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MODULATION OF DOPAMINE RECEPTOR AGONIST BINDING SITES BY CATIONS AND ESTRADIOL IN INTACT PITUITARY AND 7315a TUMORS

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Abstract—The effects of Na^+, K^+, Mg^{2+} and Ca^{2+} on the agonist binding sites of D_2 dopamine (DA) receptors were studied in 7315a pituitary tumors. The agonist high and low affinity states of the D_2 receptors were investigated with apomorphine competition for [^{3}H]spiperone binding at 25°. In the tumor, all cations markedly increased the affinity of the high affinity binding site, while the affinity of the low affinity binding site was increased only by Na^+. The proportion of high to low affinity states was not affected significantly by K^+ and Ca^{2+}, whereas it was decreased by Na^+ and increased by Mg^{2+}; none of these cations affected the total density of the D_2 receptors. The *in vitro* regulation of D_2 receptors by 17β-estradiol (E_2) was next studied in 7315a tumors and bovine intact adenohypophysis. In intact anterior pituitary, a partial conversion of the high to the low affinity state was obtained in the presence of GTP, while in tumoral pituitary, a complete conversion was observed. Addition of 1 nM E_2 to the *in vitro* incubation mixture prevented these conversions and resulted in a partial return of the high affinity state of the D_2 receptors to their control values in both normal and tumoral pituitary. In another experiment, using increasing concentrations of E_2 (0.01 to 100 nM) and GTP (10^{-8} to 10^{-3} nM) on [^{3}H]*n*-propylnorapomorphine ([^{3}H]NPA) binding to the D_2 receptors in bovine intact adenohypophysis, 1 and 10 nM E_2 doubled the IC_{50} of GTP to decrease [^{3}H]NPA binding. The results show that agonist high and low affinity states of D_2 receptors in 7315a tumors are regulated normally by cations. In addition, E_2 inhibited the effect of GTP on the agonist sites of the D_2 receptors in both intact anterior pituitary tissue and 7315a tumors.

Dopamine (DA†) agonists recognize high and low affinity states of the D_2 DA receptor in the normal anterior pituitary [1]. The high affinity state is considered as functional, and mediates the inhibition of prolactin (PRL) release [1]. Guanine nucleotides have been shown to modulate the conversion of the high affinity state to the low in the normal adenohypophysis, suggesting the involvement of a guanine nucleotide regulatory protein with the DA receptor complex [2]. It has also been shown that monovalent and divalent cations modulate D_2 receptors in intact porcine and steer anterior pituitaries [2–5]. PRL-secreting 7315a transplantable pituitary tumors possess DA receptors, although DA or its agonists do not decrease PRL release from these tumors [6]. However, 7315a tumors have been shown to have normal DA receptor regulation by sodium ion and guanine nucleotides [7] but abnormal calcium metabolism under basal conditions [8]. In the present study, the effects of cations, 17β-estradiol (E_2) and guanine nucleotides on the high and low affinity states of the D_2 receptors were examined in 7315a tumors and compared to the normal anterior pituitary, in order to gain more insight into the modulation of DA receptor agonist sites in both normal and neoplastic tissues.

MATERIALS AND METHODS

Animals. Bovine anterior pituitaries, freshly frozen on dry ice, were purchased from a local slaughterhouse and stored at −90° until used. Adult female Buffalo rats weighing 175–200 g were housed two per cage and kept in controlled temperature (22–23°) and light (monitored light–dark cycles with lights on from 5:00 a.m. to 7:00 p.m.) and received rat chow and water *ad lib*. Rats were inoculated s.c. with 0.4 to 0.6 mL of a suspension of 7315a tumors under the back skin prepared as previously described [7, 9]. Tumors were left to grow for about 3–4 weeks before decapitating the animals. Tumors were then quickly removed, immediately frozen, and kept at −90° until assayed.

PRL determination. Trunk blood was collected into heparinized tubes at the time of decapitation of the rats, and plasma was separated by centrifugation at 4000 *g* for 10 min and kept at −20° until assayed for PRL. PRL was measured in duplicate by a double-antibody radioimmunoassay [10].

Binding experiments. Membrane preparation was identical for intact bovine anterior pituitaries and 7315a tumors grown in intact female Buffalo rats. Tissue preparation for DA receptor assays was done at 0–4° as previously described [7], with the following modifications: the control buffer used in experiments to study cations contained 0.25 M sucrose, 50 mM

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† Abbreviations: DA, dopamine; PRL, prolactin; E_2, 17β-estradiol; [^{3}H]NPA, [^{3}H]*n*-propylnorapomorphine; and PLG, L-prolyl-L-leucyl glycinamide.

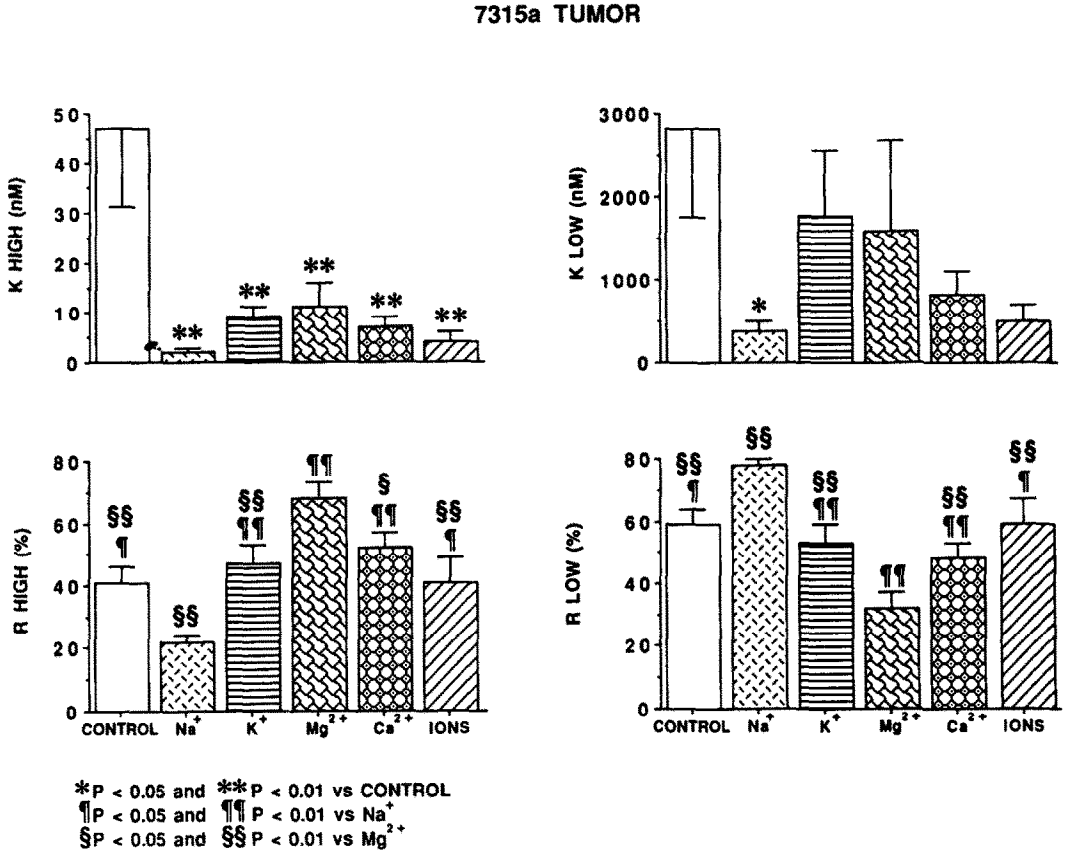

Fig. 1. Effects of cations on agonist competition of antagonist binding in rat 7315a tumors. The effects of cations on the inhibition constant (*K* HIGH, *K* LOW) and proportion (*R* HIGH, *R* LOW) of the D_2 receptor in the agonist affinity states were assessed by apomorphine competition for [^{3}H]spiperone (0.2 to 0.3 nM) binding to rat 7315a tumors at 25°. Values are means ± SEM from 4–9 separate determinations. The buffer used in the "control" conditions was 50 mM Tris–HCl, 100 μM EDTA, 12.5 μM nialamide and 0.01% ascorbic acid at pH 7.45.

Tris–HCl, 100 μM EDTA, 12.5 μM nialamide and 0.01% ascorbic acid at pH 7.45. In the experiments studying E_2 and GTP, 120 mM NaCl, 4 mM $CaCl_2$ and 5 mM KCl were also included in the control buffer. DA receptor agonist affinity states were investigated by competition of [^{3}H]spiperone (0.2 to 0.3 nM) binding by (−)-apomorphine (twenty-eight concentrations, 10^{-12} to 10^{-4} M); 1 μM (intact anterior pituitary) or 2 μM (7315a tumor) (+)-butaclamol was used to estimate nonspecific binding. [^{3}H]Spiperone (Amersham; 71.5 to 112 Ci/mmol) was incubated in triplicate for 30 min (intact anterior pituitary) or 60 min (7315a tumor) at 25° in a total volume of 2.0 mL. Incubation was initiated by the addition of membrane suspension (0.2 mL containing an average of 0.2 mg of protein) to tubes containing 0.05 mL [^{3}H]spiperone and 0.2 mL (+)-butaclamol or apomorphine or 0.1% ascorbic acid and 1.55 mL of control buffer without sucrose [or 1.50 mL of buffer plus 0.05 mL of guanosine-5′-triphosphate (GTP) dissolved in buffer or 1.50 mL of buffer plus 0.05 mL of E_2 for experiments using GTP and E_2]. Na^+ (100 mM NaCl), or K^+ (100 mM KCl), or Mg^{2+} (10 mM $MgCl_2$), or Ca^{2+} (10 mM $CaCl_2$), or all these cations together, i.e. buffer named "IONS" (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$), were added to the control buffer to study cation effects. DA receptor agonist high affinity state was also investigated by saturation of [^{3}H]*n*-propylnorapomorphine ([^{3}H]NPA) binding (ten concentrations, 0.02 to 1 nM [^{3}H]NPA). The effect of increasing concentrations of E_2 (10^{-11} to 10^{-7} M) on the effect of GTP on [^{3}H]NPA binding to the D_2 receptor was studied in competition experiments of [^{3}H]NPA (0.5 nM) binding by GTP (six concentrations, 10^{-8} to 10^{-3} M); 1 μM (+)-butaclamol was used to estimated nonspecific binding. [^{3}H]NPA (NEN/du Pont; 66 Ci/mmol) was incubated in triplicate for 30 min at 25° in a total volume of 2.0 mL. Incubation was initiated by the addition of membrane suspension (0.2 mL) to tubes containing 0.05 mL [^{3}H]NPA, 0.05 mL (+)-butaclamol or GTP or 0.1% ascorbic acid, 0.05 mL E_2 and 1.65 mL of control buffer without sucrose. At the end of incubation, samples were diluted with 2.0 mL of cold buffer and rapidly filtered through Whatman (GF/C) glass fiber filters under reduced pressure using a cell harvester (Brandel). Following two 10-sec

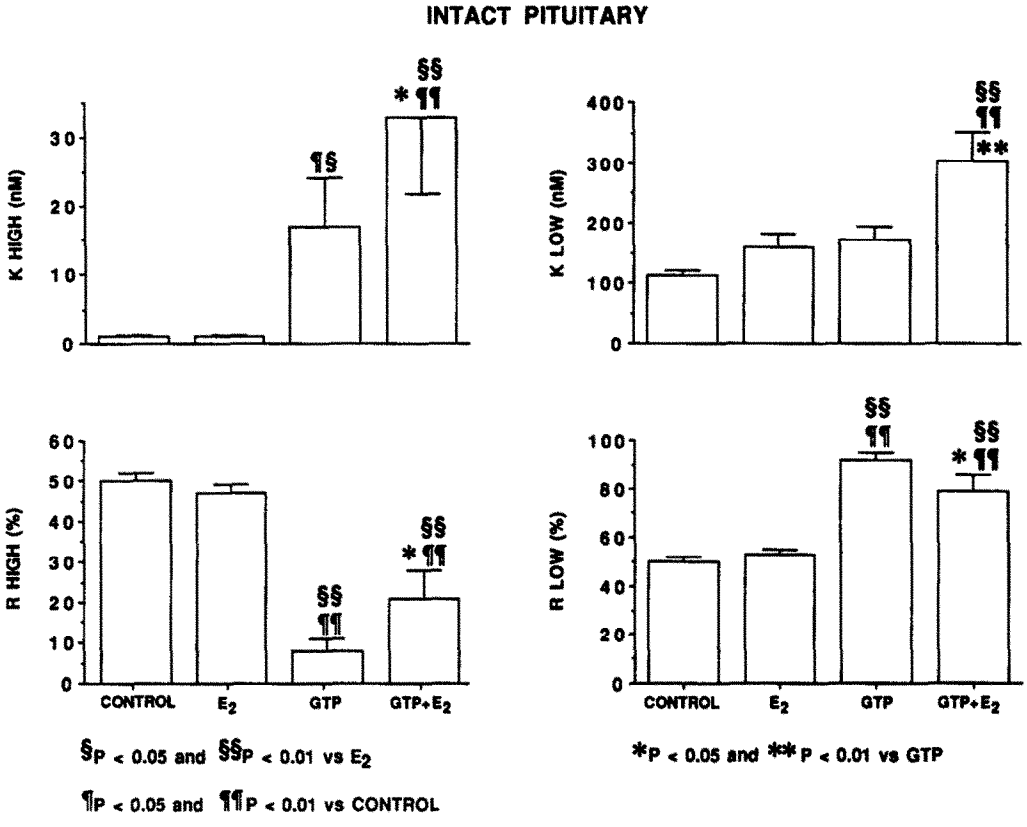

Fig. 2. Effects of 17β-estradiol and guanine nucleotides on agonist competition of antagonist binding in bovine anterior pituitary. The effects of 1 nM E_2 and 100 μM GTP on the inhibition constant (K HIGH, K LOW) and proportion (R HIGH, R LOW) of the D_2 receptor in the agonist affinity states were determined by apomorphine competition for [^{3}H]spiperone (0.2 to 0.3 nM) binding to bovine anterior pituitary at 25°. Values are means ± SEM from 5–16 separate determinations.

washes (4–6 mL each), the filters were placed into vials with 10 mL of scintillation fluid (Formula-963, NEN/du Pont). Radioactivity was counted in a Beckman LS3801 instrument (efficiency at 56%). Protein concentration was determined by the method of Lowry *et al.* [11]. Binding studies in 7315a tumors were assayed in individual tumors, while pooled anterior pituitaries of three to four animals were used for competition studies in intact anterior pituitary.

Statistical analysis. Competition binding curves were analysed with the non-linear least squares curve-fitting program LIGAND [12], a generalized model for complex ligand–receptor systems. Data were fit successively to one or two populations of binding sites. A two-site model was adopted as appropriate only when a statistically significant improvement of the fit to the data was obtained over a one-site model, as judged by the F statistics. Scatchard plots were constructed from the saturation data, and a least square linear regression analysis was performed to calculate the dissociation constant (K_D) and the maximum number of binding sites (B_{max}). Statistical evaluation of the inhibition constant (K_i) and B_{max} values, as well as proportions of receptor in the two states, were performed by the Duncan–Kramer multiple range test, while the IC_{50} values of GTP were compared with the Wilcoxon signed-rank test.

RESULTS

DA receptors were investigated in 7315a tumors which actively secrete PRL. The PRL plasma level in rats bearing these tumors was 600 ± 100 ng/mL (N = 16).

Effects of cations on agonist competition of antagonist binding. The effects of Na^+, K^+, Mg^{2+} and Ca^{2+} on D_2 receptor agonist states as assessed by apomorphine competition for [^{3}H]spiperone binding in 7315a tumors are shown in Fig. 1. Each cation individually, or all these cations together (IONS) markedly decreased the inhibition constant of the high affinity state whereas the inhibition constant of the low affinity state was decreased significantly only by Na^+. The proportions of high to low affinity apomorphine sites remained unchanged in the presence of K^+, Ca^{2+}, and IONS, while Na^+ decreased the proportion of the high affinity state, and Mg^{2+} increased it significantly. Total D_2 receptor density was not affected by the cations.

Effects of E_2 and GTP on agonist competition of antagonist binding. As shown in Fig. 2, in bovine

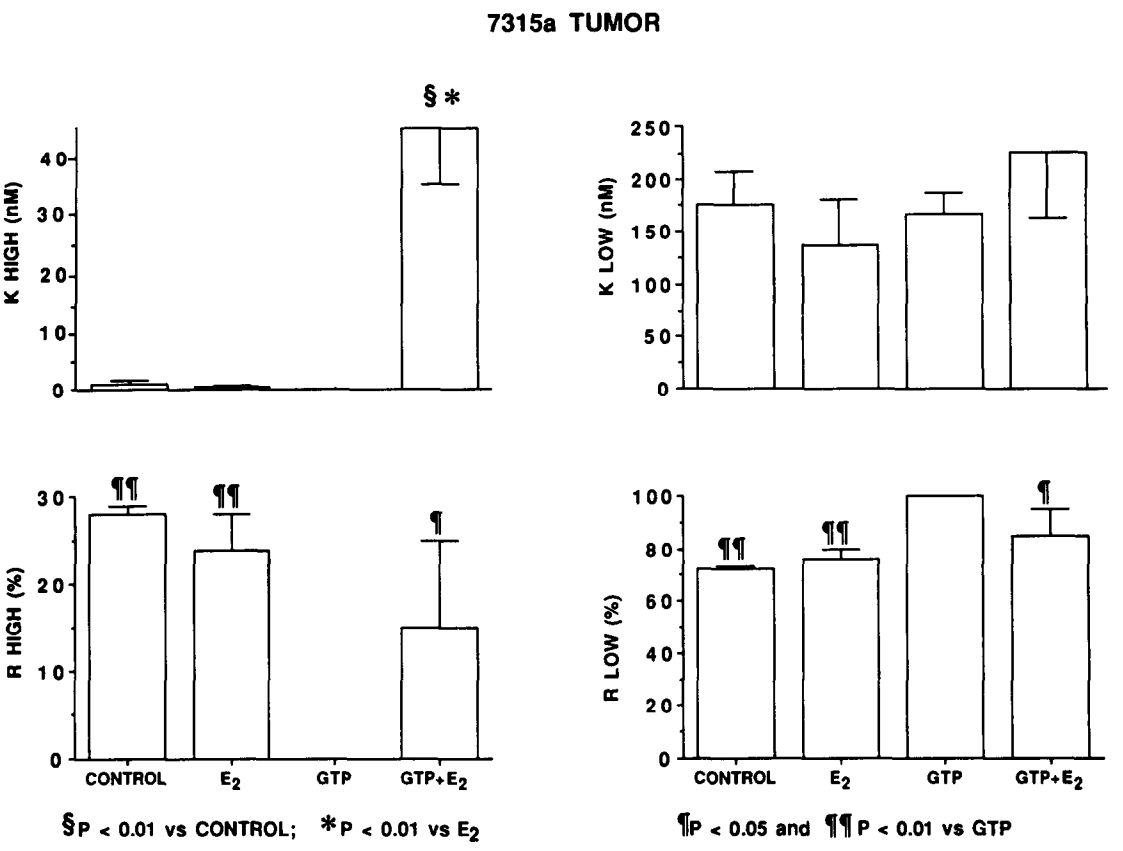

Fig. 3. Effects of 17β-estradiol and guanine nucleotides on agonist competition of antagonist binding in rat 7315a tumors. The effects of 1 nM E_2 and 100 μM GTP on the inhibition constant (K HIGH, K LOW) and proportion (R HIGH, R LOW) of the D_2 receptor in the agonist affinity states were assessed by apomorphine competition for [^{3}H]spiperone (0.2 to 0.3 nM) binding to rat 7315a tumors at 25°. Values are means ± SEM from 3–7 separate determinations.

intact anterior pituitary, E_2 alone had no effect on affinity and proportion of apomorphine binding sites to D_2 receptors, while GTP decreased both the affinity and the proportion of high affinity sites. E_2 in combination with GTP prevented the GTP shift, and partly returned the proportion of D_2 receptors to the high affinity state, while it decreased the affinities of high and low binding sites, compared to the values obtained with GTP alone.

In both 7315a tumors (Fig. 3) and intact tissue, E_2 alone did not change the affinity, total density or the proportion of apomorphine binding sites to D_2 receptors. With GTP, a complete conversion of high into low apomorphine sites was obtained. As for the intact anterior pituitary, E_2 in combination with GTP prevented the GTP shift and returned the proportion of D_2 receptors to the high affinity state; however, the affinity of the high affinity site was significantly lower than control, or than that obtained in the presence of E_2 alone.

Effects of E_2 and GTP on agonist binding. Figure 4 shows an example of [^{3}H]NPA binding to the high affinity D_2 receptor site in bovine intact anterior pituitary. This experiment was repeated twice; the equilibrium dissociation constant (K_D) value and the maximal binding capacity (B_{max}) of the two experiments were, respectively, 0.07 ± 0.01, 0.06 ± 0.003 nM, and 60 ± 10, 70 ± 7 fmol/mg of protein. Modulation of the coupling of the DA receptor to its second messenger was assessed with the inhibition of [^{3}H]NPA binding by increasing concentrations of GTP. An example of this experiment is shown in the lower right portion of Fig. 4. This experiment was repeated seven times and the mean IC_{50} value obtained from these experiments was 10 ± 2 μM. The concentration–response curve of E_2 on the IC_{50} of GTP to decrease [^{3}H]NPA binding to D_2 receptors is shown in Fig. 5. E_2 at concentrations of 1 and 10 nM increased the IC_{50} of GTP to decrease [^{3}H]agonist binding, while lower or higher concentrations were ineffective.

DISCUSSION

With competition experiments of apomorphine for [^{3}H]spiperone binding to D_2 receptors, we observed the presence of high and low agonist states of the D_2 receptors in 7315a tumors as previously observed [7]. The apomorphine inhibition constants for D_2 receptors in 7315a tumor and in intact adenohypophysis [4, 5] are high in the absence of cations. The proportion of [^{3}H]spiperone binding in

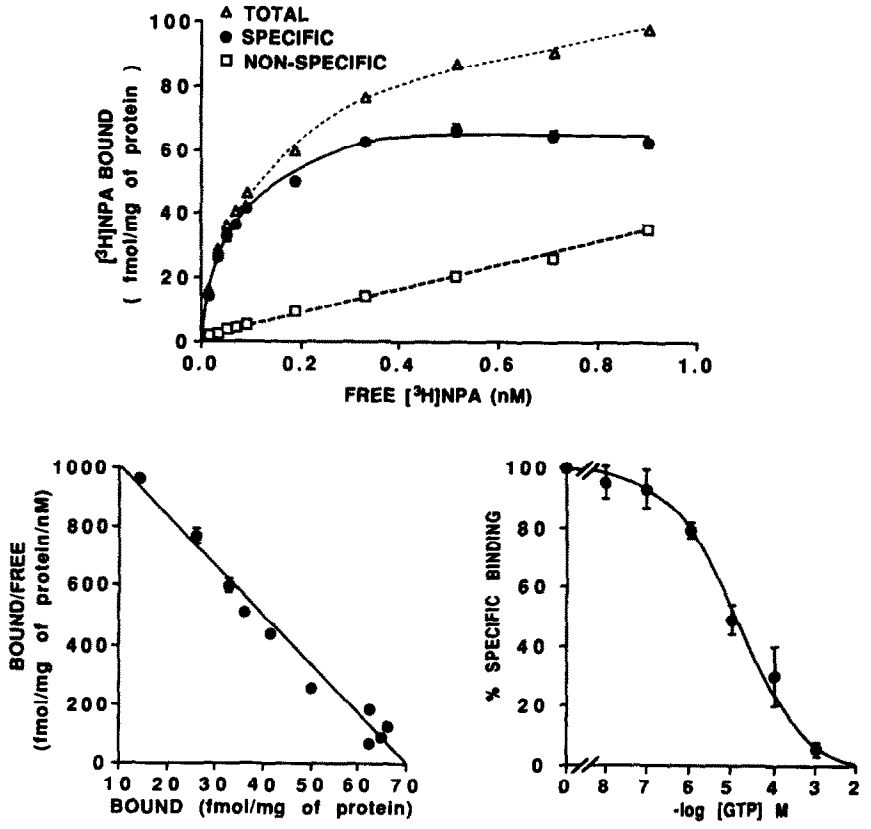

Fig. 4. Representative saturation experiment of [^{3}H]NPA binding to D_2 receptors in bovine anterior pituitary assayed at 25° (top). Scatchard plot showing BOUND/FREE as a function of BOUND is included (lower left). The equilibrium dissociation constant (K_D) value and the maximal binding capacity (B_{max}) shown were, respectively, 0.06 ± 0.003 nM and 70 ± 7 fmol/mg of protein. A concentration–response curve for the effect of guanine nucleotides (GTP) on [^{3}H]NPA binding is also presented (lower right). The IC_{50} of the inhibition of [^{3}H]NPA binding by GTP shown was 10 ± 3 μM. Values are the means ± SEM of triplicate determinations.

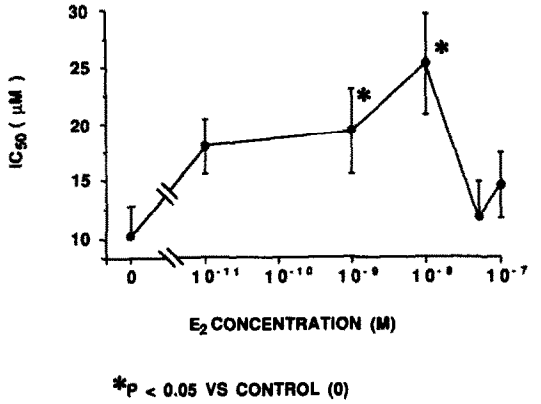

Fig. 5. Modulation by 17β-estradiol (E_2) of the effect of guanine nucleotides on the D_2 receptor. The effect of increasing concentrations of E_2 on the IC_{50} of GTP to decrease [^{3}H]NPA binding to the dopamine receptor was determined in bovine anterior pituitary homogenates at 25°. Values are means ± SEM from 5–7 separate determinations except for the 10^{-11} M concentration which was obtained from 2 determinations (mean ± range).

the high affinity state as recognized by apomorphine and DA in the steer anterior pituitary is 29% [5], 27% in the porcine anterior pituitary [4], and somewhat higher in 7315a tumors in the absence of cations (present study).

In 7315a tumors, as in the intact anterior pituitary [2–5], Na^+ decreased while Mg^{2+} increased the proportion of binding sites in the high affinity state. It has been proposed that Na^+ and Mg^{2+} may exert opposing effects on the guanine nucleotide regulatory protein in intact adenohypophysis [4]. However, as previously reported for the intact pituitary [4], Na^+ affects the inhibition constant of the low affinity state in 7315a tumors. Thus, the results indicate that modulation by Na^+ and Mg^{2+} of DA receptors was seemingly normal in 7315a tumors.

In intact rat striatum, K^+ has no effect on the conversion of the D_2 high affinity state to its low affinity state [13]. DA was shown to increase K^+ permeability [14]. It has been suggested that DA may inhibit PRL release by increasing K^+ permeability of the cell through a "receptor–potassium channel" complex located in the cytoplasmic membranes. In 7315a tumors, as for the intact striatum [13], the proportion of high affinity

apomorphine binding sites to D_2 receptors was not affected by K^+.

DA receptor activation has been shown to block influx of extracellular calcium, thus reducing the concentration of intracellular-free calcium, which in turn inhibits PRL release [15–17]. The mechanisms by which DA blocks calcium influx may be multiple, involving direct inhibition of a calcium channel associated with the D_2 receptor and/or indirect effects mediated through adenylate cyclase, phospholipid metabolism or the plasma membrane K^+ channel [15] increasing the K^+ permeability of the cell [14]. It has also been shown that Ca^{2+} is the least potent divalent cation, or is essentially without effect to increase the potency of agonists to compete for [^{3}H]antagonist binding to DA receptors [5, 18]. In 7315a tumors, we have shown that Ca^{2+} left unchanged the proportion of the agonist sites of the DA receptors. It has been suggested that under basal conditions in 7315a cells, Ca^{2+} uptake appears insufficient to maintain a level of PRL secretion upon which DA inhibition may be manifest [8, 19, 20].

In 7315a tumors, complete conversion of the high to the low affinity state was obtained in the presence of both NaCl and GTP [7], whereas in intact bovine anterior pituitary (present results) as in porcine anterior pituitary [21], it was sometimes, but not always, obtained.

It was shown in anterior pituitary cells that estradiol selectively affects the coupling mechanism of DA receptors without affecting the binding sites [22]. Accordingly, we observed that E_2 *in vitro* did not affect the inhibition constants or the proportions of the D_2 receptors in 7315a tumors and in intact adenohypophysis. One of the mechanisms by which estrogen exerts a modulatory influence on the DA regulation of PRL synthesis and release seems to be the attenuation of the functional coupling of the D_2 receptor with it G-protein. It could also affect other mechanisms within the plasma membrane, such as methyltransferase activity, a process which could affect coupling, either by modifying membrane viscosity or by changing substrate concentrations of various enzymes [22]. As for striatal tissue [23, 24], E_2 in the presence of GTP increased and returned in part the high affinity state of D_2 receptors to their control values in intact anterior pituitary and in 7315a tumors. These results suggest that 7315a tumor D_2 receptors have a normal regulation by E_2.

We have shown, in accordance with previous reports [21], that [^{3}H]NPA labels the agonist high affinity state of the pituitary D_2 receptors. The IC_{50} of the inhibition of [^{3}H]NPA binding by GTP is 17 μM in the porcine anterior pituitary [21] in agreement with our observation (10 μM) in the bovine adenohypophysis. For the first time, we report that in the intact bovine anterior pituitary, 1–10 nM E_2 about doubled the IC_{50} of GTP to decrease [^{3}H]NPA binding to DA receptors. Paul and Axelrod [25] reported a concentration of E_2 of approximately 10 nM in the rat anterior pituitary. In addition, *in vitro* the maximal effect of E_2 to decrease [^{3}H]-spiperone binding capacity (B_{max}) to anterior pituitaries from ovariectomized rats in primary culture is at 10 nM [26]. This is in agreement with our results where we observed a maximal effect of E_2 at 10 nM on the IC_{50} of GTP to decrease [^{3}H]-NPA binding to DA receptors. This suggests that specific E_2 concentrations in the range occurring in physiological conditions are able to influence the pituitary DA system. The limited range of effective hormone concentrations has also been observed with peptide hormones; indeed, Srivastava *et al.* [27] have demonstrated that L-prolyl-L-leucyl glycinamide (PLG) was effective in causing a significant change in the IC_{50} value of $Gpp(NH)_p$ to decrease [^{3}H]NPA binding to striatal DA receptors at a concentration of 10^{-6} M while lower (10 nM) or higher (100 μM) concentrations were ineffective. The effect of E_2 *in vitro* observed on the GTP shift measured with tritiated agonist binding or competition of tritiated antagonist binding by unlabelled agonist both show that this steroid opposed the effect of the guanine nucleotide, thus hindering the coupling of the DA receptor to its second messenger system.

In conclusion, the results suggest that, *in vitro*, 7315a tumors have normal high and low DA agonist affinity states and regulation by cations and E_2. In addition, E_2 is able to inhibit in part the effect of GTP on the agonist sites of D_2 receptors both in the intact anterior pituitary and 7315a tumors.

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