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Journal

Journal of neurochemistry, 64(6)

ISSN

0022-3042

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Publication Date

1995-06-01

DOI

10.1046/j.1471-4159.1995.64062765.x

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Dopamine D₂ Receptors Potentiate Arachidonate Release via Activation of Cytosolic, Arachidonate-Specific Phospholipase A₂

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Abstract: Several G_i-linked neurotransmitter receptors, including dopamine D₂ receptors, act synergistically with Ca²⁺-mobilizing stimuli to potentiate release of arachidonic acid (AA) from membrane phospholipids. In brain, AA and its metabolites are thought to act as intracellular second messengers, suggesting that receptor-dependent potentiation of AA release may participate in neuronal transmembrane signaling. To study the molecular mechanisms underlying this modulatory response, we have now used Chinese hamster ovary cells transfected with rat D₂-receptor cDNA, CHO(D₂). Two antisense oligodeoxynucleotides corresponding to distinct cDNA sequences of cytosolic, AA-specific phospholipase A₂ (cPLA₂) were synthesized and added to cultures of CHO(D₂) cells. Incubation with antisense oligodeoxynucleotides inhibited D₂ receptor-dependent release of AA but had no effect on D₂-receptor binding or D₂ inhibition of cyclic AMP accumulation. In addition, pharmacological experiments showed that D₂ receptor-dependent AA release was prevented by nonselective phospholipase inhibitors (such as mepacrine) but not by inhibitors of membrane-bound, non-AA-specific PLA₂ (such as *p*-bromophenacyl bromide). cPLA₂ is expressed in brain tissue. The results, showing that cPLA₂ participates in receptor-dependent potentiation of AA release in CHO(D₂) cells, suggest that this phospholipase may serve a similar signaling function in brain. **Key Words:** Chinese hamster ovary cells—Transfected cells—Second messengers—G protein-linked receptors. *J. Neurochem.* **64**, 2765–2772 (1995).

Several neurotransmitters evoke the receptor-dependent hydrolysis of membrane phospholipids and the release of free arachidonic acid (AA) from neurons and astrocytes. In many cases, this reaction is thought to occur through the activation of phospholipase A₂ (PLA₂), a family of structurally heterogeneous lipases that catalyze the hydrolytic cleavage of glycerophospholipid at the *sn*-2 position (where AA is most often esterified) yielding free fatty acid and lysophospholipid (for review, see Piomelli, 1993).

Based on their molecular structure, subcellular distribution, and phospholipid selectivity, members of the PLA₂ family may be divided into two groups, high molecular weight, cytosolic PLA₂ and low molecular weight, membrane-bound (secretory) PLA₂ (Glaser et al., 1993; Mayer and Marshall, 1993). A cytosolic PLA₂ with an apparent molecular mass of 100–110 kDa (by sodium dodecyl sulfate–polyacrylamide gel electrophoresis) that selectively hydrolyzes AA-containing phospholipids has been purified from several sources, and a full-length cDNA encoding it has been isolated and sequenced (Clark et al., 1990, 1991). In vitro, the activity of this PLA₂, termed cPLA₂, is stimulated by free Ca²⁺ at concentrations (0.1–1 μ M) that are likely to be reached when Ca²⁺-mobilizing receptors are stimulated in intact cells, suggesting that this activity may participate in receptor-dependent AA release (Clark et al., 1991). In support of this possibility, it was shown that when Chinese hamster ovary (CHO) cells overexpressing transfected cPLA₂ were stimulated with extracellular ATP, an agonist of P₂ receptors, release of AA was enhanced if compared with wild-type CHO cells (Clark et al., 1992). In contrast with cPLA₂, low molecular weight PLA₂ (14–18 kDa) are either secretory or membrane-bound, hydrolyze phospholipids with lesser degree of selectivity, and are activated in the presence of millimolar concentrations (Waite et al., 1991). Their possible participation in receptor-stimulated release of AA and of other fatty acids has also been suggested (Pernas et al., 1991).

A group of G_i protein-coupled neurotransmitter re-

Received September 1, 1994; revised manuscript received November 29, 1994; accepted December 5, 1994.

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Abbreviations used: AA, arachidonic acid; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; ODN, deoxyoligonucleotide; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D; RT, reverse transcriptase.

ceptors, including D₂-dopaminergic, α_2 -adrenergic, and m₂- and m₄-muscarinic receptors, act synergistically with Ca²⁺-mobilizing stimuli to produce AA release. For example, in transfected CHO cells labeled by incubation with [³H]AA, stimulation of D₂ receptors potentiates the release of [³H]AA evoked by application of ATP or Ca²⁺ ionophores but has no effect on basal [³H]AA release (Felder et al., 1991; Piomelli et al., 1991). Like adenylyl cyclase inhibition, this response is likely to involve a protein of the G_i/G_o family, as suggested by its sensitivity to pertussis toxin, and by the ability of GTP- γ -S, a nonhydrolyzable GTP analogue to mimic it (Piomelli et al., 1991; Di Marzo et al., 1993).

Receptor-dependent potentiations of AA release similar to those seen in transfected CHO cells have been demonstrated in neurons and astrocytes (Marin et al., 1991; Schinelli et al., 1994). Despite its potentially important role in signal transduction, however, the molecular mechanism underlying receptor-dependent potentiation of AA release is still poorly understood. In the present study, we used CHO cells transfected with rat D₂-receptor cDNA, CHO(D₂), to examine the possible participation of AA-specific, high molecular weight cPLA₂ in this facilitatory response. We report that antisense deoxyoligonucleotides (ODNs) directed against specific sequences of cPLA₂ inhibit D₂ receptor-dependent potentiation of AA release, without affecting inhibition of adenylyl cyclase activity or other second messenger pathways. The results, showing that cPLA₂ may mediate a signal transduction pathway activated by D₂ receptors, support a role for this phospholipase in brain signaling.

EXPERIMENTAL PROCEDURES

Cell cultures

CHO cells were transfected with rat D₂₍₄₄₄₎ receptor (also termed D_{2L}) cDNA as described previously (Giros et al., 1989). 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% CO₂. CHO(D₂) cells expressed 1.3×10^5 D₂ receptors/cell.

Receptor binding

CHO(D₂) 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, intact cells were collected by centrifugation, resuspended in DMEM, and binding measurements were performed using [¹²⁵I]-sulpride (Amersham) as described (Martres et al., 1984). Curves were analyzed by nonlinear regression.

RNA isolation, and analyses by northern blot and reverse transcriptase (RT)/PCR

RNA was isolated from CHO(D₂) cells by lysing the cells in 5 M guanidium isothiocyanate, followed by centrifugation in a cesium chloride gradient. Poly(A)⁺ RNA was prepared from total cellular RNA by fractionation over an oligo(dT) column (GIBCO-BRL). A sample of poly(A)⁺ RNA (4

μ g) was then subjected to gel electrophoresis on agarose (1%) containing formaldehyde (5%) and transferred onto nitrocellulose membranes. Blots were hybridized with a cDNA probe corresponding to amino acid residues 218–749 of human cPLA₂ (Clark et al., 1991), prepared by cutting a human cPLA₂ expression vector with *Eco*RI (kindly provided by Dr. L. L. Lin, Genetics Institute), and labeled by nick-translation. Samples of total cellular RNA (20 μ g), prepared as described (Chomczynski and Sacchi, 1987) were subjected to RT/PCR according to standard procedures, using the following primers: (1) 5'-TTC TTC ATT AAT CTC CTC TGG C-3'; (2) 5'-CCT AAT CAG GAA AAT GTT TTG G-3'. An additional amplification of the RT/PCR products was performed using the following primers: (1) 5'-TTG GAG ATT ACG TTA ATG GAT-3'; (2) 5'-TTT CTC TGG AAA ATC AGG GTG A-3'. PCR products were subjected to agarose gel (1.7%) electrophoresis, transferred onto nylon membranes, and hybridized with an ODN probe (5'-C AAC GTA GGT AGC ACA ATC CAG AAT TCC GTA TAA TGC CTT-3'), labeled by terminal transferase reaction.

Treatment with ODNs

All ODNs were prepared by solid-phase synthesis using a DNA synthesizer (Applied Biosystems). After overnight deprotection with ammonium hydroxide (30%) at 65°C, the ODNs were brought to dryness by centrifugation under reduced pressure, redissolved in sterile water to give a final concentration of 1 mM, and stored at –20°C until used. The following ODNs were used: antisense cPLA₂-1, 5'-CAT TTT GGT TTC AAT TAC AGG-3'; sense cPLA₂-1, 5'-CCT GTA ATT GAA ACC AAA ATG-3'; missense cPLA₂-1, 5'-GTA ACC CCT ATG AAA GAA ATT-3'; antisense cPLA₂-2, 5'-TTC TTC ATT CTC CTC TGG C-3'; sense cPLA₂-2, 5'-G CCA GAG GAG ATT GAA GAA-3'; missense cPLA₂-2, 5'-ATC CCC GTT TTT TCT TCG C-3'. CHO(D₂) cells were washed and incubated in DMEM for 1 h at 37°C, before exposing them for 24 h to DMEM containing final concentrations of the ODNs.

[³H]AA release

After washing residual ODNs, cells (24-well plates) were labeled by incubation with [³H]AA (Amersham; 200–220 Ci/mmol, 0.25 μ Ci/ml) in DMEM (1 ml) containing 0.2% fatty acid-free bovine serum albumin (BSA) for 2 h 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. [³H]AA release was determined by liquid scintillation counting of samples (0.5 ml) of the incubation medium.

Cyclic AMP accumulation

ODN-containing medium was removed, and cells (96-well plates) were incubated for 10 min in 0.15 ml of DMEM containing isobutylmethylxanthine (0.1 mM) plus forskolin (10 μ M) and final concentrations of drugs. After extraction in 0.1 M HCl (0.1 ml), sonication, and neutralization of the tissue extracts, cyclic AMP concentrations were determined using a radioimmunoassay kit (Amersham), following the manufacturer's instructions.

Intracellular Ca²⁺ measurements

After removing the ODN-containing medium, cells were suspended by treatment with trypsin (see above) and incubated for 1 h at 37°C in DMEM containing BSA (0.2%)

and fura-2 acetoxymethyl ester (1 μ M). Cells were washed, resuspended in HEPES-buffered Krebs solution (containing, in mmol/L, NaCl 125, KCl 5, KH₂PO₄, and MgSO₄ 1.2, CaCl₂ 2, glucose 6, HEPES-NaOH buffer, pH 7.4) and Ca²⁺ levels were determined using a Hitachi F-2000 fluorescence spectrophotometer.

[³H]Choline release

CHO(D₂) cells (24-well plates) were labeled overnight by incubation with [³H]choline (Amersham, 1 μ Ci/ml), in the presence of ODNs, washed, and incubated for 1 h at 37°C in DMEM containing choline (1 mM). After 30-min exposure to drugs, radioactivity was determined in samples (0.5 ml) of incubation medium. ODN treatment had no effect on [³H]choline incorporation into CHO(D₂) cells, as determined by measuring total radioactivity in cell extracts (data not shown).

[³H]AA uptake

Cells, labeled by incubation with [³H]AA as described above, were washed with Ca²⁺/Mg²⁺-free phosphate-buffered saline (GIBCO) and sonicated briefly in 0.25 ml of the same medium. Radioactivity was determined in samples (25 μ l) of cell extracts.

Materials

Ca²⁺ ionophore A23187, BSA, forskolin, isobutylmethylxanthine, *p*-bromophenacyl bromide, choline, and mepacrine were obtained from Sigma (France). Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR, U.S.A.), dimethyleicosadienoic acid and octadecylbenzoacrylic acid from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.), and quinpirole was a generous gift of Eli Lilly (Indianapolis, IN, U.S.A.).

RESULTS

An antisense ODN directed against cPLA₂ prevents ATP-stimulated [³H]AA release

In CHO cells, the stimulation of constitutive P₂-type purinergic receptors with extracellular ATP produces three prominent responses, activation of phosphoinositide-specific phospholipase C (PLC), elevation of intracellular Ca²⁺ levels, and release of free AA from phospholipids (Gupta et al., 1990). Evidence indicates that P₂ receptor-dependent AA release is mediated through the activation of cPLA₂. First, CHO(D₂) cells express constitutively an mRNA encoding for cPLA₂, as revealed by northern blot and RT/PCR analyses. Samples of poly(A)⁺ RNA from CHO(D₂) cells were subjected to agarose gel electrophoresis, transferred onto nitrocellulose, and incubated with a ³²P-labeled cDNA probe for human cPLA₂ (see Experimental Procedures). The probe hybridized with a transcript of the expected size (3.4 kb) (Fig. 1A). RT/PCR analysis, performed on samples of total RNA from CHO(D₂) cells, revealed the presence of a 0.5-kb product whose identity was confirmed by an additional PCR (Fig. 1B). Second, when cPLA₂ expression was enhanced in CHO cells by transfection with a cDNA encoding for human cPLA₂, extracellular ATP was much more potent in evoking AA release than in nontransfected

FIG. 1. Expression of an mRNA transcript encoding cPLA₂ in CHO(D₂) cells. **A:** Northern blot analysis of poly(A)⁺ RNA from CHO(D₂), RBL, and U937 cells in culture. **B:** Analysis by RT-PCR. Total RNA obtained from CHO(D₂) cells was subjected to (1) one or (2) two consecutive PCR with nested oligonucleotide primers (see Experimental Procedures for further details). Results are from one experiment typical of two (northern blot) or three (PCR).

cells, or in cells transfected with a secretory PLA₂ (Lin et al., 1992).

Antisense ODNs are effective in preventing expression of select target proteins, when they are either injected intracellularly or added to the medium of cells in culture (Sorscher et al., 1991; Wang et al., 1992; Holopainen and Wojcik, 1993; Wahlestedt et al., 1993). To determine whether antisense ODNs may inhibit expression of cPLA₂, CHO(D₂) cells were incubated for 24 h in culture medium containing one of three distinct 21-mer ODNs, termed antisense cPLA₂-1 (complementary to a 5'-untranslated sequence in human and rat cPLA₂ cDNA but including the ATG initiation codon), sense cPLA₂-1 (homologous to the same cDNA sequence), and missense cPLA₂-1 (random nucleotide sequence) (50 μ M, see Experimental Procedures). To limit degradation of the ODNs, the cells were maintained in serum-free medium during treatment and labeled with a short (2 h) incubation in the presence of [³H]AA.

Although these labeling conditions resulted in an incorporation of [³H]AA into CHO(D₂) lipids that was only 10% of that obtained with overnight labeling, the application of ATP (100 μ M) was found to produce a significant release of [³H]AA from cells that had been exposed to either sense cPLA₂-1 or missense cPLA₂-1 (Fig. 2). In the absence of any addition, quinpirole plus ATP produced on average a 400% increase of basal [³H]AA release (Piomelli et al., 1991). In contrast, the effect of ATP on [³H]AA release was completely abolished in cells treated with antisense cPLA₂-1 (Fig. 2).

The ability of antisense cPLA₂-1 to prevent ATP-induced release of [³H]AA may have resulted from a nonspecific action of this ODN leading to defective signaling at the P₂ receptor. To address this possibility, we examined the effect of ATP on intracellular Ca²⁺ levels in CHO(D₂) cells that had been exposed to

FIG. 2. Effects of antisense cPLA₂-1 on P₂-receptor signaling in CHO(D₂) cells. Treatment with antisense cPLA₂-1 inhibits ATP-induced release of [³H]AA. Cells were incubated for 24 h with antisense, sense, or missense cPLA₂-1 (50 μM), labeled by incubation with [³H]AA, and stimulated with ATP (100 μM) (open bars), or with ATP plus quinpirole (1 μM) (hatched bars). ATP and ATP plus quinpirole were less effective in producing [³H]AA release in antisense- than in sense- or missense-treated cells. Results are expressed as mean ± SEM values in four independent experiments. **p* < 0.05, ***p* < 0.01, by two-way analysis of variance. Values of basal [³H]AA release were for antisense-treated cells, 306 ± 10 cpm; for sense-treated cells, 314 ± 14 cpm; for missense-treated cells, 392 ± 17 cpm.

antisense, sense, or missense cPLA₂-1 (24 h, 50 μM). No difference was seen in the Ca²⁺ response to ATP (100 μM) after these treatments, suggesting that antisense cPLA₂-1 does not interfere either with the binding of ATP to the P₂ receptor or with receptor coupling to phosphoinositide-specific PLC and intracellular Ca²⁺ rises (Table 1).

The activities of arachidonoyl-CoA synthetase and arachidonoyl-CoA lysophospholipid transferase are necessary for the esterification of AA into phospholipid (Waku, 1992). An undesired effect of antisense cPLA₂-1 on these enzyme activities may affect phospholipid labeling with [³H]AA and result in diminished receptor-dependent [³H]AA release. To control for this possibility, we measured the uptake of [³H]AA

in ODN-treated CHO(D₂) cells. As shown in Table 1, no significant difference was seen in labeling among cells exposed to antisense, sense, or missense cPLA₂-1.

Antisense ODNs against cPLA₂ prevent D₂ receptor-dependent [³H]AA release

In CHO(D₂) cells, D₂-receptor agonists are potent in enhancing [³H]AA release, when such release is evoked by stimulating Ca²⁺-mobilizing receptors (such as P₂ receptors) or by applying a Ca²⁺ ionophore (Felder et al., 1991; Piomelli et al., 1991; Di Marzo et al., 1993). Although these results had suggested the participation of a PLA₂ activity in this response, the lack of selective PLA₂ inhibitors has hindered the characterization of this activity.

To examine a possible involvement of cPLA₂, we first studied the effect of antisense cPLA₂-1 on D₂-receptor potentiation of ATP-induced [³H]AA release. As shown in Fig. 2, incubation with antisense cPLA₂-1 markedly inhibited the release of [³H]AA produced by the application of ATP plus quinpirole (1 μM). In contrast, treatment with sense or missense ODNs had no effect. A similar inhibition of the response to quinpirole was obtained when [³H]AA release was evoked by applying the Ca²⁺ ionophore, A23187 (Fig. 3).

The inhibitory effect of antisense cPLA₂-1 did not result from a nonspecific action on D₂-receptor function. In support of this conclusion we found, first, that D₂-receptor binding was not affected by incubation with the antisense ODN. In two separate experiments, [¹²⁵I]-sulpride binding to intact CHO(D₂) cells was displaced by quinpirole with a K_D of 0.21 ± 0.02 nM in cells exposed to antisense cPLA₂-1, and 0.18 ± 0.05 nM in cells exposed to sense cPLA₂-1. Likewise, no significant difference in B_{max} was seen with the two ODNs (antisense, 1,606 ± 274 sites/cell; sense, 1,804

TABLE 1. Effects of synthetic ODNs on various signaling pathways in CHO(D₂) cells

	Antisense	Sense	Missense	n
Cyclic AMP (pmol/well)				
Control	13 ± 2	5 ± 2	5 ± 2	4
Forskolin	30 ± 6	29 ± 2	51 ± 8	4
Forskolin/quinpirole	13 ± 2	11 ± 4	8 ± 2	4
Ca ²⁺ (nM)				
Control	170 ± 30	135 ± 30	134 ± 49	9
ATP	578 ± 53	483 ± 92	566 ± 116	9
[³ H]Choline (cpm/well)				
Control	1,551 ± 106	1,850 ± 100	1,223 ± 99	8
A23187	3,297 ± 318	3,620 ± 218	2,477 ± 56	8
[³ H]AA uptake (cpm/well/h)	77,327 ± 4,067	65,695 ± 5,067	84,265 ± 6,725	12

CHO(D₂) cells were maintained in culture, stimulated, and analyzed as described in Experimental Procedures. When compared with sense-oriented or missense controls, treatment with anti-cPLA₂ antisense did not significantly inhibit forskolin-stimulated adenylyl cyclase activity, ATP-stimulated intracellular Ca²⁺ increases, Ca²⁺ ionophore-stimulated [³H]choline release, or [³H]AA uptake. Data are expressed as mean ± SEM values of n separate determinations performed in two to four independent experiments.

FIG. 3. Antisense cPLA₂-1 inhibits D₂ receptor-dependent potentiation of [³H]AA release. CHO(D₂) cells were stimulated with the Ca²⁺ ionophore A23187 (4 μ M) (open bars), or with a combination of A23187 plus quinpirole, a D₂-receptor agonist (1 μ M) (hatched bars). Results are expressed as mean $\pm$ SEM values of four separate experiments. * p < 0.05, by two-way analysis of variance. For values of basal [³H]AA release, see Fig. 1 legend.

$\pm$ 305 sites/cell). In addition, the ability of quinpirole to reduce forskolin-induced accumulation of cyclic AMP was not modified by treatment with any of the ODNs used (Table 1).

It is interesting that although antisense cPLA₂-1 inhibited the response to quinpirole, it did not completely abolish the release of [³H]AA induced by A23187 alone. Like other Ca²⁺ ionophores, A23187 activates in a nonspecific manner several Ca²⁺-dependent phospholipases whose activities may catalyze [³H]AA deacylation, including PLC and phospholipase D (PLD) (Piomelli, 1993).

Next, we examined the effect of an additional antisense ODN, named antisense cPLA₂-2, complementary to a translated sequence of cPLA₂ cDNA, which we used as a primer for RT/PCR analysis (see Experimental Procedures). The results, depicted in Fig. 4, show that treatment with antisense cPLA₂-2 (24 h, 50 μ M) decreased the release of [³H]AA produced by quinpirole plus A23187, without affecting the response to A23187 alone. Sense and missense cPLA₂-2 had no effect (Fig. 3).

FIG. 4. Antisense cPLA₂-2 inhibits D₂ receptor-dependent potentiation of [³H]AA release. CHO(D₂) cells were incubated with antisense, sense, or missense cPLA₂-2 (50 μ M) for 24 h, labeled by incubation with [³H]AA, and stimulated with A23187 (4 μ M) (open bars) or with A23187 plus quinpirole (1 μ M) (hatched bars). The results, expressed as mean $\pm$ SEM values, are from one experiment performed in quadruplicate. Similar results were obtained in an additional experiment (data not shown). Values of basal [³H]AA release were for antisense-treated cells, 380 $\pm$ 62 cpm; for sense-treated cells, 388 $\pm$ 16 cpm; for missense-treated cells, 455 $\pm$ 48 cpm.

FIG. 5. Effects of various PLA₂ inhibitors on [³H]AA release from CHO(D₂) cells. Cells were labeled by incubation with [³H]AA and stimulated with A23187 (4 μ M) (open bars) or with A23187 plus quinpirole (1 μ M) (hatched bars). Drugs used: Mep, mepacrine (50 μ M); ArisA, aristolochic acid (50 μ M); DEDA, dimethyleicosadienoic acid (60 μ M); OBAA, octadecylbenzoic acid (15 μ M); BPB, *p*-bromophenacyl bromide (20 μ M).

Pharmacological blockade of cPLA₂

Several alkylating agents that inhibit secretory PLA₂ by modifying covalently the enzyme at its active site have little or no effect on cPLA₂ activity. For example, the histidine reagent, *p*-bromophenacyl bromide, which is potent in inhibiting the activity of secretory PLA₂, is ineffective on purified cPLA₂ (Mayer and Marshall, 1993). In contrast, mepacrine, which interferes with the availability of phospholipid substrate, acts on cPLA₂ with an efficacy comparable with that observed with secretory lipases (Chang et al., 1987; Xin and Mattera, 1992).

We examined the effects of a series of chemically unrelated PLA₂ inhibitors on the release of [³H]AA from CHO(D₂) cells, stimulated either with A23187 or with a combination of A23187 and quinpirole (Fig. 5). [³H]AA release was prevented most effectively by mepacrine (50 μ M), and only partially reduced by aristolochic acid, dimethyleicosadienoic acid, and octadecylbenzoic acid. *p*-Bromophenacyl bromide augmented rather than inhibited [³H]AA release from CHO(D₂) cells (Fig. 4), possibly by interfering with reuptake of [³H]AA into cell lipids.

DISCUSSION

The experiments described above suggest that, in CHO(D₂) cells, the facilitation of Ca²⁺-evoked [³H]AA release produced by stimulating dopamine D₂ receptors results from activation of AA-specific, high molecular weight cPLA₂. Three observations support this conclusion. First, cPLA₂ is constitutively expressed in CHO(D₂) cells, as indicated by northern blot and RT/PCR detection of the mRNA coding for this protein. Next, incubation of CHO(D₂) cells with two distinct antisense ODNs directed against cPLA₂ markedly reduced D₂ receptor-dependent potentiation of [³H]AA release. Finally, the response was prevented by mepacrine, a nonselective PLA₂ blocker, but not by *p*-bromophenacyl bromide, a drug that inhibits membrane-bound PLA₂ selectively over cPLA₂.

As the conclusion of the present study relies mainly upon results obtained in knockout experiments with antisense ODNs, it will be important to examine, at the outset, the efficacy and selectivity of these agents. Next, we will discuss the potential significance of the results for signal transduction in the CNS.

Efficacy and selectivity of antisense ODNs against cPLA₂

Synthetic ODNs in antisense orientation, i.e., designed to hybridize to complementary sequences of mRNA, are widely used as inhibitors of translation in intact cells. Their inhibitory effects are thought to result either from translational block, by interference with ribosomal processing, or from induced degradation of target mRNA, by stimulation of ribonuclease H activity, which cleaves the RNA moiety of RNA/DNA hybrids (for review, see Colman, 1990; Hélène and Toulmé, 1990).

In cells in culture, two routes of ODN administration have been used successfully, i.e., intracellular injection and addition to the culturing medium. Intracellular injection offers the advantage of a quantitative delivery of the intact ODN to its site of action, limiting the losses associated with incomplete membrane permeation and cellular degradation (Kleuss et al., 1991). Unfortunately, this technique is not suitable for many biochemical determinations in which the quantity of cells is a limiting factor. In such cases, long-term incubation of cells in a medium containing relatively high ODN concentrations may represent a feasible alternative approach, provided that appropriate controls for possible nonspecific effects of the ODN are performed (Sorscher et al., 1991; Wang et al., 1992; Holopainen and Wojcik, 1993).

In the present study, CHO(D₂) cells were incubated for 24 h in serum-free medium containing different ODNs at concentrations up to 50 μ M. Under these conditions, the release of [³H]AA induced by stimulating D₂ receptors in the presence of ATP was found to be inhibited. To determine the selectivity of this effect, the following control experiments were performed: (1) the ODN antisense cPLA₂-1, directed against a 5' untranslated sequence of human and rat cPLA₂ cDNA sequence (Clark et al., 1991), prevented ATP-induced [³H]AA release, mediated by cPLA₂ (Lin et al., 1992), but had no effect on ATP-induced elevation of intracellular Ca²⁺ levels (Gupta et al., 1990); (2) neither a sense-oriented ODN directed against the same cPLA₂ sequence nor a missense ODN (random nucleotide sequence) had such effect; (3) treatment with antisense cPLA₂-1 did not affect D₂ receptor binding, inhibition of forskolin-stimulated cyclic AMP accumulation, [³H]AA incorporation into lipid or Ca²⁺ ionophore-induced [³H]choline release (a measure of PLC and PLD activities); (4) an additional ODN, antisense cPLA₂-2, designed to hybridize to nucleotides 741–763 of cPLA₂, was also effective in reducing D₂ receptor-dependent [³H]AA release, whereas its

sense homologue and an additional missense ODN were not. Together, the results suggest strongly that the antisense ODNs used in this study exert their actions on [³H]AA release through a selective inhibition of cPLA₂ expression.

Possible signaling functions of cPLA₂ in the brain

Several forms of receptor-dependent regulation of AA release have been described (see, for review, Piomelli, 1993). Some neurotransmitter receptors are positively coupled to AA release. For example, glutamate acting at *N*-methyl-D-aspartate receptors evokes AA release from a variety of neuronal cell types (Dumuis et al., 1988; Lazarewicz et al., 1988; Sanfeliu et al., 1990). Other receptors may be linked to the inhibition of AA release. For example, in transfected CHO cells, activation of rat H₂ receptors prevents the release of AA induced by the Ca²⁺ ionophore A23187 (Traiffort et al., 1992). A third group of receptors facilitates the release of AA evoked by stimuli (neurotransmitters or drugs) that elevate intracellular Ca²⁺ levels. This permissive effect has been demonstrated in CHO cells transfected with G_i-coupled receptors, such as D₂-dopaminergic, α_2 -adrenergic, m₂- and m₄-muscarinic, and 5-HT_{1A} serotonin receptors (Felder et al., 1991; Piomelli et al., 1991; Raymond et al., 1992). A G_i/G_o protein may participate in transducing this effect, as indicated by the ability of pertussis toxin to inhibit the response and of a nonhydrolyzable GTP analogue, GTP- γ -S, to mimic it (Piomelli et al., 1991; Di Marzo et al., 1993). Although the precise molecular mechanism underlying the potentiating effect of D₂ receptors on AA release remains unknown, two hypotheses are compatible with the available experimental evidence. G_i-coupled receptors, such as the D₂ receptor, may activate a "permissive" α G_i-protein that would enhance, but not directly evoke, cPLA₂ activity. Alternatively, G_i-coupled receptors may release "free" β - γ subunits, which have been reported to stimulate PLA₂ activity and AA release (Jelsema and Axelrod, 1987).

Permissive actions of G_i-coupled receptors on AA release have been shown to occur in neural cells. In primary cultures of striatal neurons, the release of AA evoked by applications of ATP or A23187 may be enhanced by addition of D₂-receptor agonists. In contrast, these drugs have no effect when applied alone to the neurons (Schinelli et al., 1994). Furthermore, in striatal astrocytes, AA release may be evoked by the combined application of somatostatin, a neuropeptide, and methoxamine, an α_1 -adrenergic agonist, drugs that have no effect when applied alone (Marin et al., 1991).

Despite its potentially important role in intracellular signaling, the molecular mechanism underlying receptor-dependent facilitation of AA release remains unknown. Central to the understanding of such mechanism is the unequivocal identification of the phospholipase activity involved. This identification is made difficult, however, by the existence of multiple path-

ways of AA release and by the lack of selective enzyme inhibitors for each of these pathways. At least three esterase activities participate in deacylating AA-containing phospholipids, PLA₂, PLC, and PLD (Piomelli, 1993). In addition, most tissues, including the brain, express a variety of PLA₂ activities distinguished by their different subcellular distribution and regulatory properties (Woelk et al., 1974; Gray and Strickland, 1982a,b; Yoshihara and Watanabe, 1990; Hirashima et al., 1992).

The results reported in this study, obtained in a heterologous expression system, indicate that a single PLA₂ isoform, AA-selective cPLA₂, may mediate, on the one hand, receptor-stimulated AA release (Lin et al., 1992), and participate, on the other, in receptor-dependent facilitation of such release. Expression of cPLA₂ in brain tissue (Yoshihara and Watanabe, 1990; Hirashima et al., 1992) supports a similar role in neural cells.

Acknowledgment: This study was supported in part by a Young Investigator Award from the National Alliance for Research on Schizophrenia and Depression (NARSAD). We are grateful to Dr. L. Lin for providing us with cPLA₂ plasmid.

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