Comparison of the Forms of the Dopamine D_2 Receptor Expressed in GH_4C_1 Cells

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Abstract. The effects of dopamine (DA) on prolactin (PRL) secretion, phosphoinositide metabolism, cytosolic calcium concentrations ($[Ca^{2+}]_i$), and cAMP production in GH_4C_1 cells expressed either the short (GH_4ZR_7 cells) or long (GH_4I_{12} cells) form of the rat DA D_2 receptor were compared in this study. The GH_4C_1 cell line is derived from the rat anterior pituitary gland and lacks functional DA receptors. The GH_4ZR_7 and GH_4I_{12} cell lines have been transfected with either the short or the long form of the D_2 receptor, respectively. In this study, the functional coupling of these receptors to both basal and stimulated PRL secretion and to common second messenger systems was examined. Both cell types expressing DA receptors exhibited similar saturable binding to the D_2 antagonist [^{3}H]spiperone (K_d GH_4ZR_7 = 96 ± 8 p*M*, K_d GH_4I_{12} = 107 ± 49 p*M*). In GH_4ZR_7 cells, 1 and 10 μ*M* DA inhibited basal PRL secretion by 37% and 58%, respectively. In GH_4I_{12} cells, 1 and 10 μ*M* DA inhibited basal PRL secretion by 63% and 54%, respectively. In GH_4ZR_7 cells, 10 μ*M* DA completely reversed the stimulatory effects of 1–100 n*M* thyrotropin-releasing hormone (TRH), and 1 μ*M* DA attenuated the stimulatory effects of 10 and 100 n*M* TRH. Interestingly, GH_4I_{12} cells were not responsive to TRH. In both cell lines, the inhibitory effects of DA were blocked by the specific D_2 antagonist, eticlopride. The stimulatory effects of TRH on $[Ca^{2+}]_i$ were dose dependent and could be blocked (at least in GH_4ZR_7 cells) by prior treatment of the cells with 1 μ*M* DA. The ability of dopamine to block the TRH-mediated increase in $[Ca^{2+}]_i$ was attenuated by eticlopride. DA (1 μ*M*) had no effect on resting $[Ca^{2+}]_i$ in either cell line expressing DA receptor. TRH (100 n*M*) maximally stimulated total inositol phosphate (IP) accumulation to values approximately three times greater than controls in GH_4C_1 and GH_4ZR_7 cells only. DA had no effect on basal or TRH-stimulated IP accumulation in any of the cell lines. DA (1 and 10 μ*M*) inhibited cAMP production by 40% and 39%, respectively, in GH_4ZR_7 cells. Similarly, in GH_4I_{12} cells, DA (1 and 10 μ*M*) inhibited cAMP production by 48% and 60%, respectively. These data indicate that both the long and short forms of the D_2 receptors play similar roles when expressed in GH_4C_1 cells. However, maximal inhibition of PRL in these cell lines is approximately 20% less than that of normal lactotrophs. [P.S.E.B.M. 1994, Vol 205]

Dopamine (DA) is the major hypothalamic hormone inhibiting prolactin (PRL) secretion from the anterior pituitary gland (1). The PRL secreting cell type of the anterior pituitary gland, the lactotroph, expresses specific DA receptors (2) which mediate inhibition of both basal and stimulated PRL secretion (1). Lactotrophs only express the DA D_2 receptor subtype (3, 4) which is coupled to multiple signal transducing cascades (5). In lactotrophs, the DA D_2 receptor, a G-protein coupled receptor, is able to mediate a reduction in both basal and stimulated ade-

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nylyl cyclase activity (6, 7). DA has also been shown to decrease (8–12) or have no effect on phosphatidylinositol (PI) turnover (13). Both basal and stimulated cytosolic calcium concentrations $[Ca^{2+}]_i$ are reduced by DA (14–18). DA decreases two distinct calcium currents (19, 20) and increases three distinct potassium currents (20, 21).

The myriad of effects induced by DA in the lactotrophs raises the question as to whether all these effects can be mediated by a single G-protein. A D_2 receptor purified from bovine anterior pituitary has been shown to effectively couple to a G_i subtype, G_{i2}, but not to G_{i1} or to G_{i3} (22). However, Lledo *et al.* have shown that DA-induced decreases in calcium current are, at least in part, mediated by G_o, whereas the increase in potassium current is mediated by G_{i3} (23). As previously described for the acetylcholine M2 muscarinic receptor, one receptor may be coupled to more than one G-protein (24), or alternatively, multiple D_2 receptors may be mediating the multitude of effects.

Multiple D_2 receptor subtypes have been identified by molecular cloning and designated D_{2short}, D_{2long}, D_3, and D_4 (25, 26). At least two of these members (D_{2short} and D_{2long}) are expressed in the pituitary gland (27–30) and the single difference between these two receptors is a 29 amino acid insert in the long form within the third cytoplasmic domain, a region thought to interact with G-proteins (25–30). Therefore, the possibility exists that these receptors may be coupled to distinct G-proteins thus giving rise to the numerous effects of DA on lactotrophs.

In this study, we examined the effects of DA on the pituitary cell lines expressing either the short form or the long form of the rat D_2 receptor. This cell line is derived from the mammosomatotroph line, GH_4C_1, which secretes both PRL and growth hormone, but does not express functional DA receptors (31, 32). Albert *et al.* transfected GH_4C_1 cells with the rat D_{2short} cDNA (33, 34), designated GH_4ZR_7, and Caron *et al.* (unpublished) transfected GH_4C_1 cells with rat D_{2long} cDNA, designated GH_4I_{12}, thus providing models that allow us to examine the effects of DA acting at single DA D_2 receptor subtypes in PRL secreting cells.

Materials and Methods

GH Cell Culture. GH_4C_1 cells (obtained from Dr. John Ramsdell, Medical University of South Carolina), GH_4I_{12} cells (Dr. Marc Caron, Duke University Medical Center), and GH_4ZR_7 cells (obtained from Dr. Harry Elsholtz, University of Toronto) were grown in Ham's F-10 media (Gibco) supplemented with 12.5% horse serum (HS) and 2.5% fetal calf serum (FCS) without antibiotics at 37°C in 5% CO_2. All cell lines were passed once per week and after approximately three months each line was replaced with cells from stocks kept in liquid N_2. For studies examining PRL secretion in response to DA (Calbiochem) and/or TRH (Sigma), cells were plated at a density of 28,000 cells/well in Dulbecco's Modified Eagles Medium (DMEM; supplemented with 10% FCS) in 96-well microtiter plates (Corning). Cells were challenged for 6 hr with DA and/or TRH (in DMEM without serum) four days after plating. PRL in the media was assessed by RIA using reagents provided by the NIDDK according to previously published procedures (35).

Ligand Binding. These studies were performed to confirm that both stably transfected cell types express functional DA receptors. Cells were grown in T-75 tissue culture flasks until confluent. Media were removed from the flasks, and cells were washed with ice cold wash buffer (50 m*M* Tris, 100 m*M* NaCl, pH 7.4) twice. Ten milliliters of cold lysis buffer (5 m*M* Tris, 2 m*M* EDTA, pH 7.4) was then added to each flask and the flasks were agitated for 10 min. The cell suspension was collected and the flasks were rinsed with 5 ml of lysis buffer and the cell suspension pooled. The cells were homogenized with a 15-sec burst on a Teckmar Tissuemizer (Setting 7) and then centrifuged at 14,500*g* for 20 min. The pellet was collected and resuspended in 10 ml of lysis buffer. The pellet was homogenized with a 15-sec burst on a Teckmar Tissuemizer (Setting 7) and then centrifuged at 14,500*g* for 20 min. The resulting pellet was then resuspended in binding buffer (0.75 ml/T-75 flask; 50 m*M* Tris, 100 m*M* NaCl, 12.5 m*M* $MgCl_2$, 2 m*M* EDTA, pH 7.4). Fifty microliter aliquots of membrane (20–30 μg of protein using BCA method) were added to tubes containing 1 ml of binding buffer containing various concentrations of $[^3H]$spiperone. Five μ*M* eticlopride was used to define nonspecific binding which was less than 10% of total binding at spiperone concentrations near the K_d. The tubes were incubated for 1 hr at 37°C and the reaction terminated by addition of 2.5 ml of ice-cold wash buffer. The contents of each tube was then decanted through a 3.5-cm Beckman Readyfilter (prewashed with 10 ml of 0.5% bovine serum albumin) supported on a Millipore manifold. Each tube was rinsed with an additional 5 ml of ice-cold wash buffer and each filter was washed with 10 ml of ice-cold wash buffer. Binding assays were performed in duplicate. The filters were dried overnight at 50°C, and radioactivity retained on the filters was counted using a Beckman LS6000IC scintillation counter.

Phosphatidylinositol Turnover Assay. Cells were plated at a density of 500,000 cells/dish in 3.5-cm tissue culture dishes (Corning) in Medium 199 (M199; Gibco) supplemented with 5% HS and myo-[2-^{3}H]inositol (1 μCi/ml). After a two-day incubation, the cells were washed two times with Hank's Balanced Salt Solution (HBSS) and the cells were incubated for 15 min in HBSS supplemented with 10 m*M* LiCl and

0.3% BSA. The cells were then challenged for varying amounts of time with TRH and/or dopamine dissolved in the supplemented HBSS. The challenge was terminated by replacement of the challenge media with 350 μl of distilled water. The cells were then scraped from the dishes and placed into tubes each containing 1 ml of a chloroform/methanol (1:2) solution. After an incubation of approximately 1 hr, 350 μl of chloroform and 350 μl of water was added to each tube. The tubes were centrifuged at 2500*g* for 10 min after which the top (water) phase was collected from each tube and applied to a Dowex AG 1-X8 anion exchange column (formate form; 2 ml bed volume). Free [^{3}H]inositol was eluted with four bed volumes of distilled water and total [^{3}H]inositol phosphates (IPs) were eluted with four bed volumes of 1 *M* ammonium formate/0.1 *N* formic acid. Radioactivity of aliquots of the eluents was quantitated by liquid scintillation counting.

Single Cell Microfluorimetric Imaging of Intracellular Calcium Concentrations $[Ca^{2+}]_i$. Cells were plated on poly-L-lysine-coated (Sigma; 0.25 mg/ml) coverslips in DMEM supplemented with 10% FCS. After approximately 24 hr, cells were loaded with fura-2/AM (Molecular Probes). Coverslips with the cells attached were removed from DMEM and washed with HBSS without phenol red and placed in 10-cm tissue culture plates. Approximately 250 μl of fura-2/AM, at a concentration of 1 μ*M* in HBSS, was then placed on top of each coverslip and incubated for 30 min at 37°C. The cells were washed twice with HBSS after fura-2/AM treatment and incubated another 30 min at room temperature to allow de-esterifaction of the fura-2/AM in the cytoplasm. After loading, coverslips were placed in a specially constructed microscope stage adapter that creates a chamber above the coverslip with a capacity of approximately 500 μl. Two hundred microliters of HBSS were added to the chamber and the adapter was placed on the stage of a Zeiss Axiovert 35 microscope prepared for epifluorescence microscopy. All recordings were conducted at room temperature. Excitation light was provided by an Optiquip model 770 Xenon arc lamp. Excitation wavelengths were selected by 350 and 380 nm filters mounted in a Ludl dual filter wheel rotating controller (also containing neutral density filters) between the Xenon light source and the microscope. Fluorescence emitted by fura-2 was collected by a Zeiss Plan-Neofluor 63 DIC oil immersion objective and passed through the barrier filter (500–535 nm transmission) before reaching a Quantex intensified CCD camera. A Universal Imaging Corp. 486 personal computer was used in concert with Image1/FL software provided by the same company to analyze the video signal. The signal was viewed on a Sony Trinitron monitor interfaced with a Sony Mavigraph color video printer. Ten to 30 cells were selected for analysis during each data acquisition session. Each cell was selected individually and average intensities of cells were recorded for each excitation. Average intensities for each cell were collected for the two excitations independently and the resulting ratio (350 nm/380 nm) which is proportional to Ca^{2+} (36) was saved to an ASCII file. While recordings were in progress, various secretagogues were added to the chamber above the cells. Ca^{2+} concentrations were calibrated by the *in vivo* method using 8-bromo-A23187 (36). Approximately 50–200 cells were measured for each experimental treatment, and at least three separate data acquisitions were performed for each treatment.

cAMP Extraction and Radioimmunoassay. Cells were plated at a density of 500,000 cells/35-mm dish (Corning) in DMEM (10% FCS). After approximately two days, cells were challenged with various concentrations of DA or forskolin. Cells were first washed twice with F-10 media (5% FCS, 0.5 m*M* 3-isobutyl-1-methyl-xanthine (IBMX), then 400 μl of F-10 media (5% FCS, 0.5 m*M* IBMX) containing DA or forskolin was added to the dish. After 30 min, the challenge was stopped by addition of 400 μl of 100% ethanol. The plates were placed on ice and the cells were removed from the surface of the plates by scraping. After three freeze-thaw cycles to lyse the cells, the samples were spun at 5000*g* for 20 min, and the 400 μl of supernatant was collected and lyophilized in a speed vac. The samples were then reconstituted in 50 μl of 0.05 *M* acetate buffer, pH 6.2. The cAMP concentration in the samples was then estimated using a specific double antibody radioimmunoassay for cAMP. The cAMP RIA protocol was modified from the methods provided with the NIDDK cAMP reagents and the methods of Steiner (37). The cAMP antiserum (CV-27 pool) was obtained from the NIDDK and diluted 1:14,000 with 0.05 *M* sodium acetate (0.1% gelatin, pH 6.2). cAMP (Sigma) was diluted to the appropriate concentrations (2 fmol/tube–60 pmol/tube) in 0.05 sodium acetate buffer, pH 6.2, for standard curves. The sensitivity of the assay was increased by three orders of magnitude by acetylating both the cAMP standards and samples (37). The interassay coefficient of variation was 7.6% and the intraassay coefficient of variation was 8.0%. The sensitivity of the assay was approximately 12 pmol cAMP/tube.

Statistics. Differences between control and experimental treatment values were assessed by analysis of variance followed by Scheffe's post hoc test for multiple comparisons. Differences were considered significant if $P < 0.05$.

Results

Characterization of DA D_2 receptor binding. [^{3}H]Spiperone showed no specific binding to GH_4C_1 cells. Specific binding of [^{3}H]spiperone to the D_{2short}

receptor of GH_4ZR_7 cell membranes was saturable and was displaced by 5 μ*M* eticlopride. Scatchard analysis indicated a K_d of 96 ± 8 p*M* (Fig. 1). These values agree with previously published values (33). Specific binding of [^{3}H]spiperone to the DA D_{2long} receptor in membrane preparations of GH_4I_{12} cells was saturable and was displaced by 5 μ*M* eticlopride. Scatchard analysis indicated a K_d of 107 ± 49 p*M* (Fig. 1).

DA-Mediated Inhibition of Basal and Stimulated PRL Secretion. DA, over wide concentrations, had no effect on PRL secretion from GH_4C_1 cells (Fig. 2). However, DA at concentrations of 1 and 10 μ*M* significantly ($P < 0.02$) inhibited basal PRL secretion from GH_4ZR_7 cells to 63% and 42% of control values, respectively (Fig. 2). DA at concentrations of 1 and 10 μ*M* significantly ($P < 0.02$) inhibited basal PRL secretion from GH_4I_{12} cells to 37% and 46% of control values, respectively (Fig. 2). Both the GH_4C_1 and the GH_4ZR_7 cell line secreted similar basal amounts of PRL (25–35 ng/ml over a 6-hr incubation), but the GH_4I_{12} line secreted a significantly lower basal amount

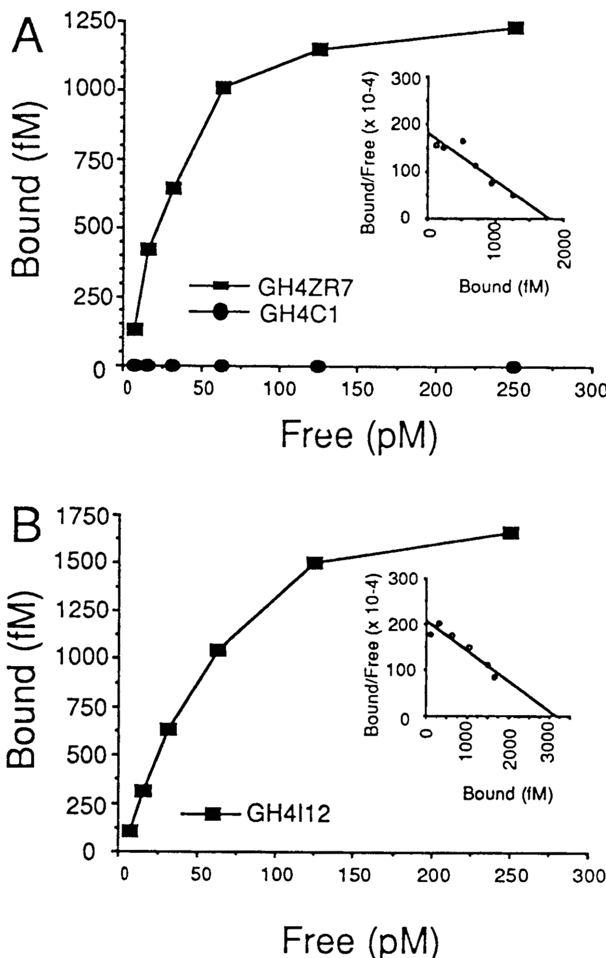

Figure 1. A) Binding of [^{3}H]spiperone to GH_4C_1 (circles) and GH_4ZR_7 (squares) cell membranes. B) Binding of [^{3}H]spiperone to GH_4I_{12} cell membranes. Saturation isotherms of specific binding of [^{3}H]spiperone to membranes from all cell lines are shown. GH_4C_1 cell membranes demonstrate no specific binding, whereas GH_4ZR_7 cell membranes bound [^{3}H]spiperone in a saturable manner with nonspecific binding of less than 10% of total binding near the K_d for [^{3}H]spiperone. The results shown are a representative of three independent experiments each completed in duplicate. A Scatchard plot of the data from the GH_4ZR_7 and GH_4I_{12} cells is shown in their respective insets. The K_d and B_{max} for [^{3}H]spiperone were 96 ± 8 p*M* and 1700 ± 300 fmol/mg protein, respectively, for GH_4ZR_7 cells. The K_d and B_{max} for [^{3}H]spiperone were 107 ± 49 p*M* and 272 ± 91 fmol/mg protein, respectively, for GH_4I_{12} cells.

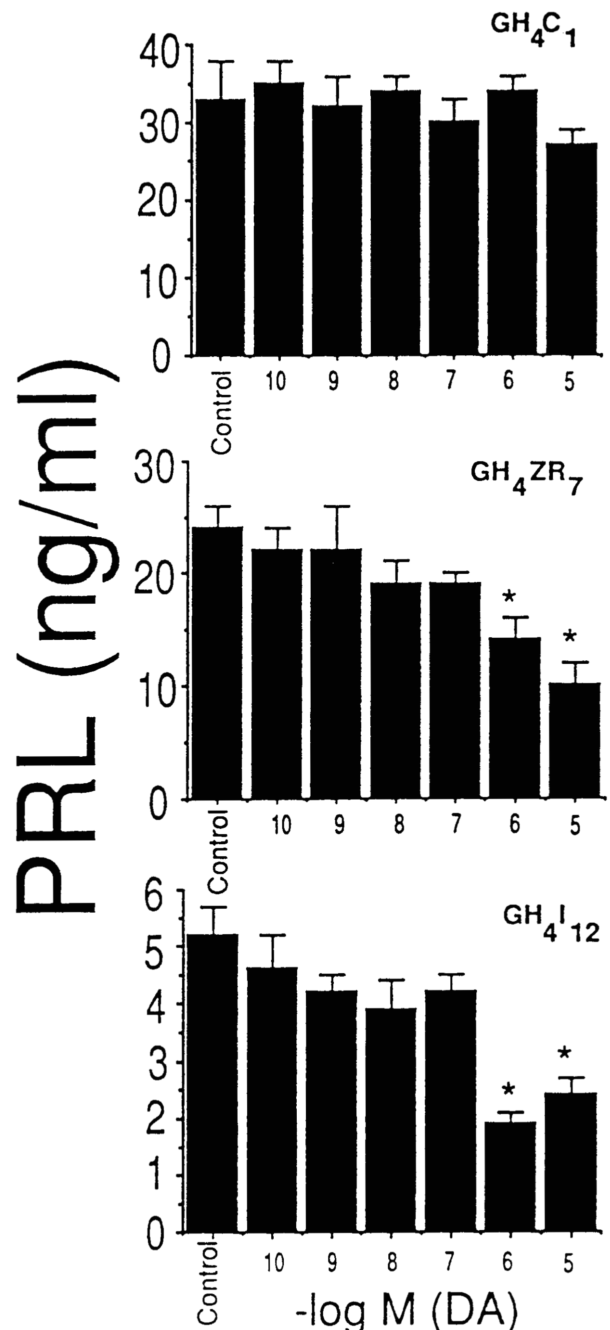

Figure 2. Effects of dopamine on basal PRL secretion from GH_4C_1, GH_4ZR_7, or GH_4I_{12} cells. Cells were challenged with various concentrations of dopamine for 6 hr. Dopamine had no effect on basal PRL secretion from GH_4C_1 cells. Dopamine at concentrations of 1 and 10 μ*M* significantly ($P < 0.02$) inhibited basal PRL secretion from both GH_4ZR_7 and GH_4I_{12} cell lines.

of PRL over the same time period (5 ng/ml). DA (1 and 10 μ*M*) had no effect on PRL secretion stimulated by various concentrations of thyrotropin-releasing hormone (TRH; 1–100 n*M*) in GH_4C_1 cells (Fig. 3). In GH_4ZR_7 cells, however, 10 μ*M* DA completely reversed the stimulatory effects of 1–100 n*M* TRH on PRL secretion ($P < 0.05$; Fig. 3), and 1 μ*M* DA significantly attenuated the stimulatory effects of 10 and 100 n*M* TRH on PRL secretion ($P < 0.05$; Fig. 3). Interestingly, TRH did not stimulate PRL secretion in GH_4I_{12} cells (data not shown). The inhibitory effects of DA on basal PRL secretion in both GH_4ZR_7 and GH_4I_{12} cells was significantly blocked by the DA D_2 receptor antagonist, eticlopride (ETI; 1 μ*M*). DA-mediated inhibition of TRH-stimulated PRL secretion in GH_4ZR_7 cells was also blocked by ETI (1 μ*M*).

Effects of TRH on $[Ca^{2+}]_i$. The effects of various concentrations of TRH (10–1000 n*M*) on $[Ca^{2+}]_i$, measured by single cell microfluorimetry in GH_4ZR_7 cells, is shown in Figure 4. In each case, the fluorescence from approximately 20 cells was recorded at each time, and each figure illustrates three representative cells. Ten nanomolar TRH increased $[Ca^{2+}]_i$ by 50 n*M* above resting levels and reached a peak within 2 sec of administration of TRH (Fig. 4). The response to 100

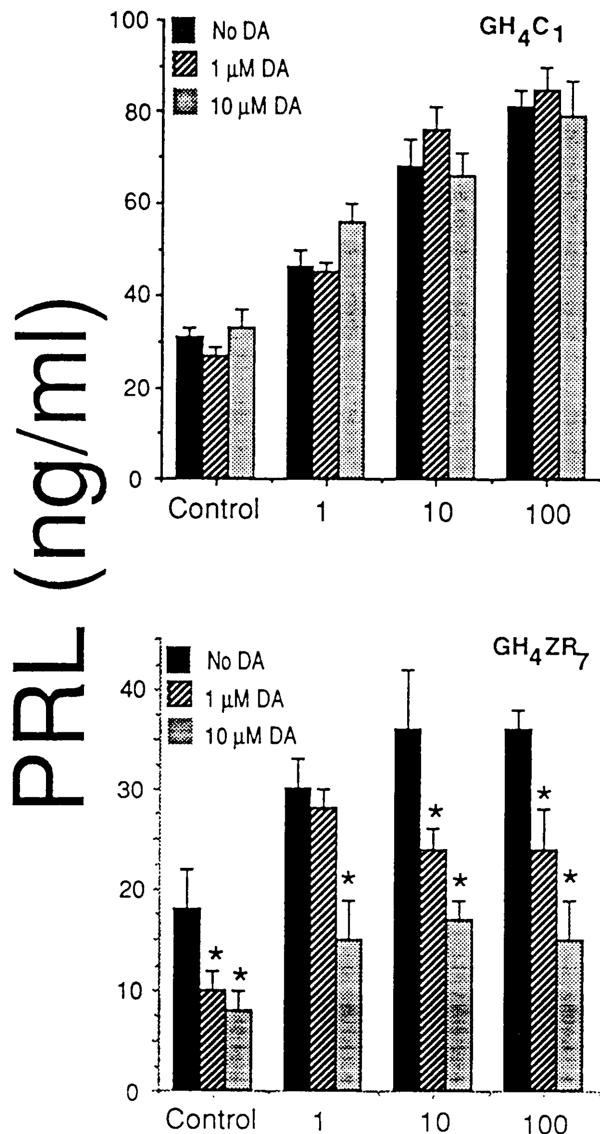

Figure 3. Effects of dopamine on TRH-stimulated PRL secretion from GH_4C_1 and GH_4ZR_7 cells. Dopamine had no effect on TRH-stimulated PRL secretion from GH_4C_1 cells. Ten micromolar dopamine completely reversed the stimulatory effects of 1–100 n*M* TRH on PRL secretion ($P < 0.05$) and 1 μ*M* dopamine significantly ($P < 0.05$) attenuated the stimulatory effects of 10 and 100 n*M* TRH on PRL secretion.

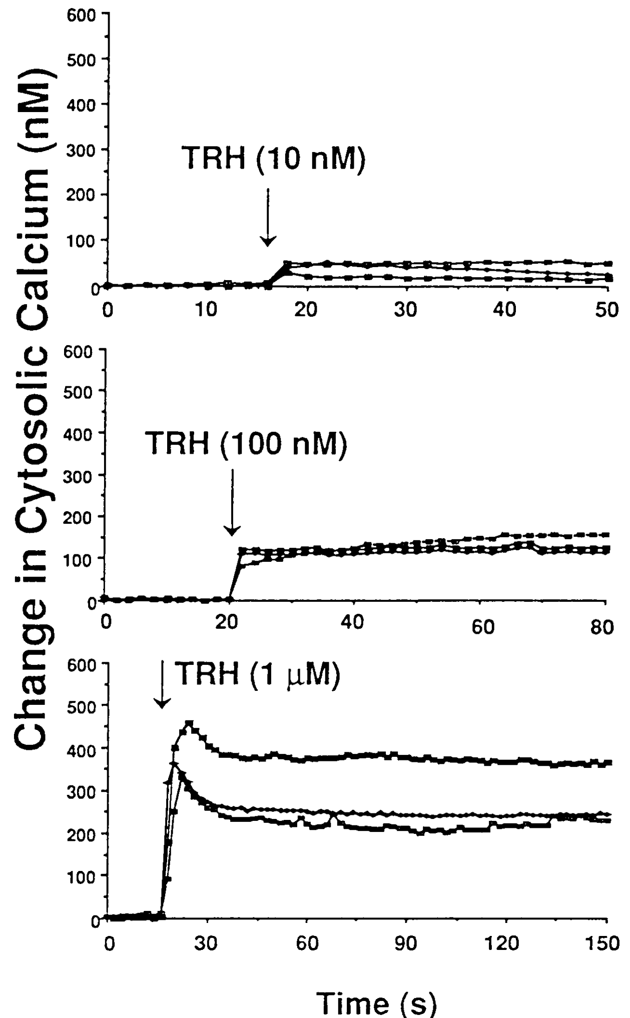

Figure 4. Dose-dependent effects of TRH on $[Ca^{2+}]_i$ in single GH_4ZR_7 cells. The effects of TRH were simultaneously measured in approximately 20 cells, and in each figure three representative cells are shown. Each symbol depicts a representative cell. Ten nanomolar TRH induced a monophasic response in which $[Ca^{2+}]_i$ increased approximately 50 n*M* within 2 sec of administration of TRH. One hundred nanomolar TRH induced a monophasic response in which $[Ca^{2+}]_i$ increased approximately 100 n*M* within 2 sec of administration of TRH. One thousand nanomolar TRH induced a biphasic response in which $[Ca^{2+}]_i$ first increased by 350 to 450 n*M* above resting $[Ca^{2+}]_i$ within 2–4 sec of administration of TRH. This response was short lived and lasted for approximately 14–50 sec and was followed by a sustained phase in which $[Ca^{2+}]_i$ was maintained 250–400 n*M* above resting $[Ca^{2+}]_i$.

nM TRH was also monophasic and reached a peak $[Ca^{2+}]_i$ approximately 100 nM greater than basal within 2 sec after TRH treatment, and this concentration was sustained indefinitely (Fig. 4). The biphasic effect of TRH on $[Ca^{2+}]_i$ is clearly demonstrated in Figure 4 (38). After application of 1000 nM TRH, each cell increased $[Ca^{2+}]_i$ by approximately 350–450 nM within 2–4 sec (Fig. 4). This first phase was relatively short lived and lasted for approximately 14–50 sec depending on the cell. $[Ca^{2+}]_i$ during the second phase was sustained and was approximately 250–400 nM above basal $[Ca^{2+}]_i$. The TRH dose response in GH_4C_1 cells was indistinguishable from GH_4ZR_7 cells (data not shown). TRH had no effect on $[Ca^{2+}]_i$ in GH_4I_{12} cells (data not shown). Resting $[Ca^{2+}]_i$ was normally in the 100–200 nM range.

Effects of DA on TRH-Stimulated $[Ca^{2+}]_i$. In this experiment, TRH (1000 nM) administration increased $[Ca^{2+}]_i$ in GH_4ZR_7 cells as previously shown (Fig. 5). If GH_4ZR_7 cells were treated with 1 μM DA prior to TRH administration, the TRH induced increase in $[Ca^{2+}]_i$ was entirely abolished (Fig. 5). As expected, DA had no effect on TRH-induced increases in $[Ca^{2+}]_i$ in GH_4C_1 cells (data not shown). Figure 5 also illustrates the lack of effect of DA on basal $[Ca^{2+}]_i$ prior to TRH treatment. DA did not reduce $[Ca^{2+}]_i$ in any of the cells examined. If the cells were treated with the DA D_2 receptor antagonist, eticlopride (ETI; 1 μM), before addition of DA, an attenuated TRH-induced increase in $[Ca^{2+}]_i$ reappeared (Fig. 5). Similar to GH_4ZR_7 cells, DA had no effect on basal $[Ca^{2+}]_i$ in GH_4I_{12} cells (data not shown).

Effects of DA and TRH on PI Turnover. DA, administered over a wide range of concentrations (1 pM–10 μM) and times (30 sec–20 min), had no effect on PI turnover in GH_4C_1 or GH_4ZR_7 cells (data not shown). The responses of the GH_4C_1 and GH_4ZR_7 cell lines to TRH, in terms of total IP accumulation after 1-, 10-, and 20-min incubation was similar. A TRH dose-dependent increase in IP accumulation was noted in both the GH_4C_1 and GH_4ZR_7 cell lines. In the GH_4I_{12} cell line, TRH had no effect on IP accumulation. Ten micromolar DA had no effect on IP accumulation after 1-, 10-, or 20-min incubations in GH_4C_1 cells (Fig. 6). In these cells, TRH (100 nM) increased IP accumulation by 2.4-, 3.6-, and 4.1-fold after incubations of 1-, 10-, and 20-min, respectively (Fig. 6). Coincubation of DA (10 μM) with TRH (100 nM) did not affect the normal TRH response (Fig. 6). Similar results were obtained for GH_4ZR_7 cells (Fig. 6). DA (10 μM) had no effect on IP accumulation after 1-, 10-, or 20-min incubations. In GH_4ZR_7 cells, TRH (100 nM) increased IP accumulation by 3.7-, 3.7-, and 5.3-fold after incubations of 1, 10, and 20 min, respectively. Coincubation of DA (10 μM) with TRH (100 nM) did not affect the normal TRH response. DA had no effect on IP accumulation in GH_4I_{12} cells (Fig. 6).

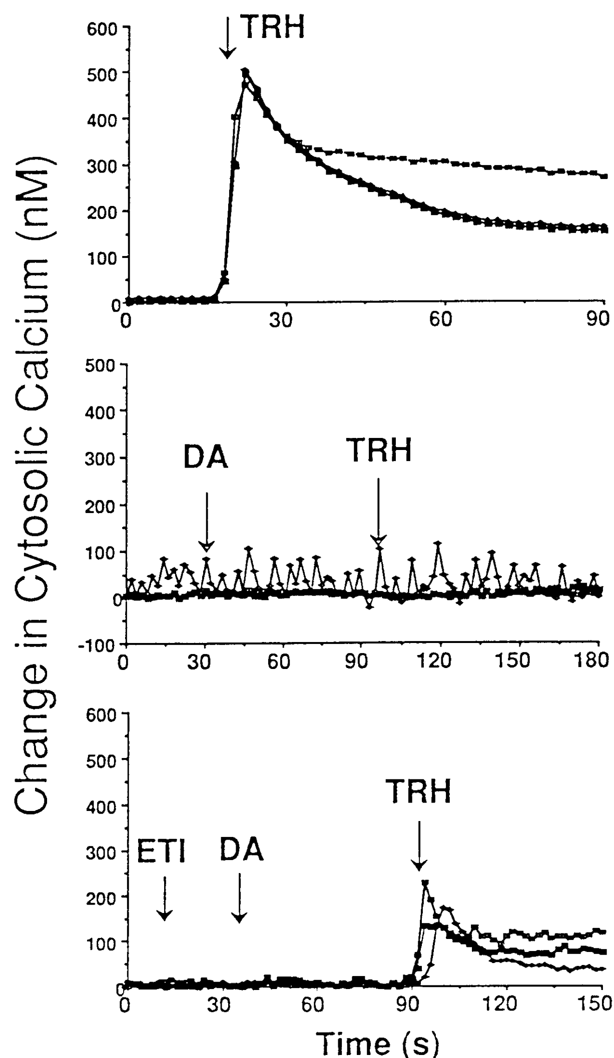

Figure 5. Effects of dopamine on TRH-stimulated $[Ca^{2+}]_i$ in GH_4ZR_7 cells. Each symbol depicts a representative cell. A typical response to 1000 nM TRH which was recorded during the same session as the following experiments shown in this figure. Administration of 1 μM dopamine immediately prior to treatment with 1000 nM TRH completely abolished the TRH-mediated increase in $[Ca^{2+}]_i$. If the cells received the D_2 receptor antagonist, eticlopride (1 μM; ETI), the ability of dopamine to abolish the TRH mediated increase in $[Ca^{2+}]_i$ was attenuated.

Effects of DA on cAMP Production. DA had no effect on cAMP production in the GH_4C_1 cell line (Fig. 7). However, in both GH_4ZR_7 and GH_4I_{12} lines, DA had significant inhibitory effects on cAMP production. In GH_4ZR_7 cells, DA at concentrations of 1 and 10 μM inhibited cAMP production by 40% and 39%, respectively (Fig. 7). In GH_4I_{12} cells, DA at concentrations of 1 and 10 μM inhibited cAMP production by 48% and 60%, respectively (Fig. 7). As with basal PRL secretion, both the GH_4C_1 and the GH_4ZR_7 lines had similar basal concentrations of cAMP (88–124 pmol/ml), and

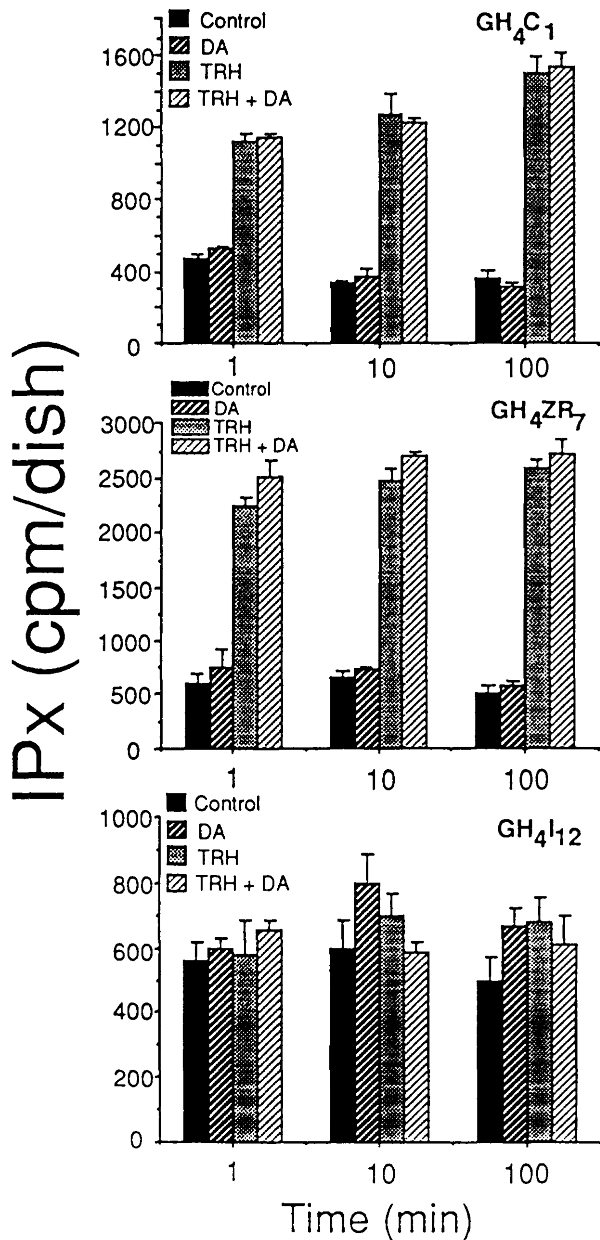

Figure 6. The effects of dopamine (10 μM) on TRH-stimulated (100 nM) PI turnover in GH_4C_1, GH_4ZR_7, and GH_4I_{12} cells. Dopamine did not affect basal or TRH-stimulated IP accumulation in any of the cell lines.

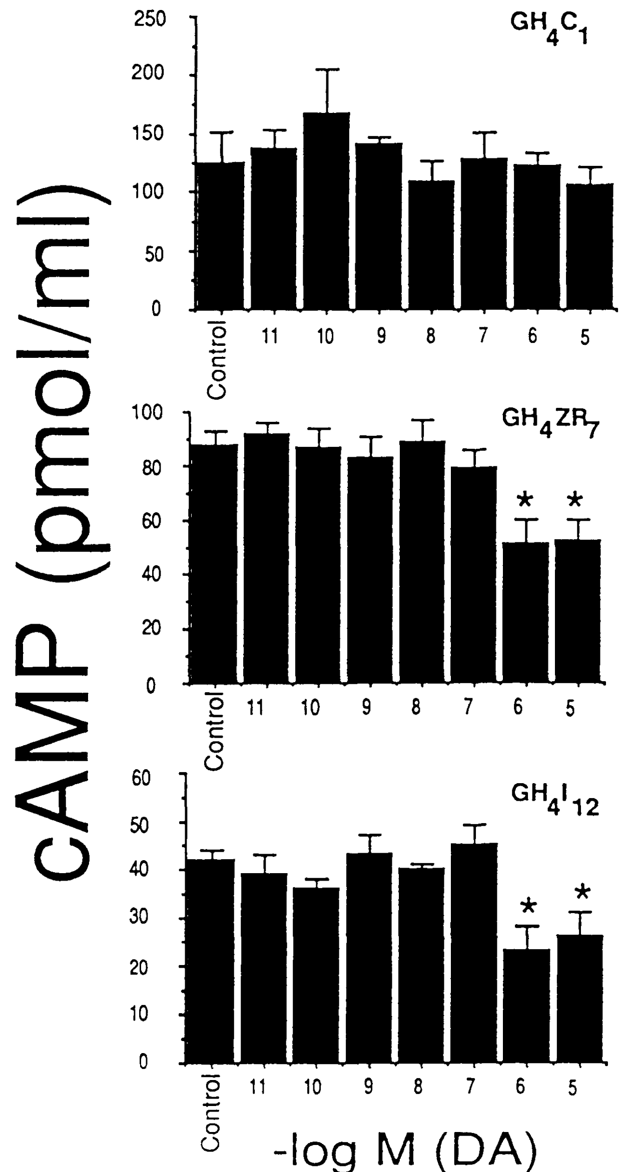

Figure 7. The effects of various concentrations of dopamine on cAMP production in GH_4C_1, GH_4ZR_7, or GH_4I_{12} cells. Dopamine had no effect on cAMP production in GH_4C_1 cells. Dopamine at concentrations of 1 and 10 μM inhibited cAMP production by 40% and 39%, respectively ($P < 0.05$) in GH_4ZR_7 cells. Dopamine at concentrations of 1 and 10 μM inhibited cAMP production by 48% and 60%, respectively ($P < 0.05$) in GH_4I_{12} cells.

the GH_4I_{12} line had greatly reduced basal concentrations of cAMP (42 pmol/ml).

Discussion

Both the GH_4ZR_7 and the GH_4I_{12} cell lines are derived from the GH_4C_1 line, a PRL-secreting cell line which normally lacks functional DA receptors (31, 32). The GH_4ZR_7 cell line has been transfected with the cDNA of the short form of the DA D_2 receptor and has been demonstrated to express a functional D_2 receptor (33). The GH_4I_{12} cell line has been stably transfected with the cDNA of the long form of the DA D_2 receptor (M. Caron, unpublished). The coupling of the DA receptor to various signal transduction mechanisms in GH_4I_{12} cells has not been thoroughly examined, however, in GH_4ZR_7 cells, the D_2 receptor is coupled to a pertussis toxin–sensitive G-protein which presumably mediates inhibition of VIP or TRH-stimulated PRL secretion, inhibition of both basal and stimulated cAMP accumulation, decreases in $[Ca^{2+}]_i$, and membrane hyperpolarization (33, 34). One important action of DA in the anterior pituitary gland is inhibition of basal secretion of PRL. However, in one study, DA did not inhibit basal PRL secretion in GH_4ZR_7 cells (33). These investigators used short incubations (30 min) which may not allow observation of the inhibitory ef-

fects of DA on PRL secretion (33). Using the same cell line (expressing D_{2short}), in this study, we found that DA could in fact inhibit basal PRL secretion by 58% during a 6-hr incubation. In the GH_4I_{12} cell line (expressing D_{2long}), similar results were obtained: DA maximally inhibited PRL secretion by 47% after 6 hr. DA was also able to completely reverse the stimulatory effects of TRH on PRL secretion as has been demonstrated previously in GH_4ZR_7 cells (33). However, since the GH_4I_{12} cells lacked a response to TRH, similar experiments were not conducted in these cells. The lack of effect of TRH was not due to expression of the D_{2long} receptor, as a GH_4C_1 cell line expressing the human D_{2long} receptor is responsive to both DA and TRH (unpublished). These effects of DA were absent in the GH_4C_1 cell line, which lacks functional DA receptors.

Examination of $[Ca^{2+}]_i$ in single GH_4ZR_7 or GH_4I_{12} cells revealed that DA had no effect on resting $[Ca^{2+}]_i$. This is in contrast to the observation that DA lowers $[Ca^{2+}]_i$ in GH_4ZR_7 cells (34). The reason for the discrepancy between the two observations is unknown, but may be due to the fact that our recordings were conducted at room temperature whereas those of Vallar *et al.* (34) were conducted at 37°C. However, in our system, DA was able to completely abolish the TRH-mediated increase in $[Ca^{2+}]_i$ in GH_4ZR_7 cells. Furthermore, if the D_2 receptor is blocked by the antagonist, eticlopride, an attenuated increase in $[Ca^{2+}]_i$ in response to TRH is seen even in the presence of DA. These data indicate that the D_2 receptor is functional in this system and that the lack of effect of DA on resting $[Ca^{2+}]_i$ is not artifactual. This experiment could not be reproduced in GH_4I_{12} cells since they lacked any response to TRH. Interestingly, although DA has no acute effect on resting $[Ca^{2+}]_i$, DA is still able to maximally inhibit basal PRL secretion by 58% in GH_4ZR_7 cells and 47% in GH_4I_{12} cells. This inhibition is probably linked to the ability of DA to potently inhibit cAMP production in both normal lactotrophs (6, 7) and in the GH_4ZR_7 and GH_4I_{12} cell lines (33, 34, 39, this study). The inability of DA to inhibit basal PRL secretion after 30 min (33) is supportive of the fact that we do not see an acute reduction in $[Ca^{2+}]_i$ by that time. At least a fraction of the total inhibition of PRL secretion after a 6-hr incubation may be attributable to the DA-mediated reduction in PRL mRNA transcription (39, 40), an event which would probably not occur within 30 min (33).

In spite of several reports of DA being negatively coupled to PI turnover in lactotrophs (8–12), our data indicate, at least in GH_4ZR_7 and GH_4I_{12} cells, that DA has no effect on basal or stimulated IP accumulation. Others have also suggested that DA is not coupled to PI turnover in normal lactotrophs (13, 14). It is possible that a D_2 receptor subtype other than the short or long form examined in this and in previous studies (33, 34) may be coupled to a decrease in PI turnover. Alternatively, the cell line in which the D_2 short form was examined may be lacking the necessary G-protein or components of the effector system required for coupling of the receptor to inhibition of PI turnover. Vallar *et al.* (12) have suggested that DA-mediated inhibition of TRH-stimulated PI turnover is not due to direct coupling of D_2 receptors to phospholipase C, but rather is a late Ca^{2+}-dependent effect of D_2 receptor activation. However, our data indicate that DA has no effect on TRH-stimulated PI turnover. One would expect that if the attenuation in TRH-stimulated PI turnover by DA was a relatively non-specific late Ca^{2+}-dependent effect of D_2 receptor activation, the effects would be apparent in our system. Thus, phospholipase C may be negatively linked to a DA D_2 receptor other than the D_2 short or long subtypes. It is interesting to note that DA is able to completely abolish the TRH-induced increase in cytosolic Ca^{2+} without affecting TRH-induced increase in PI turnover. This suggests that DA is able to interfere with cellular signals induced by TRH at a point between the production of inositol phosphates and Ca^{2+} release from intracellular stores. The mechanism by which DA accomplishes this is unknown, but may be due to signal transduction "cross talk" between the PI turnover and cAMP production pathways. In one study using pituitary cells enriched for lactotrophs, DA had no effect on TRH-induced mobilization of Ca^{2+} from intracellular stores (14). However, the pituitary cells used in these studies were enriched for lactotrophs by induction of pituitary tumors with estrogen treatment (14). Estrogen has been demonstrated to reverse the inhibitory effects of DA on PRL secretion and may therefore account for the lack of effect of DA on TRH-induced mobilization of Ca^{2+} (41, 42).

DA induces a variety of effects in lactotrophs which may be mediated by multiple DA D_2 receptor subtypes and/or multiple G-protein subtypes. In this study, we demonstrated that both the long and short form of the D_2 receptor, when expressed in GH_4C_1 cells, are coupled to an inhibition of both basal and stimulated PRL secretion. Our data indicate that these receptors cannot mediate an acute reduction in $[Ca^{2+}]_i$, although TRH-stimulated levels of $[Ca^{2+}]_i$ can be completely reduced (at least in GH_4ZR_7 cells). This reduction of TRH-stimulated $[Ca^{2+}]_i$ is not due to a reduction in TRH-stimulated PI turnover, as DA has no effect on either basal or TRH-stimulated PI turnover. One major role of these receptors is to mediate a decrease in adenylyl cyclase activity (33, 34, 39, this study). In normal lactotrophs, DA has been demonstrated to be coupled to a decrease in PI turnover and to a decrease in resting $[Ca^{2+}]_i$ (15–18). Although both the long and short forms of the D_2 receptor mediate

several events which occur in normal lactotrophs, these two effects were not observed in GH_4ZR_7 or GH_4I_{12} cells. The maximal inhibition of basal PRL secretion caused by DA in GH_4ZR_7 cells (58%) or GH_4I_{12} cells (47%) was less than that in normal lactotrophs (80%) (43). These data, taken together, indicate that multiple DA D_2 receptors may be required to act in concert in order to mediate the multitude of effects caused by DA in normal pituitary lactotrophs. One must also consider that these GH_4C_1 cell lines may lack some effector systems required for normal physiological function in normal lactotrophs.

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