Differential Coupling of Dopaminergic D₂ Receptors Expressed in Different Cell Types

STIMULATION OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE HYDROLYSIS IN Ltk⁻ FIBROBLASTS, HYPERPOLARIZATION, AND CYTOSOLIC-FREE Ca²⁺ CONCENTRATION DECREASE IN GH₃C₁ CELLS

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Lucia Vallar, Claudia Muca, Michele Magni, Paul Albert†‡, James Bunzow‡, Jacopo Meldolesi†, and Olivier Civelli‡

From the Department of Pharmacology, Consiglio Nazionale delle Ricerche Center of Cytopharmacology and Scientific Institute San Raffaele, University of Milano, 20132 Milano, Italy and the ‡Vollum Institute for Advanced Biochemical Research, LTH, Oregon Health Sciences University, Portland, Oregon 97201

Dopaminergic D₂ receptors are widely regarded as typical inhibitory receptors, as they both inhibit adenylyl cyclase and decrease the cytosolic free Ca²⁺ concentration ([Ca²⁺]) by activating K⁺ channels. A D₂ receptor has recently been cloned (Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M. D., Machida, C. A., Neve, K. A., and Civelli, O. (1988) Nature 336, 783-787) and expressed in two different cell lines, pituitary GH₃C₁ cells and Ltk⁻ fibroblasts, where it has been shown to induce inhibition of adenylyl cyclase. We have investigated the additional effector systems coupled to this receptor. The responses observed in the two cell lines, which express similar levels of receptors (0.5-1 x 10⁶/cell), were surprisingly different. In GH₃C₁ cells, D₂ receptors failed to affect phosphoinositide hydrolysis and induced a decrease of [Ca²⁺]. This latter effect appears to be mediated by hyperpolarization, most likely due to the activation of K⁺ channels. In striking contrast, in Ltk⁻ fibroblasts the D₂ receptor induced a rapid stimulation of inositol(1,4,5)-trisphosphate (+73% at 15 s) followed by the other inositol phosphates, and an immediate increase of [Ca²⁺], due to both Ca²⁺ mobilization from internal stores and influx from the extracellular medium. In both GH₃C₁ and Ltk⁻ cells, the D₂ receptor response was mediated by G protein(s) sensitive to pertussis toxin. The increases of inositol trisphosphate and [Ca²⁺], observed in Ltk⁻ cells required dopamine concentrations only slightly higher than those inhibiting adenylyl cyclase (EC₅₀ = 25, 29, and 11 nM, respectively) and were comparable in magnitude to the responses induced by the endogenous stimulatory receptor agonists, thrombin and ATP. The results demonstrate that in certain cells D₂ receptors are efficiently coupled to the stimulation of phosphoinositide hydrolysis. The nature of receptor responses appears therefore to depend on the specific properties not only of the receptor molecule but also of the cell type in which it is expressed.

The receptors for many hormones and neurotransmitters transduce their signals by coupling to GTP binding (G) proteins, which in turn regulate the activity of effector molecules, such as adenylyl cyclase, phospholipase C, and K⁺ and Ca²⁺ channels (1-3). Each of these receptors was initially believed to selectively activate a single effector pathway. However, results accumulated during the last few years clearly indicate that individual receptor molecules can generate multiple signals by coupling to more than one effector system. Strong evidence in favor of this possibility comes from the study of a group of receptors, which were first demonstrated to inhibit adenylyl cyclase via G₁ (4). In some systems agonists to these inhibitory receptors also induce, by means of PTx-sensitive G protein(s), opening of K⁺ channels and/or inhibition of Ca²⁺ channels, both resulting in the reduction of [Ca²⁺], (1-3, 5-9). In the case of the heart muscarinic M₂ receptor, the current evidence unambiguously indicates that the inhibition of adenylyl cyclase and the activation of K⁺ channels are mediated by the same receptor molecule rather than by different coexisting muscarinic receptor subtypes with specialized functions (5, 8, 10-12). In this and in other systems, the multiple events triggered by inhibitory receptors appear to cooperate in inducing a negative effect on cell function via the decrease of second messenger levels. It was therefore rather unexpected that the cloned muscarinic M₂ and M₃ receptors stimulate phosphoinositide hydrolysis in various transfected cells by coupling to G proteins sensitive to PTx (11-13). This response, however, required much higher receptor densities and/or agonist concentrations in comparison to the inhibition of adenylyl cyclase and, even under optimal conditions, was rather weak (11-13). It would therefore appear that, although inhibitory receptors can mediate stimulation of phosphoinositide hydrolysis, such a coupling is inefficient and of uncertain biological significance.

Further insight into the multiple effector coupling mechanisms of inhibitory receptors and their relative roles in mediating the final response in the target cells can be provided by the extension of these studies to other members of the same receptor family expressed in various cell types. Dopaminergic D₂ receptors are known to inhibit adenylyl cyclase as well as

The abbreviations used are: G₁, the inhibitory regulator of adenylyl cyclase; PTx, pertussis toxin; [Ca²⁺], cytosolic free Ca²⁺ concentration; PtdInsP₂, phosphatidylinositol 4,5-bisphosphate; TRH, thyrotropin-releasing hormone; InsP₃, inositol trisphosphate; InsP₂, inositol bisphosphate; InsP₁, inositol monophosphate; HPLC, high performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ins(1,4,5)P₃ and Ins(1,3,4)P₃, inositol 1,4,5- and 1,3,4-trisphosphate.

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† Present address: Dept. of Pharmacology, McGill University, Montreal, Quebec H3A 2B2, Canada.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, Scientific Institute San Raffaele, Via Olgettina 60, 20132 Milano, Italy.

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to activate K+ channels (14–17). However, the characterization of the transduction pathways used by this inhibitory receptor has been limited by the lack of convenient experimental cell models, and our present knowledge results from the study of very few cell systems (17). A D2 receptor has recently been cloned from a rat brain cDNA library and expressed in two cell lines, pituitary GH4C1 cells and Ltk- mouse fibroblasts (18–20). In both cell types this receptor induces inhibition of adenylyl cyclase, and in the GH4C1 line it also blocks prolactin secretion (19, 20). We report here the effects of receptor activation on phosphoinositide hydrolysis, [Ca2+]i, and membrane potential. The results demonstrate that in GH4C1 cells the D2 receptors do not affect phosphoinositide metabolite, most likely via a K+ channel-dependent hyperpolarization, whereas in Ltk- cells it efficiently stimulates PtdInsP2 hydrolysis with consequent increase of [Ca2+]i. The properties of this latter response indicate that the stimulation of phosphoinositide hydrolysis is a coupling of primary relevance of the D2 receptor in certain cells. Thus, an individual receptor molecule can induce profoundly different responses depending on the cell type in which it is expressed.

**EXPERIMENTAL PROCEDURES**

Transfection of Ltk- and GH4C1 cells with the D2 receptor cDNA RGB-2 has been described elsewhere (18–20). Wild-type and transfected Ltk- and GH4C1 cells were maintained in monolayer culture in Dulbecco's modified Eagle's medium + 10% fetal calf serum and Ham's F-10 medium + 15% horse serum and 2.5% fetal calf serum, respectively. To increase D2 receptor expression (see "Results"), the transfected GH4C1 cells were exposed for 16 h to culture medium supplemented with 100 μM ZnSO4, which was replaced with fresh standard medium for 24 h before the experiments. All the cell lines were used after detachment from the dishes by a brief exposure (1–2 min) to Ca2+ and Mg2+-free phosphate buffer containing 0.02% EDTA. Before [Ca2+]i and membrane potential measurements, GH4C1 and Ltk- cells were kept for 3 h in spinner culture in Ham's F-10 medium + 1% fetal calf serum and 10 μM Heps.

**Inositol Phosphate Measurements**—For these experiments GH4C1 and Ltk- cells were labeled with 5 μCi/ml myo-[2-3H]inositol for 24 and 48 h, respectively. Cells were washed three times with a modified Krebs-Ringer buffer (KRH) (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 5 mM glucose, 25 mM Hepes/NaOH, pH 7.4) resuspended in the same buffer, distributed to the reaction tubes (1 × 106 cells in 400 μl), and preincubated for 10 min at 37 °C. Incubations were started by adding 100 μl of prewarmed KRH containing dopamine and/or other test substances, and were stopped with 900 μl of ice-cold 20% trichloroacetic acid. The trichloroacetic extractable fractions were washed three times with diethyl ether; other inositol phosphates were separated by either anion-exchange chromatography on Dowex 1-X8 columns, or HPLC on a Partisil SAX-10 column (Technicon Ltd, Stockport, United Kingdom) exactly as previously described (21).

**[Ca2+]i Measurements**—[Ca2+]i was measured with the fluorescent probe fura-2 (22). GH4C1 and Ltk- cells were washed with the respective culture medium + 1% fetal calf serum and 10 μM Heps, resuspended in the same medium, allowed to equilibrate for 10 min at 37 °C, and then loaded with 4.5 μM fura-2/acetoxymethyl ester for 30 min at 37 °C. The loaded cells were washed and kept at room temperature until use. Approximately 1 × 106 cells were resuspended in 1.5 ml of KRH and placed in the cuvette of a thermostatically controlled (37 ± 1 °C) cell holder. Fluorescence recording and calibration in terms of [Ca2+]i were as previously described (23).

**Measurements of Membrane Potential**—Membrane potential was qualitatively assessed with the slow response fluorescent dye bis-oxonol (93, 94). GH4C1 and Ltk- cells were washed as for [Ca2+]i measurements and resuspended in KRH. 1–5 × 105 cells in 1.5 ml of KRH were transferred into the spectrophotometric cuvette. 100 nM bis-oxonol was added from a concentrated stock solution, and equilibration and redistribution of the dye was allowed to proceed for at least 8 min. Fluorescence was recorded as described in Ref. 24. Downward or upward deflections of the fluorescence tracings represent hyper- or depolarizations, respectively.

cAMP Measurements—Cells were washed, resuspended, and incubated as described for inositol phosphate measurements. At the end of the incubation times, the sample were diluted with 1 ml of acetate buffer (0.5 mM sodium acetate, pH 6.2) and immediately transferred to boiling water. After 30 min, the extracts were centrifuged to remove cell debris, and the supernatant was stored at −20 °C until assayed. cAMP was determined by radioimmunoassay using an commercial kit (Du Pont-New England Nuclear).

**Materials**—Dopamine, somatostatin, thrombin, ATP, verapamil, quinidine, 8-Br-cAMP, and forskolin were purchased from Sigma. Purified charybdotoxin was from Latoxan (Rosans, France). Dopaminergic antagonists and affinity purified PTX were kind gifts of Drs. M. Parenti (Department of Pharmacology, Milano, Italy) and R. Rappuoli (Sclavo, Sienna, Italy), respectively. Myo-[2-3H]inositol (80–120 Ci/mmol) was from Amersham Corp., fura-2 acetoxyethyl ester was from Calbiochem and bis-oxonol was from Molecular Probes. Culture media were from Flow Laboratories; all other chemicals were reagent grade.

**RESULTS**

**Expression of D2 Receptors**—The cell lines employed in this study were wild-type GH4C1 and Ltk- cells, which are devoid of endogenous D2 receptors, and the previously described GH4ZR and LZR, (formerly referred as L-RGB2-zen-1) clones transfected with D2 receptor cDNA (18–20). In GH4ZR, cells transfection of the D2 receptor cDNA is regulated by a zinc-sensitive metallothionein promoter (20). The experiments were therefore carried out after treatment of the cells with ZnSO4, as described under "Experimental Procedures." Under these conditions GH4ZR cells express approximately 1 × 105 D2 receptors/cell. The LZR1 clone stably expresses about 0.5 × 107 D2 receptors/cell.

**Effects of D2 Receptor Activation in GH4ZR Cells**—We first examined the ability of the D2 receptor to affect phosphoinositide hydrolysis. As shown in Fig. 1A, in GH4ZR cells D2 receptor activation failed to induce a detectable change in inositol phosphate production, even in the presence of very high dopamine concentrations (1 mM). In the same experi-

**FIG. 1. Effects of dopamine (DA) in GH4ZR cells. A**, inositol phosphate production in the presence of dopamine. Cells were labeled with myo-[2-3H]inositol as described under "Experimental Procedures." After 10 min of preincubation at 37 °C in KRH containing 10 mM LiCl, the cells were treated with dopamine for 10 min. [3H]Inositol phosphates were determined by anion-exchange chromatography on Dowex 1-X8 formate columns. The results (means ± S.E. of nine observations) are given as percentage of the values obtained in control cells, which were 703, 908, and 9,029 cpm for InsP3, InsP2, and InsP1, respectively. In the same experiments 100 mM TRH increased InsP3 to 2,005, InsP2 to 2,859, and InsP1 to 23,199 cpm. B, CAMP formation obtained with forskolin alone (259 ± 34 pmol of cAMP).
ments endogenous TRH receptors efficiently stimulated this response (see legend to Fig. 1).

Despite the lack of effect on phosphoinositide hydrolysis, the D2 receptor modified [Ca$$^{2+}$$]i in GH4ZR cells. In the presence of 1 $$\mu$$M dopamine [Ca$$^{2+}$$], measured in cells loaded with the [Ca$$^{2+}$$]-indicator fura-2, rapidly decreased from 155 $$\pm$$ 8 nM to 109 $$\pm$$ 7 nM (n = 9) (Fig. 2A). This dopamine effect was abolished by the selective D2 antagonists 1-sulpiride and butaclamol and was not detected in wild-type GH4C1 cells (Fig. 2A and results not shown).

Endogenous D2 receptors in pituitary lactotrophs, and somatostatin receptors in cells of the GH1 lines, reduce [Ca$$^{2+}$$]i by inhibiting Ca$$^{2+}$$ entry through voltage-dependent Ca$$^{2+}$$ channels (25-27). The latter effect is in turn mediated, at least in part, by hyperpolarization due to the opening of K$$^+$$ channels (15, 16, 25–28). The following results indicate that the action on [Ca$$^{2+}$$]i of the D2 receptor transfected into GH4ZR cells can be accounted for by the same mechanism.

(a) The effect of DA on [Ca$$^{2+}$$]i was similar, and not additive to those induced by withdrawal of external Ca$$^{2+}$$ and inhibition of voltage-gated Ca$$^{2+}$$ channels by verapamil (Fig. 2B and C). (b) When the fluorescent probe bis-oxonol was used to evaluate changes in membrane potential dopamine was found to cause hyperpolarization of GH4ZR cells (Fig. 3A). This effect was antagonized by 1-sulpiride and butaclamol (Fig. 3A and results not shown). Raising the extracellular K$$^+$$ concentration (Fig. 3B) abolished the dopamine hyperpolarization, indicating that the latter process is sustained by the activation of K$$^+$$ current(s).

As shown in Fig. 1, the dopamine concentrations required for both [Ca$$^{2+}$$]i decrease and membrane hyperpolarization (EC$$_{50}$ = 9 $$\pm$$ 2 and 12 $$\pm$$ 2 nM, respectively, n = 3) were similar to those inducing inhibition of cAMP production (EC$$_{50}$ = 14 $$\pm$$ 4 nM, n = 3) (see also Ref. 20). The first two effects, however, were independent of the action of DA on adenyl cyclase, as they were unaffected by the addition of the membrane permeant cAMP analogue, 8-BrcAMP (Figs. 2E and 3E).

**Fig. 2.** [Ca$$^{2+}$$]i decrease induced by dopamine (DA) in GH4ZR cells. Cells were loaded with fura-2 and analyzed as described in detail under “Experimental Procedures.” Dopamine, butaclamol (Bt), verapamil, and somatostatin (SRIF) concentrations were 1 $$\mu$$M, 500 nM, 100 $$\mu$$M, and 100 nM, respectively. Preincubation with 8-BrcAMP (1 mM) was for 10 min. Trace C was obtained with cells incubated in Ca$$^{2+}$$-free medium; trace F was obtained with cells pretreated with 100 ng/ml PTx for 3 h before loading with fura-2. The traces are representative of three to six experiments.

3E). Pretreatment of GH4ZR cells with 100 ng/ml PTx abolished the D2 receptor induced changes of membrane potential and [Ca$$^{2+}$$]i. (Figs. 2F and 3F), indicating that these responses are coupled by PTx-sensitive G protein(s). These results are similar to those reported for endogenous D2 receptors and other classical inhibitory receptors (1–3, 25).

**D2 Receptor Stimulation of Phosphoinositide Hydrolysis in LZR Cells—**A completely different set of results was obtained in LZR cells. In fact in these cells the activation of the transfected D2 receptor induced phosphoinositide hydrolysis. Fig. 4A shows the time course of the stimulation of inositol phosphate production observed in the presence of 1 $$\mu$$M dopamine. Total InsP$$^3$$, measured by conventional anion-exchange chromatography on Dowex columns, showed a relatively slow increase, which was clearly detectable only at 1 min and maximal at 10 min (+55%, n = 30). However, analysis of the individual isomers by HPLC (Fig. 4A, inset) revealed that Ins(1,4,5)P$$^3$$, i.e. the inositol phosphate which originates directly from PtdInsP$$_2$$ hydrolysis, was promptly stimulated by dopamine (at 15 s: +73%, n = 6). This rapid increase of Ins(1,4,5)P$$^3$$ was accompanied by a delayed and gradual rise of Ins(1,3,4)P$$_3$$. It should be noted that in resting LZR cells Ins(1,3,4)P$$^3$$ is by far the predominant InsP$$^3$$ isomer (see legend to Fig. 4). This easily explains why the early increase of Ins(1,4,5)P$$^3$$ is not reflected by a parallel change of the total InsP$$^3$$ fraction separated by Dowex chromatography. Dopamine also caused accumulation of InsP$$^2$$ (maximal increase +75%, n = 30, at 10 min) and InsP$$_1$$ (at 10 min: +24, n = 30; at 20 min: +70%, n = 6) (Fig. 4A). Dopamine stimulation of inositol phosphate production in LZR cells was unaffected
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FIG. 4. Stimulation of inositol phosphate production by dopamine (DA), thrombin, and ATP in LZR1 cells. Panels A, C, and D: time course of the effects of dopamine (1 μM), thrombin (7 units/ml), and ATP (100 μM), respectively. The results are expressed as means ± S.E. of the increases above control values at each time point (n = 6). Basal levels averaged 6,440, 9,754, and 22,986 cpm at 20 min. for InsP3, InsP4, and InsP1, respectively. Panel A, inset: stimulation of Ins(1,4,5)P3 and Ins(1,3,4)P4 by 1 μM dopamine. Data (means ± S.E. of six observations) are given as percentage of the basal values at each time point (at zero time: Ins(1,4,5)P3 = 152 ± 5 cpm; Ins(1,3,4)P4 = 1,353 ± 74 cpm). Panel B, effects of 1-sulpiride (1-Su) butaclamol (Bt), and SCH 23390 (SCH) on the DA stimulation of inositol phosphate production. The results (means ± S.E. of six observations) are expressed as percentage of the response obtained at 10 min with dopamine alone. Concentrations were: 1-sulpiride, 10 μM; butaclamol, 1 μM; SCH 23390, 1 μM.

Inositol phosphates were separated by anion-exchange chromatography or HPLC as described under "Experimental Procedures." by the selective D1 antagonist SCH 23390 and blocked by the D2 antagonists, 1-sulpiride and butaclamol (Fig. 4B). In wild-type Ltk− cells, DA had no effect on inositol phosphate generation (not shown).

In keeping with the ability of D2 receptors to stimulate PtdInsP2 hydrolysis, dopamine increased [Ca2+]i in LZR1 cell (Fig. 5). In the presence of extracellular Ca2+ an immediate rise, going on the average from 191 ± 15 to 664 ± 78 nM (n = 15), was observed. After this initial peak [Ca2+]i gradually decreased to reach a lower plateau that was maintained for several minutes (trace A). The ability of dopamine to increase [Ca2+]i was retained in Ca2+-free medium, although under these conditions the rise was short lived, with return to resting levels within 1–2 min (trace B). Thus, the D2 receptor expressed in LZR1 cells induce the [Ca2+]i response described as "Experimental Procedures" and incubated as GH,ZR1 cells (see legend to Fig. 1), except that LiCl was omitted in the samples in which InsP2 isomers were analyzed. Inositol phosphates were separated by anion-exchange chromatography or HPLC as described under "Experimental Procedures.

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Fig. 5. [Ca2+]i increase induced by dopamine (DA) in LZR1 cells. [Ca2+]i was monitored with fura-2 as described for GH,ZR1 cells in the legend to Fig. 2. Traces A, C, and D were obtained in complete KRH medium and trace B in Ca2+-free medium. The concentrations of dopamine, SCH 23390 (SCH), 1-sulpiride (1-Su), and butaclamol (Bt) were the same as in the experiments shown in Fig. 4.

FIG. 6. Effect of dopamine (DA) on membrane potential in LZR1 cells. Membrane potential was monitored with bis-oxonol as described in the legend to Fig. 2. Trace A was obtained in complete KRH medium and traces B and C in Ca2+-free medium. Dopamine and thrombin concentrations were 1 μM and 5 units/ml, respectively.

(not shown). The effects of dopamine on [Ca2+]i, and membrane potential were antagonized by 1-sulpiride and butaclamol and were not observed in non-transfected Ltk− cells (Fig. 5, C and D, and results not shown).

Fig. 7 shows the dose response analysis of the stimulation of inositol phosphate production and [Ca2+]i, in LZR1 cells.
The effect of dopamine on inositol phosphate generation was clearly detectable at 10 nM and maximal between 100 nM and 1 µM, with EC50 values of 25 ± 4, 29 ± 2, and 18 ± 3 nM for InsP3, InsP2, and InsP1, respectively (n = 3). Similar dopamine concentrations were required for raising [Ca2+]i (EC50 = 29 ± 3 nM), respectively (n = 3). These values are only slightly higher than those estimated for the dopamine inhibition of cAMP production in LZR1 cells (EC50 = 11 ± 3, n = 3) (Fig. 7) as well as in GH3ZRT cells (see above and Fig. 1).

The above experiments indicate that stimulation of PtdInsP2 hydrolysis and inhibition of cAMP production by the dopamine D2 receptor expressed in LZR1 cells are both PTx-sensitive receptors coupled to PtdInsP2 hydrolysis. Screening with a number of agonists revealed that these cells are endowed with both thrombin and ATP receptors. Fig. 4 (panels C and D) shows the effect of maximal concentration of thrombin and ATP on inositol phosphate production. With thrombin the maximal stimulations observed over 10-min time course experiments were 36% for InsP3 (at 15 s), 39% for InsP2 (at 1 min), and 16% for InsP1 (at 10 min), (n = 12); with ATP 34, 53 and 26% for InsP3, InsP2, and InsP1, respectively (at 10 min, n = 12). Although the responses induced by the three agonists are difficult to compare because they exhibit different time courses, it is clear from the data that the effects of endogenous thrombin and ATP receptors do not differ substantially in terms of magnitude from that mediated by the transfected D2 receptor (see Fig. 1A and text above). Also the maximal [Ca2+]i peak responses induced by thrombin (from 192 ± 18 to 784 ± 131 nM, n = 5), ATP (from 192 ± 16 to 731 ± 101 nM, n = 7) and dopamine (see above and Fig. 5A) were comparable.

All the effects so far attributed to endogenous D2 receptors, as well as those now observed with the cloned D2 receptor expressed in GH3ZRT cells, are mediated by PTx-sensitive G protein(s). Whether this is the case also for the novel D2 response observed in LZR1 cells it was interesting to establish because recent results in various systems have revealed that receptors can employ different G proteins, either sensitive or insensitive to PTx, to stimulate PtdInsP2 hydrolysis (13, 31). As shown in Fig. 8, dopamine lost its ability to stimulate inositol phosphate generation in cells pretreated with 100 ng/ml PTx for 4.5 h. Fig. 8 also shows that the PTx treatment which abolished the D2 effect reduced by only about 50% the thrombin stimulation and left unchanged the ATP response.

**DISCUSSION**

The present knowledge of the effector systems coupled to dopaminergic D2 receptors has been inferred from studies carried out exclusively on pituitary and brain tissue and primary culture preparations (17). Even in the few cases in which a homogenous cell system, such as purified pituitary lactotrophs, was employed the interpretation of the results
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remains problematical due to the possible existence of multiple D<sub>2</sub> receptor subtypes that might have differentially contributed to the overall response. Indeed, although pharmacological studies failed so far to provide conclusive evidence for receptor subtypes, the molecular biology approach has revealed that at least two forms of D<sub>2</sub> receptor exist (18, 32, 33). These molecules, which appear to be generated from the same gene by alternative splicing, differ from each other only for the insertion of a stretch of 29 amino acids in the third cytoplasmic loop (18, 32, 33). Whether or not the "short" and the "long" receptor represent functionally distinct subtypes remains to be elucidated. In this report we characterize the effects of the short receptor and demonstrate that this molecule can transduce with similar efficiency at least three distinct responses: the inhibition of adenylyl cyclase, a K<sup>+</sup> channel-dependent hyperpolarization, and the stimulation of PtdInsP<sub>2</sub> hydrolysis.

The results obtained in the GH<sub>3</sub> cells clearly indicate that in these cells dopamine concentrations similar to those required for the inhibition of adenylyl cyclase induce hyperpolarization. The dependence of this effect on K<sup>+</sup> channel activation is demonstrated by the blockade observed with high extracellular K<sup>+</sup> and the K<sup>+</sup> channel blocker, quinidine. Hyperpolarization was independent of the effect of D<sub>2</sub> receptors on adenylyl cyclase, as shown by the experiments with 8-BrcAMP, and was prevented by pretreatment of the cells with PTx. Taken as a whole, these results appear remarkably similar to those previously obtained in pituitary lactotrophs (15, 16, 25) and confirm the inference (17) that a single D<sub>2</sub> receptor may mediate, by coupling to PTx-sensitive G proteins, both the inhibition of adenylyl cyclase and the activation of K<sup>+</sup> channels. The latter effect can explain the ability of D<sub>2</sub> receptors to decrease the resting [Ca<sup>2+</sup>]<sub>i</sub>, in GH<sub>3</sub> cells. Indeed, hyperpolarization is expected to prevent the firing of spontaneous Ca<sup>2+</sup> action potentials, as previously described in both individual lactotrophs and cells of the GH lines treated with dopamine and somatostatin, respectively (25, 34).

Whether the hyperpolarization mechanism accounts entirely for the [Ca<sup>2+</sup>]<sub>i</sub> decrease observed in GH<sub>3</sub> cells or whether, in addition, the D<sub>2</sub> receptor is also negatively coupled to voltage-gated Ca<sup>2+</sup> channels, as suggested based on indirect evidence in lactotrophs and striatal neurons (17), cannot be established from our present data. The ability of D<sub>2</sub> receptors to stimulate PtdInsP<sub>2</sub> hydrolysis is demonstrated by the experiments in GH<sub>3</sub> fibroblasts where dopamine induced 1) a rapid increase of Ins(1,4,5)P<sub>3</sub>, followed by the other inositol phosphates; together with 2) a [Ca<sup>2+</sup>]<sub>i</sub> rise due to both Ca<sup>2+</sup> mobilization from internal stores and influx from the extracellular medium. The experiments with 8-BrcAMP quickly ruled out the possibility that this D<sub>2</sub> response is the simple consequence of the decrease of cAMP concomitantly induced by dopamine. On the other hand, the stimulation of PtdInsP<sub>2</sub> hydrolysis can account for the hyperpolarization observed in GH<sub>3</sub> cells, as the latter effect appears to be due primarily to the activation of Ca<sup>2+</sup>-dependent K<sup>+</sup> channels, triggered by the [Ca<sup>2+</sup>]<sub>i</sub> increase. The evidence includes that hyperpolarization was abolished by both high extracellular [K<sup>+</sup>] and charybdotoxin (an inhibitor of Ca<sup>2+</sup>-activated K<sup>+</sup> channels); most important, it was greatly shortened by withdrawal of external Ca<sup>2+</sup> and almost abolished under conditions which prevent the dopamine-induced [Ca<sup>2+</sup>]<sub>i</sub> increase (i.e. in cells incubated in Ca<sup>2+</sup>-free medium and pretreated with thrombin). Although the D<sub>2</sub> receptor induces hyperpolarization in both GH<sub>3</sub> and LZR cells, different mechanisms appear to be involved in this response in the two cell lines.

The stimulation of PtdInsP<sub>2</sub> hydrolysis by the the D<sub>2</sub> receptor transfected into LZR<sub>1</sub> cells should be compared with the results obtained with other inhibitory receptors expressed in various cell types. M<sub>1</sub> and M<sub>3</sub> muscarinic receptors in human embryonic kidney cells and Chinese hamster ovary cells (11-13), 5HT<sub>1A</sub> serotoninergic receptors in HeLa cells (35), and α<sub>1</sub>-adrenergic receptors in Chinese hamster lung fibroblasts (36), all stimulate phosphoinositide hydrolysis. However, their coupling appears rather inefficient. Thus, in all these studies the agonist concentrations required for PtdInsP<sub>2</sub> hydrolysis were much higher than those able to inhibit adenylyl cyclase, with EC<sub>50</sub> values in the micromolar and nanomolar range, respectively. In the case of the M<sub>3</sub> and M<sub>5</sub> receptors expressed in Chinese hamster ovary cells, the response also required high levels of receptor, with maximal stimulation at 1.5-2.5 × 10<sup>6</sup> receptors/cell (11). Moreover, in the cells in which the various types of muscarinic receptors have been expressed, the PtdInsP<sub>2</sub> hydrolysis responses induced by M<sub>3</sub> and M<sub>5</sub> subtypes were distinctly weaker than those triggered by the typical stimulatory receptors, M<sub>1</sub> and M<sub>4</sub> (12, 18). A similar difference in the magnitude of the response was observed when the transfected M<sub>3</sub> and M<sub>5</sub> receptors were compared to some endogenous stimulatory receptors (13). The coupling of D<sub>2</sub> receptors to PtdInsP<sub>2</sub> hydrolysis that we have observed in LZR fibroblasts displays definitely different features. 1) Compared with the inhibition of adenylyl cyclase, only slightly higher EC<sub>50</sub> values were estimated for both the stimulation of InsP<sub>3</sub> production and the [Ca<sup>2+</sup>]<sub>i</sub> increase (11 versus 25 and 29 nM, respectively). The significance of these small differences is doubtful. 2) The stimulation of PtdInsP<sub>2</sub> hydrolysis was observed in cells expressing relatively low receptor levels (0.5 × 10<sup>6</sup> receptors/cell). 3) The D<sub>2</sub> response was comparable in magnitude to those induced by the endogenous Ltk<sup>−</sup> cell receptors coupled to PtdInsP<sub>2</sub> hydrolysis, i.e. thrombin and ATP receptors. Taken all together these findings indicate that the stimulation of PtdInsP<sub>2</sub> hydrolysis cannot be regarded as a coupling of secondary relevance of the D<sub>2</sub> receptor. Further work is needed to establish whether the difference between the D<sub>2</sub> receptor investigated in this study and other inhibitory receptors is due to intrinsic functional properties of the different receptor molecules or whether similar results can be obtained with all these receptors when expressed in Ltk<sup>−</sup> cells (and possibly in other cell types).

The response induced by D<sub>2</sub> receptors in LZR<sub>1</sub> cells is shown here to be mediated by G protein(s) sensitive to PTx. This appears to be a general feature when receptors normally considered to inhibit cell activation are coupled to the stimulation of PtdInsP<sub>2</sub> hydrolysis (11-13, 35, 36). Recent work has demonstrated that in Chinese hamster ovary cells, where receptors can stimulate PtdInsP<sub>2</sub> hydrolysis via G proteins PTx-sensitive, PTx-insensitive or both, the M<sub>3</sub> and M<sub>5</sub> muscarinic receptors selectively use the first alternative (13). From this point of view, the results now obtained with Ltk<sup>−</sup> cells appear similar, as PTxs abolished the D<sub>2</sub> response, but affected only partially or not at all those of thrombin and ATP.

The results obtained in LZR<sub>1</sub> cells suggest that a similar response may occur also in some of the cell types which endogenously express the same D<sub>2</sub> receptor. However, such a possibility needs to be substantiated. So far D<sub>2</sub> receptors have been shown to stimulate [Ca<sup>2+</sup>]<sub>i</sub>, only in a subpopulation of pituitary lactotrophs cells in which, however, PtdInsP<sub>2</sub> hydrolysis was not investigated (37). In other cell systems D<sub>2</sub> receptors have been reported either to have no effect on inositol phosphate generation or even to inhibit the response induced by stimulatory agonists, such as TRH (21, 38-40). In pituitary lactotrophs the latter effect is, at least in part,
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indirect depending on the \([Ca^{2+}]\), decrease induced by DA (21). While we report here that dopamine does not modify basal inositol phosphate production in GH2ZR cells, the possibility (and the mechanisms) of a D2-mediated inhibition of the TRH response has not been investigated yet.

A major finding of the present work is that, with the exception of the inhibition of adenyl cyclase, the D2 receptor appears to selectively activate different effector pathways in the two cell types investigated. In fact, the coupling to K+ channels observed in GH2ZR cells most probably does not operate in LZR cells. On the other hand, the D2 receptor stimulates PtdInsP2 hydrolysis in the latter cell type but not in GH2ZR cells. A similar heterogeneity of responses most likely occurs also with other inhibitory receptors. Indeed, the transfected M4 and M5 receptors, which stimulate PtdInsP2 hydrolysis in various cell types (see above), fail to induce such an effect in neuroblastoma × glioma cells (41). As the GH2ZR- and LZR- cells employed in the present study express similar numbers of D2 receptors, the simplest interpretation of our results is a different expression in the two cell types of the post-receptor molecules required for the response, either the G proteins or the effectors themselves. In particular, in the case of GH2C cells the PTx-sensitive G protein mediating PtdInsP2 hydrolysis may be not (or not sufficiently) expressed. Alternatively, it can be imagined that despite the availability of the relevant G protein these cells lack its effector, i.e. a specific phospholipase C different from the enzyme activated by TRH via a PTx-insensitive G protein. In this respect it is worth to emphasize that (i) none of the stimulatory receptors identified so far in GH2C cells operate through a PTx-sensitive pathway, and (ii) as many as five hypothesis could be proposed about the G protein and/or its effector, K+ channel target to explain the dopamine effect observed in GH2ZR but not in LZR cells. Regardless of their actual explanation, these results indicate that the responses induced by an individual D2 receptor molecule differs not simply in degree but even in nature depending on the cell type. Whereas in GH2C cells this receptor appears to function in complete agreement with its definition of “inhibitory” receptor, in Ltk- cells it clearly activates a “stimulatory” pathway. We conclude therefore that the role of a given receptor in transmembrane signaling is determined not only by the functional properties of the receptor molecule itself but also by the specific features of the cell in which it does operate.

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L Vallar, C Muca, M Magni, P Albert, J Bunzow, J Meldolesi and O Civelli

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