basal TH activity in striatal slices (13). Also, Ca^{2+}-dependent phosphorylation processes mediated by Ca^{2+}-CaM-dependent kinase, and/or protein kinase C, are primarily responsible for the in vivo and in situ activation of TH (34).

Several lines of evidence suggest that the activation of TH following neuroleptic administration might also be due to phosphorylation of the enzyme (13). Therefore, neuroleptic-induced changes in TH activity might reflect the state of equilibrium between phosphorylated and dephosphorylated forms of the enzyme (13,34). In this regard, it was postulated that HAL-activated TH represents a mixture of phosphorylated and nonphosphorylated forms of the enzyme (23). The interpretation of these results is complicated by the possibility that TH may be regulated differently in different systems. For example, phorbol esters increase TH activity in adrenal cromaffin cells and superior cervical ganglion but not in striatal slices (32).

Luteinizing hormone-releasing hormone, like noradrenaline, can mediate the lordosis-facilitating effect of P (11, 12,35), and its release seems to be stimulated by the steroid activity on the Ca^{2+}-CaM system. Progesterone applied in vivo to estrogen-primed ovariectomized rats is able to stimulate LHRH release from mediobasal hypothalamus (25). In vitro administration of P is able to produce a marked increase in LHRH release by mediobasal hypothalamic slices obtained from adult Ovx rats, provided the animals has received E_2 before sacrifice (10). It has been shown by changing membrane properties, E_2 appears selectively and specifically to be involved in the process coupling nerve-ending depolarization and LHRH release (9). As in all other instances of depolarization-induced secretion, enhancement of LHRH release must be Ca^{2+}-dependent. In effect, the omission of Ca^{2+} in the medium or the addition of a Ca^{2+}-channel blocker (D600, 10^{-4} M) or a CaM-dependent protein kinase inhibitor (trifluoperazine, 30 μM) results in a complete cancellation of the stimulator effect of P on LHRH release. When sodium channels were blocked with tetrodotoxin (5×10^{-7} M), the stimulatory effect of the steroid was completely abolished (10). These results indicate that the secretor response of LHRH to P requires priming with estrogens, is Ca^{2+}-dependent, involves the mediation of CaM and a CaM-dependent kinase system, and may be mediated through a nongenomic process.

In addition to the potential assisting role of P-induced LHRH release on lordosis behavior (31), it is possible that the direct participation of P on the facilitation of sexual behavior may be realized through nongenomic mechanisms similar to those mentioned earlier. Direct membrane recognition of the steroid (8) would be followed by modifications of the intracellular Ca^{2+} metabolism mediated by CaM. If this is so, we suggest that the delayed response to the facilitating effect of P could be explained by the participation of a complex process involving: a) coupling of recognition sites of the steroid to enzymes and/or receptors directly involved in the regulation of ionic fluxes; and b) the subsequent modification of intracellular Ca^{2+}-dependent processes. One of these processes may be the Ca^{2+}-CaM-dependent phosphorylation of the neurocytoskeleton protein tubulin.

In excitable tissues, changes in the intracellular Ca^{2+} concentration, frequently accompanied by changes in cyclic nucleotides, provide an important mechanism for nerve transmission and signal transduction (3). Among the systems that are Ca^{2+}-dependent, those mediated by calmodulin appear to be particularly important in neuronal function (21). Blockade of axoplasmic transport by the intracranial infusion of colchicine disrupts both estrogenic induction and maintenance of lordotic responsiveness (16,43). It is thus possible to propose that, similarly to the mechanism of action of colchicine, inhibitors of the Ca^{2+}-CaM system disrupt lordotic responsiveness by interfering with cytoskeletal functions, including Ca^{2+}-CaM-regulated neurotransmitter turnover and release (7,40, 47,49), cytoeskeletal dynamics (46,29), synaptic vesicle function (including exocytosis), and synaptic protein phosphorylation (34). The Ca^{2+}-CaM system may also interfere, at least partially, with the fast axoplasmic transport of substances from the medial hypothalamus to the dorsal midbrain, a process that has been proposed to be important for estrogenic action on lordosis (16). Also, inhibition of CaM-dependent protein kinase II blocks the induction of long-term potentiation of synaptic transmission (30,45), and the Ca^{2+}-CaM-stimulated cGMP phosphodiesterase plays an important role in the regulation of nitric oxide/cGMP-mediated excitatory signal transduction (50).

Although the results presented here may support the hypothesis that the activity of P on the facilitation of rodent sexual behavior may be realized through nongenomic mechanisms, it has been shown that CaM has some important genetic activities (29,38). Of these, the most crucial is the inhibition of protein synthesis produced by phosphorylation of elongation factor 2, a process that is catalyzed by the CaM-dependent protein kinase III (33). This CaM-related activity must be carefully considered as an important way of regulating protein synthesis, particularly in CaM-rich brain tissues, and therefore, as supporting the possibility that anticalmodulin drugs act by interfering with the genomic protein synthesis-stimulating properties of the steroid hormones.

In summary, our results suggest that modifications to the function of the Ca^{2+}-CaM system may have important effects on the sexual behavior facilitatory activity of P on estrogen-primed rats.

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