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Differential Coupling of Dopaminergic D_2 Receptors Expressed in Different Cell Types

STIMULATION OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE HYDROLYSIS IN LtK$^-$ FIBROBLASTS, HYPERPOLARIZATION, AND CYTOSOLIC-FREE Ca^{2+} CONCENTRATION DECREASE IN GH_4C_1 CELLS*

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Dopaminergic D_2 receptors are widely regarded as typical inhibitory receptors, as they both inhibit adenylyl cyclase and decrease the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by activating K^+ channels. A D_2 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 GH_4C_1 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 cells lines, which express similar levels of receptors (0.5–1 × 10^5/cell), were surprisingly different. In GH_4C_1 cells D_2 receptors failed to affect phosphoinositide hydrolysis and induced a decrease of $[Ca^{2+}]_i$. 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 D_2 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 $[Ca^{2+}]_i$ due to both Ca^{2+} mobilization from internal stores and influx from the extracellular medium. In both GH_4C_1 and Ltk$^-$ cells, the D_2 receptor response was mediated by G protein(s) sensitive to pertussis toxin. The increases of inositol trisphosphate and $[Ca^{2+}]_i$ observed in Ltk$^-$ cells required dopamine concentrations only slightly higher than those inhibiting adenylyl cyclase (EG_{50} = 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 D_2 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 Ca^{2+} 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 G_i[1] (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 Ca^{2+} channels, both resulting in the reduction of $[Ca^{2+}]_i$ (1–3, 5–9). In the case of the heart muscarinic M_2 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 M_2 and M_3 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 D_2 receptors are known to inhibit adenylyl cyclase as well as

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[1] The abbreviations used are: G_i, the inhibitory regulator of adenylyl cyclase; PTx, pertussis toxin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate; TRH, thyrotropin-releasing hormone; $InsP_3$, inositol trisphosphate; $InsP_2$, inositol bisphosphate; $InsP_1$, inositol monophosphate; HPLC, high performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ins(1,4,5)P_3 and Ins(1,3,4)P_3, inositol 1,4,5- and 1,3,4-trisphosphate.

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 D_2 receptor has recently been cloned from a rat brain cDNA library and expressed in two cell lines, pituitary GH_4C_1 cells and Ltk^- mouse fibroblasts (18–20). In both cell types this receptor induces inhibition of adenylyl cyclase, and in the GH_4C_1 line it also blocks prolactin secretion (19, 20). We report here the effects of receptor activation on phosphoinositide hydrolysis, $[Ca^{2+}]_i$, and membrane potential. The results demonstrate that in GH_4C_1 cells the D_2 receptor does not affect phosphoinositide hydrolysis and decreases $[Ca^{2+}]_i$ most likely via a K^+ channel-dependent hyperpolarization, whereas in Ltk^- cells it efficiently stimulates $PtdInsP_2$ hydrolysis with consequent increase of $[Ca^{2+}]_i$. The properties of this latter response indicate that the stimulation of phosphoinositide hydrolysis is a coupling of primary relevance of the D_2 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 GH_4C_1 cells with the D_2 receptor cDNA RGB-2 has been described elsewhere (18–20). Wild-type and transfected Ltk^- and GH_4C_1 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 D_2 receptor expression (see "Results"), the transfected GH_4C_1 cells were exposed for 16 h to culture medium supplemented with 100 μM $ZnSO_4$, which was replaced with fresh standard medium for 24 h before the experiments. All the cells lines were used after detachment from the dishes by a brief exposure (1–2 min) to Ca^{2+} and Mg^{2+}-free phosphate buffer containing 0.02% EDTA. Before $[Ca^{2+}]_i$ and membrane potential measurements, GH_4C_1 cells were kept for 3 h in spinner culture in Ham's F-10 medium + 1% fetal calf serum and 10 mM Hepes.

Inositol Phosphate Measurements—For these experiments GH_4C_1 and Ltk^- cells were labeled with 5 μCi/ml *myo*-[^{3}H]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 $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 6 mM glucose, 25 mM Hepes/NaOH, pH 7.4), resuspended in the same buffer, distribuited to the reaction tubes (1×10^6 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 500 μl of ice-cold 20% trichloroacetic acid. The trichloroacetic extractable material was washed three times with diethyl ether; inositol phosphates were separated by either anion-exchange chromatography on Dowex 1-X8 columns, or HPLC on a Partisil SAX-10 column (Technicol Ltd, Stockport, United Kingdom) exactly as previously described (21).

$[Ca^{2+}]_i$ Measurements—$[Ca^{2+}]_i$ was measured with the fluorescent probe fura-2 (22). GH_4C_1 and Ltk^- cells were washed with the respective culture medium + 1% fetal calf serum and 10 mM Hepes, 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 1×10^6 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 $[Ca^{2+}]_i$ were as previously described (21).

Measurements of Membrane Potential—Membrane potential was qualitatively assessed with the slow response fluorescent dye bis-oxonol (23, 24). GH_4C_1 and Ltk^- cells were washed as for $[Ca^{2+}]_i$ measurements and resuspended in KRH. $1–3 \times 10^6$ cells in 1.5 ml of KRH were transferred into the spectrofluorimetric 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 where washed, resuspendend, 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 20 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 a commercial kit (Du Pont-New England Nuclear).

Materials—Dopamine, somatostatin, thrombin, ATP, verapamil, quinidine, 8-BrcAMP, 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, Siena, Italy), respectively. *Myo*-[2-^{3}H]inositol (80–120 Ci/mmol) was from Amersham Corp., fura-2 acetoxymethyl 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 D_2 Receptors—The cell lines employed in this study were wild-type GH_4C_1 and Ltk^- cells, which are devoid of endogenous D_2 receptors, and the previously described GH_4ZR_7 and LZR_1 (formerly referred as L-RGB2Zem-1) clones transfected with D_2 receptor cDNA (18–20). In GH_4ZR_7 cells transcription of the D_2 receptor cDNA is regulated by a zinc-sensitive metallothionein promoter (20). The experiments were therefore carried out after treatment of the cells with $ZnSO_4$ as described under "Experimental Procedures." Under these conditions GH_4ZR_7 cells express approximately 1×10^5 D_2 receptors/cell. The LZR_1 clone stably expresses about 0.5×10^5 D_2 receptors/cell.

Effects of D_2 Receptor Activation in GH_4ZR_7 Cells—We first examined the ability of the D_2 receptor to affect phosphoinositide hydrolysis. As shown in Fig. 1*A*, in GH_4ZR_7 cells D_2 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-

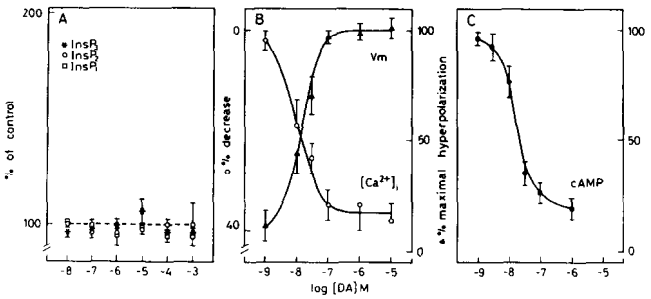

FIG. 1. **Effects of dopamine (*DA*) in GH_4ZR_7 cells.** *A*, inositol phosphate production in the presence of dopamine. Cells were labeled with *myo*-[2-^{3}H] inositol as described under "Experimental Procedures." After 10 min of preincubation at 37 °C in KRH containing 10 mM LiCl, the cells were treated with dopamine for 10 min. [^{3}H] 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 2,622 cpm for $InsP_3$, $InsP_2$, and $InsP_1$, respectively. In the same experiments 100 nM TRH increased $InsP_3$ to 2,005, $InsP_2$ to 2,859, and $InsP_1$ to 23,199 cpm. *B*, $[Ca^{2+}]_i$ decrease and hyperpolarization. $[Ca^{2+}]_i$ and membrane potential (*Vm*) were measured with fura-2 and bis-oxonol, respectively, as described under "Experimental Procedures." Values (means of three experiments ± S.E.) are expressed as percentage decrease of $[Ca^{2+}]_i$ from resting levels and as percentage of the hyperpolarization observed with maximal concentrations of dopamine. *C*, inhibition of cAMP levels. Cells were preincubated for 10 min in KRH containing 100 μM 3-isobutyl-1-methylxanthine and then treated with 10 μM forskolin and the indicated dopamine concentrations for 20 min. Values (n = 9) are given as percentage of the increase of 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 D_2 receptor modified $[Ca^{2+}]_i$ in GH_4ZR_7 cells. In the presence of 1 μM dopamine $[Ca^{2+}]_i$, measured in cells loaded with the $[Ca^{2+}]_i$ indicator fura-2, rapidly decreased from 155 ± 8 nM to 109 ± 7 nM ($n = 9$) (Fig. 2*A*). This dopamine effect was abolished by the selective D_2 antagonists 1-sulpiride and butaclamol and was not detected in wild-type GH_4C_1 cells (Fig. 2*A* and results not shown).

Endogenous D_2 receptors in pituitary lactotrophs, and somatostatin receptors in cells of the GH 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 D_2 receptor transfected into GH_4ZR_7 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. 2,*B* and *C*). (*b*) When the fluorescent probe bis-oxonol was used to evaluate changes in membrane potential dopamine was found to cause hyperpolarization of GH_4ZR_7 cells (Fig. 3*A*). This effect was antagonized by 1-sulpiride and butaclamol (Fig. 3*A* and results not shown). Raising the extracellular $[K^+]$ (Fig. 3*B*) and treatment with the K^+ channel inhibitor quinidine (Fig. 3*C*) abolished the dopamine hyperpolarization, indicating that the latter process is sustained by the activation of K^+ current(s). (*c*) GH_4ZR_7 cells possess endogenous somatostatin receptors. The effects of maximal concentrations of dopamine and somatostatin on $[Ca^{2+}]_i$ and membrane potential were comparable and not additive (Figs. 2*D* and 3*D*).

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 ± 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 adenylyl cyclase, as they were unaffected by the addition of the membrane permeant cAMP analogue, 8-BrcAMP (Figs. 2*E* and 3*E*). Pretreatment of GH_4ZR_7 cells with 100 ng/ml PTx abolished the D_2 receptor induced changes of membrane potential and $[Ca^{2+}]_i$ (Figs. 2*F* and 3*F*), indicating that these responses are coupled by PTx-sensitive G protein(s). These results are similar to those reported for endogenous D_2 receptors and other classical inhibitory receptors (1–3, 25).

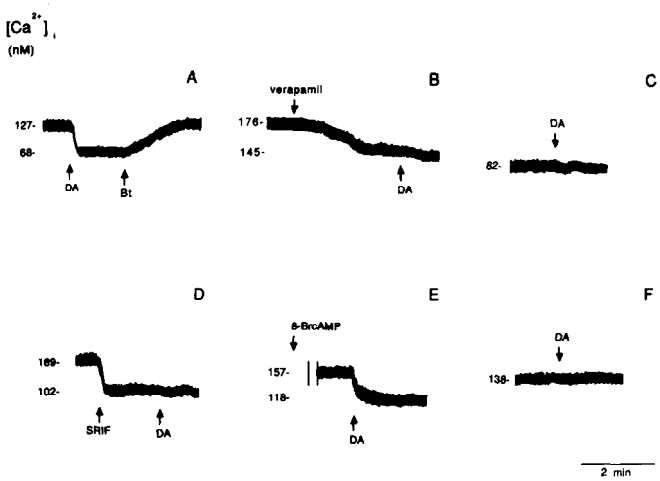

FIG. 2. **$[Ca^{2+}]_i$ decrease induced by dopamine (*DA*) in GH_4ZR_7 cells.** Cells were loaded with fura-2 and analyzed as described in detail under "Experimental Procedures." Dopamine, butaclamol (*Bt*), verapamil, and somatostatin (*SRIF*) concentrations were 1 μM, 500 nM, 100 μM, and 100 nM, respectively. 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.

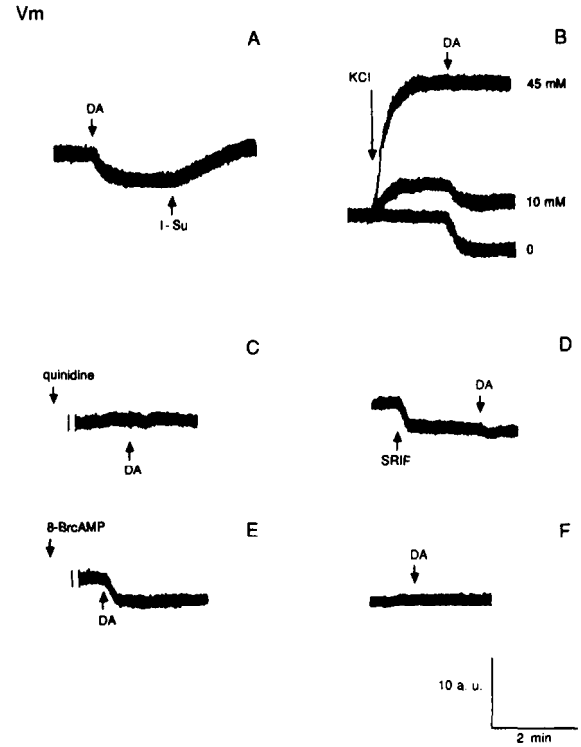

FIG. 3. **Dopamine (*DA*) induced hyperpolarization in GH_4ZR_7 cells.** Membrane potential was monitored by the bis-oxonol fluorescence technique as described in detail under "Experimental Procedures." Fluorescence is expressed in arbitrary units (*a.u.*). Notice that hyperpolarization is indicated by a downward movement in the traces. 1-sulpiride (*1-Su*) was used at 1 μM; quinidine (1 mM) was added 5 min before dopamine; all the other conditions were as in Fig. 2. *Trace F* was obtained with cells pretreated with PTx as described in the legend to Fig. 2. The traces are representative of two to four experiments.

D_2 Receptor Stimulation of Phosphoinositide Hydrolysis in LZR_1 Cells—A completely different set of results was obtained in LZR_1 cells. In fact in these cells the activation of the transfected D_2 receptor induced phosphoinositide hydrolysis. Fig. 4*A* shows the time course of the stimulation of inositol phosphate production observed in the presence of 1 μM 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. 4*A*, *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_1 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. 4*A*). Dopamine stimulation of inositol phosphate production in LZR_1 cells was unaffected

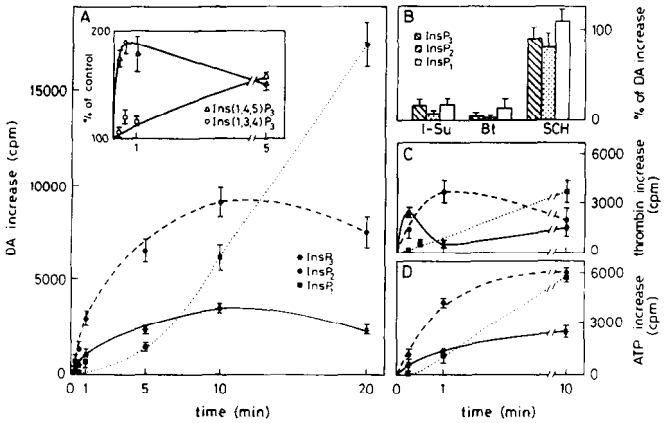

FIG. 4. **Stimulation of inositol phosphate production by dopamine (*DA*), thrombin, and ATP in LZR_1 cells.** *Panels A, C,* and *D*: time course of the effects of dopamine (1 μM), thrombin (7 units/ml), and ATP (100 μM), respectively. The results are expressed as means ± S.E. of the increases above control values at each time point (n = 6). Basal levels averaged 6,440, 9,754, and 12,361 cpm at zero time, and 6,478, 10,210, and 22,986 cpm at 20 min, for $InsP_3$, $InsP_2$, and $InsP_1$, respectively. *Panel A, inset*: stimulation of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ 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: $Ins(1,4,5)P_3$ = 152 ± 5 cpm; $Ins(1,3,4)P_3$ = 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. Cells were labeled with *myo*-[2-^{3}H]inositol as described under "Experimental Procedures" and incubated as GH_4ZR_7 cells (see legend to Fig. 1), except that LiCl was omitted in the samples in which $InsP_3$ isomers were analyzed. Inositol phosphates were separated by anion-exchange chromatography or HPLC as described under "Experimental Procedures."

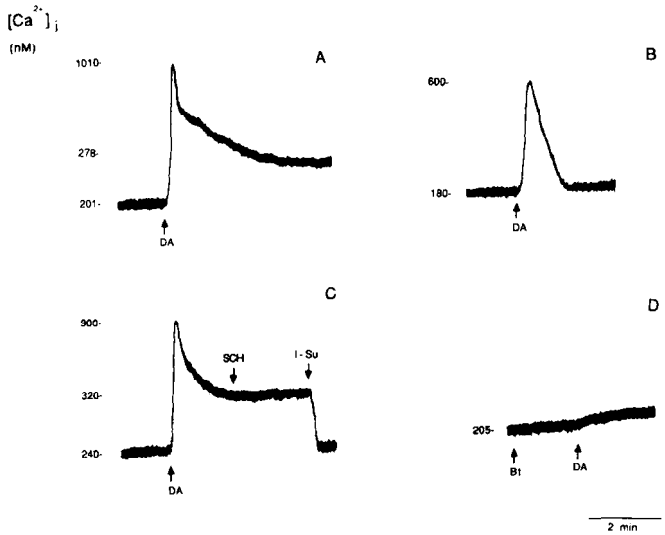

FIG. 5. **$[Ca^{2+}]_i$ increase induced by dopamine (*DA*) in LZR_1 cells.** $[Ca^{2+}]_i$ was monitored with fura-2 as described for GH_4ZR_7 cells in the legend to Fig. 2. *Traces A, C,* and *D* were obtained in complete KRH medium and *trace B* in Ca^{2+}-free medium. The concentrations of dopamine, SCH 23390 (*SCH*), 1-sulpiride (*1-Su*), and butaclamol (*Bt*) were the same as in the experiments shown in Fig. 4.

by the selective D_1 antagonist SCH 23390 and blocked by the D_2 antagonists, 1-sulpiride and butaclamol (Fig. 4*B*). In wild-type Ltk^- cells, DA had no effect on inositol phosphate generation (not shown).

In keeping with the ability of D_2 receptors to stimulate $PtdInsP_2$ hydrolysis, dopamine increased $[Ca^{2+}]_i$ in LZR_1 cell (Fig. 5). In the presence of extracellular Ca^{2+} an immediate rise, going on the average from 191 ± 15 to 664 ± 78 nM (n = 15), was observed. After this initial peak $[Ca^{2+}]_i$ gradually decreased to reach a lower plateau that was maintained for several minutes (*trace A*). The ability of dopamine to increase $[Ca^{2+}]_i$ was retained in Ca^{2+}-free medium, although under these conditions the rise was short-lived, with return to resting levels within 1–2 min (*trace B*). Thus, the D_2 receptor expressed in LZR_1 cells induce the $[Ca^{2+}]_i$ response described for many classical receptors coupled to $PtdInsP_2$ hydrolysis: an initial rapid release of Ca^{2+} from intracellular stores, which is believed to be triggered by $Ins(1,4,5)P_3$, accompanied by sustained influx of external Ca^{2+} (29). Many Ca^{2+} mobilizing receptors induce a concomitant hyperpolarization due to the opening of Ca^{2+}-activated K^+ channels (30). Hyperpolarization was observed also in LZR_1 cells when the effect of dopamine on membrane potential was investigated (Fig. 6*A*). Consistent with the involvement of Ca^{2+}-dependent K^+ channels the dopamine-induced hyperpolarization 1) was shortened in the absence of external Ca^{2+} and nearly abolished when cells incubated in Ca^{2+}-free medium were pretreated with another $PtdInsP_2$ hydrolysis stimulating agonist, *i.e.* thrombin (see below), to deplete intracellular stores (Fig. 6, *B* and *C*); 2) was blocked by high extracellular $[K^+]$ and by the inhibitor of Ca^{2+}-activated K^+ channels, charybdotoxin

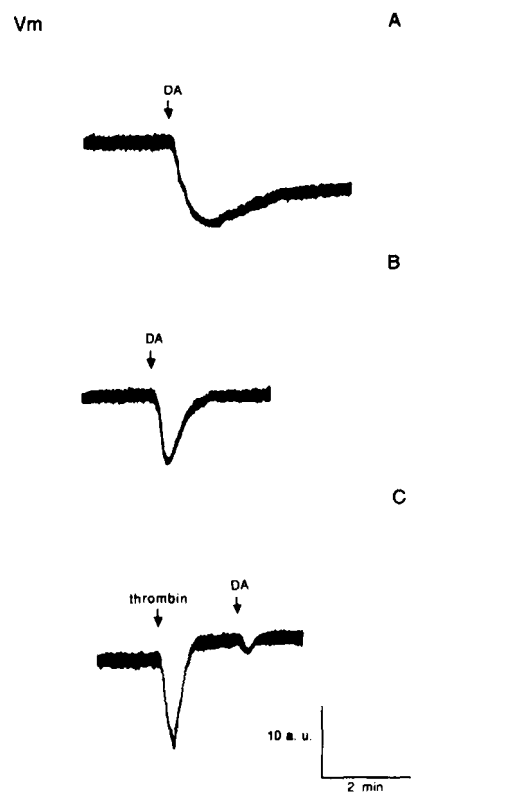

FIG. 6. **Effect of dopamine (*DA*) on membrane potential in LZR_1 cells.** Membrane potential was monitored with bis-oxonol as described in the legend to Fig. 2. *Trace A* was obtained in complete KRH medium and *traces B* and *C* in Ca^{2+}-free medium. Dopamine and thrombin concentrations were 1 μM and 5 units/ml, respectively.

(not shown). The effects of dopamine on $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ in LZR_1 cells.

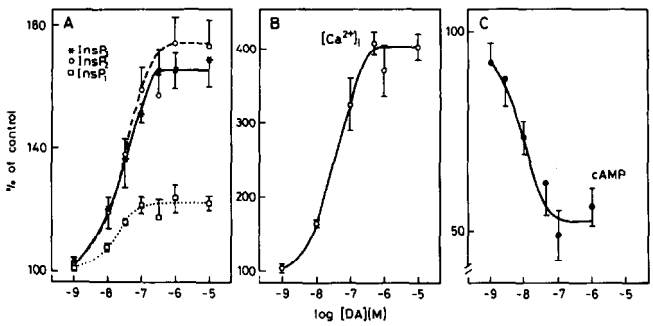

FIG. 7. **Concentration dependence of stimulation of inositol phosphate production (*A*), $[Ca^{2+}]_i$ increase (*B*), and inhibition of cAMP formation (*C*) induced by dopamine (*DA*) in LZR_1 cells.** Inositol phosphate production was determined after 10-min incubations with the indicated dopamine concentrations as described in the legend to Fig. 1. Results are means ± S.E. of six to nine observations, expressed as percentage of basal values ($InsP_3$ = 4452; $InsP_2$ = 6520; $InsP_1$ = 9719 cpm). $[Ca^{2+}]_i$ measurements were as in Fig. 5. Values, which are given as percentage of the resting levels, are means of three experiments ± S.E. cAMP formation was determined as described in the legend to Fig. 1. The results (n = 9) are expressed as percentage of the increase induced by 10 μM forskolin during 20 min incubations (32 ± 3 pmol of cAMP).

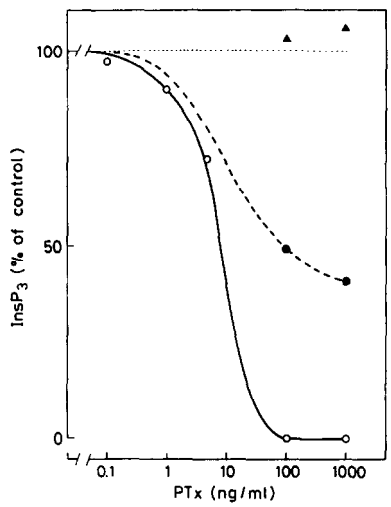

FIG. 8. **Effect of PTx on the stimulation of $InsP_3$ induced by dopamine (○), thrombin (●), and ATP (▲) in LZR_1 cells.** Cells were labeled and incubated as described in the legend to Fig. 4. The results (n = 6–9) are presented as percentage of the increase of $InsP_3$ obtained in the absence of PTx after 15-s incubations with thrombin (7 units/ml) and 10-min incubations with dopamine (1 μM) and ATP (100 μM). Cells were pretreated with the indicated PTx concentrations for 4.5 h.

The effect of dopamine on inositol phosphate generation was clearly detectable at 10 nM and maximal between 100 nM and 1 μM, with EC_{50} values of 25 ± 4, 29 ± 2, and 18 ± 3 nM for $InsP_3$, $InsP_2$, and $InsP_1$, respectively (n = 3). Similar dopamine concentrations were required for raising $[Ca^{2+}]_i$ (EC_{50} = 29 ± 3 nM, n = 3). These values are only slightly higher than those estimated for the dopamine inhibition of cAMP production in LZR_1 cells (EC_{50} = 11 ± 3, n = 3) (Fig. 7) as well as in GH_4ZR_7 cells (see above and Fig. 1).

The response of LZR_1 cells to dopamine was compared with those induced in the same cell type by endogenous receptors coupled to $PtdInsP_2$ 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 $InsP_3$ (at 15 s), 39% for $InsP_2$ (at 1 min), and 16% for $InsP_1$ (at 10 min), (n = 12); with ATP 34, 53 and 26% for $InsP_3$, $InsP_2$, and $InsP_1$, 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 D_2 receptor (see Fig. 1*A* and text above). Also the maximal $[Ca^{2+}]_i$ 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. 5*A*) were comparable.

All the effects so far attributed to endogenous D_2 receptors, as well as those now observed with the cloned D_2 receptor expressed in GH_4ZR_7 cells, are mediated by PTx-sensitive G protein(s). Whether this is the case also for the novel D_2 response observed in LZR_1 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 $PtdInsP_2$ 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 D_2 effect reduced by only about 50% the thrombin stimulation and left unchanged the ATP response.

TABLE I

Effects of 8-BrcAMP and forskolin on the dopamine stimulation of inositol phosphate production in LZR_1 cells

Myo-[2-^{3}H]inositol-labeled cells were incubated with 1 μM dopamine for 10 min as described in the legends to Figs. 4 and 7. Preincubations with 8-BrcAMP (1 mM) and forskolin (10 μM) were for 10 min. The results are means ± S.E. of six observations.

Treatment	^{3}H radioactivity ($InsP_3$ + $InsP_2$ + $InsP_1$)	
	Control	Dopamine
	cpm	
	16,918 ± 224	26,811 ± 582 + 58.4
8-BrcAMP	13,517 ± 334	21,114 ± 204 + 56.2
Forskolin	13,671 ± 841	21,027 ± 34 + 53.8

These last results were confirmed also when using a higher toxin concentration (1 μg/ml).

The above experiments indicate that stimulation of $PtdInsP_2$ hydrolysis and inhibition of cAMP production by the D_2 receptor expressed in LZR_1 cells are both PTx-sensitive responses and require similar agonist concentrations. To rule out the possibility of a major interference of the cAMP decrease in the effect of dopamine on phosphoinositide hydrolysis, we carried out experiments in which either the intracellular cAMP levels were increased by forskolin, or the cAMP analogue 8-BrcAMP was administered to the cells (Table I). Both agents slightly decreased basal inositol phosphates (about −20%) in LZR_1 cells. Under these conditions the net increase of inositol phosphates induced by dopamine was proportionally reduced, so that the fold stimulation was the same as in control cells. Similar results were obtained with thrombin and ATP (not shown).

DISCUSSION

The present knowledge of the effector systems coupled to dopaminergic D_2 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 homogeneous cell system, such as purified pituitary lactotrophs, was employed the interpretation of the results

remains problematical due to the possible existence of multiple D_2 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_2 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^+ channel-dependent hyperpolarization, and the stimulation of $PtdInsP_2$ hydrolysis.

The results obtained in the GH_4ZR_7 clone 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^+ channel activation is demonstrated by the blockade observed with high extracellular K^+ and the K^+ channel blocker, quinidine. Hyperpolarization was independent of the effect of D_2 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_2 receptor can mediate, by coupling to PTx-sensitive G protein(s), both the inhibition of adenylyl cyclase and the activation of K^+ channels. The latter effect can explain the ability of D_2 receptors to decrease the resting $[Ca^{2+}]_i$ in GH_4ZR_7 cells. Indeed, hyperpolarization is expected to prevent the firing of spontaneous Ca^{2+} 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 $[Ca^{2+}]_i$ decrease observed in GH_4ZR_7 cells or whether, in addition, the D_2 receptor is also negatively coupled to voltage-gated Ca^{2+} channels, as suggested based on indirect evidence in lactotrophs and striatal neurons (17), cannot be established from our present data. The ability of D_2 receptors to stimulate $PtdInsP_2$ hydrolysis is demonstrated by the experiments in LZR_1 fibroblasts where dopamine induced 1) a rapid increase of $Ins(1,4,5)P_3$, followed by the other inositol phosphates; together with 2) a $[Ca^{2+}]_i$ rise due to both Ca^{2+} mobilization from internal stores and influx from the extracellular medium. The experiments with 8-BrcAMP quickly ruled out the possibility that this D_2 response is the simple consequence of the decrease of cAMP concomitantly induced by dopamine. On the other hand, the stimulation of $PtdInsP_2$ hydrolysis can account for the hyperpolarization observed in LZR_1 cells, as the latter effect appears to be due primarily to the activation of Ca^{2+}-dependent K^+ channels, triggered by the $[Ca^{2+}]_i$ increase. The evidence includes that hyperpolarization was abolished by both high extracellular $[K^+]$ and charybdotoxin (an inhibitor of Ca^{2+} activated K^+ channels); most important, it was greatly shortened by withdrawal of external Ca^{2+} and almost abolished under conditions which prevent the dopamine-induced $[Ca^{2+}]_i$ increase (*i.e.* in cells incubated in Ca^{2+}-free medium and pretreated with thrombin). Although the D_2 receptor induces hyperpolarization in both LZR_1 and GH_4ZR_7 cells, different mechanisms appear therefore to be involved in this response in the two cell lines.

The stimulation of $PtdInsP_2$ hydrolysis by the the D_2 receptor transfected into LZR_1 cells should be compared with the results obtained with other inhibitory receptors expressed in various cell types. M_2 and M_3 muscarinic receptors in human embryonic kidney cells and Chinese hamster ovary cells (11–13), 5HT1A serotoninergic receptors in HeLa cells (35), and α_2-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 $PtdInsP_2$ hydrolysis were much higher than those able to inhibit adenylyl cyclase, with EC_{50} values in the micromolar and nanomolar range, respectively. In the case of the M_2 and M_3 receptors expressed in Chinese hamster ovary cells, the response also required high levels of receptor, with maximal stimulation at 1.5–2.5 $\times 10^6$ receptors/cell (11). Moreover, in the cells in which the various types of muscarinic receptors have been expressed, the $PtdInsP_2$ hydrolysis responses induced by M_2 and M_3 subtypes were distinctly weaker than those triggered by the typical stimulatory receptors, M_1 and M_4 (12, 13). A similar difference in the magnitude of the response was observed when the transfected M_2 and M_3 receptors were compared to some endogenous stimulatory receptors (13). The coupling of D_2 receptors to $PtdInsP_2$ hydrolysis that we have observed in LZR_1 fibroblasts displays definitely different features. 1) compared with the inhibition of adenylyl cyclase, only slightly higher EC_{50} values were estimated for both the stimulation of $InsP_3$ production and the $[Ca^{2+}]_i$ increase (11 *versus* 25 and 29 nM, respectively). The significance of these small differences is doubtful. 2) The stimulation of $PtdInsP_2$ hydrolysis was observed in cells expressing relatively low receptor levels (0.5×10^5 receptors/cell). 3) The D_2 response was comparable in magnitude to those induced by the endogenous Ltk^- cell receptors coupled to $PtdInsP_2$ hydrolysis, *i.e.* thrombin and ATP receptors. Taken all together these findings indicate that the stimulation of $PtdInsP_2$ hydrolysis cannot be regarded as a coupling of secondary relevance of the D_2 receptor. Further work is needed to establish whether the difference between the D_2 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^- cells (and possibly in other cell types).

The response induced by D_2 receptors in LZR_1 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 $PtdInsP_2$ hydrolysis (11–13, 35, 36). Recent work has demonstrated that in Chinese hamster ovary cells, where receptors can stimulate $PtdInsP_2$ hydrolysis via G proteins PTx-sensitive, Ptx-insensitive or both, the M_2 and M_3 muscarinic receptors selectively use the first alternative (13). From this point of view, the results now obtained with Ltk^- cells appear similar, as PTx abolished the D_2 response, but affected only partially or not at all those of thrombin and ATP.

The results obtained in LZR_1 cells suggest that a similar response may occur also in some of the cell types which endogenously express the same D_2 receptor. However, such a possibility needs to be substantiated. So far D_2 receptors have been shown to stimulate $[Ca^{2+}]_i$ only in a subpopulation of pituitary lactotrophs cells in which, however, $PtdInsP_2$ hydrolysis was not investigated (37). In other cell systems D_2 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,

indirect depending on the $[Ca^{2+}]_i$ decrease induced by DA (21). While we report here that dopamine does not modify basal inositol phosphate production in GH_4ZR_7 cells, the possibility (and the mechanisms) of a D_2-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 adenylyl cyclase, the D_2 receptor appears to selectively activate different effector pathways in the two cell types investigated. In fact, the coupling to K^+ channels observed in GH_4ZR_7 cells most probably does not operate in LZR_1 cells. On the other hand, the D_2 receptor stimulates $PtdInsP_2$ hydrolysis in the latter cell type but not in GH_4ZR_7 cells. A similar heterogeneity of responses most likely occurs also with other inhibitory receptors. Indeed, the transfected M_2 and M_3 receptors, which stimulate $PtdInsP_2$ hydrolysis in various cell types (see above), fail to induce such an effect in neuroblastoma × glioma cells (41). As the GH_4ZR_7 and LZR_1 cells employed in the present study express similar numbers of D_2 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 GH_4C_1 cells the PTx-sensitive G protein mediating $PtdInsP_2$ 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 GH_4C_1 cells operates through a PTx-sensitive pathway, and (ii) as many as five different phospholipases C are known to exist, which might be selectively activated by specific G proteins (42). Analogous hypothesis could be proposed about the G protein and/or its K^+ channel target to explain the dopamine effect observed in GH_4ZR_7 but not in LZR_1 cells. Regardless of their actual explanation, these results indicate that the responses induced by an individual D_2 receptor molecule differs not simply in degree but even in nature depending on the cell type. Whereas in GH_4C_1 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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