0026-895X/95/010131-09$03.00/0

MOLECULAR PHARMACOLOGY, 47:131–139, 1995.

Characterization of Cloned Human Dopamine D1 Receptor-Mediated Calcium Release in 293 Cells

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Received April 29, 1994; Accepted October 5, 1994

SUMMARY

Dopamine (DA) D1 receptors are generally known to couple only to G_s and cAMP production. Recently, D1 receptors expressed in mouse Ltk$^-$ cells have been shown to induce cAMP production, phosphoinositide (PI) hydrolysis, and calcium mobilization [*Mol. Endocrinol.* 6:1815–1824 (1992)]. To further evaluate second messenger systems that could be activated by the D1 receptor, we examined the effects of DA, (*R*)-(+)-SKF-38393, and DA antagonists on cAMP production and calcium release in human embryonic kidney 293 cells stably expressing three different levels (B_{max} = 0.12, 1.4, and 23 pmol/mg of protein) of the human D1 receptor. DA and (*R*)-(+)-SKF-38393 activated cAMP production and calcium release in all three D1-293 clones, and their potency was proportional to receptor density. The efficacy of SKF-38393 was also increased with receptor density in both cAMP and calcium studies. The effect of DA on calcium release consisted of a transient peak response (<20 sec) that declined to an ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid-sensitive plateau level above the base-line (>5 min). The effect of DA on cAMP and calcium release was selectively inhibited by SCH23390, a selective D1 antagonist, and not by spiperone, a selective D2 antagonist. DA did not induce PI hydrolysis in any of the three receptor-expressing clones. A 24-hr pretreatment with cholera toxin (2 μg/ml) greatly attenuated the effect of DA on cAMP formation and calcium release. To address how DA could activate calcium release without enhancing PI hydrolysis, the effects of forskolin, thapsigargin, and isoproterenol (Iso) were studied. Similarly to the effects of DA, forskolin and Iso stimulated cAMP production and calcium release from D1-293 cells. Cells that were stimulated with Iso or forskolin showed a reduced response to subsequent addition of DA. Pretreatment of D1-293 cells with thapsigargin, a selective Ca^{2+}-ATPase inhibitor, elicited calcium release from the inositol-1,4,5-trisphosphate-sensitive calcium store and attenuated the response to subsequent addition of DA. Carbachol stimulated PI hydrolysis and calcium release but had little effect on cAMP production. Prestimulation with carbachol abolished the calcium response to DA, Iso, or forskolin. These studies indicate that D1 receptor-mediated calcium mobilization in 293 cells is dependent on cAMP production and the cAMP-dependent calcium store is part of the inositol-1,4,5-trisphosphate-sensitive calcium pool.

DA is a major neurotransmitter in brain that regulates motor, affect, and reward behaviors. Receptors for DA are classified as D1 or D2 subtypes (1). Activation of D1 receptors results in stimulation of AC and cAMP production, whereas activation of D2 receptors inhibits cAMP production. Recently, five distinct DA receptors have been cloned and characterized. Two of these, D1 and D5 (or D1b), have been shown to stimulate AC, whereas the D2 and D4 receptors are coupled to inhibition of AC. Little is known about second messengers elaborated by the D3 receptor (2–4).

Studies in rat striatal slices, rat kidney membranes, and goldfish retina have shown that a separate D1-like receptor in those tissues is coupled to PI hydrolysis, in addition to cAMP production (5–7). However, until the recent study by Liu *et al.* (8), such dual coupling to AC and PI had never been reported for the cloned mammalian D1 or D5 receptors. Liu *et al.* (8) have shown that the human D1 receptor expressed in Ltk$^-$ cells stimulates PI hydrolysis, calcium release, and cAMP production, whereas the D1 receptor expressed in GH_4 cells stimulates cAMP production, which, in turn, elevates $[Ca^{2+}]_i$ by activation of voltage-senstive calcium channels. The dependence of receptor response on the functional repertoire of host cells is also seen with the D2 receptor (9). When expressed in Ltk cells D2 receptors stimulate PI hydrolysis and calcium mobilization, but when expressed in GH_4 cells D2 receptors induce hyperpolarization and decrease $[Ca^{2+}]_i$. In recent studies we observed that both the goldfish D1 and human D1 receptors, when transiently expressed in 293 cells, are able to stimulate AC as well as calcium mobilization (10, 11). This suggests that D1 receptors may indeed be able to elaborate both cAMP and PI/calcium

ABBREVIATIONS: DA, dopamine; Iso, isoproterenol; HEK, human embryonic kidney; PI, phosphoinositide; IP_3, inositol-1,4,5-trisphosphate; AC, adenylate cyclase; BSA, bovine serum albumin; Carb, carbachol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; 5-HT, 5-hydroxytryptamine; CHO, Chinese hamster ovary; RIA, radioimmunoassay.

signaling pathways. We therefore have further characterized the DA-induced cAMP production and calcium release in 293 cells stably expressing different densities of the D1 receptor. We show here that the D1 receptor could indeed activate both cAMP and calcium release, even at low receptor density (~0.1 pmol/mg of protein). However, the D1-induced calcium release is not mediated via PI hydrolysis. Rather, the 293 cells possess a cAMP-dependent calcium release mechanism that could account for the DA-induced calcium response.

Materials and Methods

(*R*)-(+)-SKF-38393, spiperone, and SCH23390 were obtained from Research Biochemicals (Natick, MA). DA and fetal bovine serum were purchased from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium with 25 mM HEPES, L-glutamine, antibiotics/antimycotics, and Geneticin was obtained from GIBCO (Grand Island, NY). [^{3}H]-SCH23390 was from DuPont-NEN (Boston, MA). The acetoxymethyl ester of indo-1 was purchased from Molecular Probes (Eugene, OR). The IP_3 RIA kit was obtained from Amersham (Arlington Heights, IL).

Cell culture. HEK 293 cells (CRL 1573; American Type Culture Collection) were cultured in minimal essential medium containing 25 mM HEPES, 10% fetal bovine serum, 1% L-glutamine, 0.5 mg/ml Geneticin, and 1% antibiotics/antimycotics, at 37° under an atmosphere of 5% CO_2/95% O_2. Cells were harvested for radioligand binding, cAMP, and calcium assays when they reached ~90–100% confluency.

Human D1 receptor-expressing clones. A 2.6-kilobase *Eco*RI-*Xba*I fragment containing the coding region of the human D1 receptor (provided by Dr. B. F. O'Dowd, University of Toronto) (12) was cloned into the eukaryotic expression vector Rc/CMV (Invitrogen) and transfected into HEK 293 cells using a modified calcium phosphate procedure (13), as described previously (14). Three days after transfection, Geneticin was added to the culture medium to select for cells that contained the plasmid. In time, colonies of cells appeared and were individually selected and cultured further. The colonies were then tested for their ability to accumulate cAMP in response to a D1 agonist and were subjected to saturation binding assays using the D1-specific radioligand [^{3}H]SCH23390 (see below).

Radioligand binding studies. D1-293 cells were homogenized with a Polytron homogenizer (setting 6) in 10 mM Tris·HCl, pH 7.4 at 4°, for 10 sec and centrifuged at 39,000 × *g* for 20 min, and cell pellets were resuspended in 20 ml of Tris·HCl and centrifuged again at 39,000 × *g* for 20 min. The final pellets were then resuspended in 10 mM Tris·HCl, 5 mM $MgCl_2$, pH 7.4 at room temperature. Protein was determined by the Bradford method (Bio-Rad), using BSA as the standard. The amounts of membranes used in each assay tube for the low, high, and very high expression clones were 100, 40, and 4 μg of protein, respectively. Saturation binding assays were performed using [^{3}H]SCH23390, in 1.5-ml polystyrene tubes arranged in 96-well formats. The final assay volume was 0.25 ml, and membranes were incubated at room temperature for 40 min. Bound ligand was separated from free by filtration using a Skatron (Sterling, VA) cell harvester. Radioactivity was determined with a Betaplate scintillation counter (LKB, Gaithersburg, MD). Nonspecific binding was defined as binding of the radioligand in the presence of 1 μM SCH23390.

Determination of cAMP levels. Assays were conducted in 24-well tissue culture plates. The incubation buffer consisted of Dulbecco's PBS containing $CaCl_2$ and $MgCl_2$, 0.1% glucose, 0.5 mM isobutylmethylxanthine, and 0.004% ascorbic acid. Except for studies with Iso, 10 μM propranolol was routinely used. Culture medium was removed, D1-293 cells were washed once with assay buffer, and 400 μl of assay buffer were added for 10 min. One hundred microliters of DA and other DA compounds, dissolved in assay buffer, were added for an additional 15 min and incubations were terminated with 500 μl of 0.2 N HCl. cAMP contents were determined by RIA, using the automated Attoflo system (Rockville, MD).

Indo-1/calcium fluorescence. Fifty microliters of 1 mM indo-1/acetoxymethyl ester dissolved in dimethylsulfoxide were added directly to D1-293 cells cultured in 162-cm^2 flasks containing 35 ml of culture medium (the final concentration was 1.4 μM). Cells were incubated for 1 hr at 37° in a humidified atmosphere of 5% CO_2/95% O_2. Culture medium was then removed and cells were detached by addition of PBS, pH 7.4, without $CaCl_2$ or $MgCl_2$. After ~5 min, cells were harvested by centrifugation and resuspended in 50–100 ml of assay buffer (Dulbecco's PBS, pH 7.4, containing 0.1% BSA and 0.1% glucose). The cell density was 2–4 × 10^6 cells/ml. $[Ca^{2+}]_i$ levels were monitored with a spectrofluorimeter (model 8000C; SLM, Rochester, NY), with excitation setting of 350 nm and emission settings of 405 and 480 nm. Calibrations of $[Ca^{2+}]_i$ were as previously described by Grynkiewicz *et al.* (15), i.e., $[Ca^{2+}]_i = K_d(R - R_{min})/[(R_{max} - R)(F_{480,min}/F_{480,max})]$, where K_d is assumed to be 240 nM and R is the fluorescence ratio (480/405 nm) obtained in the presence and absence of DA and other agonists. R_{max} is the ratio obtained in the presence of digitonin (50 μM). After the addition of digitonin, R_{min} is determined with the addition of EGTA (10 mM). $F_{480,min}$ is the fluorescence value of the calcium-free dye at 480 nm, and $F_{480,max}$ is the fluorescence value of the calcium-bound dye at 480 nm.

PI hydrolysis and [^{3}H]inositol phosphate measurements. The PI assay was conducted based on the modification of lithium amplification method of Berridge *et al.* (16), as described previously (17). D1-293 cells were cultured in 12-well culture plates and [^{3}H]inositol (1 μCi/ml, 2 ml/well) was added 14–18 hr before the experiment. Culture medium was removed and cells were rinsed once with 1 ml of incubation buffer (Dulbecco's PBS containing $CaCl_2$, $MgCl_2$, 10 mM LiCl, 0.1% glucose, and 0.008% ascorbic acid); 0.63 ml of incubation buffer was then added to each well and cells were incubated for 10 min at 37° in an atmosphere of 5% CO_2/95% O_2. Afterward, 70 μl of DA, Carb, or other agents were added and cells were further incubated for 60 min. Methanol (0.7 ml) was added to terminate the incubation. Cells were detached with a cell scraper, and the entire contents were transferred to glass tubes containing 2.3 ml of chloroform and 0.5 ml of water. Cell wells were rinsed with 1 ml of water and the rinse solution was added to the glass tubes. Contents were vortex-mixed and centrifuged at 1000 × *g* for 10 min. Two milliliters of the upper phase were combined with 4 ml of water and applied to 1 ml of Dowex AG1-X8 ion exchange resin. After rinsing with 15 ml of 5 mM inositol and 6 ml of 5 mM sodium borate/50 mM sodium formate, inositol phosphates were eluted with two 3.5-ml fractions of 1 M ammonium formate/0.1 N formic acid. To the fractions were added 15 ml of scintillation fluid (Insta-gel; Packard, Meriden, CT). Radioactivity was measured by scintillation counting.

IP_3 determination. Studies were conducted in six-well plates. Incubation buffer consisted of PBS containing 10 μM propranolol. Culture medium was removed, and cells were rinsed once with 1 ml of PBS. Cells were preincubated for 10 min in 0.9 ml of incubation buffer at 37°, and 0.1 ml of DA was added for 5, 30, or 60 sec. Then, 0.2 ml of 20% perchloric acid was added to stop the reaction. Cells were harvested, transferred to 12- × 75-mm glass culture tubes on ice, and centrifuged at 2200 × *g*, and 1 ml of supernatant was saved and neutralized with ice-cold 1.5 M KOH in 60 mM HEPES. Tubes were centrifuged and 1 ml of supernatant was lyophilized and reconstituted in 0.25 ml of water. Insoluble particulates were removed by centrifugation, and the amount of IP_3 in the samples was determined with the IP_3 RIA kit.

Results

Fig. 1 shows the Scatchard plot of saturation experiments for [^{3}H]SCH23390 binding to the three D1-293 clones. The apparent maximal binding capacity for the very high, high, and low expression D1 clones were 23 ± 2.9 pmol/mg of protein (three experiments), 1.4 ± 0.12 pmol/mg of protein (three experiments), and 0.12 ± 0.056 pmol/mg of protein (three experiments), respectively. The K_d values of [^{3}H]SCH23390 for

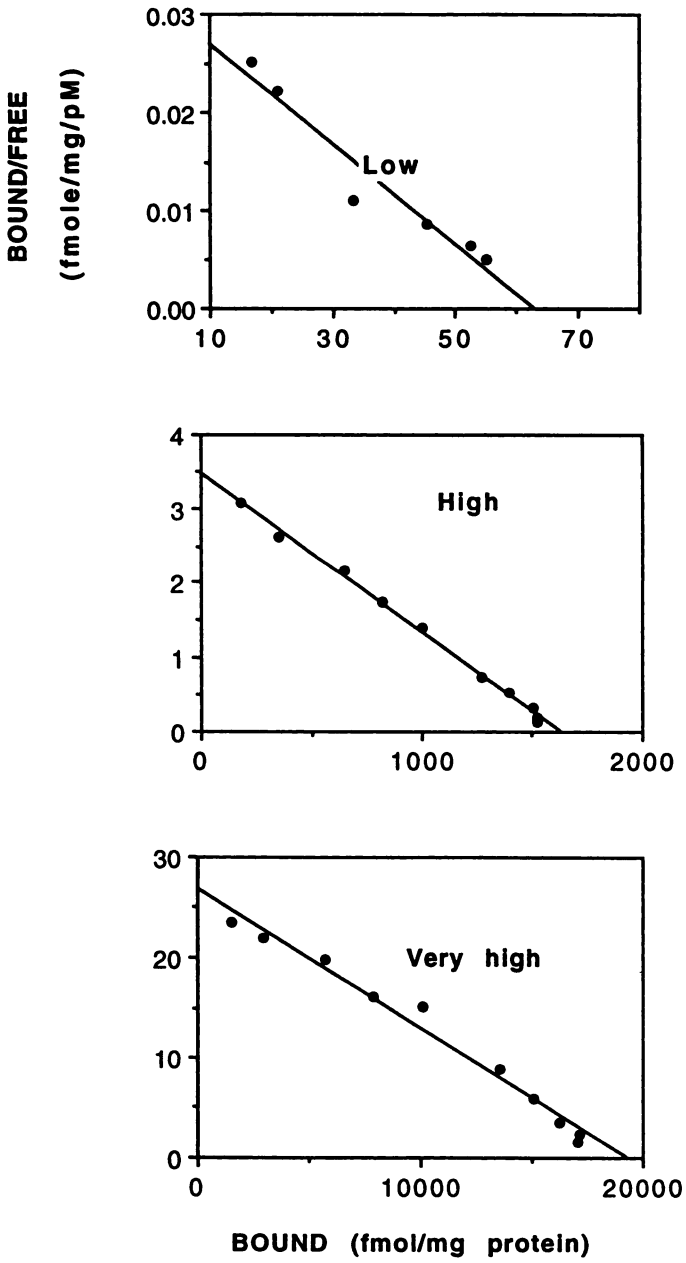


Fig. 1. Scatchard plots for the three 293 cell lines stably expressing different levels of the human D1 receptor. Saturation binding experiments using [^{3}H]SCH23390 were conducted as described in Materials and Methods. Data shown were from one of the experiments conducted in triplicate tubes and repeated at least two times. The D1 receptor densities of the low, high, and very high expression clones were 0.12 ± 0.056 pmol/mg of protein (three experiments), 1.4 ± 0.12 pmol/mg of protein (three experiments), and 23 ± 2.9 pmol/mg of protein (three experiments), respectively. The K_d values of [^{3}H]SCH23390 for the three clones were 3.7 ± 0.91 nM (three experiments), 0.69 ± 0.2 nM (three experiments), and 0.97 ± 0.25 nM (three experiments), respectively.

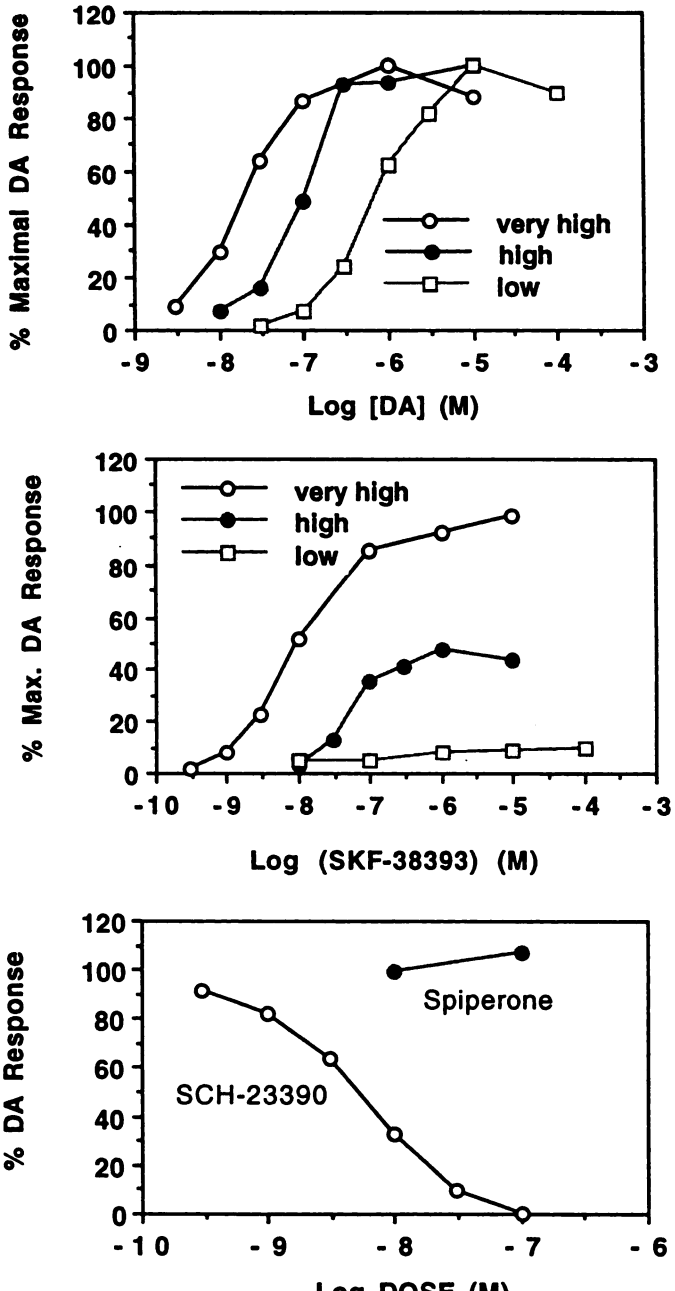


Fig. 2. Stimulation of cAMP production by DA and (*R*)-(+)-SKF-38393 in the three D1-293 cell lines. *Top* and *middle*, DA (*top*) and SKF-38393 (*middle*) were incubated for 15 min in the presence of isobutylmethylxanthine (0.5 mM). The incubations were terminated with 0.1 N HCl (final concentration), and cAMP levels were determined with an automated RIA Attoflo system. The maximal response produced by DA in the low expression cell line was 45 ± 5.4 nM (six experiments), in the high expression cell line was 320 ± 64 nM (five experiments), and in the very high expression cell line was 420 ± 91 nM (four experiments). *Bottom*, the selective inhibition of DA-induced increases in cAMP by a DA D1 receptor antagonist in the high expression cell line is shown. SCH23390 and spiperone were added 5 min before DA (300 nM).

these three clones were 0.97 ± 0.25 nM (three experiments), 0.69 ± 0.2 nM (three experiments), and 3.7 ± 0.91 nM (three experiments), respectively. Because the reported levels of D1 receptors in rat striatum are 0.1–1 pmol/mg of protein, levels of D1 receptors in the low and high expression clones are comparable to levels found in tissues (18, 19).

Potency of DA and (*R*)-(+)-SKF-38393 is related to receptor number in AC studies. Fig. 2, *top*, shows the ability of DA to stimulate AC in the three clones. The potency of DA was proportional to receptor density. The EC_{50} value of DA was 570 ± 66 nM (four experiments) in the low expression clone, 160 ± 32 nM (five experiments) in the high expression clone, and 13 ± 3.8 nM (four experiments) in the very high

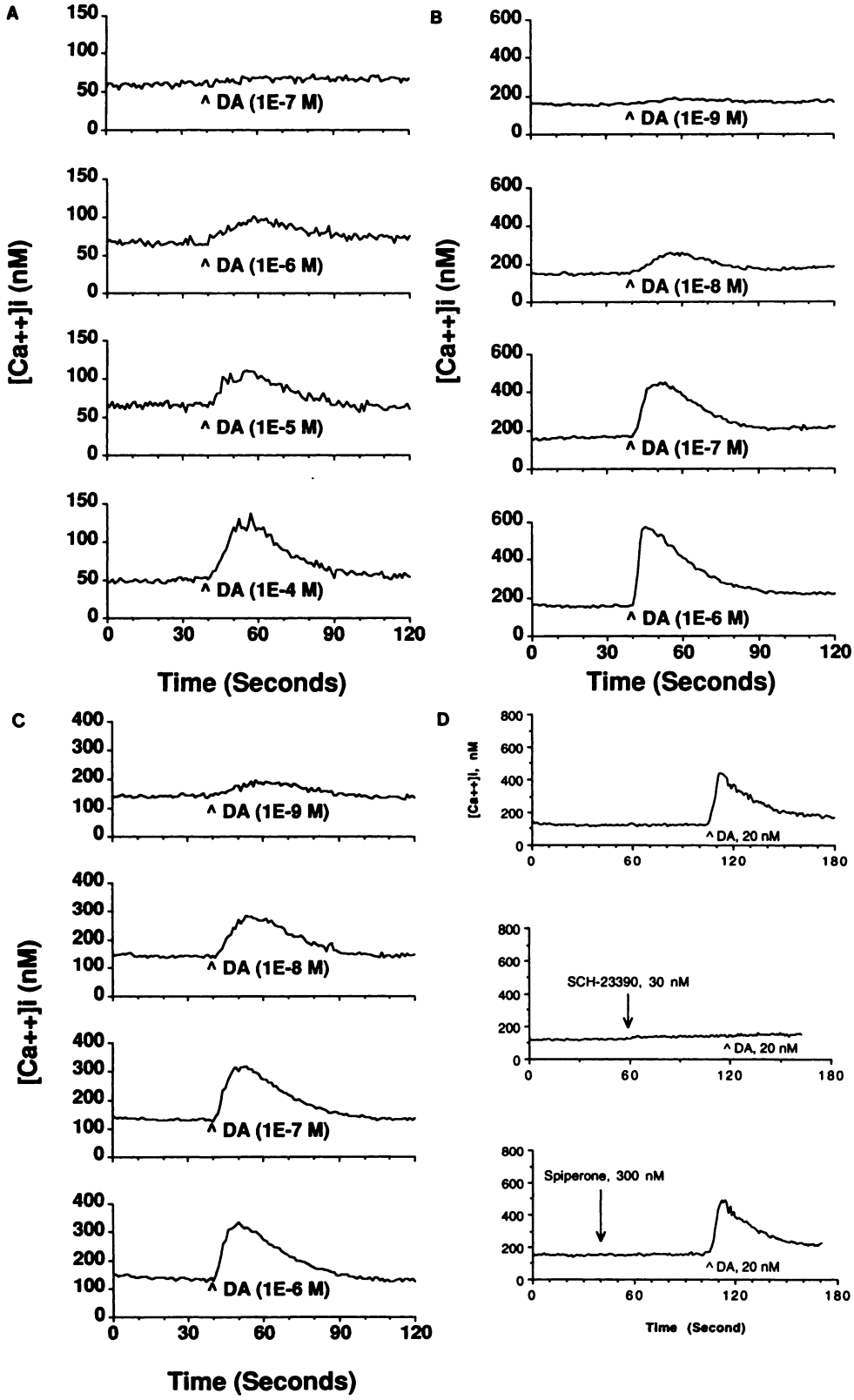


Fig. 3. Stimulation of indo-1/calcium fluorescence by DA in the three D1-HEK cell lines. D1-HEK cells were incubated with 1.4 μM indo-1/acetoxymethyl ester for 1 hr at 37°, centrifuged, and resuspended at a density of 2–4 × 10^6 cells/ml in Dulbecco's PBS, pH 7.4, containing 0.1% BSA and 0.1% glucose. A-C, The effects of DA on calcium fluorescence in the low (A), high (B), and very high (C) expression clones were monitored with a SLM 8000C spectrofluorimeter, with excitation setting of 350 nm and emission settings of 405 and 480 nm. The effect of DA at each concentration was monitored with fresh aliquots (2 ml) of cells. *Tracings* are from a representative experiment that was repeated at least three times. D, Selective antagonism of DA-induced calcium fluorescence by the D1 antagonist SCH23390 in the 293 cells expressing 1.4 pmol/mg of protein levels of the D1 receptor is shown. Cells were exposed to either DA alone (*top*), SCH23390 before DA (*middle*), or spiperone before DA (*bottom*).

expression clone. DA (3 μM) did not stimulate cAMP production in mock-transfected or native HEK cells.

Like that of DA, the potency of SKF-38393 (Fig. 2, *middle*) also increased with receptor density. Its EC_{50} value was 74 ± 14 nM (three experiments) in the high expression clone and 8.8 ± 0.94 nM (four experiments) in the very high expression clone. The efficacy of SKF-38393, relative to DA, increased from 7 ± 0.7% (four experiments) in the low expression clone to 49 ± 4.2% (three experiments) in the high expression clone and to 105 ± 5% (six experiments) in the very high expression clone.

Fig. 2, *bottom*, shows that the DA (300 nM)-induced cAMP production in the high expression clone (1.4 pmol/mg of protein) was sensitive to SCH23390, a D1 antagonist, whereas spiperone, a D2 antagonist, did not antagonize DA-stimulated cAMP production. Selective D1 antagonist blockade of DA-induced cAMP production was also seen for the low and very high expression clones (data not shown).

DA D1 receptor-mediated intracellular calcium mobilization is observed in low as well as high receptor expression clones. DA stimulated intracellular Ca^{2+} mobilization in all three D1-293 clones (Fig. 3, A-C). Like that for DA-stimulated cAMP production, the potency of DA in affecting calcium release increased with receptor density, with EC_{50} values of 4200 ± 1200 nM (six experiments) for the low expression clone (Fig. 3A), 76 ± 7 nM (21 experiments) for the high expression clone (Fig. 3B), and 5.2 ± 0.7 nM (six experiments) for the very high expression clone (Fig. 3C). The maximal DA-induced calcium release was increased from low expression (130 ± 25 nM, six experiments) to high expression (410 ± 22 nM, 21 experiments) clones, but a reduced maximal DA response was seen with the very high expression clone (230 ± 32 nM, six experiments), compared with the high expression clone. DA (3 μM) did not elicit calcium responses in mock-transfected or native 293 cells.

Fig. 3D shows that the DA-induced calcium release in the high expression clone (1.4 pmol/mg of protein) was sensitive to SCH23390 and insensitive to spiperone. Selective D1 antagonist blockade was also obtained with the low and very high expression clones. These results indicate that DA D1 receptors could indeed mediate calcium release in 293 cells and that this is not an artifact of high receptor transfection levels.

In each of the D1-293 clones studied, the DA-induced calcium response consisted of a transient peak response (<20 sec) that declined to a sustained phase (>5 min) above the basal level (Fig. 4, *left*). In the presence of 1.5 mM EGTA, which chelates extracellular Ca^{2+} (1.28 mM) in the assay buffer, most (~60–70%) of the DA-induced transient response was maintained, whereas the plateau phase was eliminated (Fig. 4, *right*). These results indicate that the DA-induced transient response was due mostly to mobilization of intracellular Ca^{2+}, whereas the sustained phase was dependent on extracellular Ca^{2+}. The DA-induced calcium response was insensitive to nifedipine (1 μM), an L-type, voltage-gated, calcium channel blocker. Addition of the calcium channel agonist (±)-Bay K 8644 (10 μM) did not elicit a calcium response in 293 cells, suggesting the absence of Bay K 8644-sensitive voltage-dependent calcium channels.

D1 receptor-mediated intracellular calcium release is independent of PI hydrolysis. Because the pattern of DA-induced calcium responses is similar to that for receptors that are coupled to PI hydrolysis, the effect of DA on PI hydrolysis was examined. As shown in Table 1, DA (0.1 μM to 1 mM) did

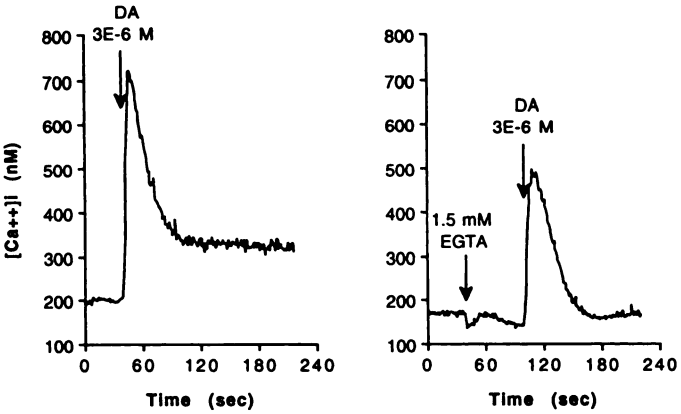


Fig. 4. Characteristics of the DA-induced calcium response. *Left*, the DA-induced calcium response was biphasic, composed of a transient peak response that declined to a sustained phase above the base-line. *Right*, the sustained response was sensitive to removal of extracellular calcium, whereas most of the transient response was insensitive to calcium chelation.

not stimulate PI hydrolysis in the three D1-293 clones. Carb, through activation of endogenous muscarinic receptors, induced a robust PI response in the low and high expression clones, but its response was greatly attenuated in the very high expression clone, suggesting reduced muscarinic receptor or phospholipase C levels in the very high expression clone. Forskolin, thapsigargin (3 μM), and Iso (10 μM) did not stimulate PI hydrolysis in the three clones (data not shown).

To eliminate the possibility that D1 receptors might transiently activate phospholipase C, IP_3 levels were determined after 5-, 30-, and 60-sec additions of DA (10 μM) in the high expression clone. Similarly to results obtained from [^{3}H]inositol phosphate studies, there was no significant ($p > 0.05$, Student's paired t test) increase in IP_3 formation for any of the time periods studied. The amounts of IP_3 in the control and DA-treated groups were 20.3 ± 2.6 (11 experiments) and 25.2 ± 3.2 pmol/well (11 experiments), respectively, at 5 sec, 16.2 ± 3.5 (seven experiments) and 21.0 ± 6.0 pmol/well (seven experiments), respectively, at 30 sec, and 19.0 ± 4.8 (seven experiments) and 20.3 ± 5.4 pmol/well (seven experiments), respectively, at 60 sec. In comparison, Carb significantly ($p < 0.05$) increased IP_3 production (28.2 ± 5.9 pmol/well, seven experiments) after 30 sec. These data therefore indicate that the D1 receptor-mediated calcium response is independent of PI hydrolysis.

Forskolin and Iso stimulate calcium release in 293 cells. To investigate the potential mechanisms of the D1-induced intracellular Ca^{2+} release that is independent of PI hydrolysis, the effects of forskolin (an activator of AC), Iso (a β-adrenergic receptor agonist), and thapsigargin (a selective inhibitor of reticular Ca^{2+}-ATPase) on intracellular Ca^{2+} release were evaluated. As shown in Fig. 5, 3 μM forskolin (Fig. 5, *top*) and 10 μM Iso (Fig. 5, *third*) activated intracellular Ca^{2+} release, in a manner similar to that of DA. Pretreatment with forskolin or Iso attenuated the response to subsequent additions of DA. Conversely, preactivation of calcium release by DA attenuated release of intracellular Ca^{2+} by forskolin (Fig. 5, *second*) and Iso (Fig. 5, *bottom*). The effect of Iso on calcium release was sensitive to propranolol (1 μM), a β receptor antagonist, and not to SCH23390 (3 μM). The effect of DA was insensitive to propranolol (data not shown). 1,9-Dideoxyforsko-

TABLE 1

Effects of DA, Carb, and forskolin on [^{3}H]inositol phosphate formation in the three D1-HEK clones

The basal level of [^{3}H]inositol phosphate formation was 1293 ± 374 dpm (four experiments) in the clone with 0.12 pmol/mg of protein, 1199 ± 223 dpm (10 experiments) in the clone with 1.4 pmol/mg of protein, and 2260 ± 430 dpm (five experiments) in the clone with 23 pmol/mg of protein.

	Receptor Density		
	Low expression	High expression	Very high expression
Treatment	0.12 ± 0.056 (3)[a]	*pmol/mg of protein* 1.4 ± 0.12 (3) Ratio (drug/vehicle)	23 ± 2.9 (3)
	Low expression	High expression	Very high expression
0.1 μM DA	1.12 (1)	1.06 ± 0.07 (5)	
1 μM DA	1.02 (1)	1.12 ± 0.05 (5)	
10 μM DA	0.93 ± 0.09 (3)	1.01 ± 0.04 (6)	
0.1 mM DA	0.92 (2)	0.95 ± 0.05 (5)	0.94 ± 0.01 (3)
1 mM DA	1.02 ± 0.06 (3)	0.92 ± 0.06 (5)	1.10 ± 0.08 (5)
1 mM Carb	23.63 ± 6.83 (4)	21.86 ± 3.74 (10)	1.20 ± 0.11 (4)
10 μM Forskolin	1.07 ± 0.05 (4)	0.90 ± 0.06 (6)	NA[b]

[a] Numbers in parentheses, numbers of experiments.
[b] NA, not applicable.

lin (3 μM), a forskolin analog that does not stimulate AC, did not induce calcium release (data not shown). These studies indicate that agents that increase intracellular cAMP levels are capable of inducing intracellular Ca^{2+} release in 293 cells. The ability of DA to stimulate calcium release is therefore largely dependent on cAMP production.

The DA-activated calcium store is part of the IP_3-sensitive calcium pool. To further examine the source of the intracellular Ca^{2+} pool mobilized by DA, we studied the effects of thapsigargin, which causes calcium release through inhibition of Ca^{2+}-ATPase (20), as well as Carb, which stimulates PI hydrolysis. Carb greatly enhanced intracellular Ca^{2+} release and abolished the calcium response to subsequent addition of DA (Fig. 6, *top*). Pretreatment of D1-293 cells with DA partially inhibited the calcium response to Carb (Fig. 6, *second*). The very high $[Ca^{2+}]_i$ observed immediately after Carb addition is probably an overestimation derived from the calibration procedure.

Thapsigargin induced intracellular Ca^{2+} release and attenuated the response to DA (Fig. 6, *third*). Conversely, prior addition of DA also attenuated the response to thapsigargin (Fig. 6, *bottom*). These results indicate that the source of intracellular Ca^{2+} mobilized by DA is part of the IP_3-sensitive calcium pool.

Cholera toxin attenuates DA-induced cAMP production and calcium release. If the observed DA-induced calcium release is due primarily to DA-induced cAMP production, then treatments that modify DA-induced cAMP production should also alter the calcium response. To test this, D1-293 cells were treated with cholera toxin (2 μg/ml) and pertussis toxin (25 ng/ml) for 24 hr. Fig. 7, *top*, shows that DA-stimulated cAMP production was greatly diminished by cholera toxin treatment. Similarly, the DA-induced calcium release (Fig. 7, *bottom*) was also attenuated by cholera toxin treatment. In contrast, cholera toxin did not affect forskolin-induced cAMP production or calcium response (data not shown). Pertussis toxin enhanced (~30%) DA-induced cAMP production and calcium release in some experiments; however, the enhancement of calcium release was variable.

Calcium release is independent of cAMP levels *per se*. Although DA-mediated calcium release may be dependent on cAMP production, calcium release clearly is not directly related to levels of cAMP. This is illustrated by the time-course experiment shown in Fig. 8. Whereas DA-induced calcium release peaked at <20 sec and declined to a sustained level in 2 min (Fig. 4), cAMP production stimulated by DA continued to increase throughout the 15-min period. Thus, calcium release is regulated by the early rise in cAMP levels, which in turn activates a portion of the IP_3-sensitive calcium pool, possibly via cAMP-dependent kinases in 293 cells.

Discussion

The D1 receptor is generally known to couple only to stimulation of AC. However, recent studies in brain slices, kidney membranes, and retina have indicated the existence of other D1-like receptors linked to PI hydrolysis (5–7). The cloned D1 receptors are reported to stimulate AC, but not PI hydrolysis, when expressed in COS, CHO, or 293 cells (12, 21, 22). In contrast, the human D1 receptor expressed in Ltk^- fibroblasts is coupled to both PI and cAMP stimulation (8). Recently, we showed that the cloned goldfish D1 and human D1 receptors, when transiently expressed in 293 cells, are able to stimulate calcium mobilization, besides cAMP production (10). To further document the possibility of dual signaling by the human D1 receptor, we examined in greater detail the functional responses of stably expressed D1-293 cells. To ensure that the observed response is not due to high levels of receptor expression, we evaluated clones expressing low, high, and very high levels of the D1 receptor.

As shown in Figs. 2 and 3, the human D1 receptor is indeed capable of activating both cAMP production and calcium mobilization in 293 cells, even at low receptor density (~100 fmol/mg of protein). The finding that the calcium responses are sensitive to D1 receptor antagonists and not D2 or β receptor blockade provides additional evidence for involvement of the D1 receptor. The potency of DA increases with increased receptor density, in both the cAMP and calcium assays (Figs. 2 and 3). In addition, the potency and efficacy of SKF-38393 also increase with receptor number. The direct relationship between potency and receptor number is consistent with models of receptor activation (23) and has been previously observed in other systems, such as the 5-HT receptor system (24). The large difference in efficacy, 7 and 100%, relative to DA, of SKF-38393 between the low and very high expression clones indi-

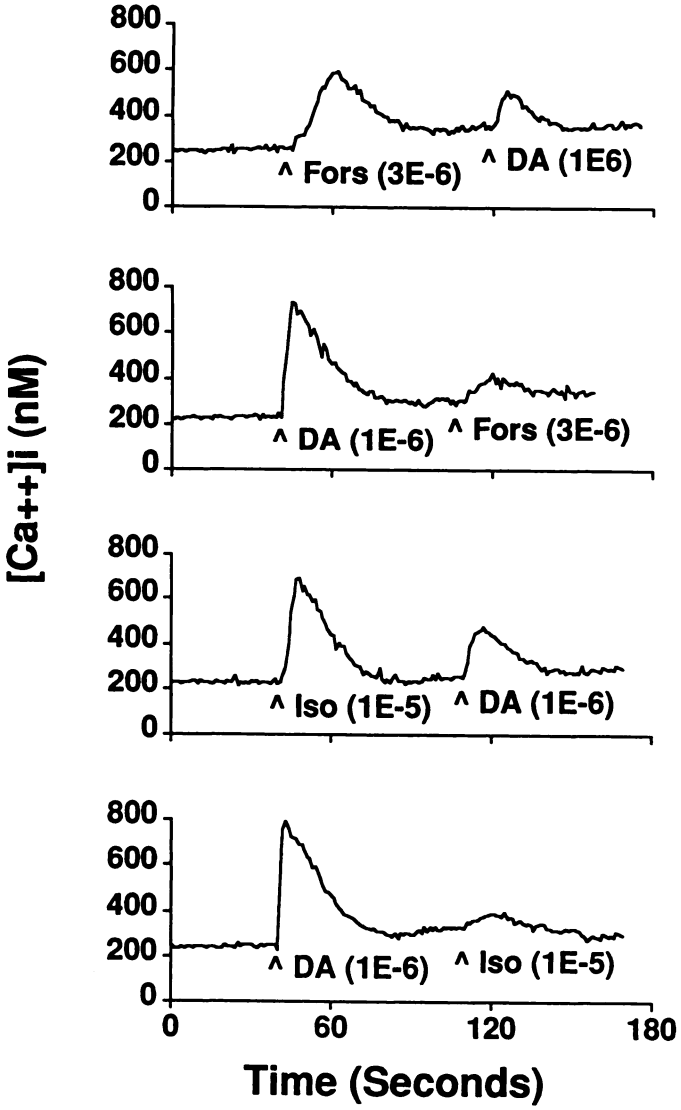


Fig. 5. Evidence that forskolin (*Fors*) and Iso mimic the effect of DA on calcium release in D1-293 cells. Cells were exposed to forskolin (3 μM) (*top*) or 10 μM Iso (*third*) before the addition of DA (1 μM). Prior addition of forskolin or Iso reduced the response to subsequent addition of DA. Similarly, prior addition of DA reduced the response to subseqent exposure to forskolin (*second*) or Iso (*bottom*).

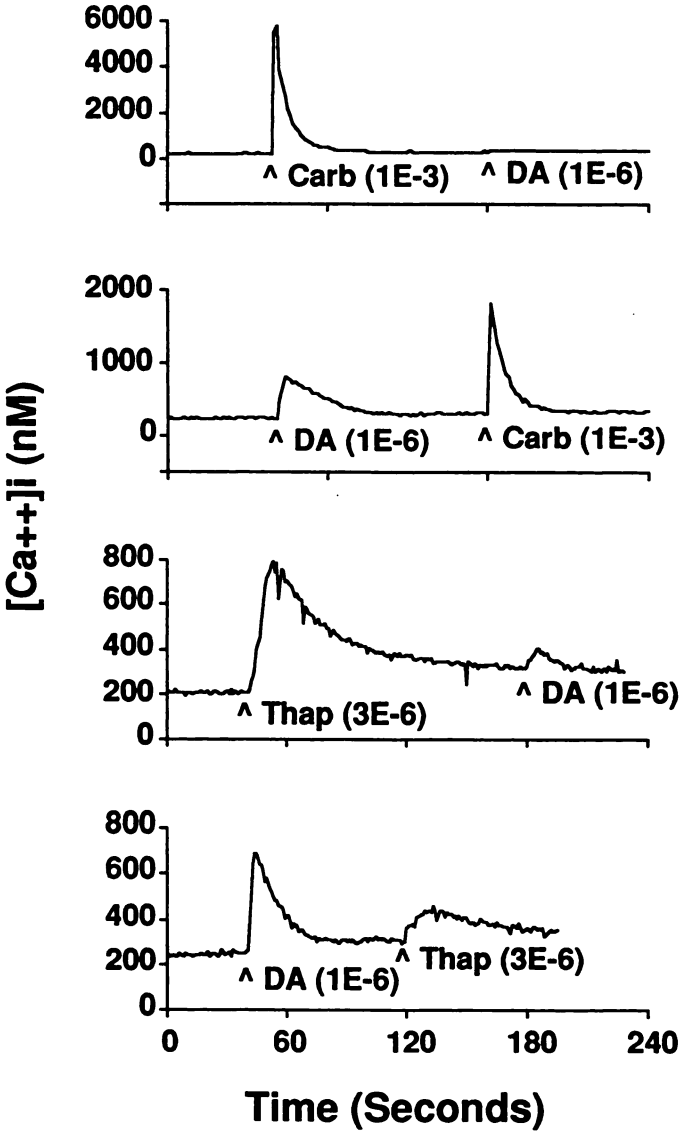


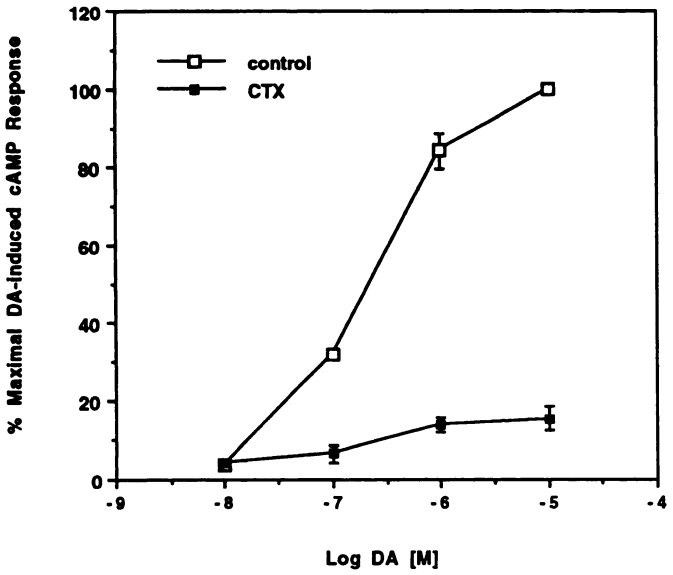
Fig. 6. Effects of Carb and thapsigargin (*Thap*) in D1-293 cells. Carb (*top*) and thapsigargin (*third*) stimulated calcium release and attenuated the response to subseqent addition of DA in D1-293 cells. Conversely, prior addition of DA reduced the response to subsequent addition of Carb (*second*) or thapsigargin (*bottom*).

cates the complexity of determining the efficacy of drugs in tissues with different receptor densities. The partial efficacy of SKF-38393 in the high expression cells is similar to that seen in tissues. We selected the cell line expressing high levels of the D1 receptor (1 pmol/mg of protein) for further characterization of calcium response because 1) it shows the most pronounced calcium response to DA and 2) its expression level is similar to that reported by Liu *et al.* (8).

In contrast to D1-Ltk$^-$ cells (8), DA does not stimulate PI hydrolysis in D1-293 cells. This raises the issue of the mechanisms of D1-mediated calcium release. As shown in Figs. 4–6, Iso, a β-adrenergic receptor agonist, and forskolin, an activator of AC, both activate calcium release in 293 cells. Like DA, both agents increase cAMP production without affecting PI hydrolysis. 1,9-Dideoxyforskolin, an inactive analog of forskolin, fails to increase cAMP production and calcium release. These results suggest that calcium release induced by DA, forskolin, and Iso is dependent on cAMP production. This is further strengthened by the cholera toxin studies. Pretreatment of cells with cholera toxin uncouples the DA receptor from G_s, thereby reducing DA-stimulated cAMP production and calcium release (Fig. 7).

The D1 receptor-activated calcium pool in 293 cells appears to be part of the IP_3-sensitive pool. This is shown by the cross-attenuation experiments with Carb and thapsigargin. As shown in Fig. 6, pretreatment with thapsigargin, a Ca^{2+}-ATPase inhibitor, or Carb attenuates the subsequent response to DA. Conversely, pretreatment with DA reduces the response to subsequent addition of Carb or thapsigargin. The muscarinic and β-adrenergic receptor subtypes that mediate the PI and calcium responses to Carb and Iso in 293 cells have not been defined at present.

At expression levels of ~1 pmol/mg of protein, the DA-mediated calcium response in 293 cells is the most robust (>200% stimulation), compared with that observed in GH_4 or Ltk$^-$ cells (~30–40% above base-line levels). The calcium re-



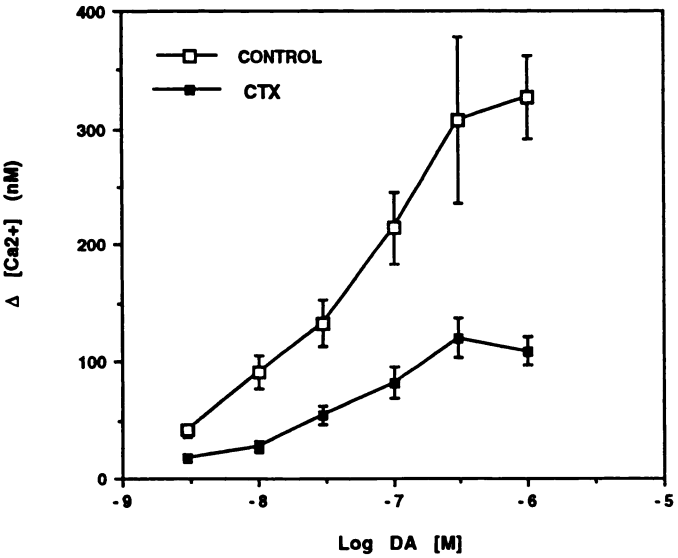


Fig. 7. Attenuation by cholera toxin (*CTX*) (2 μg/ml) pretreatment of DA-induced cAMP production (*top*) and calcium release (*bottom*) in D1-293 cells. 293 cells expressing 1.4 pmol/mg of protein levels of D1 receptor were exposed to cholera toxin (2 μg/ml) for 24 hr, rinsed, and then harvested for cAMP or calcium measurements. Values represent the mean ± standard error of three experiments, conducted in triplicate, for cAMP studies and at least three experiments, performed singly, for calcium experiments.

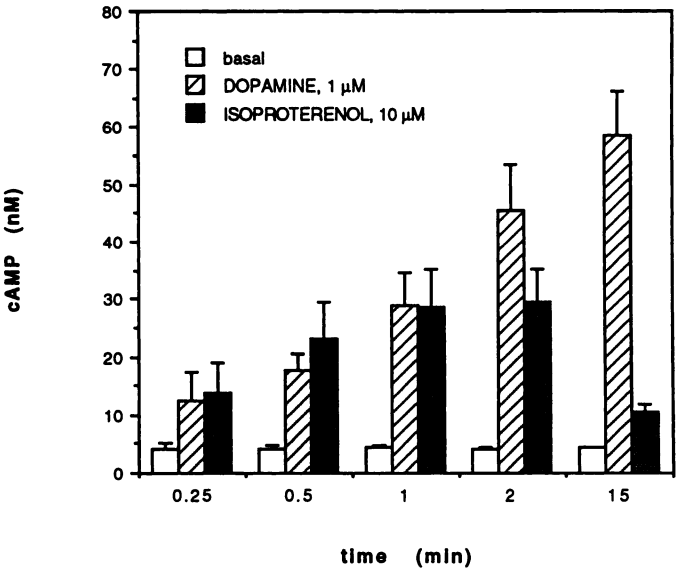


Fig. 8. Time course of cAMP accumulation in response to DA (1 μM) or Iso (10 μM) for the 293 cells expressing 1.4 pmol/mg of protein levels of the D1 receptor. Values represent the mean ± standard error of three experiments conducted in triplicate. For direct comparison with calcium studies (Figs. 3–5), isobutylmethylxanthine was not added for these experiments. In its presence, cAMP levels were 3–4-fold higher than in its absence.

sponse in Ltk$^-$ cells is mediated by PI hydrolysis, whereas cAMP-dependent voltage-sensitive L channels are involved in calcium influx in GH$_4$ cells (8). In contrast, the DA-induced calcium response is independent of PI hydrolysis in 293 cells, which do not appear to contain Bay K-sensitive L channels. D1 receptors expressed in COS and CHO cells have not been shown to elevate calcium release. Although cAMP appears to be responsible for intracellular Ca^{2+} release, the DA-induced calcium response in 293 cells mimics that produced by phospholipase C-linked receptors, such as the muscarinic receptor. The calcium and cAMP responses in 293, Ltk$^-$, and GH$_4$ cells are all sensitive to cholera toxin pretreatment, suggesting that some cholera toxin substrates (e.g., G$_s$) mediate the described actions of DA. It is of interest to note that, when D2 receptors are expressed in GH$_4$ and Ltk$^-$ cells, only the Ltk$^-$ cells show enhanced PI hydrolysis (9). In comparisons of the properties of D1 receptors expressed in COS, CHO, 293, GH$_4$, and Ltk$^-$ cells (12, 21, 22, 25), Ltk$^-$ is the only cell line that exhibits dual cAMP and PI signaling. Taken together, these results indicate that the cloned D1 receptor is unlikely to mediate PI hydrolysis in such tissues as retina, striatum, and kidney. Rather, it is more likely that other D1-like receptors exist and are responsible for the reported PI response in those tissues (5–7). In this regard, a D1-like receptor mRNA isolated from rat striatum has been shown to express PI activity (26).

Although cAMP may account for much of the D1 receptor-induced calcium release in 293 cells, the kinetics of cAMP and calcium release suggest that cAMP levels *per se* do not appear to correspond to the calcium response. DA-induced calcium release is fast (<20 sec) and rapidly declines (<1 min) to a plateau above basal level (Figs. 3 and 4), whereas cAMP production induced by DA continues to increase from 1 to 15 min (Fig. 8). This indicates that cAMP-stimulated calcium release from the IP$_3$-sensitive pool is rapid, is limited in capacity, and may undergo desensitization, as shown for other IP$_3$-sensitive calcium response (27). The mechanisms of calcium release produced by cAMP in 293 cells are unknown but may involve protein kinase A-dependent phosphorylation and sensitization of the IP$_3$ receptor to resting IP$_3$ levels.

To our knowledge, this is the first demonstration of a cAMP-dependent $[Ca^{2+}]_i$ response in 293 cells. Because 293 cells are widely used as a recipient cell line for expression and characterization of a number of G$_s$-linked receptors, including the D5, 5-HT$_6$, 5-HT$_7$, and growth hormone receptors, cAMP-dependent intracellular Ca^{2+} release from the IP$_3$-sensitive pool should also be seen in these cells in response to the respective ligands (28–32). In addition, the calcium response of receptors that are

known to couple to both PI and cAMP pathways, such as calcitonin and leuteinizing hormone receptors, should be the integrative responses of both pathways in 293 cells (33, 34). Finally, Iso-induced calcium release in 293 cells is different from that observed for other G_s-linked receptor agonists. In 293 cells Iso stimulates calcium release through cAMP production that is independent of extracellular calcium. In hepatocytes, Iso alone does not stimulate calcium release, but it potentiates the calcium response to IP_3-mobilizing agonists (35), whereas glucagon elevates both cAMP and IP_3 production, with the latter being responsible for calcium mobilization (36). In AtT-20 cells, corticotropin-releasing factor induces extracellular calcium entry through voltage-sensitive L channels, a mechanism similar to that of the D1 receptor expressed in GH_4 cells (8, 37). The unique intracellular Ca^{2+} release mechanism in 293 cells therefore represents an attractive model to study the regulation of IP_3-sensitive calcium channels by cAMP-dependent processes.

Acknowledgments

We thank Dr. R. G. MacKenzie and M. E. Steffey for assistance in establishing the three D1 receptor-expressing clones used in this study and Dr. Chi-Ming Lee for his suggestions.

References

1. Kebabian, J. W., and D. B. Calne. Multiple receptors for dopamine. *Nature (Lond.)* **227**:93–96 (1979).
2. Civelli, O., J. R. Bunzow, and D. K. Grandy. Molecular diversity of the dopamine receptors. *Annu. Rev. Pharmacol. Toxicol.* **33**:281–307 (1993).
3. Gingrich, J. A., and M. G. Caron. Recent advances in the molecular biology of dopamine receptors. *Annu. Rev. Neurosci.* **16**:299–321 (1993).
4. Sibley, D. R., and F. J. Monsma, Jr. Molecular biology of dopamine receptors. *Trends Pharmacol Sci.* **13**:61–69 (1992).
5. Felder, C. C., M. Belcher, and P. A. Jose. Dopamine-1-mediated stimulation of phospholipase C activity in rat renal cortical membranes. *J. Biol. Chem.* **264**:8739–8745 (1989).
6. Rodrigues, P. D. S., and J. E. Dowling. Dopamine induces neurite retraction in retinal horizontal cells via diacylglycerol and protein kinase C. *Proc. Natl. Acad. Sci. USA* **87**:9693–9697 (1990).
7. Undie, A. S., and E. Friedman. Stimulation of a dopamine D1 receptor enhances inositol phosphates formation in rat brain. *J. Pharmacol. Exp. Ther.* **253**:987–992 (1990).
8. Liu, Y. F., O. Civelli, Q. Y. Zhou, and P. R. Albert. Cholera toxin-sensitive 3′,5′-cyclic adenosine monphosphate and calcium signals of the human dopamine-D1 receptor: selective potentiation by protein kinase A. *Mol. Endocrinol.* **6**:1815–1824 (1992).
9. Vallar, L., C. Muca, M. Magni, P. Albert, J. Bunzow, J. Meldolesi, and O. Civelli. Differential coupling of dopaminergic D2 receptors expressed in different cell types: stimulation of phosphatidylinositol 4,5-biphosphate hydrolysis in Ltk^- fibroblasts, hyperpolarization, and cytosolic free Ca^{2+} concentration decrease in GH_4C_1. *J. Biol. Chem.* **265**:10320–10326 (1990).
10. Frail, D. E., A. M. Manelli, D. G. Witte, C. W. Lin, M. E. Steffey, and R. G. MacKenzie. Cloning and characterization of a truncated DA D1 receptor from goldfish retina: stimulation of cAMP production and calcium mobilization. *Mol. Pharmacol.* **44**:1113–1118 (1993).
11. Lin, C. W., D. G. Witte, T. R. Miller, B. R. Bianchi, M. A. Stashko, A. M. Manelli, and D. E. Frail. Further characterization of dopamine (DA)-stimulated cyclic AMP formation and calcium mobilization in 293 cells transfected with the D1 DA receptor. *Soc. Neurosci. Abstr.* **19**:78 (1993).
12. Sunahara, R. K., H. B. Niznik, D. M. Weiner, T. M. Stormann, M. R. Brann, J. L. Kennedy, J. E. Gelernter, R. Rozmahel, Y. L. Yang, Y. Isreail, P. Seeman, and B. O'Dowd. Human dopamine D1 receptor encoded by an intronless gene on chromosome 5. *Nature (Lond.)* **347**:80–83 (1990).
13. Gorman, C. M., R. Padmannathan, and B. Howard. High efficiency DNA-mediated transformation of primate cells. *Science (Washington D. C.)* **221**:551–553 (1983).
14. Pollock, N. J., A. M. Manelli, C. W. Hutchins, M. E. Steffey, R. G. MacKenzie, and D. E. Frail. Serine mutations in transmembrane V of the dopamine D1 receptor affect ligand interactions and receptor activation. *J. Biol. Chem.* **267**:17780–17786 (1992).
15. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
16. Berridge, M. J., C. P. Downes, and M. R. Hanley. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.* **206**:587–595 (1982).
17. Lin, C. W., B. R. Bianchi, D. Grant, T. Miller, E. A. Danaher, M. D. Tufano, H. Kopecka, and A. M. Nadzan. Cholecystokinin receptors: relationships among phosphoinositide breakdown, amylase release and receptor affinity in pancreas. *J. Pharmacol. Exp. Ther.* **236**:729–734 (1986).
18. Alburges, M. E., N. Narang, and J. K. Wamsley. Alterations in the dopaminergic receptor system after chronic administration of cocaine. *Synapse* **14**:314–323 (1993).
19. Kirch, D. G., T. R. Taylor, I. Creese, S.-X. Xu, and J. Wyatt. Effect of chronic nicotine treatment and withdrawal on rat striatal D1 and D2 dopamine receptors. *J. Pharm. Pharmacol.* **42**:89–92 (1992).
20. Lytton, J., M. Westlin, and M. R. Hanley. Thapsigargin inhibits the sarcoplasmic and endoplasmic reticulum Ca-ATPase family of calcium pumps. *J. Biol. Chem.* **266**:17067–17071 (1992).
21. Dearry, A., J. A. Gingrich, P. Falardeau, R. T. Fremeau, Jr., M. D. Bates, and M. G. Caron. Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature (Lond.)* **347**:72–76 (1990).
22. Monsma, F. J., L. C. Mahan, L. D. McVitte, C. R. Gerfen, and D. R. Sibley. Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. *Proc. Natl. Acad. Sci. USA* **87**:6723–6727 (1990).
23. Kenakin, T. P., and P. H. Morgan. Theoretical effects of single and multiple transducer receptor coupling proteins on estimates of the relative potency of agonists. *Mol. Pharmacol.* **35**:214–222 (1989).
24. Varrault, A., L. Journot, Y. Audigier, and J. Bockaert. Transfection of human of 5-hydroxytryptamine$_{1A}$ receptors in NIH-3T3 fibroblasts: effects of increasing receptor density on the coupling of 5-hydroxytryptamine$_{1A}$ receptors to adenylyl cyclase. *Mol. Pharmacol.* **41**:999–1007 (1992).
25. Zhou, Q. Y., D. K. Grandy, L. Thambi, J. A. Kushner, H. H. Van Tol, R. Cone, D. Pribnow, J. Salon, J. R. Bunzow, and O. Civelli. Cloning and expression of human and rat D1 dopamine receptors. *Nature (Lond.)* **347**:76–80 (1990).
26. Mahan, L. C., R. M. Burch, F. J. Monsma, Jr., and D. R. Sibley. Expression of striatal D1 dopamine receptors coupled to inositol phosphate production and Ca^{2+} mobilization in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **87**:2196–2200 (1990).
27. Bezprozvanny, I., J. Watras, and B. E. Ehrlisch. Bell-shaped calcium-response curves of Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature (Lond.)* **351**:751–754 (1991).
28. Tiberi, M., K. R. Jarvie, C. Silvia, P. Falardeau, J. A. Gingrich, N. Godinot, L. Bertrand, T. L. Yang-Feng, R. T. Fremeau, Jr., and M. G. Caron. Cloning, molecular characterization and chromosomal assignment of a gene encoding a novel D_1 dopamine receptor subtype: differential expression pattern in rat brain compared to the D_{1A} receptor. *Proc. Natl. Acad. Sci. USA* **88**:7491–7495 (1991).
29. Grandy, D. K., Y. A. Zhang, C. Bouvier, Q. Y. Zhou, R. A. Johnson, L. Allen, K. Buck, J. R. Bunzow, J. Salon, and O. Civelli. Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes. *Proc. Natl. Acad. Sci. USA* **88**:9175–9179 (1991).
30. Shen, Y., F. J. Monsma, Jr., M. A. Metcalf, P. A. Jose, M. W. Hamblin, and D. R. Sibley. Molecular cloning and expression of a 5-hydroxytryptamine$_7$ serotonin receptor subtype. *J. Biol. Chem.* **268**:18200–18204 (1993).
31. Monsma, F. J., Jr., Y. Shen, R. P. Ward, M. W. Hamblin, and D. R. Sibley. Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol. Pharmacol.* **43**:320–327 (1993).
32. Mayo, K. E. Molecular cloning and expression of a pituitary-specific receptor for growth hormone-releasing hormone. *Mol. Endocrinol.* **6**:1734–1744 (1992).
33. Chabre, O., B. R. Conklin, H. Y. Lin, H. F. Lodish, E. Wilson, H. E. Ives, L. Catanzariti, B. A. Hemmings, and H. R. Bourne. A recombinant calcitonin receptor independently stimulates 3′,5′-cyclic adenosine monophosphate and Ca^{2+}/inositol phosphate signaling pathways. *Mol. Endocrinol.* **6**:551–556 (1992).
34. Gudermann, T., M. Birnbaumer, and L. Birnbaumer. Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca^{2+} mobilization. *J. Biol. Chem.* **267**:4479–4488 (1992).
35. Burgess, G. M., G. S. J. Bird, J. F. Obie, and J. W. Putney, Jr. The mechanism for synergism between phopholipase C- and adenyl cyclase-linked hormones in liver. *J. Biol. Chem.* **266**:4772–4781 (1991).
36. Blackmore, P. F., and J. H. Exton. Studies on the hepatic calcium-mobilizing activity of aluminum fluoride and glucagon. *J. Biol. Chem.* **261**:11056–11063 (1986).
37. Liuni, A., D. Lewis, S. Guild, D. Corda, and J. Axelrod. Hormone secretagogues elevate cytosolic calcium by increasing cAMP in corticortropin-secreting cells. *Proc. Natl. Acad. Sci. USA* **82**:8034–8038 (1985).

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