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Bidirectional control of phospholipase A_2 activity by Ca^{2+}/calmodulin-dependent protein kinase II, cAMP-dependent protein kinase, and casein kinase II

(Eicosanoids/nerve terminals/neurotransmitter release)

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ABSTRACT **In preparations of synaptic terminals (synaptosomes) isolated from rat brain, the activity of phospholipase A_2 (PLA_2), a phospholipid hydrolase that serves a central function in signal transduction, was inhibited in a Ca^{2+}-dependent manner by incubation with 60 mM K^+ or with the Ca^{2+}-selective ionophore ionomycin. Reversal by alkaline phosphatase treatment suggested that this inhibitory effect resulted from phosphorylation of a synaptosomal protein substrate. When lysed synaptosomes were incubated with Ca^{2+}/calmodulin (CaM), purified Ca^{2+}/CAM-dependent protein kinase II (Ca^{2+}/CaM-dependent PK II) and ATP, PLA_2 activity in lysates was nearly abolished within 10 min. This effect was accompanied by a marked decrease in the V_{max} of the enzyme and little or no change in the K_m. Furthermore, Ca^{2+}/CaM with ATP but without exogenous Ca^{2+}/CaM-dependent PK II partially inhibited PLA_2 activity, and this effect was prevented by treating the lysates with a selective peptide inhibitor of Ca^{2+}/CaM-dependent PK II. In contrast, incubation of intact synaptosomes with 4β-phorbol 12-myristate 13-acetate or of lysed synaptosomes with purified protein kinase C had little or no effect on PLA_2 activity. The results strongly suggest that the Ca^{2+}-dependent inhibition of PLA_2 activity observed in intact nerve endings was produced by activation of the multifunctional Ca^{2+}/CaM-dependent PK II. A membrane-permeable adenylyl cyclase activator, forskolin, enhanced PLA_2 activity in intact synaptosomes, and cAMP-dependent protein kinase potentiated PLA_2 activity in lysed synaptosomes. Furthermore, another broad-spectrum protein kinase present in synaptic terminals, casein kinase II, also potentiated PLA_2 activity in lysed synaptosomes. The effects of both protein kinases were associated with a decrease in K_m and no change in V_{max}. The results suggest that PLA_2 activity in synaptic terminals is subject to bidirectional control by distinct signal transduction pathways. Moreover, mutually antagonistic effects of the Ca^{2+}/CaM-dependent PK II and PLA_2 pathways provide a possible molecular mechanism for bidirectional modulation of neurotransmitter release.**

Phospholipase A_2 (PLA_2) catalyzes the receptor-stimulated hydrolysis of membrane phospholipids, providing substrates for the biosynthesis of potent cellular regulators, which include arachidonic acid metabolites (eicosanoids) and platelet-activating factor (1–3). In neurons, the eicosanoids may participate in various forms of signal transduction and synaptic plasticity, including K^+ channel modulation, presynaptic inhibition, and long-term potentiation (for review, see ref. 4). Despite these important functions in intracellular signaling, the mechanism of regulation of PLA_2 activity is only poorly understood. In many tissues, a membrane-bound, Ca^{2+}-dependent form of PLA_2, which is thought to be responsible for the receptor-mediated liberation of arachidonic acid, is stimulated directly by free Ca^{2+} (1, 2). The high (millimolar) concentration of Ca^{2+} required for stimulation *in vitro* suggests, however, that a different mechanism may be responsible for PLA_2 activation in intact cells. In many tissues, guanine nucleotide-binding proteins (G proteins) coupled to neurotransmitter and hormone receptors have been shown to activate PLA_2 and promote arachidonic acid liberation (5), possibly by reducing the enzyme requirement for free Ca^{2+} (6, 7). In agreement with this interpretation, G protein-mediated activation of PLA_2 by certain hormones was shown to occur independently of hormone-induced increases in Ca^{2+} (8).

In this study, we have investigated the possible role of protein phosphorylation in the regulation of PLA_2 activity in neural tissue. Using a preparation of intact synaptic nerve endings, we found that membrane depolarization produces a Ca^{2+}- and phosphorylation-dependent inhibition of PLA_2 activity. Studies using purified protein kinases indicate that this inhibitory effect results from activation of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (Ca^{2+}/CaM-dependent PK II). Conversely, two other broad-spectrum protein kinases, casein kinase II and cAMP-dependent protein kinase, were found to potentiate PLA_2 activity in synaptic endings.

EXPERIMENTAL PROCEDURES

Synaptosome Preparation. Synaptosomes, prepared from rat cerebral cortex by discontinuous density gradient centrifugation as described (9), were resuspended in oxygenated Hepes-buffered saline (142 mM NaCl/2.4 mM KCl/1.2 mM K_2HPO_4/1 mM $MgCl_2$/5 mM D-glucose/10 mM Hepes buffer, pH 7.4) to 1 mg of protein per ml. Standard incubations were carried out at 37°C in the presence of 1 mM $CaCl_2$ and stopped by the addition of 5 volumes of an ice-cold hypotonic buffer (lysis buffer) containing 1 mM EDTA, 1 mM EGTA, 10 mM sodium pyrophosphate, 10 mM Hepes (pH 7.4), and protease inhibitors (10).

PLA_2 Assay. The fluorogenic PLA_2 substrate 1,2-bis-(1-pyrenedecanoyl) phosphatidylcholine (2 μM for standard assays) was added to 1.5 ml of 0.1 M Tris·HCl (pH 9.0) in a stirred quartz cuvette maintained at 37°C within a Perkin–Elmer LS-5B luminescence spectrometer. After 2 min, samples of the lysed synaptosomes (0.2 mg of protein) were transferred to the cuvette, and the rate of increase in fluorescence due to the liberation of pyrenedecanoic acid was monitored (excitation wavelength, 340 nm; emission wave-

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Abbreviations: PLA_2, phospholipase A_2; CaM, calmodulin; Ca^{2+}/CAM-dependent PK, Ca^{2+}/CAM-dependent protein kinase; PKC, protein kinase C; G protein, guanine nucleotide-binding protein.

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length, 380 nm). The changes in fluorescence due to released pyrenedecanoate were calibrated against known concentrations of the unesterified fatty acid. Both Ca^{2+}-dependent and Ca^{2+}-independent PLA_2 activities were present in the lysed tissue. Ca^{2+}-dependent PLA_2 activity was maximal at alkaline pH (pH optimum, 9.0–9.5) in the presence of 2 mM Ca^{2+}. In addition, this activity was inhibited by the histidine reagent *p*-bromophenacyl bromide (10 μM) and entirely associated with membranes. In contrast, Ca^{2+}-independent PLA_2 activity was optimal at acidic pH (4–4.5), 1/10th as sensitive to inhibition by *p*-bromophenacyl bromide, and distributed approximately equally in both soluble and particulate fractions. Ca^{2+}-independent and Ca^{2+}-dependent PLA_2 activities were measured before and after the addition of Ca^{2+} (2 mM final concentration), respectively. Ionomycin, forskolin, and 4β-phorbol 12-myristate 13-acetate were added to the synaptosomes from dimethyl sulfoxide stock solutions kept at −20°C. 1,2-Bis-(1-pyrenedecanoyl) phosphatidylcholine and pyrenedecanoic acid were stored in dimethyl sulfoxide at −20°C for up to 1 month. Final dimethyl sulfoxide concentration in the assay buffer was 0.2%. No change in activity occurred in lysed synaptosomes that had been incubated in control conditions in the absence or presence of this concentration of dimethyl sulfoxide for up to 10 min.

Protein Purifications. Highly purified preparations of rat forebrain Ca^{2+}/CaM-dependent PK II, Ca^{2+}/CaM-dependent PK I, and protein kinase C (PKC); rat pancreas Ca^{2+}/CaM-dependent PK III; rabbit brain CaM; and bovine heart catalytic subunit of cAMP-dependent protein kinase were used (11). Casein kinase II was purified from bovine brain (12).

Materials. Ca^{2+}/CaM-dependent PK II inhibitory peptide was synthesized and purified as described (13). Ionomycin and forskolin were obtained from Calbiochem. 4β-phorbol 12-myristate 13-acetate, from LC Services (Woburn, MA); 1,2-bis-(1-pyrenedecanoyl) phosphatidylcholine and pyrenedecanoic acid, from Molecular Probes; bovine intestinal alkaline phosphatase and porcine pancreatic PLA_2, from Boehringer Mannheim; and *p*-bromophenacyl bromide from Sigma.

RESULTS

The effect of depolarization of intact nerve terminals on PLA_2 activity, measured subsequently in synaptosomal lysates, is shown in Fig. 1 (line a). K^+-induced depolarization reduced Ca^{2+}-dependent PLA_2 activity to 50% of its initial value within 1 min of incubation. Inhibition was complete within 10 min. When the synaptosomes were depolarized with K^+ in the absence of external Ca^{2+}, PLA_2 activity remained unchanged (Fig. 1, line d). When intact synaptosomes were incubated with the Ca^{2+} ionophore ionomycin (10 μM), PLA_2 activity was inhibited to an extent comparable to that observed with the elevated K^+ level and with a similar time course (Fig. 1, line b). In contrast to these actions on Ca^{2+}-dependent PLA_2 activity, neither K^+ nor ionomycin had any effect on Ca^{2+}-independent PLA_2 activity; thus, after incubation of synaptosomes with Ca^{2+} and a high level of K^+ or ionomycin for 10 min, Ca^{2+}-independent PLA_2 activity was 110 ± 9% of control (n = 8) or 114 ± 30% of control (n = 3), respectively.

The depolarization-induced inhibition of PLA_2 activity might have been caused by Ca^{2+}-dependent protein kinase, Ca^{2+}-dependent protein phosphatase, or Ca^{2+}-dependent protease activity. To determine whether protein phosphorylation was responsible for this effect, lysates of K^+-depolarized synaptosomes (10 min) were incubated in the presence of alkaline phosphatase before measuring PLA_2 activity. Ca^{2+}-dependent PLA_2 activity, which had been undetectable (the 10-min time point of line a in Fig. 1)

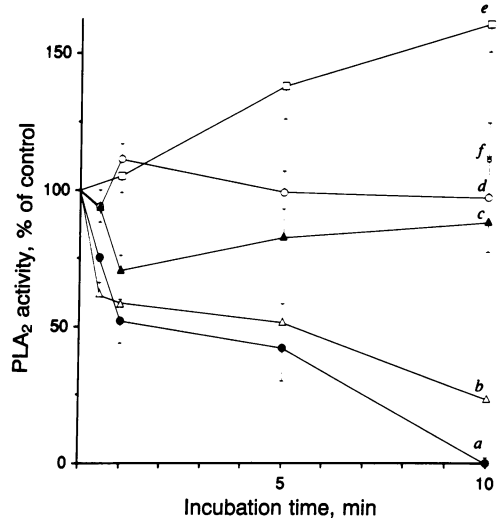

FIG. 1. Modulation of Ca^{2+}-dependent PLA_2 activity in intact synaptosomes. Synaptosomes were incubated under standard conditions in the presence of 60 mM KCl (line a), 10 μM ionomycin (line b), 1 μM 4β-phorbol 12-myristate 13-acetate (line c), 60 mM KCl in the absence of external Ca^{2+} (line d), or 50 μM forskolin (line e). Reactions were stopped by lysing the synaptosomes, and PLA_2 activity was measured fluorometrically (see *Experimental Procedures*). In some experiments, lysed K^+-depolarized synaptosomes were incubated with intestinal alkaline phosphatase for 10 min at 37°C before measuring PLA_2 activity (point f). PLA_2 activity was calculated as a percentage of the activity at zero time and is expressed as the mean ± SEM (vertical bars) of 7–10 experiments. PLA_2 activity was not changed by incubation of the synaptosomes for 10 min under control conditions (not shown).

returned upon treatment with alkaline phosphatase to the value of control, unstimulated synaptosomes (Fig. 1, point f). Alkaline phosphatase treatment did not affect PLA_2 activity of control synaptosomes (98 ± 8%, n = 3). These results suggest that inhibition of PLA_2 activity results from Ca^{2+}-dependent phosphorylation of a protein substrate rather than from the activation of a protease or a protein phosphatase.

In synaptic terminals, voltage-dependent or receptor-regulated Ca^{2+} influx activates two broad-spectrum protein kinases, Ca^{2+}/CaM-dependent PK II and Ca^{2+}/phospholipid-dependent PKC (14, 15). No specific, membrane permeable activator of Ca^{2+}/CaM-dependent PK II has been described. Therefore, to test the possibility that this protein kinase participates in the K^+-induced regulation of PLA_2 activity, we incubated lysed synaptosomes in the presence of purified Ca^{2+}/CaM-dependent PK II (in a reaction medium containing Ca^{2+}, CaM, and ATP). Within 2 min of incubation, PLA_2 activity was reduced to 50% of its control value (Fig. 2, line a). Almost complete inhibition of activity occurred after 10 min of incubation. A kinetic analysis indicated that Ca^{2+}/CaM-dependent PK II inhibited PLA_2 activity by decreasing the V_{max} of the enzyme, with no significant change in the apparent K_m (Table 1).

PLA_2 activity was not affected when Ca^{2+}/CaM, ATP (Fig. 3), Ca^{2+}, or CaM (not shown) was added separately to lysed synaptosomes. In contrast, addition of a combination of Ca^{2+}, CaM, and ATP resulted in a 70% inhibition of Ca^{2+}-dependent PLA_2 activity, most likely through activation of endogenous Ca^{2+}/CaM-dependent PK II (which is abundant in synaptic terminals; ref. 15). The inhibition by Ca^{2+}, CaM, and ATP in combination could be enhanced further by the addition of exogenous Ca^{2+}/CaM-dependent PK II (Fig. 3) but not by the addition of Ca^{2+}/CaM-dependent PK I or Ca^{2+}/CaM-dependent PK III (data not shown). Furthermore, inhibition of PLA_2 by combined Ca^{2+} CaM, and ATP was prevented by treatment with a selective peptide inhibitor of

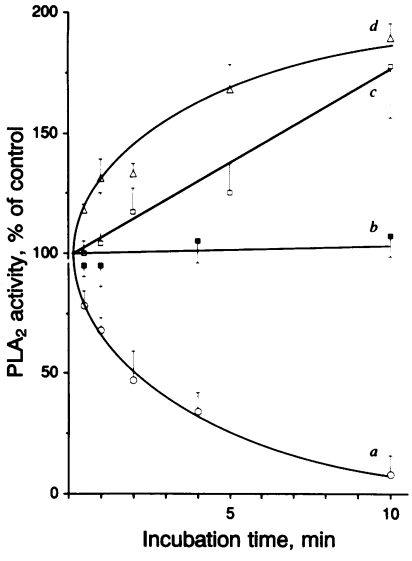

FIG. 2. Effects of purified protein kinases on Ca^{2+}-dependent PLA_2 activity in lysed synaptosomes. Synaptosomes, purified and lysed as described, were incubated at 37°C in 0.1 ml of lysis buffer containing 5 mM magnesium acetate, 0.5 mM ATP, and one of the following protein kinases: ≈10 nM Ca^{2+}/CaM-dependent protein kinase II with 1 mM $CaCl_2$ and 1 μM CaM (curve a), 100 nM PKC with 1 mM $CaCl_2$ and 1 μM 4β-phorbol 12-myristate 13-acetate (line b), 150 nM catalytic subunit of cAMP-dependent protein kinase (line c), or 10 nM casein kinase II (curve d). At the indicated times PLA_2 activity was measured. Results were calculated as the percentage of activity in the absence of added protein kinase and are expressed as means ± SEM (vertical bars) of three to nine experiments. Ca^{2+}-dependent PLA_2 activity was 6.5 ± 1 nmol/min per mg of protein (n = 27) at 0 min and was not affected by incubation for 10 min under control conditions (not shown).

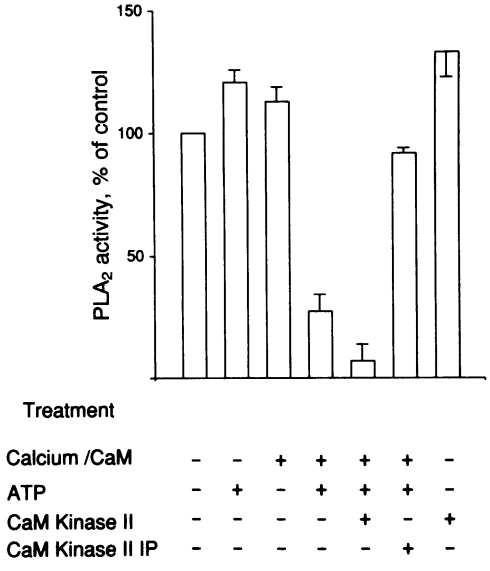

FIG. 3. Regulation of Ca^{2+}-dependent PLA_2 activity in synaptosomal lysates. Synaptosomes were lysed and incubated at 37°C for 10 min in 0.1 ml of lysis buffer containing the indicated combinations of the following agents: 1 mM Ca^{2+}, 1 μM CaM, 0.5 mM ATP, ≈10 nM Ca^{2+}/CaM-dependent PK II (CaM Kinase II), and 75 μM Ca^{2+}/CaM-dependent PK II inhibitory peptide (IP). Ca^{2+}-dependent PLA_2 activity was calculated as the percentage of the activity in the absence of any additions and is expressed as the mean ± SEM (vertical bars) of three to eight experiments.

Ca^{2+}/CaM-dependent PK II (13) (Fig. 3). Addition of exogenous Ca^{2+}/CaM-dependent PK II in the absence of Ca^{2+}/CaM/ATP did not inhibit PLA_2 activity (Fig. 3).

Ca^{2+}-dependent PLA_2 activity of rat cortical synaptosomes is entirely membrane bound (see *Experimental Procedures*). We prepared membranes from lysed synaptosomes and incubated them in the presence of purified Ca^{2+}/CaM-dependent PK II, Ca^{2+}/CaM, and ATP. After 10 min of incubation (37°C) membrane-associated PLA_2 activity was reduced from 44 ± 3 to 14 ± 0.4 nmol/min per mg of protein (n = 4). In contrast to these results with synaptosomal PLA_2, Ca^{2+}/CaM-dependent PK II had no effect on an extracellular form of Ca^{2+}-dependent PLA_2, purified from porcine pancreas (data not shown).

Table 1. Kinetic analysis of the regulation of Ca^{2+}-dependent PLA_2 activity by various protein kinases in lysed synaptosomes

	K_m, μM	V_{max}, nmol/min per mg of protein
Control	4.1 ± 0.8	167 ± 16
Ca^{2+}/CaM-dependent PK II*	5.7 ± 2.0	58 ± 6
cAMP-dependent PK catalytic subunit	2.0 ± 0.2	133 ± 5
Casein kinase II	1.3 ± 0.1	134 ± 3

Lysed synaptosomes were incubated for 10 min at 37°C in lysis buffer (1 mg of protein per ml) supplemented with 5 mM magnesium acetate, 0.5 mM ATP, and the purified protein kinase (PK) indicated. Incubations with Ca^{2+}/CaM-dependent PK II were carried out in the presence of 1 mM Ca^{2+} and 1 μM CaM. PLA_2 activity was measured as described with concentrations of substrate ranging from 2 to 10 μM. Values are means ± SEM of three to eight experiments.

*To determine the kinetics of inhibition by Ca^{2+}/CaM-dependent PK II, an amount of kinase was used that resulted in only partial (≈65%) inhibition under V_{max} conditions.

Activators of PKC were previously shown to enhance PLA_2 activity in various tissues (16, 17). When we incubated intact synaptosomes with the tumor-promoting phorbol ester 4β-phorbol 12-myristate 13-acetate (1 μM), which is very potent in stimulating PKC-dependent protein phosphorylation in intact synaptosomes (14), only a small and transient inhibition of Ca^{2+}-dependent PLA_2 activity was seen (Fig. 1, line c). Moreover, purified PKC had no effect on PLA_2 activity in synaptosomal lysates (Fig. 2, line b). These findings indicate that PKC-dependent protein phosphorylation does not contribute to the Ca^{2+}-dependent modulation of PLA_2 activity in synaptic nerve endings.

To investigate further the role of protein phosphorylation in the regulation of PLA_2 activity, we examined cAMP-dependent protein kinase and casein kinase II, two other broad-spectrum protein kinases present in synaptic endings (12, 18), for their effects on this phospholipase. To study the possible action of cAMP-dependent protein kinase on PLA_2 activity in intact synaptosomes, we used the membrane-permeable adenylate cyclase activator forskolin. When synaptosomes were treated with 50 μM forskolin, Ca^{2+}-dependent PLA_2 activity was potentiated in 10 min to 160% of control (Fig. 1, line e). Consistent with this finding, the addition of the purified catalytic subunit of cAMP-dependent protein kinase to lysed synaptosomes enhanced PLA_2 activity (Fig. 2, line c), an effect that was dependent on the presence of magnesium and ATP (not shown). Kinetic analysis of this response showed that cAMP-dependent protein kinase potentiated PLA_2 activity by decreasing the K_m, with little effect on the V_{max} of the enzyme (Table 1). In contrast to Ca^{2+}/CaM-dependent PK II, cAMP-dependent protein kinase did not affect PLA_2 activity when added to isolated synaptosomal membranes (not shown).

Purified casein kinase II also enhanced PLA_2 activity of synaptosomal lysates (Fig. 2, line d). This increase in enzyme activity was associated with a decrease in the K_m of PLA_2 for its substrate by a factor of 3 (Table 1). Like cAMP-dependent protein kinase, casein kinase II had no effect on isolated synaptosomal membranes (data not shown).

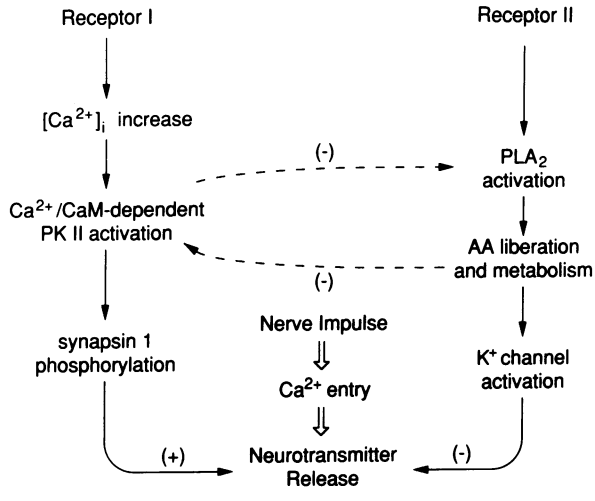

FIG. 4. Mutually antagonistic effects of the Ca^{2+}/CaM-dependent PK II and PLA_2 pathways: a possible role in bidirectional modulation of neurotransmitter release. This hypothetical scheme illustrates how nerve impulse-induced neurotransmitter release (open arrows) may be subject to bidirectional regulation by the Ca^{2+}/CaM-dependent and PLA_2 pathways. According to this model, neurotransmitters that increase intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels or repetitive nerve activity [which also increases intracellular Ca^{2+} (not shown)] may stimulate Ca^{2+}/CaM-dependent PK II activity, enhance phosphorylation of synapsin 1 (a synaptic vesicle-associated phosphoprotein), and potentiate release of neurotransmitter evoked by a nerve impulse (for a review, see ref. 21). Conversely, neurotransmitters that activate PLA_2 cause the formation of free arachidonic acid (AA) and its metabolites, which have been shown to enhance the opening of K^+ channels and reduce neurotransmitter release (4, 23, 24). Thus, these two receptor-operated cascades, may exert opposing functions on transmitter release (solid arrows). In addition, they may also exhibit mutually antagonistic actions (broken arrows), with Ca^{2+}/CaM-dependent PK II inhibiting PLA_2 activity (present study) and arachidonic acid metabolites inhibiting Ca^{2+}/CaM-dependent PK II activity (11). These antagonistic effects would be expected to amplify the effects of the relevant receptors on neurotransmitter release.

DISCUSSION

Our experiments suggest that Ca^{2+} entry into presynaptic terminals inhibits Ca^{2+}-dependent PLA_2 activity through activation of Ca^{2+}/CaM-dependent PK II and subsequent phosphorylation of a membrane protein substrate. This substrate may be PLA_2 itself or an associated PLA_2 regulatory protein (19, 20). The ability of Ca^{2+}/CaM-dependent PK II to inhibit PLA_2 activity was unexpected in view of the stimulatory action of Ca^{2+} on this phospholipase (for review, see refs. 1 and 2). However, even though free Ca^{2+} is known to enhance PLA_2 activity *in vitro*, increasing evidence now indicates that, in intact cells, receptor-dependent activation of PLA_2 may occur via a G protein-mediated mechanism and may take place at physiological Ca^{2+} concentrations (5–8). Our results further underscore the dissociation between increases in intracellular Ca^{2+} and PLA_2 activation, suggesting that, in tissues containing high levels of Ca^{2+}/CaM-dependent PK II, such as the brain, the predominant effect of increasing Ca^{2+} levels may be to inhibit, rather than to stimulate, PLA_2 activity.

A variety of evidence indicates that activation of Ca^{2+}/CaM-dependent PK II enhances neurotransmitter release (see ref. 21 for a review). For instance, purified Ca^{2+}/CaM-dependent PK II injected into the presynaptic digit of the squid giant synapse (22) or introduced by freeze–thaw cycles into rat cortical synaptosomes (13) potentiated the depolarization-induced release of neurotransmitter. In contrast, arachidonic acid and its lipoxygenase metabolites, products of PLA_2 activity, have been shown to stimulate K^+ channels and reduce neurotransmitter release in both mollusk and mammalian neurons (4, 23). Thus, at least in certain instances, these two receptor-operated cascades appear to exert opposing actions on neurosecretion. The inhibition by Ca^{2+}/CaM-dependent PK II of PLA_2 activity, observed in the present study, and the reciprocal inhibition of Ca^{2+}/CaM-dependent PK II activity by arachidonic acid and its metabolites (11), products of PLA_2 activity, suggest that these two receptor-operated cascades exert mutually antagonistic actions. These actions, in turn, may contribute to the opposing effects of the two signal-transduction pathways on neurotransmitter release (Fig. 4).

Using lysed synaptosomes, we found that cAMP-dependent protein kinase and casein kinase II potentiate the activity of Ca^{2+}-dependent PLA_2. In agreement, incubation with the catalytic subunit of cAMP-dependent protein kinase has been shown to increase PLA_2 activity in lysed peritoneal macrophages (25). The lack of effect of cAMP-dependent protein kinase and casein kinase II on isolated synaptosomal membranes suggests that these protein kinases may act by stimulating the phosphorylation of a cytosolic PLA_2-modulating protein. The identity of the hypothetical activating factor(s) remains to be determined, however.

The opposing effects of Ca^{2+}/CaM-dependent PK II, cAMP-dependent protein kinase and casein kinase II on the activity of Ca^{2+}-dependent PLA_2, shown in the present study, suggest that various signal-transduction pathways may have opposing effects on the activity of this phospholipase (and therefore on the release of arachidonic acid and platelet-activating factor) by modulating the activities of distinct protein kinases. Thus, neurotransmitters and neurohormones that activate cAMP-dependent protein kinase (26) or casein kinase II (27) may potentiate PLA_2 activity and enhance arachidonic acid release, whereas repetitive nerve activity and neurotransmitters that activate Ca^{2+}/CaM-dependent PK II (15) may inhibit it. This bidirectional control of PLA_2 activity may represent a final common pathway for interactions amongst a variety of first messengers.

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