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Dopamine D_2 Receptors Potentiate Arachidonate Release via Activation of Cytosolic, Arachidonate-Specific Phospholipase A_2

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Abstract: Several G_i-linked neurotransmitter receptors, including dopamine D_2 receptors, act synergistically with Ca^{2+}-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 D_2-receptor cDNA, CHO(D_2). Two antisense oligodeoxynucleotides corresponding to distinct cDNA sequences of cytosolic, AA-specific phospholipase A_2 (cPLA$_2$) were synthesized and added to cultures of CHO(D_2) cells. Incubation with antisense oligodeoxynucleotides inhibited D_2 receptor–dependent release of AA but had no effect on D_2-receptor binding or D_2 inhibition of cyclic AMP accumulation. In addition, pharmacological experiments showed that D_2 receptor–dependent AA release was prevented by nonselective phospholipase inhibitors (such as mepacrine) but not by inhibitors of membrane-bound, non–AA-specific PLA$_2$ (such as *p*-bromophenacyl bromide). cPLA$_2$ is expressed in brain tissue. The results, showing that cPLA$_2$ participates in receptor-dependent potentiation of AA release in CHO(D_2) 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.
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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 A_2 (PLA$_2$), 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 PLA$_2$ family may be divided into two groups, high molecular weight, cytosolic PLA$_2$ and low molecular weight, membrane-bound (secretory) PLA$_2$ (Glaser et al., 1993; Mayer and Marshall, 1993). A cytosolic PLA$_2$ 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 PLA$_2$, termed cPLA$_2$, is stimulated by free Ca^{2+} at concentrations (0.1–1 μM) that are likely to be reached when Ca^{2+}-mobilizing receptors are stimulated in intact cells, su esting that this activity may participate in receptor- endent AA release (Clark et al., 1991). In suppor his possibility, it was shown that when Chinese ha ovary (CHO) cells overexpressing transfected were stimulated with extracellular ATP, an a at purinergic P_2 receptors, release of AA was enhanced if compared with wild-type CHO cell et al., 1992). In contrast with cPLA$_2$, low molec ight forms of PLA$_2$ (14–18 kDa) are either secr or membrane-bound, hydrolyze phospholipids w lesser degree of selectivity, and are activated in by free Ca^{2+} at millimolar concentrations (Waite, 7). Their possible participation in receptor-stimul release of AA and of other fatty acids has also been s ggested (Pernas et al., 1991).

A group of G_i protein–coupled neurotransmitter re-

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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; PLA$_2$, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; RT, reverse transcriptase.

ceptors, including D_2-dopaminergic, α_2-adrenergic, and m_2- and m_4-muscarinic receptors, act synergistically with Ca^{2+}-mobilizing stimuli to produce AA release. For example, in transfected CHO cells labeled by incubation with [^{3}H]AA, stimulation of D_2 receptors potentiates the release of [^{3}H]AA evoked by application of ATP or Ca^{2+} ionophores but has no effect on basal [^{3}H]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 D_2-receptor cDNA, CHO(D_2), to examine the possible participation of AA-specific, high molecular weight $cPLA_2$ in this facilitatory response. We report that antisense deoxyoligonucleotides (ODNs) directed against specific sequences of $cPLA_2$ inhibit D_2 receptor–dependent potentiation of AA release, without affecting inhibition of adenylyl cyclase activity or other second messenger pathways. The results, showing that $cPLA_2$ may mediate a signal transduction pathway activated by D_2 receptors, support a role for this phospholipase in brain signaling.

EXPERIMENTAL PROCEDURES

Cell cultures

CHO cells were transfected with rat $D_{2(444)}$ 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% CO_2. CHO(D_2) cells expressed 1.3×10^5 D_2 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, intact cells were collected by centrifugation, resuspended in DMEM, and binding measurements were performed using ^{125}I-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(D_2) 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 $cPLA_2$ (Clark et al., 1991), prepared by cutting a human $cPLA_2$ 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 μg), 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 m*M*, and stored at −20°C until used. The following ODNs were used: antisense $cPLA_2$-1, 5′-CAT TTT GGT TTC AAT TAC AGG-3′; sense $cPLA_2$-1, 5′-CCT GTA ATT GAA ACC AAA ATG-3′; missense $cPLA_2$-1, 5′-GTA ACC CCT ATG AAA GAA ATT-3′; antisense $cPLA_2$-2, 5′-TTC TTC ATT CTC CTC TGG C-3′; sense $cPLA_2$-2, 5′-G CCA GAG GAG ATT GAA GAA-3′; missense $cPLA_2$-2, 5′-ATC CCC GTT TTT TCT TCG C-3′. CHO(D_2) 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.

[^{3}H]AA release

After washing residual ODNs, cells (24-well plates) were labeled by incubation with [^{3}H]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. [^{3}H]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 m*M*) 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 Ca^{2+} 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 μM). Cells were washed, resuspended in HEPES-buffered Krebs solution (containing, in mmol/L, 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 using a Hitachi F-2000 fluorescence spectrophotometer.

[^{3}H]Choline release

$CHO(D_2)$ cells (24-well plates) were labeled overnight by incubation with [^{3}H]choline (Amersham, 1 μCi/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 [^{3}H]choline incorporation into $CHO(D_2)$ cells, as determined by measuring total radioactivity in cell extracts (data not shown).

[^{3}H]AA uptake

Cells, labeled by incubation with [^{3}H]AA as described above, were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (GIBCO) and sonicated briefly in 0.25 ml of the same medium. Radioactivity was determined in samples (25 μl) of cell extracts.

Materials

Ca^{2+} 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 $cPLA_2$ prevents ATP-stimulated [^{3}H]AA release

In CHO cells, the stimulation of constitutive P_2-type purinergic receptors with extracellular ATP produces three prominent responses, activation of phosphoinositide-specific phospholipase C (PLC), elevation of intracellular Ca^{2+} levels, and release of free AA from phospholipids (Gupta et al., 1990). Evidence indicates that P_2 receptor–dependent AA release is mediated through the activation of $cPLA_2$. First, $CHO(D_2)$ cells express constitutively an mRNA encoding for $cPLA_2$, as revealed by northern blot and RT/PCR analyses. Samples of poly(A)$^+$ RNA from $CHO(D_2)$ cells were subjected to agarose gel electrophoresis, transferred onto nitrocellulose, and incubated with a ^{32}P-labeled cDNA probe for human $cPLA_2$ (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 $CHO(D_2)$ cells, revealed the presence of a 0.5-kb product whose identity was confirmed by an additional PCR (Fig. 1B). Second, when $cPLA_2$ expression was enhanced in CHO cells by transfection with a cDNA encoding for human $cPLA_2$, extracellular ATP was much more potent in evoking AA release than in nontransfected cells, or in cells transfected with a secretory PLA_2 (Lin et al., 1992).

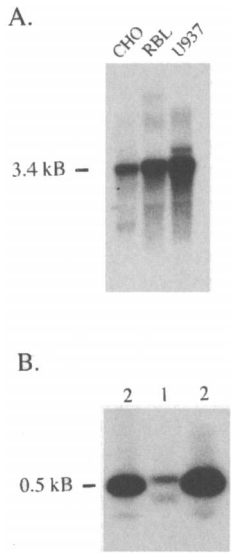

FIG. 1. Expression of an mRNA transcript encoding $cPLA_2$ in $CHO(D_2)$ cells. **A**: Northern blot analysis of poly(A)$^+$ RNA from $CHO(D_2)$, RBL, and U937 cells in culture. **B**: Analysis by RT-PCR. Total RNA obtained from $CHO(D_2)$ cells was subjected to (1) one or (2) two consecutive PCR with nested oligonucleotide primers (see Experimental Procedures for further details). Results are from one experiment typical of two (northern blot) or three (PCR).

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 $cPLA_2$, $CHO(D_2)$ cells were incubated for 24 h in culture medium containing one of three distinct 21-mer ODNs, termed antisense $cPLA_2$-1 (complementary to a 5′-untranslated sequence in human and rat $cPLA_2$ cDNA but including the ATG initiation codon), sense $cPLA_2$-1 (homologous to the same cDNA sequence), and missense $cPLA_2$-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 [^{3}H]AA.

Although these labeling conditions resulted in an incorporation of [^{3}H]AA into $CHO(D_2)$ 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 [^{3}H]AA from cells that had been exposed to either sense $cPLA_2$-1 or missense $cPLA_2$-1 (Fig. 2). In the absence of any addition, quinpirole plus ATP produced on average a 400% increase of basal [^{3}H]AA release (Piomelli et al., 1991). In contrast, the effect of ATP on [^{3}H]AA release was completely abolished in cells treated with antisense $cPLA_2$-1 (Fig. 2).

The ability of antisense $cPLA_2$-1 to prevent ATP-induced release of [^{3}H]AA may have resulted from a nonspecific action of this ODN leading to defective signaling at the P_2 receptor. To address this possibility, we examined the effect of ATP on intracellular Ca^{2+} levels in $CHO(D_2)$ cells that had been exposed to

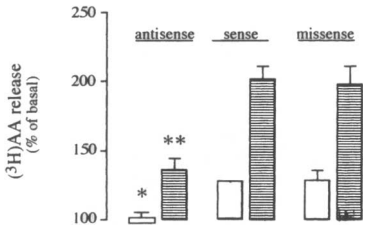

FIG. 2. Effects of antisense $cPLA_2$-1 on P_2-receptor signaling in $CHO(D_2)$ cells. Treatment with antisense $cPLA_2$-1 inhibits ATP-induced release of [^{3}H]AA. Cells were incubated for 24 h with antisense, sense, or missense $cPLA_2$-1 (50 μM), labeled by incubation with [^{3}H]AA, and stimulated 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 [^{3}H]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 [^{3}H]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 $cPLA_2$-1 (24 h, 50 μM). No difference was seen in the Ca^{2+} response to ATP (100 μM) after these treatments, suggesting that antisense $cPLA_2$-1 does not interfere either with the binding of ATP to the P_2 receptor or with receptor coupling to phosphoinositide-specific PLC and intracellular Ca^{2+} 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 $cPLA_2$-1 on these enzyme activities may affect phospholipid labeling with [^{3}H]AA and result in diminished receptor-dependent [^{3}H]AA release. To control for this possibility, we measured the uptake of [^{3}H]AA in ODN-treated $CHO(D_2)$ cells. As shown in Table 1, no significant difference was seen in labeling among cells exposed to antisense, sense, or missense $cPLA_2$-1.

Antisense ODNs against $cPLA_2$ prevent D_2 receptor–dependent [^{3}H]AA release

In $CHO(D_2)$ cells, D_2-receptor agonists are potent in enhancing [^{3}H]AA release, when such release is evoked by stimulating Ca^{2+}-mobilizing receptors (such as P_2 receptors) or by applying a Ca^{2+} ionophore (Felder et al., 1991; Piomelli et al., 1991; Di Marzo et al., 1993). Although these results had suggested the participation of a PLA_2 activity in this response, the lack of selective PLA_2 inhibitors has hindered the characterization of this activity.

To examine a possible involvement of $cPLA_2$, we first studied the effect of antisense $cPLA_2$-1 on D_2-receptor potentiation of ATP-induced [^{3}H]AA release. As shown in Fig. 2, incubation with antisense $cPLA_2$-1 markedly inhibited the release of [^{3}H]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 [^{3}H]AA release was evoked by applying the Ca^{2+} ionophore, A23187 (Fig. 3).

The inhibitory effect of antisense $cPLA_2$-1 did not result from a nonspecific action on D_2-receptor function. In support of this conclusion we found, first, that D_2-receptor binding was not affected by incubation with the antisense ODN. In two separate experiments, ^{125}I-sulpride binding to intact $CHO(D_2)$ cells was displaced by quinpirole with a K_D of 0.21 ± 0.02 nM in cells exposed to antisense $cPLA_2$-1, and 0.18 ± 0.05 nM in cells exposed to sense $cPLA_2$-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 $CHO(D_2)$ 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

$CHO(D_2)$ cells were maintained in culture, stimulated, and analyzed as described in Experimental Procedures. When compared with sense-oriented or missense controls, treatment with anti-$cPLA_2$ antisense did not significantly inhibit forskolin-stimulated adenylyl cyclase activity, ATP-stimulated intracellular Ca^{2+} increases, Ca^{2+} ionophore–stimulated [^{3}H]choline release, or [^{3}H]AA uptake. Data are expressed as mean ± SEM values of n separate determinations performed in two to four independent experiments.

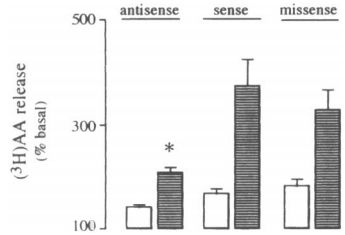

FIG. 3. Antisense $cPLA_2$-1 inhibits D_2 receptor–dependent potentiation of [^{3}H]AA release. CHO(D_2) cells were stimulated with the Ca^{2+} ionophore A23187 (4 μM) (open bars), or with a combination of A23187 plus quinpirole, a D_2-receptor agonist (1 μM) (hatched bars). Results are expressed as mean ± SEM values of four separate experiments. *$p < 0.05$, by two-way analysis of variance. For values of basal [^{3}H]AA release, see Fig. 1 legend.

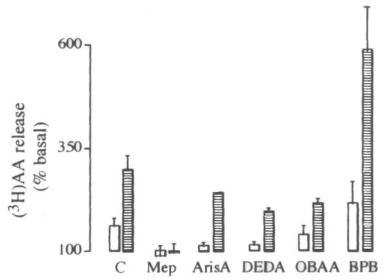

FIG. 5. Effects of various PLA_2 inhibitors on [^{3}H]AA release from CHO(D_2) cells. Cells were labeled by incubation with [^{3}H]AA and stimulated with A23187 (4 μM) (open bars) or with A23187 plus quinpirole (1 μM) (hatched bars). Drugs used: Mep, mepacrine (50 μM); ArisAc, aristolochic acid (50 μM); DEDA, dimethyleicosadienoic acid (60 μM); OBAA, octadecylbenzoacrylic acid (15 μM); BPB, *p*-bromophenacyl bromide (20 μM).

± 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 $cPLA_2$-1 inhibited the response to quinpirole, it did not completely abolish the release of [^{3}H]AA induced by A23187 alone. Like other Ca^{2+} ionophores, A23187 activates in a nonspecific manner several Ca^{2+}-dependent phospholipases whose activities may catalyze [^{3}H]AA deacylation, including PLC and phospholipase D (PLD) (Piomelli, 1993).

Next, we examined the effect of an additional antisense ODN, named antisense $cPLA_2$-2, complementary to a translated sequence of $cPLA_2$ 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 $cPLA_2$-2 (24 h, 50 μM) decreased the release of [^{3}H]AA produced by quinpirole plus A23187, without affecting the response to A23187 alone. Sense and missense $cPLA_2$-2 had no effect (Fig. 3).

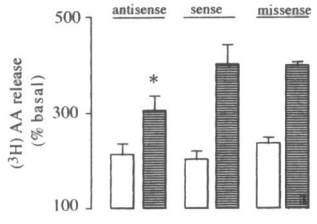

FIG. 4. Antisense $cPLA_2$-2 inhibits D_2 receptor–dependent potentiation of [^{3}H]AA release. CHO(D_2) cells were incubated with antisense, sense, or missense $cPLA_2$-2 (50 μM) for 24 h, labeled by incubation with [^{3}H]AA, and stimulated with A23187 (4 μM) (open bars) or with A23187 plus quinpirole (1 μM) (hatched bars). The results, expressed as mean ± SEM values, are from one experiment performed in quadruplicate. Similar results were obtained in an additional experiment (data not shown). Values of basal [^{3}H]AA release were for antisense-treated cells, 380 ± 62 cpm; for sense-treated cells, 388 ± 16 cpm; for missense-treated cells, 455 ± 48 cpm.

Pharmacological blockade of $cPLA_2$

Several alkylating agents that inhibit secretory PLA_2 by modifying covalently the enzyme at its active site have little or no effect on $cPLA_2$ activity. For example, the histidine reagent, *p*-bromophenacyl bromide, which is potent in inhibiting the activity of secretory PLA_2, is ineffective on purified $cPLA_2$ (Mayer and Marshall, 1993). In contrast, mepacrine, which interferes with the availability of phospholipid substrate, acts on $cPLA_2$ 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 PLA_2 inhibitors on the release of [^{3}H]AA from CHO(D_2) cells, stimulated either with A23187 or with a combination of A23187 and quinpirole (Fig. 5). [^{3}H]AA release was prevented most effectively by mepacrine (50 μM), and only partially reduced by aristolochic acid, dimethyleicosadienoic acid, and octadecylbenzoacrylic acid. *p*-Bromophenacyl bromide augmented rather than inhibited [^{3}H]AA release from CHO(D_2) cells (Fig. 4), possibly by interfering with reuptake of [^{3}H]AA into cell lipids.

DISCUSSION

The experiments described above suggest that, in CHO(D_2) cells, the facilitation of Ca^{2+}-evoked [^{3}H]AA release produced by stimulating dopamine D_2 receptors results from activation of AA-specific, high molecular weight $cPLA_2$. Three observations support this conclusion. First, $cPLA_2$ is constitutively expressed in CHO(D_2) cells, as indicated by northern blot and RT/PCR detection of the mRNA coding for this protein. Next, incubation of CHO(D_2) cells with two distinct antisense ODNs directed against $cPLA_2$ markedly reduced D_2 receptor–dependent potentiation of [^{3}H]AA release. Finally, the response was prevented by mepacrine, a nonselective PLA_2 blocker, but not by *p*-bromophenacyl bromide, a drug that inhibits membrane-bound PLA_2 selectively over $cPLA_2$.

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 $cPLA_2$

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(D_2) 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 [^{3}H]AA induced by stimulating D_2 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 $cPLA_2$-1, directed against a 5′ untranslated sequence of human and rat $cPLA_2$ cDNA sequence (Clark et al., 1991), prevented ATP-induced [^{3}H]AA release, mediated by $cPLA_2$ (Lin et al., 1992), but had no effect on ATP-induced elevation of intracellular Ca^{2+} levels (Gupta et al., 1990); (2) neither a sense-oriented ODN directed against the same $cPLA_2$ sequence nor a missense ODN (random nucleotide sequence) had such effect; (3) treatment with antisense $cPLA_2$-1 did not affect D_2 receptor binding, inhibition of forskolin-stimulated cyclic AMP accumulation, [^{3}H]AA incorporation into lipid or Ca^{2+} ionophore–induced [^{3}H]choline release (a measure of PLC and PLD activities); (4) an additional ODN, antisense $cPLA_2$-2, designed to hybridize to nucleotides 741–763 of $cPLA_2$, was also effective in reducing D_2 receptor–dependent [^{3}H]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 [^{3}H]AA release through a selective inhibition of $cPLA_2$ expression.

Possible signaling functions of $cPLA_2$ 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 H_2 receptors prevents the release of AA induced by the Ca^{2+} 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 Ca^{2+} levels. This permissive effect has been demonstrated in CHO cells transfected with G_i-coupled receptors, such as D_2-dopaminergic, α_2-adrenergic, m_2- and m_4-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 D_2 receptors on AA release remains unknown, two hypotheses are compatible with the available experimental evidence. G_i-coupled receptors, such as the D_2 receptor, may activate a "permissive" αG_i-protein that would enhance, but not directly evoke, $cPLA_2$ activity. Alternatively, G_i-coupled receptors may release "free" β-γ subunits, which have been reported to stimulate PLA_2 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 D_2-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, PLA_2, PLC, and PLD (Piomelli, 1993). In addition, most tissues, including the brain, express a variety of PLA_2 activities distinguished by their different subcellular distribution and regulatory properties (Woelk et al., 1974; Gray and Strickland, 1982*a,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 PLA_2 isoform, AA-selective $cPLA_2$, 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 $cPLA_2$ in brain tissue (Yoshihara and Watanabe, 1990; Hirashima et al., 1992) supports a similar role in neural cells.

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