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Lordosis facilitation by LHRH, PGE_2 or db-cAMP requires activation of the kinase A signaling pathway in estrogen primed rats

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Abstract

Dose–response curves for lordosis and proceptive behaviors were obtained for luteinizing hormone releasing hormone (LHRH), prostaglandin E_2 (PGE_2) and dibutyryl cyclic AMP (db-cAMP), by infusing them in the right lateral ventricle (icv) of ovariectomized (OVX) estradiol benzoate (E_2B; 2 μg) treated rats. Two dose levels, one producing the maximal effect and the other one producing a submaximal response (~ED50) were selected for testing the capacity of Rp-cAMPS, a kinase A blocker, to modify the behavioral response to the three compounds. Icv injections of Rp-cAMPS, significantly depressed both lordosis and proceptive responses induced by LHRH, PGE_2 and db-cAMP. The results show that these agents use the cAMP-kinase A signaling pathway to elicit their stimulating effect on estrous behavior in the rat.

Keywords: Lordosis; Proceptivity; LHRH; Prostaglandin E_2; db-cAMP; Rp-cAMPS; Kinase A

1. Introduction

It is generally accepted that estrous behavior in the cycling rat is triggered by a rise in progesterone (P) occurring at proestrus, when estradiol (E_2) plasma levels are high (see Blaustein and Erskine, 2002; Moralí and Beyer, 1979; Pfaff et al., 2006). This belief is supported by the finding that the sequential administration of E_2 and P to ovariectomized (OVX) rats induces normal estrous behavior, both lordosis and proceptivity (Beach, 1942; Edwards et al., 1968; Moralí and Beyer, 1979; Yanase and Gorski, 1976) Large variety of non-steroidal agents; including peptides, amines, prostanoids and aminoacids (for review see Beyer and Gonzalez-Mariscal, 1986; Beyer et al., 2003; Blaustein, 2003; Etgen, 2003; Pfaff et al., 2006) can substitute for P to facilitate lordosis behavior in OVX rats primed with estradiol benzoate (E_2B). Particularly well studied has been the stimulatory effect on lordosis behavior of LHRH and PGE_2 (Dudley and Moss, 1976; Foreman and Moss, 1977; González-Mariscal and Beyer, 1988; Moss and McCann, 1973; Riskind and Moss, 1983; Rodriguez-Sierra and Komisaruk, 1977, 1982; Sakuma and Pfaff, 1980; Wu et al., 2006). The temporal characteristics of the responses induced by these agents, i.e., latency to display lordosis are similar to those of P, suggesting a common mechanism of action (see Beyer and Gonzalez-Mariscal, 1986). Indeed, the progesterone receptor (PR) antagonist RU486 blocks the stimulatory effect of LHRH and PGE_2 on the lordosis behavior of estrogen primed rats pointing to the participation of the PR in this event (Beyer et al., 1997). However, since these agents act on membrane receptors their effect on estrous behavior must be mediated by second messenger signaling pathways (Nestler and Duan, 1999).

Second messengers are involved in the facilitation of lordosis behavior in rodents. Thus, administration of cAMP (into the brain or subcutaneously) facilitates lordosis behavior in E_2B-primed rats (Beyer et al., 1981, 1982, 1997; Beyer and Gonzalez-Mariscal, 1986; González-Flores et al., 2006; Mani et al., 2000; Petralia and Frye, 2006). Moreover, the administration of blockers of kinase A (cAMP dependent kinase) interferes with the facilitatory effect of P in E_2B primed OVX rats (González-Flores et al., 2006; Mani et al., 2000).

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Since both LHRH and PGE_2 activate the cAMP-kinase signaling pathway in several tissues (Jabbor and Sales, 2004; Pawson and McNeilly, 2005), we tested the capacity of Rp-cAMPS, a specific blocker of kinase A, to interfere with the facilitatory effect on estrous behavior exerted by these compounds in OVX E_2B primed rats. The effect of db-cAMP on estrous behavior was also studied for comparative purposes and for verifying the capacity of our dose of RpcAMP to block the effect on estrous behavior of cAMP.

2. Methods

2.1. Animals

A total of 236 females were used in this study. Animals were sexually inexperienced female Wistar rats (240–280 g) bred in our colony. They were kept at 23±2 °C with an inverted light–dark cycle (14 h light, 10 h dark, lights on at 2300 h). They were fed with Purina rat chow and water *ad libitum*.

2.2. Surgical procedures

Females were bilaterally OVX under ether anesthesia, injected with penicillin (22,000 u.i./kg) and housed in collective cages (4 females per cage). Two weeks later, they were anesthetized with xylazine (4 mg/kg) and ketamine (80 mg/kg) and placed in a Kopf stereotaxic instrument (Tujunga, California). Females were implanted with a stainless steel cannula (22 gauge, 17 mm long) in the right lateral ventricle (icv): coordinates; interaural 8.20 mm, bregma 0.80 mm (Paxinos and Watson, 1997). A stainless steel screw was fixed to the skull and both the cannula and screw were attached to the bone with dental cement. An insert cannula (30 gauge) provided with a cap was introduced into the guide cannula to prevent clogging and contamination.

Animal care and all the experimental procedures adhered to the Mexican Law for the Protection of Animals.

2.3. Behavioral testing

Females were placed in a circular plexiglas arena (53 cm in diameter) with a vigorous male. Receptivity for each female was determined as a lordosis quotient [LQ=(number of lordosis/10 mounts)×100]. The intensity of lordosis (0 to 3) was quantified according to the lordosis score (LS) proposed by Hardy and DeBold (1977). The presence of proceptive behaviors (hopping, darting, ear-wiggling) was also recorded. A female was considered proceptive when showing any of the above mentioned behavioral patterns. Females were tested at 2 and 4 h after icv drug injections.

2.4. Experimental procedure

2.4.1. Experiment 1

2.4.1.1. Establishment of dose–response curves and effective dose 50 (ED50) for LHRH, PGE_2 and db-cAMP administered icv to E_2B (2 μg) treated OVX rats. One week after surgery, 118 females were injected s.c. with E_2B (2 μg) and 40 h later with different dosages of LHRH, PGE_2 or db-cAMP. These agents were infused through a plastic Clay Adams catheter (PE 10 No 7401), fitted to a Hamilton syringe (10 μl) that was inserted into the guide ventricular cannula. E_2B, PGE_2 and db-cAMP were purchased from Sigma (St. Louis, Missouri, USA). LHRH was purchased from Peninsula laboratories (Belmont, CA, USA).

LHRH and db-cAMP were dissolved in distilled water (1 μl volume) and PGE_2 in saline (2 μl volume). Dosages explored were: LHRH, 0.0005 μg, 0.005 μg, 0.05 μg, 0.5 μg; db-cAMP, 0.040 μg, 0.200 μg, 1 μg, 5 μg and PGE_2, 0.010 μg, 0.100 μg, 1 μg, 10 μg. Each group consisted of 8 or 9 females. Control injections (vehicle) were also performed. Observations were made at 2, and 4 h following intraventricular infusion. Infusion lasted 60 s. Animals were used only once.

2.4.2. Experiment 2

2.4.2.1. Effect of Rp-cAMPS, a kinase A blocker, on the estrous behavior induced by two selected doses of LHRH, PGE_2 or db-cAMP. Rp-cAMPS (Rp-adenosine 3′,5′-cyclic monophosphorothiate triethylamonium salt) is a specific inhibitor of kinase A (Gjertsen et al., 1995). This drug has been effective in blocking the db-cAMP signal by inhibiting protein kinase-A (PKA) "in vivo" (Botelho et al., 1988). Mani et al. (2000) reported that the icv injection of 100 ng Rp-cAMPS to E_2B-primed rats interfered with the lordosis response induced by P. Therefore, this dose was selected to assess the role of the cAMP signaling and PKA cascade, in the facilitation of lordosis by the three agents studied. A preliminary study using this treatment showed that it does not produce unspecific effects (ataxia, changes in locomotion, motor disabilities, food and water intake) which could confound interpretation of the results. Rp-cAMPS was purchased from Sigma (St. Louis, Missouri, USA).

One week after implantation of the cannula in the right lateral ventricle, 118 rats were treated with E_2B (2 μg) and 40 h later with one of the selected compounds (LHRH, PGE_2 or db-cAMP). Two dose levels for each agent were selected from experiment 1. One dose was maximal, i.e., the one producing the maximal effect for that drug, and the other one submaximal (between 50 and 60 ED). Doses for LHRH were: 0.005 and 0.05 μg; for db-cAMP, 0.2 and 1 μg, and for PGE_2, 1 and 10 μg.

Five minutes before and 15 min after the injection of the drug, Rp-cAMPS was injected (100 ng/μl) into the lateral ventricle. Behavioral observations were performed at the same time intervals as in experiment 1.

2.5. Histological study

Twenty-four hours after completion of the experiments, females were anesthetized with ether and 1% methylene blue was administered through the cannula. Rats were sacrificed with an overdose of the anesthetic. The brain was removed and sectioned in the transverse plane to verify the cannula position in the right lateral ventricle. Those animals with the cannula outside the ventricle were discarded from the experiment.

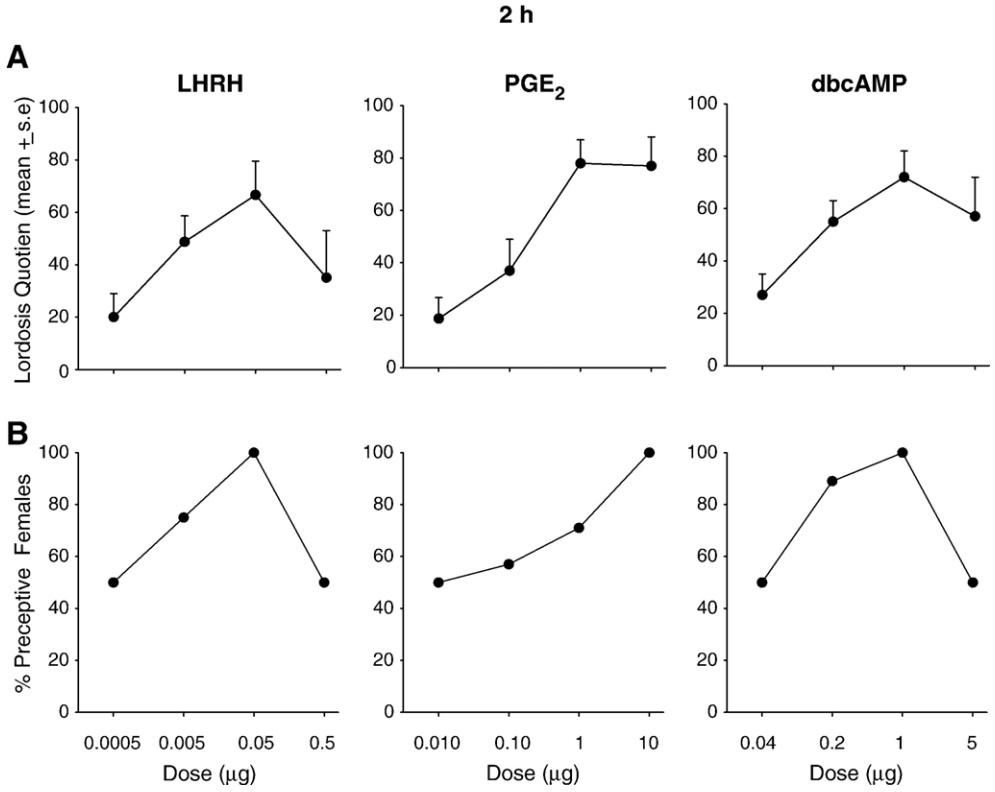

Fig. 1. Effect of the icv injection of four doses of LHRH (0.0005–0.5 µg), PGE_2, (0.010–10 µg) db-cAMP (0.040–5 µg) to OVX E_2B primed rats on the lordosis quotient (panel A) and % proceptive females (panel B).

2.6. Statistical analysis

Regression lines for the dose–response curves of the three agents explored in this study and ED50s were calculated according to Tallarida and Murray (1987).

The effect of the kinase A blocker (Rp-cAMPS) on the behavioral action of the LHRH, db-cAMP and PGE_2 (experiment 2) was assessed by comparing the LQs obtained with these agents alone versus those obtained when Rp-cAMPS was added. Since the distribution of LQ values in same groups were not normal a Wilcoxon–Mann–Whitney test was used to compare two independent groups (Bruning and Kintz, 1987; Siegel and Castellan, 1988). This test is an excellent alternative to the *t*-test with a power efficiency of 95.5% of the parametric test (Siegel and Castellan, 1988). Fischer's exact probability test was used to compare the proportion of proceptive females among experimental groups (Bruning and Kintz, 1987).

3. Results

3.1. Experiment 1

3.1.1. Establishment of dose–response curves and effective dose 50 (ED50) for LHRH, PGE_2 and db-cAMP

Fig. 1 shows dose–response curves for lordosis behavior produced by the icv infusion of four dosages of LHRH, PGE_2 and db-cAMP (panel A) infused into the lateral ventricle of E_2B-primed rats (2 µg E_2B s.c.). The control group, which received only vehicle into the lateral ventricle showed very low levels of lordosis at both testing intervals and did not display proceptivity. As can be seen in Fig. 1 though the responses were not linear across the range of dosages used a linear part of the curve occurred with the three chemicals, allowing regression analysis and the establishment of the ED values. ED50 values for lordosis behavior were as follows: LHRH = 0.001 µg, PGE_2 = 0.29 µg and db-cAMP = 0.1 µg. The dose–response curve for lordosis behavior of LHRH had a U inverted shape, i.e., larger doses induced smaller responses. LHRH was the most potent of the chemicals used to elicit lordosis. However, the greatest efficacy, i.e., the largest response, was observed with PGE_2.

Clear dose–response curves to the three chemicals used were also observed for proceptive behaviors (Fig. 1B). With adequate dosages, proceptivity was already manifested at the 2 h testing interval, though slightly higher proportions of responding females were seen at the 4 h interval (data not shown). Regarding proceptivity LHRH was the most potent and efficacious of the three chemicals used. Yet, the largest dose of both LHRH and db-cAMP failed to produce a significant effect on proceptive behaviors, indicating that these drugs elicit a dualistic type of response, with an inverted U shape i.e., larger doses producing smaller or null responses (Ariens et al., 1964).

3.2. Experiment 2

3.2.1. Effect of Rp-cAMPS on the stimulatory effect of LHRH, PGE_2 and db-cAMP on estrous behavior of E_2B-pretreated rats

Fig. 2 shows the effect on lordosis and proceptive behaviors of the icv administration of two selected doses of

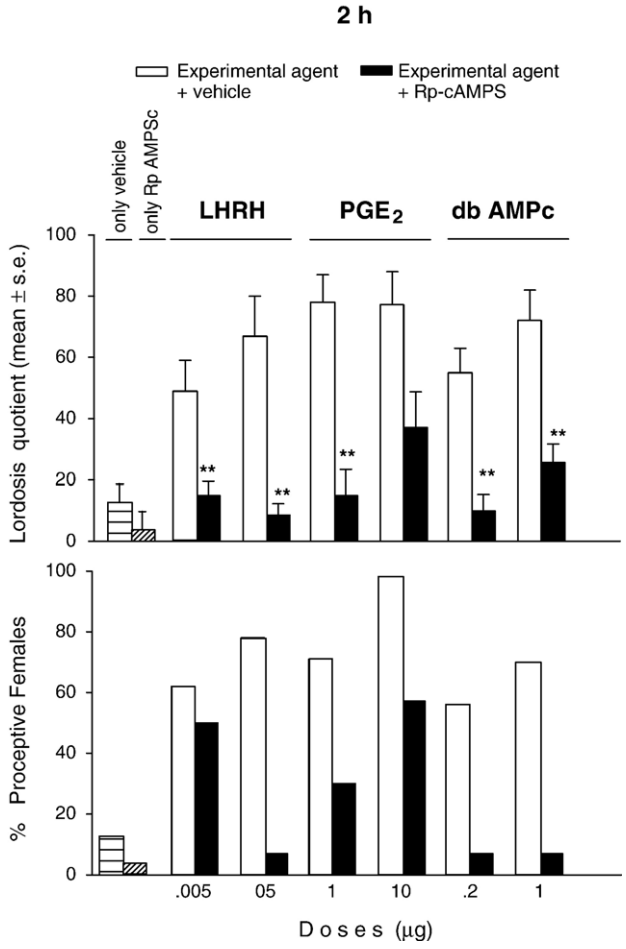

Fig. 2. Efect of the icv injection of 100 ng of RpcAMPS on the stimulatory effect of LHRH (0.005 and 0.05 μg), db-cAMP (0.2 and 1 μg) and PGE_2 (1 and 10 μg) on lordosis and proceptive behavior of OVX E_2B-treated rats. Facilitation of lordosis and proceptivity by LHRH, db-cAMP, PGE_2 at 2 h was inhibited by Rp-cAMPS administration. $^{**}P<0.001$; $^{*}P<0.01$ vs. corresponding group receiving drugs plus vehicle.

LHRH, PGE_2 and db-cAMP Administration of Rp-cAMPS significantly decreased the lordosis quotient induced by LHRH, PGE_2 and db-cAMP at 2 h post injection: LQ values were not different from those observed in the vehicle only group. This effect was transitory since at 4 h after testing no significant differences between Rp-cAMPS treated groups and those receiving only LHRH, PGE_2 or db-cAMP were noted.

The magnitude of the inhibitory effect of Rp-cAMPS on proceptivity varied with the chemical tested. Thus, Rp-cAMPS significantly suppressed proceptive behaviors induced by both dosages of db-cAMP. In the case of the LHRH the inhibitor significantly blocked the proceptive response to the higher dose of the peptide but only slightly reduced the effect of the lower dose. A decrease in the proportion of proceptive animals was also observed in the two groups treated with PGE_2 and Rp. cAMP but this decrease did not reach statistical significance. The inhibitory effect of Rp-cAMPS on proceptivity was transitory since at 4 h values were comparable to those obtained in the control group.

4. Discussion

The present study shows that the icv infusion of LHRH or PGE_2 elicits lordosis and proceptive behaviors in rats pretreated with E_2B. The temporal characteristics of the response were similar to those obtained with the icv infusion of db-cAMP. These results agree with previous data using these chemicals both through the intracerebral and the sc routes (Beyer et al., 1982, 1997; González-Mariscal et al., 1993; Hall and Luttge, 1977; Hall et al., 1975; Moss and McCann, 1973; Moss and Foreman, 1976; Rodriguez-Sierra and Komisaruk, 1977, 1982; Sakuma and Pfaff, 1980; Wu et al., 2006).

Previous work has shown that the cAMP-kinase A system is important, if not essential, for the expression of estrous behavior in E_2B primed rats treated with P. Treatment with Rp-cAMPS, a specific antagonist of kinase A, prevents the stimulatory effect of P or its 5α reduced metabolite 5α-pregnanedione (5α-DHP) on lordosis behavior (González-Flores et al., 2006; Mani et al., 2000). Our data strongly suggest that LHRH and PGE_2 also elicit estrous behavior in E_2B primed rats by activating the cAMP-kinase A cascade. Thus, Rp-cAMPS, interfered with the behavioral action of both LHRH and PGE_2. Several data indirectly support the participation of the cAMP-kinase A pathway in the facilitation of estrous behavior by LHRH and PGE_2 in E_2B-treated rats (Beyer et al., 1982, 1997; Beyer and Gonzalez-Mariscal, 1986; González-Mariscal and Beyer, 1988; González-Mariscal et al., 1993). Thus, both LHRH and PGE_2 have been reported to activate the cAMP kinase-A cascade in several tissues (Ojeda et al., 1986, 1988; Starzec et al., 1989; Waring and Turgeon, 1992). Moreover, the action of LHRH on lordosis behavior is potentiated by the administration of phosphodiesterase inhibitors (theophylline, methyl isobutyl-xanthine) which prevent the degradation of cAMP (Beyer and Canchola, 1981; Beyer et al., 1982).

It is only possible to speculate on the identity of the molecules phosphorylated by kinase A for eliciting estrous behavior. Fig. 3 shows some of the cellular mechanism through which cAMP-kinase cascade can stimulate lordosis. Since the antiprogestin RU486 interferes with the facilitatory effect of LHRH and PGE_2 on lordosis behavior (Beyer et al., 1997) it appears likely that kinase A acted by phosphorylating the PR itself. However, none of the phosphorylation sites so far reported in the PR are targets of kinase A (Bai et al., 1997; Chauchereau et al., 1994). Therefore, the participation of kinase A in the facilitation of estrous behavior must be mediated by molecules other than PR. It is well established that PR-mediated transcription depends on the recruitment of groups of coactivator proteins (Giangrande et al., 2000, Liu et al., 1999; Rowan and O'Malley, 2000). The PR has two activation domains to which activators must incorporate: 1) a ligand (P) dependent activation function 2 (AF-2), located in the C terminal ligand binding domain of the receptor and 2), a ligand independent activation function 1 (AF-1) located in the N-terminal region. Both AF-1 and AF-2 require for their transcriptional activity to recruit distinct groups of coactivators (Oñate et al., 1998; Wärnmark et al., 2003). Coactivators affect the transcriptional activity of the PR through a variety of

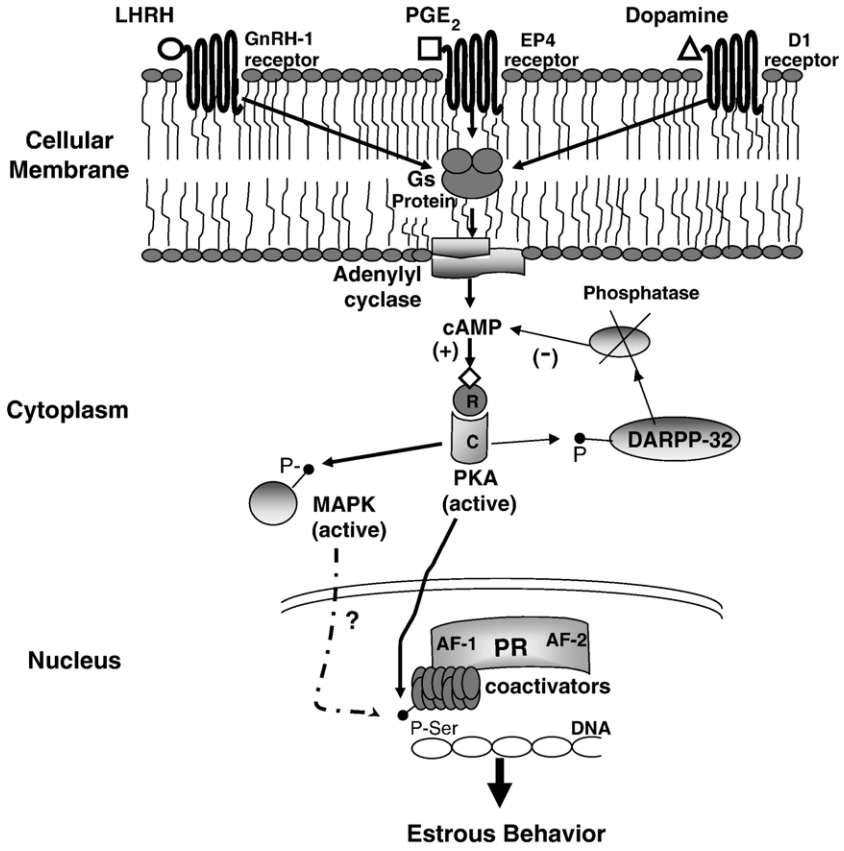

Fig. 3. By binding to membrane receptors, LHRH (O), PGE_2 (□) and dopamine (△) activate the enzyme adenylyl cyclase to generate cAMP (◇). cAMP in turn, by binding to the regulatory (R) subunit of protein kinase A (PKA) allows release of its catalytic (C) subunit. PKA does not directly phosphorylate the progesterone receptor (PR) but can modulate PR activity by either phosphorylating coactivators essential for PR transcriptional activity or indirectly stimulating MAPK that facilitates estrous behavior through a still undefined process that also requires the PR. PKA also phosphorylates DARPP-32 which enhances and prolongs cAMP action (+) by inhibiting the phosphatase involved in its enzymatic degradation.

cellular processes: some of them promote transcription by acting as adaptors between the receptor and the transcription machinery while others facilitate this process by remodeling chromatin. Since kinase A can modulate the activity of some coativators by phosphorylating specific sites in these proteins (Lonard and O'Malley, 2005, Rowan et al., 2000) this process could be involved in the facilitation of estrous behavior, as has been suggested by some investigators (Auger et al., 2000; Auger, 2004; Molenda et al., 2002). Recruitment of coactivators in the AF2 domain requires the binding of P to the PR, a condition absent in this experiment. On the other hand, the AF-1 domain mediates several ligand independent processes, i.e., not requiring P, including transcription, and could therefore be a candidate for the facilitatory effect on lordosis by LHRH, PGE_2 or cAMP through phosphorylation by kinase A (see Fig. 3). Kinase A signaling system has been reported to induce a facilitatory effect on the AF-1 domain such as the enhancement of the agonist effect exerted by RU486 when interacting with this site (Leonhardt and Edwards, 2002; Leonhardt et al., 2003; Meyer et al., 1990; Tetel et al., 1999). Moreover, some of the coactivators reported to interact with the AF-1 domain influence transcriptional activity in transfection assays and have been reported to be substrates of kinase A (Lonard and O'Malley, 2005; Rowan et al., 2000). Further studies should be made to establish the relevance of these processes, i.e., coactivator phosphorylation, for estrous behavior.

Mani et al. (1994, 2000) found that dopamine, acting on D1 receptors, facilitated lordosis behavior by phosphorylating DARPP-32 through the cAMP-kinase A system. DARPP-32 is a phosphatase 1 inhibitor which enhances and prolongs the effects of various second messenger kinase systems. Since LHRH and PGE_2 activate the cAMP-kinase cascade it appears likely that their effect is modulated by this process, i.e., DARPP-32 activation. This interpretation is also consistent with the finding that synthetic phosphatase inhibitors (i.e., MIX or theophylline) enhance the effect of LHRH on E_2B primed OVX rats (Beyer et al., 1982).

Several signaling systems, besides the cAMP/kinase A: DAG/KC, cGMP/kinase, MAPK also appear to be involved in the facilitation of lordosis induced by P or its ring A reduced metabolites (González-Flores and Etgen, 2004; González-Flores et al., 2006). Thus, administration of blockers of these signaling pathways interfere with the expression of lordosis behavior in OVX estrogen primed rats treated with P (Chu and Etgen, 1997; Chu et al., 1999; González-Flores and Etgen, 2004; González-Flores et al., 2004; Kow et al., 1993, 1994; Mobbs et al., 1989). Several data indicate that cAMP/kinase system cross talks with other signaling pathways including the

above mentioned systems (kinase C, kinase G, MAPK; Chin et al., 2002; Robison-White and Stratakis, 2002; Stork and Schmitt, 2002). In this context, the cAMP/kinase-A would be the initial event in a chain of processes resulting in the expression of estrous behavior.

The multiplicity of signals apparently participating in the facilitation of estrous behavior in the rat should not be surprising considering recent results both *in vivo* and *in vitro* in the functioning of intracellular signaling pathways. Thus, Nestler and Greengard (1999), conclude in a review on this topic that "individual signaling mechanism often drawn as distinct intracellular pathways normally function as complex nets with virtually every conceivable type of interaction among them".

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