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

Daniel Vial and Daniele Piomelli

Unité de Neurobiologie et Pharmacologie de l’INSERM, Paris, France

Abstract: Several G₁-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 μM) 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 cDNA were stimulated with extracellular ATP, an agonist at purinergic P₂ receptors, release of AA was enhanced compared with wild-type CHO cells (Pernas et al., 1992). In contrast with cPLA₂, low molecular weight forms of PLA₂ (14–18 kDa) are either secreted or membrane-bound, hydrolyze phospholipids with a lesser degree of selectivity, and are activated in vivo by free Ca²⁺ at millimolar concentrations (Waite et al., 1997). 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 protein–coupled neurotransmitter re-
receptors, including D₂-dopaminergic, α₂-adrenergic, and m₃- 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 adenyl cyclase inhibition, this response is likely to involve a protein of the G₁/G₃ 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 cPLAZ, in this facilitatory response. We report that antisense deoxyoligonucleotides (ODNs) directed against specific sequences of cPLAZ inhibit D₂ receptor-dependent potentiation of AA release, without affecting inhibition of adenyl cyclase activity or other second messenger pathways. The results, showing that cPLAZ 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 D₂,4 receptors (also termed D₂,4 receptors) 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 1.5 × 10⁶ 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 [³H]AA (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 μg) 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 cPLAZ (Clark et al., 1991), prepared by cutting a human cPLAZ expression vector with EcoRI (kindly provided by Dr. L. L. Lin, Genetics Institute), and labeled by nick-translation. Samples of total cellular RNA (20 μg), prepared as described (Chomczynski and Sacchi, 1987), were subjected to RT/PCR according to standard procedures, using the following primers: (1) 5'-TTT GCT GCC ACC AAA ATG GC-3'; (2) 5'-TCT 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'-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 ammonia hydroxide (10%) 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 cPLAZ-1, 5'-CAT TTT GGT TTT CTA ACC TTG TTT GTA ATT AAC AGG TTA ATG GAT-3'; sense cPLAZ-1, 5'-CCT GTA ATT GAA ACC AAA ATG TTT G-3'; antisense cPLAZ-2, 5'-TTC TTC ATT CTC CTCTGG C-3'; sense cPLAZ-1, 5'-TTC TTC ATT CTC CTC TTC CTG C-3'; antisense cPLAZ-1, 5'-TTC TTC ATT CTC CTC TTC CTG C-3'; sense cPLAZ-2, 5'-G CCA GAG GAG ATT GAA AAG-3'; sense cPLAZ-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 μCi/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 μM) 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%)
for human cPLA2, extracellular ATP was much more potent in evoking AA release than in nontransfected CHO cells, revealed the presence of a 0.5-kb product whose 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 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 μM, 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 μM) 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, quisinrole plus ATP produced on average a 400% increase in incorporation of [3H]AA into CHO(D2) lipids that had been exposed to either sense cPLA2-1 or missense cPLA2-1 (Fig. 2).

The ability of sense 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 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 intracellurally or added to the medium of cells in culture (Sorscher et al., 1991; Wang et al., 1992; Holopainen and Wojcik, 1993; Wahlstedt 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 μM, 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.

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 same cDNA sequence, and the presence of a 0.5-kb product whose identity was confirmed by an additional PCR (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 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 human cPLA2, extracellular ATP was much more potent in evoking AA release than in nontransfected 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 intracellurally or added to the medium of cells in culture (Sorscher et al., 1991; Wang et al., 1992; Holopainen and Wojcik, 1993; Wahlstedt 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 μM, 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.

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 same cDNA sequence, and the presence of a 0.5-kb product whose identity was confirmed by an additional PCR (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 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 human cPLA2, extracellular ATP was much more potent in evoking AA release than in nontransfected 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 intracellurally or added to the medium of cells in culture (Sorscher et al., 1991; Wang et al., 1992; Holopainen and Wojcik, 1993; Wahlstedt 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 μM, 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.

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 same cDNA sequence, and the presence of a 0.5-kb product whose identity was confirmed by an additional PCR (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 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 human cPLA2, extracellular ATP was much more potent in evoking AA release than in nontransfected 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 intracellurally or added to the medium of cells in culture (Sorscher et al., 1991; Wang et al., 1992; Holopainen and Wojcik, 1993; Wahlstedt 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 μM, 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 μM) 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, quisinrole 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 nM), labeled by incubation with [3H]AA, and stimulated with ATP (100 nM) (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 Kd 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 Bmax was seen with the two ODNs (antisense, 1,606 ± 274 sites/cell; sense, 1,804 sites/cell).

| 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  |
| ATP                              | 170 ± 30 | 135 ± 30 | 134 ± 49 | 9  |
| [3H]Choline (cpm/well)          | 578 ± 53 | 483 ± 92 | 566 ± 116 | 9  |
| Control                         | 1,551 ± 106 | 1,850 ± 100 | 1,223 ± 99 | 8  |
| A23187                           | 3,297 ± 318 | 3,620 ± 218 | 2,477 ± 56 | 8  |
| [3H]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 μM) (open bars), or with a combination of A23187 plus quinpirole, a D₂-receptor agonist (1 μM) (hatched bars). Results are expressed as mean ± 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.

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 μM) 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).

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 μM), and only partially reduced by aristolochic acid, dimethylleicosadienoic acid, and octadecylbenzoyl acetic 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 μM. 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 Gc-coupled receptors, such as D2 dopaminergic, α2-adrenergic, m1- and m3-muscarinic, and 5-HT1A serotonin receptors (Felder et al., 1991; Piomelli et al., 1991; Raymond et al., 1992). A Gc 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 potentiation effect of D2 receptors on AA release remains unknown, two hypotheses are compatible with the available experimental evidence. Gc-coupled receptors, such as the D2 receptor, may activate a “permissive” αG protein that would enhance, but not directly evoke, cPLA2 activity. Alternatively, Gc-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 Gc-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 α2-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 isozyme, AA-selective cPLA₂-a, 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.

REFERENCES

Chang J., Musser J. H., and McGregor H. (1987) Phospholipase A₂: function and pharmacological regulation. Biochem. Pharmacol. 36, 2429–2436.

Chomczynski P. and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Clark J. D., Milona N., and Knopf J. L. (1991) Purification of a 110-kilodalton cytosolic phospholipase A₂ from the human monocytic cell line U937. Proc. Natl. Acad. Sci. USA 87, 7708–7712.

Clark J. D., Lin L.-L., Kriz R. W., Ruedena C. S., Sultzman L. A., Lin A. Y., Milona N., and Knopf J. L. (1991) A novel, arachidonate-selective cytosolic phospholipase A₂ contains a Ca²⁺-dependent translocation domain with homology to PKC and GAP. Cell 65, 1043–1051.

Colman A. (1990) Antisense strategies in cell and developmental biology. J. Cell. Biol. 97, 399–409.

Di Marzo V., Vial D., Sokoloff P., Schwartz J. C., and Piomelli D. (1993) Selection of alternative G₂-mediated signaling pathways at the dopamine D₂ receptor by protein kinase C. J. Neurosci. 13, 4846–4853.

Dunna A., Sebben M., Haynes L., Pin J. P., and Bockaert J. (1988) NMDA receptors activate the arachidonic acid cascade system in striatal neurons. Nature 336, 68–70.

Farooqui A. A., Rammohan K. W., and Horrocks L. A. (1989) Isolation, characterization and regulation of diacylglycerol lipases from the bovine brain. Ann. NY Acad. Sci. 559, 25–36.

Felder C. C., Williams H. L., and Axelrod J. (1991) A transduction pathway associated with receptors coupled to the inhibitory guanine nucleotide binding protein G that amplifies ATP-mediated arachidonic acid release. Proc. Natl. Acad. Sci. USA 88, 6477–6480.

Gray N. C. C. and Strickland K. P. (1982a) The purification and characterization of a phospholipase A₂ activity from the 106,000 g pellet (microsomal fraction) of bovine brain acting on phosphatidylcholine. Can. J. Biochem. 60, 108–117.

Gray N. C. C. and Strickland K. P. (1982b) The specificity of phospholipase A₂ purified from the 106,000 g pellet of bovine brain. Lipids 17, 91–96.

Gronich J. H., Bonventre J. V., and Nemenoff R. A. (1990) Purification of a high molecular mass form of phospholipase A₂ from rat kidney activated at physiological Ca²⁺ concentrations. Biochim. Biophys. Acta 1049, 99–125.

Hirashima Y., Farooqui A. A., Mills J. S., and Horrocks L. A. (1992) Identification and purification of calcium-independent phospholipase A₂ from bovine brain cytosol. J. Neurochem. 59, 708–714.

Holopainen I. and Wojcik W. J. (1993) A specific antisense oligodeoxynucleotide to mRNAs encoding receptors with seven transmembrane spanning regions decreases muscarinic m₁ and γ-aminobutyric acid receptors in rat cerebellar granule cells. J. Pharmacol. Exp. Ther. 264, 423–429.

Jehena C. L. and Axelrod J. (1987) Stimulation of phospholipase A₂ activity in bovine rod outer segment by the βγ subunit of transducin and its inhibition by the α subunit. Proc. Natl. Acad. Sci. USA 84, 3625–3630.

Kleuss C., Hescheler J., Ewel C., Rosenthal W., Schultz G., and Witting B. (1991) Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature 353, 45–48.

Lazarowski J. W., Wrobleski J. T., Palmer M. E., and Costa E. (1988) Activation of N-methyl-D-aspartate-sensitive glutamate receptor stimulates arachidonic acid release in primary cultures of cerebellar granule cells. Neuropharmacology 27, 765–769.

Lin L. L., Lin A. Y., and Knopf J. L. (1992) Cytosolic phospholipase A₂ is coupled to hormonally regulated release of arachidonic acid. Proc. Natl. Acad. Sci. USA 89, 6147–6151.

Marin P., Delumeau J. C., Tence M., Cordier J., Govinski J., and Prémont J. J. (1991) Somatostatin potentiates the α₁-adrenergic activation of phospholipase C in striatal astrocytes through a mechanism involving arachidonic acid and glutamate. Proc. Natl. Acad. Sci. USA 88, 9016–9020.

Martess P. M., Sokoloff P., Delandre M., Schwartz J. C., Protass P., and Contentin J. (1984) Selection of dopamine antagonists discriminating various behavioral responses and radioligand binding sites. Synaps. Schmiedeb. Arch. Pharmak. 325, 102–115.

Mayer R. J. and Marshall L. A. (1993) New insights on mammalian phospholipase A₂; comparison of arachidonoyl-selective and non-selective enzymes. FASEB J. 7, 339–348.

Moskowitz N., Puszkin S., and Schook W. (1983) Characterization of a high-molecular mass form of phospholipase A₂ from the 106,000 g pellet (microsomal fraction) of bovine brain acting on phosphatidylcholine. Can. J. Biochem. 60, 108–117.

Piomelli D. (1993) Eicosanoids in synaptic transmission. Crit. Rev. Neurob. 8, 65–83.

Piomelli D., Pilon C., Giros B., Sokoloff P., Martess P. M., and Schwartz J. C. (1991) Dopamine activation of the arachidonic acid cascade as a basis for D₁/D₅ receptor synergism. Nature 353, 164–167.

Raymond J. R., Albers F. J., and Middleton J. P. (1992) Functional expression of human 5-HT₁A receptors and differential coupling to second messengers in CHO cells. Synaps. Schmiedeb. Arch. Pharmak. 326, 127–137.

J. Neurochem., Vol. 64, No. 6, 1995
Sanfeliu C., Hunt A., and Patel A. J. (1990) Exposure to N-methyl-D-aspartate increases release of arachidonic acid in primary cultures of rat hippocampal neurons and not in astrocytes. Brain Res. 526, 241–248.

Schinelli S., Paolillo M., and Corona G. L. (1994) Opposing actions of D₁- and D₂-dopamine receptors on arachidonic acid release and cyclic AMP production in striatal neurons. J. Neurochem. 62, 944–949.

Sharp J. D., White D. L., Chiou X. G., Goodson T., Gambha G. C., McLure D., Burgett S., et al. (1991) Molecular cloning and expression of human Ca²⁺-sensitive cytosolic phospholipase A₂. J. Biol. Chem. 266, 14850–14853.

Sorscher E. J., Kirk K. L., Weaver M. L., Jilling T., Blalock J. E., and LeBoeuf R. D. (1991) Antisense oligodeoxynucleotide to the cystic fibrosis gene inhibits anion transport in normal cultured sweat duct cells. Proc. Natl. Acad. Sci. USA 88, 7759–7762.

Traiffort E., Ruat M., Arrang J. M., Leurs R., Piomelli D., and Schwartz J. C. (1992) Expression of a cloned histamine H₁ receptor mediating inhibition of arachidonate release and activation of cAMP accumulation. Proc. Natl. Acad. Sci. USA 89, 2649–2653.

Wahlestedt C., Merlo Pich E., Koob G. F., Yee F., and Heilig M. (1993) Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. Science 259, 526–531.

Waki K. (1992) Origins and fates of fatty acyl-CoA esters. Biochim. Biophys. Acta 1124, 101–111.

Wang H., Watkins D. C., and Malbon C. C. (1992) Antisense oligodeoxynucleotides to Gs protein α-subunit sequence accelerate differentiation of fibroblast to adipocytes. Nature 358, 334–337.

Woelk H., Peiler-Ichikawa K., Binaglia L., Goracci G., and Porcellati G. (1974) Distribution and properties of phospholipases A₁ and A₂ in synaptosomes and subsynaptosomal fractions of rat brain. Hoppe Seylers Z. Physiol. Chem. 355, 1535–1542.

Xin M. and Mattera R. (1992) Phosphorylation-dependent regulation of phospholipase A₂ by G-proteins and Ca²⁺ in HL60 granulocytes. J. Biol. Chem. 267, 25966–25975.

Yoshihara Y. and Watanabe Y. (1990) Translocation of phospholipase A₂ from cytosol to membranes in rat brain induced by calcium ions. Biochem. Biophys. Res. Commun. 170, 484–490.