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Selection of Alternative G_i-mediated Signaling Pathways at the Dopamine D_2 Receptor by Protein Kinase C

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Evidence indicates that a single membrane receptor subtype may be responsible for the generation of multiple intracellular signals, but mechanisms allowing for the selection of a specific effector pathway have not yet been documented. In neurons and other cells, the stimulation of dopamine D_2 receptors produces, via G-protein activation, a spectrum of intracellular responses including inhibition of adenylyl cyclase activity, modulation of K^+ currents, and potentiation of Ca^{2+}-evoked arachidonic acid (AA) release. In this study, we report that, in Chinese hamster ovary cells, stimulation of protein kinase C (PKC) directs the preferential coupling of transfected D_2 receptors from inhibition of adenylyl cyclase to potentiation of AA release, two responses mediated by G_i. The switch between these two signaling systems is accompanied by marked changes in their GTP sensitivities, indicating that it may result from the phosphorylation of component(s) of the receptor–G_i-protein complex. Brain PKC activity is enhanced by neurotransmitters and by neuronal depolarization. Thus, the ability of this protein kinase to remodel signaling pathways at the D_2 receptor may regulate these G_i-mediated responses in an activity-dependent manner, and represent a novel form of synaptic plasticity.

[Key words: Chinese hamster ovary fibroblasts, transfected cells, second messengers, arachidonic acid, phospholipase A_2, cAMP, adenylyl cyclase]

Communication between neurons requires that the actions of neurotransmitters on their target cells be both specific and modifiable, to allow for plastic changes to occur in response to environmental demands. To achieve specificity, many neurotransmitters interact with distinct subtypes of membrane receptors coupled to specific intracellular second messengers. For example, dopamine, a major modulatory transmitter in the brain, exerts its neural actions through stimulation of at least five receptor subtypes, discretely distributed throughout the CNS, and functionally coupled either to G_s-mediated activation of adenylyl cyclase ("D_1 type") or to G_i-mediated cyclase inhibition ("D_2 type") (Sibley and Monsma, 1992; Schwartz et al., 1992).

Increasing evidence indicates, however, that a single receptor subtype may be linked to the formation of multiple, parallel intracellular signals. This spectrum of heterogeneous effectors may be produced through activation of one or several transducing G-proteins (for review, see Spiegel, 1992). For example, in anterior pituitary cells, two different G-proteins, G_o and G_{i3}, link D_2 receptors to modulation of Ca^{2+} and K^+ channel opening, respectively (Lledo et al., 1992). Likewise, in Chinese hamster ovary (CHO) cells transfected with D_2 receptor cDNA [CHO(D_2)], D_2 receptor stimulation causes both inhibition of adenylyl cyclase activity and facilitation of evoked release of arachidonic acid (AA) via pertussis toxin–sensitive G_i-proteins (Felder et al., 1991; Kanterman et al., 1991; Piomelli et al., 1991).

The functional redundancy in intracellular signaling underscored by these findings raises several important questions. Are all the signals driven by a single receptor subtype equally operative under all circumstances? Or rather, are these signals subject to cellular mechanisms of integration and regulation? Can the spectrum of second messengers in a cell be remodeled, for example, by changing the functional weight of one pathway with respect to others, to adapt to its varying needs? And if this is the case, could such a remodeling at the second messenger level affect the response to receptor stimulation? Interest in such questions lies in the possibility that, like ion channels and neurosecretory mechanisms, the pathways of transmembrane signaling may represent a potential substrate for plastic changes in neural cells.

Protein kinase C (PKC) serves important regulatory functions in the CNS, where it is thought to participate in the modulation of ion channel activity, receptor desensitization and transmitter release (Nishizuka, 1988). Using CHO(D_2) cells, we have now examined the role played by this protein kinase in controlling parallel intracellular signaling at the D_2 receptor. We report that activation of PKC switches the coupling of D_2 receptors from inhibition of adenylyl cyclase toward facilitation of AA release, possibly by phosphorylating component(s) of the receptor–G_i-protein complex. Because brain PKC activity is stimulated by neurotransmitters and neuronal depolarization (Nishizuka, 1988), the results suggest that remodeling of transmembrane signaling pattern by this protein kinase, demonstrated here in an heterologous expression system, may modify in an activity-dependent manner physiological responses to D_2 receptor stimulation.

Materials and Methods

Cell culture and transfections. CHO cells were transfected with rat $D_{2(444)}$ receptor (also termed D_{2L}) cDNA or with human $D_{2(415)}$ receptor (also termed D_{2S}) cDNA as previously described (Giros et al., 1989). CHO

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cells expressing rat $D_{2(444)}$ receptor are referred to in the text as CHO(D_2); those expressing $D_{2(415)}$ receptor, as CHO($D_{2(415)}$). Transfected clones were maintained in monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (GIBCO) at 37°C in 5% CO_2. CHO(D_2) expressed 1.3 × 10^5 receptors/cell, and CHO($D_{2(415)}$), 1.5 × 10^5 receptors/cell.

Receptor binding. CHO(D_2) cells were incubated in DMEM containing 0.25% trypsin (GIBCO) for 5 min at room temperature, and the reaction was stopped by adding 10 ml of supplemented DMEM. Detached cells were collected by centrifugation and washed in 15 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ and 10 mM sodium pyrophosphate, to inhibit protein phosphatase activity. After cell disruption, membranes were isolated by centrifugation (50,000 × *g* for 15 min) in the same buffer. Binding was determined in 50 mM Tris-HCl, pH 7.4, containing (mM) NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; ascorbic acid, 0.28; and 8-hydroxyquinoline, 0.1. Curves were analyzed by computerized nonlinear regression using a one- or two-site model (Martres et al., 1984).

^{3}H-AA release. Cells (24-well plates) were labeled by incubation with ^{3}H-AA (Amersham; 200–220 Ci/mmol, 0.25 mCi/ml) in DMEM (1 ml) containing 0.2% bovine serum albumin (BSA) for 2 hr at 37°C. To eliminate unincorporated radioactivity, cells were washed with 1 ml of DMEM plus BSA, before incubating them for 30 min at 37°C in 1 ml of DMEM, containing final concentrations of the appropriate drugs. Analysis by high-performance liquid chromatography revealed that, in BSA-containing incubations, free ^{3}H-AA constituted more than 90% of the released radioactivity in both control and stimulated samples (Di Marzo and Piomelli, 1992). To prevent dopamine oxidation, incubations with the drug were carried out in the presence of ascorbic acid (0.1%), which did not affect either basal or stimulated ^{3}H-AA release (not shown). In some experiments, cells were incubated with pertussis toxin (200 ng/ml) for 4 hr at 37°C in 1 ml of DMEM. Incubation with the toxin did not affect ^{3}H-AA labeling of phospholipids (not shown). ^{3}H-AA release was determined by liquid scintillation counting of samples (0.5 ml) of the incubation medium.

cAMP formation. Cells (96-well plates) were incubated for 10 min in 0.15 ml in DMEM containing isobutylmethylxanthine (IBMX) (0.1 mM), a phosphodiesterase inhibitor, plus forskolin (10 μM) and final concentrations of the appropriate drugs. After extraction in 0.1 M HCl (0.1 ml), sonication and neutralization of the tissue extracts, cAMP concentrations were determined using a radioimmunoassay kit (Amersham), following the manufacturer's instructions.

Measurements of intracellular free Ca^{2+} concentration. Cells were suspended by treatment with trypsin (see above), and incubated for 60 min at 37°C of DMEM containing BSA (0.2%) and fura-2 acetoxymethyl ester (1 μM). After washing, cells were resuspended in HEPES-buffered Krebs' solution (containing, in mmol/liter, NaCl, 125; KCl, 5; KH_2PO_4 and $MgSO_4$, 1.2; $CaCl_2$, 2; glucose, 6; HEPES/NaOH buffer, pH 7.4) and Ca^{2+} levels were determined by using a Hitachi F-2000 fluorescence spectrophotometer (Grynkiewicz et al., 1985).

Permeabilized cells. CHO(D_2) cells in 24-well plates were washed twice with 1 ml of Spinner MEM (with Earl's salts, without glutamine; GIBCO) with 0.2% BSA, and incubated in 1 ml of the same medium containing 50 μg/ml saponin plus final concentrations of drugs. At least 90% of the cells became permeable to trypan blue within 5 min of saponin treatment. For ^{3}H-AA release experiments, cells were previously labeled for 2 hr as described above and unincorporated radioactivity eliminated by washing.

Statistical analysis. Results are expressed as mean ± SEM of *n* experiments. EC_{50} and IC_{50} values were calculated by nonlinear regression analysis, using data obtained from at least three separate experiments. Statistical comparisons between means ± SEM were carried out using analysis of variance, unless otherwise indicated.

Materials. Ca^{2+} ionophore A23187, dopamine, saponin, 4-β-phorbol-12-myristate-13-acetate (PMA), 4-α-phorbol, bovine serum albumin (essentially fatty acid-free), pertussis toxin, staurosporine, forskolin, and isobutylmethylxanthine were from Sigma (France). 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (OMPC) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA); guanosine-5'-*O*-(3-thiotrisphosphate) (GTP-γ-S), from Boehringer Mannheim (France); fura-2 acetoxymethyl ester, from Molecular Probes (Eugene, OR); and Rp-adenosine-cAMP monophosphothioate (Rp-cAMPS), from Research Biochemicals (Natick, MA). Quinpirole was a generous gift of Eli Lilly (Indianapolis, IN), and raclopride, of Astra (Södertalje, Sweden). Verrucosin B was purified from the marine mollusk *Doris verrucosa* as described (Cimino et al., 1988).

Results

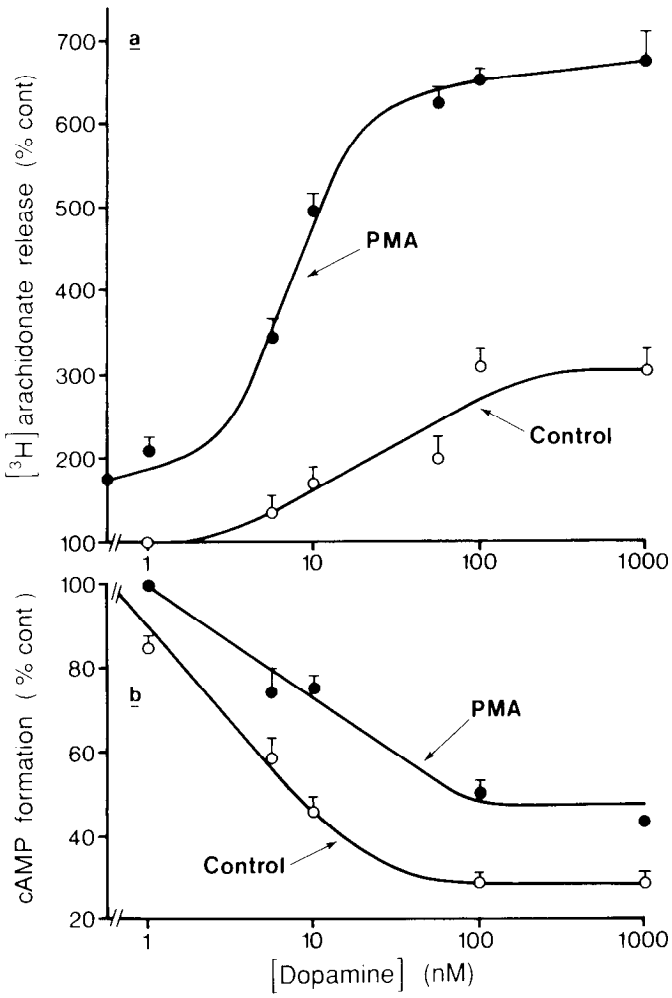
Phorbol ester switches preferential D_2 receptor signaling from inhibition of adenylyl cyclase to facilitation of AA release

In CHO(D_2) cells, dopamine enhanced the release of ^{3}H-AA stimulated by agents that elevate intracellular Ca^{2+}, such as the Ca^{2+} ionophore A23187 (Fig. 1*a*), and inhibited the accumulation of cAMP stimulated by forskolin (Fig. 1*b*). Concentrations of dopamine that produced half-maximal potentiation (EC_{50}) of ^{3}H-AA release and half-maximal inhibition (IC_{50}) of cAMP accumulation were 24 nM and 5 nM, respectively. When cells were stimulated with dopamine in the presence of the PKC activator PMA (100 nM), the resulting potentiation of A23187-evoked ^{3}H-AA release was much greater than with dopamine alone (Fig. 1*a*). Incubation with PMA affected both the maximal response to dopamine (from 300% to 700% of A23187-evoked ^{3}H-AA release) and its EC_{50} (from 24 nM to 8 nM). In contrast with this facilitating action on AA release, treatment with PMA decreased the inhibition of adenylyl cyclase activity caused by stimulation of D_2 receptors (Fig. 1*b*). In the presence of phorbol ester, maximal dopamine-induced inhibition of cAMP accumulation was reduced from 70% to 50% of control and its IC_{50} was increased from 5 nM to 30 nM. PMA was as potent in enhancing dopamine potentiation of ^{3}H-AA release (EC_{50} = 8 nM PMA) as in preventing its inhibition of adenylyl cyclase (EC_{50} = 5 nM) (Fig. 1*c*).

In unstimulated CHO(D_2) cells, PMA had no effect on either cAMP formation or ^{3}H-AA release (not shown). Likewise, levels of cAMP measured in the presence of PMA (1 μM) plus forskolin (10 μM) were 107 ± 11% of those found with forskolin alone (mean ± SEM, n = 4), indicating that the phorbol ester exerted no direct action on adenylyl cyclase activity. By contrast, PMA enhanced release of ^{3}H-AA evoked by Ca^{2+} ionophore A23187 in the absence of D_2 receptor stimulation (Figs. 1*a*, 2*A*). The result confirms previous reports showing that phorbol esters potentiate evoked ^{3}H-AA release from CHO cells, likely by stimulating phosphorylation of cytosolic phospholipase A_2 (cPLA$_2$) (Felder et al., 1991; Kanterman et al., 1991; Lin et al., 1992). However, even at maximal concentrations of drug (0.1–1 μM), the effect of PMA on A23187-evoked ^{3}H-AA release was markedly weaker than that produced by stimulating D_2 receptors with quinpirole, a D_2 receptor agonist (Fig. 2), or with dopamine (not shown). Furthermore, when PMA and quinpirole were combined, the resulting ^{3}H-AA release was much greater than the sum of the effects of the two drugs used separately (Fig. 2). The greater-than-additive release produced by concomitant activation of D_2 receptors and PKC suggests that the two stimuli may act through independent pathways to cause a synergistic facilitation of cPLA$_2$ activity.

The effects of PMA on D_2 receptor signaling are mediated by PKC

The actions of PMA on D_2 receptor signaling were produced through activation of PKC. In agreement, we observed that (1) PMA enhanced ^{3}H-AA release and prevented adenylyl cyclase inhibition with similar potencies (Fig. 1*c*); (2) the inactive analog 4-α-phorbol-12,13-didecanoate was ineffective (Table 1); (3) the effects of PMA on both cAMP accumulation and ^{3}H-AA release were prevented by the PKC inhibitors staurosporine (0.5 μM) and OMPC (50 μM) (Helfman et al., 1983); (4) a non-phorbol ester PKC activator, the marine diterpene verrucosin B (De Petrocellis et al., 1991), enhanced ^{3}H-AA release to an extent



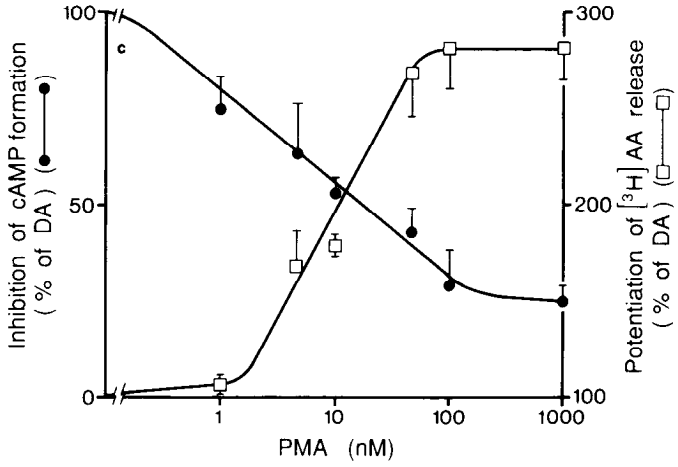


Figure 1. Effects of the PKC activator PMA on intracellular signaling in CHO cells expressing the D_2 receptor subtype. *a*, PMA potentiates D_2-dependent facilitation of ^{3}H-AA release. CHO(D_2) cells, labeled by incubation with ^{3}H-AA, were incubated for 30 min with the Ca^{2+} ionophore A23187 (2 μM), plus dopamine at the indicated concentrations, in the absence (*open circles*) or in the presence (*solid circles*) of PMA (0.1 μM). Results are expressed as percentage of the release evoked by stimulation with A23187 (2 μM) (297 ± 55 cpm/well, $n = 6$). *b*, PMA impairs D_2-dependent inhibition of cAMP accumulation. Cells were incubated for 20 min with the adenylyl cyclase activator forskolin (10 μM), plus dopamine, in the absence (*open circles*) or presence (*solid circles*) of PMA (0.1 μM, 10 min preincubation). Results are expressed as percentage inhibition of forskolin-stimulated cAMP level (51 ± 9 fmol/well, $n = 6$). *c*, Concentration-dependent effects of PMA on both D_2-dependent facilitation of ^{3}H-AA release (*open squares*) and inhibition of cAMP accumulation (*solid circles*). In both cases, results are expressed as percentage of the response elicited by dopamine (50 nM).

similar to PMA (Table 1). In the absence of PMA, staurosporine did not affect D_2 receptor–dependent inhibition of adenylyl cyclase (Table 1). By contrast, the potentiation of ^{3}H-AA release produced by D_2 receptor stimulation was reduced by PKC inhibitors (Table 1), confirming that activity of this protein kinase is an important requirement in the receptor-dependent activation of $cPLA_2$ (Felder et al., 1991; Kanterman et al., 1991; Lin et al., 1992).

Application of 8-bromo-cAMP, a membrane-permeant cAMP analog, enhances D_2 receptor–dependent potentiation of ^{3}H-AA release in CHO(D_2) cells (Piomelli et al., 1991). To examine whether enhancement of ^{3}H-AA release by PMA resulted from increased cAMP levels (subsequent to its effect on D_2 receptor–induced inhibition of adenylyl cyclase), we stimulated cells in the presence of Rp-cAMPS, a selective cAMP antagonist (Rothermel et al., 1984). Rp-cAMPS (20 μM) did not affect ^{3}H-AA release produced by PMA plus quinpirole, a D_2 receptor agonist, suggesting that cAMP does not participate in this response (Table 1).

PMA has no effect on D_2 receptor binding

In membranes prepared from rat striatum, phosphorylation by purified PKC was shown to reduce the modulatory effect of GTP-γ-S on ^{3}H-spiperone binding, suggesting that PKC-mediated mechanisms may regulate striatal D_2 receptor function (Rogue et al., 1990). Therefore, we examined the effects of PMA on D_2 receptor binding in CHO(D_2) cells, using the highly selective D_2 receptor ligand ^{125}I-iodosulpride (Martres et al., 1985). Treating cells with PMA (1 μM) had no effect either on the displacement of ^{125}I-iodosulpride binding by dopamine, measured subsequently in isolated membranes, or on the modulation of this binding by GTP-γ-S. B_{max} values were (in pmol/mg protein) 1.36 ± 0.12 in control and 1.37 ± 0.13 in PMA-treated cells. Likewise, EC_{50} values for GTP-γ-S in shifting the agonist competition curve from complex to monophasic remained unchanged (14 ± 3 nM in control and 19 ± 8 nM in PMA-treated cells, $n = 4$ or 5).

PKC enables the productive coupling of D_2 receptors to $cPLA_2$ in CHO cells expressing the $D_{2(415)}$ receptor subtype

The effects of PMA on D_2-dependent potentiation of $cPLA_2$ and inhibition of adenylyl cyclase activities were further examined using a clonal line of CHO cells expressing the human $D_{2(415)}$ receptor, CHO($D_{2(415)}$). This isoform, derived from alternative splicing of the D_2 receptor gene transcript (Schwartz et al., 1992), also termed D_{2S}, differs from the $D_{2(444)}$ (here referred to as D_2) in that it lacks a segment of 29 amino acid residues located within the third putative cytoplasmic loop of the protein, a domain that is thought to participate in G-protein coupling and in effector regulation (Dohlman et al., 1991).

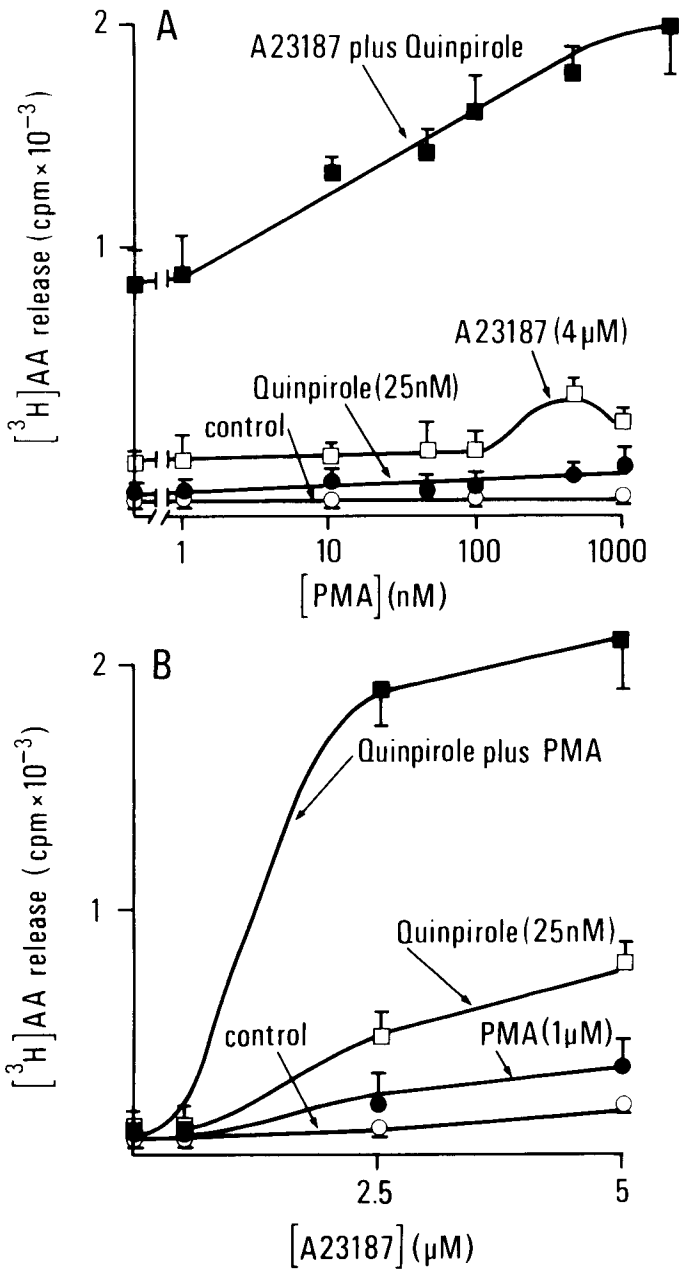


Figure 2. Effects of PMA on stimulated ^{3}H-AA release from CHO(D_2) cells. *A*, Concentration-dependent effects of PMA alone (*open squares*), PMA in the presence of A23187 (*open circles*), and PMA in the presence of A23187 plus quinpirole, a D_2 receptor agonist (200 nM) (*solid circles*). *B*, Effects of PMA (1 μM) on the release of ^{3}H-AA evoked by A23187 (0.5–5 μM) or by A23187 plus quinpirole (25 nM).

Cell lines expressing the two splicing variants at similar levels (see Materials and Methods) showed no marked difference either in their endogenous $cPLA_2$, adenylyl cyclase, and PKC activities, or in their ability to respond to the activation of a constitutive, G_i-coupled receptor. In agreement, release of ^{3}H-AA stimulated by A23187 and accumulation of cAMP stimulated by forskolin were similar in the two cell lines (not shown). Likewise, no significant difference was seen between these cell lines in the ability of PMA to potentiate A23187-evoked ^{3}H-AA release: in three experiments, potentiations by PMA (50 nM) were 177 ± 22% of A23187-evoked release in CHO(D_2) cells and 149 ± 6% in CHO($D_{2(415)}$) cells. Furthermore, the two lines did not differ in their response to the application of extracellular ATP, which elevates intracellular Ca^{2+} levels and stimulates $cPLA_2$ activity by interacting with constitutive type 2 purinergic receptors, coupled to a G_i-protein (Gupta et al., 1990). ATP was equally potent (EC_{50} = 5 μM) in elevating Ca^{2+} and in evoking ^{3}H-AA release in both CHO(D_2) and CHO($D_{2(415)}$) cells.

Table 1. The effects of PMA on D_2 receptor signaling are mediated by PKC

Treatment	^{3}H-AA release (% of A23187 plus quinpirole)	Inhibition of cAMP accumulation (% of quinpirole)
Quinpirole	100	100
+ stauro	83 ± 2	109 ± 9
+ PMA	252 ± 19*	71 ± 5*
+ 4-α-phorbol	105 ± 8	97 ± 1
+ PMA/stauro	61 ± 4*	92 ± 5
+ PMA/OMPC	130 ± 5	ND
+ verrucosin B	289 ± 20*	ND
+ verrucosin B/stauro	89 ± 4	ND
+ PMA/Rp-cAMPs	284 ± 22*	ND

For experiments on ^{3}H-AA release, prelabeled CHO(D_2) cells were incubated for 30 min with A23187 (4 μM) and quinpirole (1 μM) plus various combinations of the following drugs: PMA (0.5 μM); staurosporine (stauro) (0.5 μM); OMPC (50 μM); verrucosin B (1 μM); Rp-cAMPs (20 μM). For experiments on cAMP accumulation, cells were incubated for 20 min with quinpirole (0.1 μM) plus the drugs mentioned above. Results are expressed as percentage ± SEM of the effect produced by quinpirole in 3–10 separate experiments. ND, not determined.

* Statistically different from A23187 plus quinpirole.

By contrast, stimulation of CHO($D_{2(415)}$) cells with dopamine had little effect on A23187-evoked ^{3}H-AA release and only weakly inhibited forskolin-stimulated adenylyl cyclase activity (Fig. 3). Similar results were obtained in four of five CHO($D_{2(415)}$) cell lines (not shown), suggesting that the productive coupling of transfected $D_{2(415)}$ receptors to endogenous $cPLA_2$ and adenylyl cyclase activities was inefficient in these cells. However, when CHO($D_{2(415)}$) cells were stimulated with a combination of dopamine plus PMA (0.5 μM), release of ^{3}H-AA was enhanced to an extent comparable to that produced by the two agents in CHO(D_2) cells (Fig. 3*a*). Maximal concentrations of PMA (0.1–1 μM) increased ^{3}H-AA release by about sevenfold over dopamine alone. As seen with CHO(D_2) cells, PMA diminished dopamine-induced inhibition of adenylyl cyclase in CHO($D_{2(415)}$) cells (Fig. 3*b*). The results, showing that treatment with a PKC activator fully restores D_2 receptor–dependent potentiation of ^{3}H-AA release, suggest that stimulated PKC activity enables the productive coupling between $D_{2(415)}$ receptors and $cPLA_2$ in CHO($D_{2(415)}$) cells. They also support the idea that, where coupling to $cPLA_2$ is already functional, as in CHO(D_2) cells, PKC reinforces it, resulting in a much greater receptor–dependent facilitation of Ca^{2+}-evoked AA release.

PMA exerts opposing actions on the sensitivities of adenylyl cyclase and $cPLA_2$ to GTP-γ-S

To examine further the actions of PKC on D_2 receptor signaling, we used stably permeabilized CHO(D_2) cells, previously labeled by incubation with ^{3}H-AA. When the cells were exposed to an extracellular medium containing saponin (50 μg/ml) and no added Ca^{2+}, they released ^{3}H-AA in the medium in a Ca^{2+}-

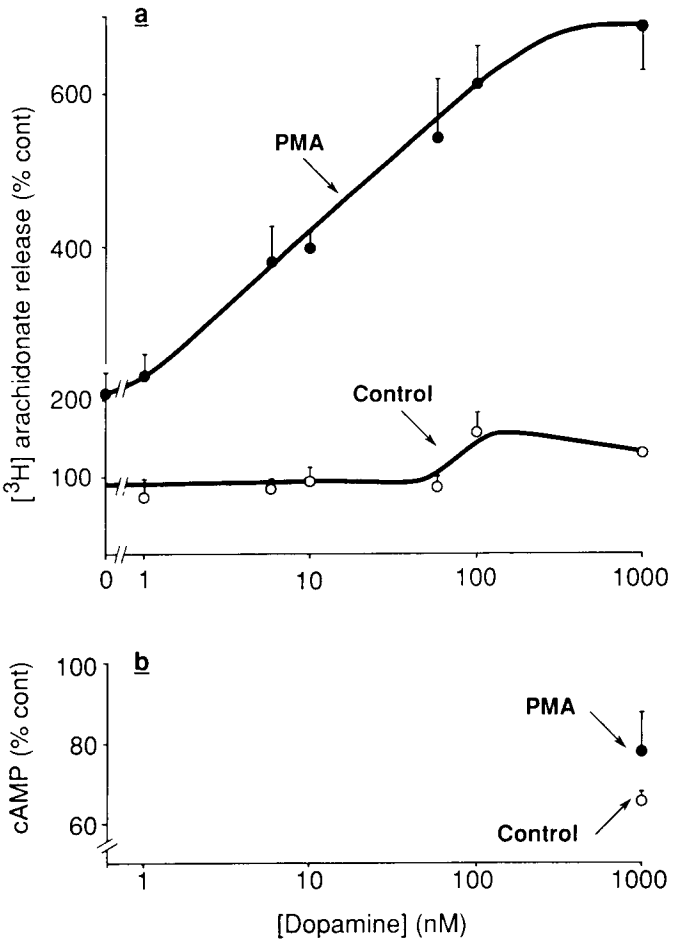


Figure 3. Effects of PMA on cAMP accumulation and ^{3}H-AA release from CHO cells expressing the $D_{2(415)}$ receptor isoform, an alternative splicing variant of the D_2 receptor. *a*, PMA enables D_2-dependent facilitation of ^{3}H-AA release in CHO($D_{2(415)}$) cells. Cells were labeled by incubation with ^{3}H-AA, and incubated for 30 min with A23187 (2 μM) plus dopamine, in the absence (*open circles*) or in the presence (*solid circles*) of PMA (0.1 μM). Results are expressed as percentage of release evoked by stimulation with A23187 (255 ± 17 cpm/well, n = 19). *b*, Weak D_2-dependent inhibition of cAMP accumulation in CHO($D_{2B(415)}$) cells, and its prevention by PMA. Cells were incubated for 20 min with the adenylyl cyclase activator forskolin (10 μM), plus dopamine, in the absence (*open circles*) or in the presence (*solid circles*) of PMA (0.1 μM, 10 min preincubation). Results are expressed as percentage inhibition of forskolin-stimulated cAMP level (99 ± 12 fmol/well, n = 3).

dependent manner, as shown by the ability of EGTA (5 mM) to inhibit the release (from 1669 ± 86 to 1209 ± 42 cpm/well; $p < 0.05$ by paired t test, n = 12). In additional experiments, quinpirole (5 μM) was found to produce a small but significant increase in ^{3}H-AA release, which was markedly enhanced by coapplication of GTP-γ-S (100 μM), a nonhydrolyzable GTP analog, and inhibited by treatment with either raclopride, a D_2 receptor antagonist (5 μM) (Table 2), EGTA (5 mM), or pertussis toxin (200 ng/ml; 4 hr) (not shown). Alone, GTP-γ-S had no effect on ^{3}H-AA release (Table 2).

The ability of GTP-γ-S to synergize with D_2 receptors in potentiating $cPLA_2$ activity in a pertussis toxin–sensitive manner confirms that this response is transduced via a G_i-protein (Felder et al., 1991; Piomelli et al., 1991). To determine the effects of PMA on this synergistic interaction, saponin-permeabilized CHO(D2) cells were stimulated with GTP-γ-S in the

Table 2. Effects of quinpirole and GTP-γ-S on Ca^{2+}-evoked ^{3}H-AA release from permeabilized CHO(D_2) cells

Treatment	^{3}H-AA release (cpm/well)
Control	2092 ± 136
Quinpirole (5 μM)	3089 ± 386*
Quinpirole + raclopride (5 μM)	2204 ± 402
GTP-γ-S (100 μM)	2284 ± 289
GTP-γ-S + raclopride	2205 ± 480
GTP-γ-S + quinpirole	3875 ± 457*

Cells were prelabeled with ^{3}H-AA and stably permeabilized by incubation in a medium containing saponin (50 μg/ml) and no added Ca^{2+}, plus the appropriate drugs (30 min). ^{3}H-AA release, measured in samples of the incubation media, is expressed as cpm/well ± SEM from a representative experiment carried out in quadruplicate. Similar results were obtained in six additional experiments.

* Statistically different from control.

presence of a concentration of dopamine (1 μM), which alone had little or no effect on ^{3}H-AA release. In control incubations, GTP-γ-S synergized with 1 μM dopamine to cause ^{3}H-AA release only at concentrations greater than 50 μM. However, the inclusion of PMA (50 nM) in the incubation medium resulted both in increased GTP-γ-S–induced ^{3}H-AA release and in enhanced sensitivity to the nucleotide analog (Fig. 4*a*). Basal, Ca^{2+}-dependent release of ^{3}H-AA was also increased by PMA (Fig. 4*a*), whereas Ca^{2+}-independent release (in the presence of 5 mM EGTA) remained unaffected (not shown).

Next, we used saponin-permeabilized CHO(D_2) cells to investigate the actions of PMA on D_2 receptor–dependent inhibition of adenylyl cyclase activity. Forskolin-stimulated release of cAMP in the medium of permeabilized cells was inhibited weakly by dopamine alone (1 μM) (44% inhibition) and by GTP-γ-S (100 μM) (49% inhibition) or, more effectively, by a combination of dopamine plus GTP-γ-S (Fig. 4*b*). Inclusion of PMA (100 nM) in the incubations prevented the ability of low concentrations of GTP-γ-S to inhibit adenylyl cyclase activity (Fig. 4*b*). In the presence of phorbol ester, the concentration of extracellular GTP-γ-S necessary to produce maximal inhibition of cAMP accumulation was increased from 10 μM to 100 μM (Fig. 4*b*).

Together, the results obtained with permeabilized CHO(D_2) cells suggest that activation of PKC may exert opposing actions on the coupling efficacies of D_2 receptors with $cPLA_2$ and adenylyl cyclase by modifying the sensitivities of these signaling pathways to intracellular GTP. These changes in coupling strength may, in turn, underlie the ability of PKC, observed in intact CHO(D_2) cells, to direct the preferential signaling at D_2 receptors from inhibition of adenylyl cyclase to facilitation of ^{3}H-AA release.

Discussion

Our experiments suggest that PKC activity may control effector coupling at the D_2 receptor by switching it from inhibition of adenylyl cyclase, prevalent in unstimulated cells, to facilitation of AA release, prevalent in cells where PKC has been activated. By remodeling the pattern of intracellular second messengers generated at the D_2 receptor, such an activity-dependent switch may modify the physiological actions of dopamine in select cells, and potentially represent, if operative in neurons, a novel form of short-term synaptic plasticity. Here we will discuss, first, the possible mechanisms underlying the regulation by PKC of D_2

receptor signaling and, next, its possible relevance and implications for the CNS.

Regulation of cPLA$_2$ activity by G$_i$-coupled receptors and by protein phosphorylation

We have shown here that (1) in intact CHO(D_2) cells, stimulation of PKC enhances the D_2 receptor–dependent facilitation of AA release; (2) in permeabilized cells, this enhancement is accompanied by a reduction in the concentration threshold of the GTP analog GTP-γ-S necessary to potentiate AA release; and (3) stimulation of PKC unmasks the coupling of $D_{2(415)}$ receptors to cPLA$_2$ in a cell line in which this coupling is normally impaired. This impairment was likely caused by inadequate recognition between transfected $D_{2(415)}$ receptor and endogenous G-protein, and does not appear to be consistently linked to the expression of this shorter receptor isoform. In agreement, one of five cell lines tested showed a normal AA response (not shown).

Recent studies on CHO cells have greatly advanced our understanding of the molecular mechanisms underlying receptor-operated release of AA from membrane phospholipids. It is now recognized that some membrane receptors (e.g., P_2-purinergic), acting through a transducing G_i-protein, can induce the Ca^{2+}-dependent translocation of an AA-specific cPLA$_2$ from the cytosol to the cell membrane, and its subsequent activation (Gupta et al., 1990; Lin et al., 1992). These events are accompanied by increased phosphorylation of cPLA$_2$ on a serine residue, and can be prevented by treatment with PKC inhibitors, suggesting that the protein kinase plays an important role in enabling activation of cPLA$_2$ (Kanterman et al., 1991; Lin et al., 1992).

Furthermore, heterologous expression of D_2 receptors in CHO cells has led to the demonstration of an additional mechanism involved in regulating cPLA$_2$ activity. In transfected CHO cells, D_2 receptor stimulation potently facilitates release of AA evoked either by stimulating constitutive P_2 receptors or by applying Ca^{2+} ionophores, but has no effect on basal release (Kanterman et al., 1991; Piomelli et al., 1991). This permissive action of D_2 receptors on cPLA$_2$ activity, which is also seen with other transfected G_i-coupled receptors, such as α2-adrenergic and m2-muscarinic (Felder et al., 1991), is transduced by a G_i-protein. In agreement, the response (1) is prevented by treatment with pertussis toxin (Felder et al., 1991; Piomelli et al., 1991) and (2) is potentiated in permeabilized cells by coapplication of GTP-γ-S (present results). Although the identity of the transducing G-protein is not known, this is unlikely to be a G_o, because these proteins are not expressed in CHO cells (Gupta et al., 1990). In addition, differences in the sensitivity to pertussis toxin suggest that the transducing G_i may be distinct from the G_i involved in coupling the D_2 receptor to inhibition of adenylyl cyclase activity (Felder et al., 1991).

In summary, three factors appear to contribute to receptor-stimulated AA release in CHO cells: first, the state of phosphorylation of cPLA$_2$, which may play an enabling role in the enzyme translocation and activation processes; second, the levels of intracellular Ca^{2+}, which may signal the turning on and off of these processes; and third, the presence of an activated G_i-protein, which may exert a permissive action on cPLA$_2$ after translocation of the phospholipase to the membrane, allowing expression of its full catalytic activity. Our findings suggest that PKC may act to reinforce the productive G_i-mediated coupling between D_2 receptors and cPLA$_2$. Potential sites of action for

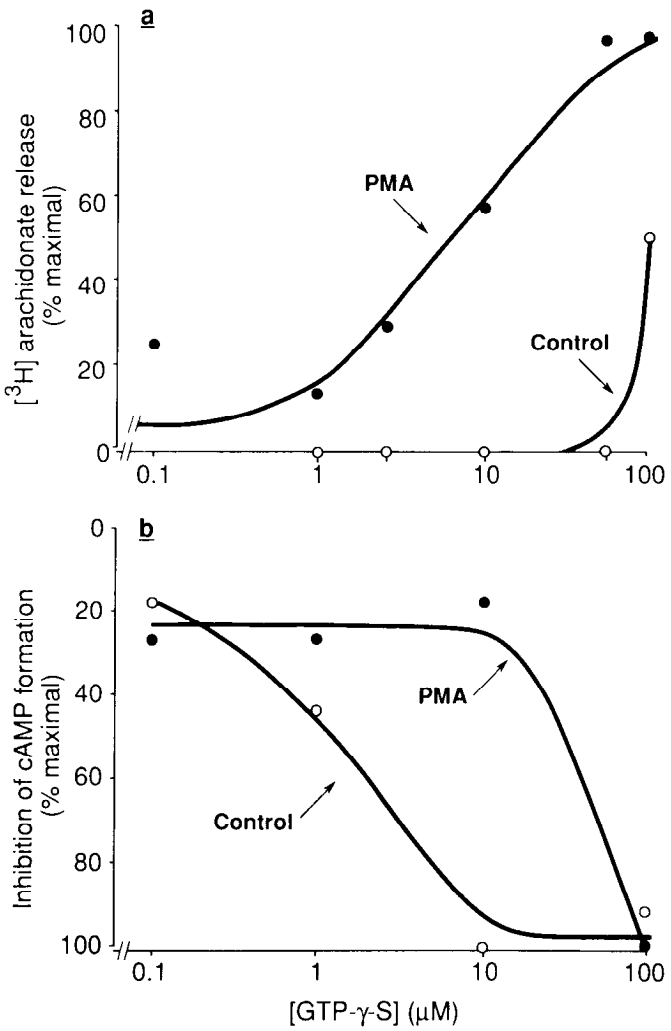


Figure 4. Effects of PMA on the sensitivity of D_2 receptor–dependent responses to GTP-γ-S in permeabilized CHO(D_2) cells. *a*, PMA enhances the sensitivity to GTP-γ-S of D_2-dependent facilitation of ^{3}H-AA release. Cells were labeled by incubation with ^{3}H-AA, and incubated (30 min) in a permeabilizing medium containing saponin (50 μg/ml), plus final concentrations of dopamine (1 μM) and GTP-γ-S (0–100 μM) in the absence (*open circles*) or in the presence (*solid circles*) of PMA (50 nM). Results are from one experiment performed in triplicate (representative of six) and are expressed as percentage of maximal ^{3}H-AA release after subtraction of Ca^{2+}-independent release (determined in the presence of 5 mM EGTA). In the experiment shown, maximal ^{3}H-AA release (obtained in the presence of dopamine, PMA, plus 50 μM GTP-γ-S) was, before background subtraction, 1390 ± 35 cpm/well. Background release (measured in the presence of 5 mM EGTA) was 1107 ± 51 cpm/well. *b*, PMA decreases the sensitivity to GTP-γ-S of D_2-mediated inhibition of cAMP accumulation. Cells were incubated for 20 min in a permeabilizing medium containing saponin plus final concentrations of dopamine (1 μM) and GTP-γ-S (0–100 μM), in the absence (*open circles*) or in the presence (*solid circles*) of PMA (100 nM). IBMX (0.1 mM) and forskolin (10 μM) were added to all incubations. Results are from one experiment performed in triplicate (representative of three) and are expressed as percentage of maximal inhibition of cAMP formation. Maximal inhibition (obtained in the presence of dopamine plus 100 μM GTP-γ-S) was 60% of forskolin alone, which produced a four- to sixfold increase in cAMP levels. In control cells, measured cAMP levels were 2.4–6.3 fmol/well, and in the presence of forskolin, 9–31.7 fmol/well.

the protein kinase include the D_2 receptor itself, which contains a consensus sequence for PKC phosphorylation (Bunzow et al., 1988); the transducing G-protein, which may be a PKC substrate, as shown for G_{i2} and G_z (Bushfield et al., 1990; Lounsbury et al., 1991); or the G-protein–regulating protein phosducin (Bauer et al., 1992).

PKC regulates D_2 receptor–dependent inhibition of cAMP accumulation

We find that stimulation of PKC reduces the inhibitory action of D_2 receptor agonists on adenylyl cyclase activity, and that this effect is accompanied by a decreased ability of GTP-γ-S to inhibit the cyclase in permeabilized cells. The findings suggest that PKC decreases the GTP sensitivity of the G_i subunit linked to adenylyl cyclase, producing, in turn, a weakened coupling of D_2 receptors with this effector system. In agreement with this possibility, phorbol esters were previously found to reduce receptor-operated inhibition of adenylyl cyclase in platelets and hepatocytes, to stimulate the phosphorylation of αG_{i2}, and to reduce its sensitivity to GTP analogs (Jakobs et al., 1985; Katada et al., 1985; Bushfield et al., 1990). A similar mechanism may be operative in CHO cells. If this is the case, it will be interesting to examine whether PKC-mediated phosphorylation of αG_{i2} may also play a role in the enhancement of $cPLA_2$ activity produced by D_2 receptor stimulation. Following phosphorylation of αG_{i2}, a greater number of D_2 receptors, which are thought to be preferentially coupled to this subunit (Senogles et al., 1990), might become free to interact with a distinct, $cPLA_2$-linked G-protein, whose existence was suggested by the work of Felder et al. (1991). Alternatively, phosphorylated αG_{i2} may interact better with $cPLA_2$ than with adenylyl cyclase. In either case, a single molecular event might affect, concomitantly and in opposite directions, the D_2-dependent regulation of both adenylyl cyclase and $cPLA_2$ activities. This intriguing possibility may find indirect support in two observations: (1) the identical potencies of PMA in enhancing $cPLA_2$ potentiation and in decreasing adenylyl cyclase inhibition and (2) the quantitatively similar, but opposing shifts produced by PMA on the sensitivities of D_2-dependent responses to GTP-γ-S.

Such a mechanism may also provide a possible explanation for the inability of PMA to affect the GTP-γ-S modulation of dopamine binding. Conceivably, no effect of PMA on this binding parameter should be seen if the linkage of D_2 receptors with G_{i2} is not affected, or if this linkage is switched onto another G-protein. A corollary to this mechanism is that PMA-induced modifications in adenylyl cyclase and $cPLA_2$ responses should be correlated: any given change produced on the modulation of $cPLA_2$ activity should result from a corresponding change in adenylyl cyclase. However, this prediction is not supported by the results obtained with CHO($D_{2(415)}$) cells, which show that activation of PKC may enhance D_2-dependent facilitation of $cPLA_2$ markedly, even when the adenylyl cyclase response is only slightly affected (Fig. 3).

Alternative hypothetical models may implicate multiple sites of action for PKC, for example, the concomitant phosphorylation of αG_{i2}, leading to decreased cyclase inhibition, and of phosducin, leading to desinhibition of other G-proteins (Bauer et al., 1992). An essential step to allow the testing of these different possibilities will be to determine the nature of the G-protein involved in mediating the actions of D_2 receptors on $cPLA_2$ activity.

Possible physiological function

Although the relevance of our findings to the CNS is not yet known, several lines of evidence suggest that coupling mechanisms similar to those observed in transfected CHO cells may be found in cells constitutively expressing D_2 receptors, including neurons. G_i-mediated cyclase inhibition is a well-established response produced by D_2 receptor occupation in various brain areas (Schwartz et al., 1992). Likewise, stimulation of D_2 receptors was shown to enhance release and metabolism of AA in neural (Hillier et al., 1976) and kidney cells (Huo et al., 1991), while activation of this signaling pathway may participate in certain neuronal D_2 responses, such as stimulation of somatostatin release from hypothalamic nerve terminals (Junier et al., 1990).

AA and its metabolites are thought to serve important functions in the nervous system, where they may act both as intracellular second messengers and as local mediators, regulating K^+ channel opening (Piomelli et al., 1987a,b; Belardetti et al., 1989; Schweitzer et al., 1990), protein kinase activity (McPhail et al., 1984; Piomelli et al., 1989), neurotransmitter release (Linch and Voss, 1990; Freeman et al., 1991), and synaptic plasticity (for review, see Fazeli, 1992). The ability of PKC to amplify D_2 receptor signaling via AA release at the expenses of cAMP inhibition may result in the recruitment of a distinct spectrum of biologically active mediators in cells in which PKC activity has been elevated. Through such a mechanism, the physiological responses to dopamine could be regulated dynamically within a neuron by stimuli promoting PKC activation, which include membrane depolarization and Ca^{2+}-mobilizing neurotransmitters (e.g., glutamate acting at metabotropic receptors) (Schoepp et al., 1990).

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