*Biochemical Pharmacology*, Vol. 40, No. 10, pp. 2233–2239, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00


# INHIBITORY EFFECT OF EDTA·$Ca^{2+}$ ON THE HYDROLYSIS OF SYNAPTOSOMAL PHOSPHOLIPIDS BY PHOSPHOLIPASE $A_2$ TOXINS AND ENZYMES

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(*Received* 16 *January* 1990; *accepted* 23 *May* 1990)

**Abstract**—Phospholipases $A_2$ ($PLA_2$) are $Ca^{2+}$-dependent enzymes that are inhibited by EDTA; this inhibition would be expected to be reversed by restoring the $Ca^{2+}$ concentration. By examining the hydrolysis of synaptosomal phospholipids by $PLA_2$ enzymes, *Naja naja atra* and *Naja nigricollis*, and by toxins with $PLA_2$ activity, β-bungarotoxin (β-BuTX) and notexin, we demonstrated a novel inhibitory action of EDTA manifested in the presence of excess $Ca^{2+}$. We postulate the formation of an EDTA·$Ca^{2+}$ complex which inhibits $PLA_2$ activity in a concentration-dependent manner. Synaptosomes in which phospholipids are hydrolyzed by $PLA_2$ have membranal damage expressed by increased acetylcholine (ACh) release and decreased osmotic activity. Addition of EDTA·$Ca^{2+}$, which inhibits phospholipid hydrolysis, also reversed the $PLA_2$ effect on ACh release, but not its effect on osmotic activity. The inhibition of $PLA_2$ was observed on membranal phospholipids as well as on an artificial substrate of phospholipid-Triton mixed micelles. Moreover, we found that another enzyme, lactate dehydrogenase, was also inhibited. Our results indicate a non-specific inhibition exerted on the enzyme rather than on the substrate.

In the course of our studies on snake venom phospholipase $A_2$ ($PLA_2$§) (EC 3.1.1.4) enzymes and neurotoxins with $PLA_2$ activity, we investigated their phospholipid hydrolyzing ability on rat brain synaptosomes, which was found to be correlated with other effects such as changes in syanaptosomal integrity and eicosanoid synthesis [1, 2]. Synaptosomes prepared from homogenates in an EDTA-containing buffer were treated with the enzymes either in the same buffer supplemented with excess $Ca^{2+}$ or in an EDTA-free buffer with the addition of $Ca^{2+}$. To our surprise, the phospholipid hydrolysis was lower when EDTA was present during the incubation with $PLA_2$. This observation suggested a direct inhibitory effect of EDTA on phospholipid hydrolysis, an effect which was independent of its $Ca^{2+}$-chelating ability since it was manifest in the presence of excess $Ca^{2+}$. These detailed findings are reported in the present paper, and their implications are discussed.

## METHODS

Ficoll (type 400-DL) and Triton X-100 were obtained from the Sigma Chemical Co. (St. Louis, MO). Lactate dehydrogenase (LDH; EC 1.1.1.27) from rabbit muscle was purchased from Boehringer Mannheim, and silica gel (Kieselgel 60 HR reinst) was purchased from Merck Darmstadt. Lyophilized *Naja nigrocollis*, *Bungarus multicinctus*, and *Notechis scutatus scutatus* snake venoms were purchased from the Miami Serpentarium Laboratories (Salt Lake City, UT). *Naja naja atra* snake venom was collected and lyophilized in Hsinchu, Taiwan, by Dr. C-C. Yang, who also prepared the purified toxins and enzymes. The most basic (pI 10.6) $PLA_2$ (CMS-9) from *N. nigricollis* snake venom and the major acidic (pI 5.2) $PLA_2$ from *N. n. atra* snake venom were purified to homogeneity as previously described. $\beta_1$-Bungarotoxin (β-BuTX) was isolated from *B. multicinctus* snake venom, and notexin was isolated from the *N. s. scutatus* snake venom. Purities of the toxins and enzymes were confirmed by amino acid analysis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [3–6]. All other reagents were of analytical grade.

### *Preparation of synaptosomes*

Cerebral cortex was dissected from the brains of decapitated male Sprague–Dawley rats (150–200 g) and homogenized in a 0.32 M sucrose solution containing 10 mM EDTA, pH 7.4, by means of a Ten Broek tissue grinder. Synaptosomes were isolated from the homogenate by differential and discontinuous sucrose–ficoll gradient centrifugations [7].

### *Incubation with enzymes and toxins*

Following isolation, the synaptosomes were resuspended in 0.32 M sucrose buffered with 10 mM Tris–HCl at pH 7.4 (Tris–sucrose) at a concentration of 2.5 mg protein/mL. This concentration was adjusted by reading the optical density of the suspension at

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§ Abbreviations: $PLA_2$, phospholipase(s) $A_2$; LDH, lactate dehydrogenase; β-BuTX, $\beta_1$-bungarotoxin; ACh, acetylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

450 nm in a Beckman spectrophotometer. The protein concentration was calculated from a standard curve of absorbance versus milligrams of synaptosomal protein. For constructing the standard curve, serial dilutions of a synaptosomal suspension were assayed for protein [8], and their optical density was read in a Beckman spectrophotometer at 450 nm. Incubations with $PLA_2$ enzymes and toxins were performed in Tris–sucrose buffer supplemented with either $Ca^{2+}$ and/or EDTA as described in the legends to the figures. Following a 10-min incubation at 37°, the reactions were terminated by rapid lipid extraction.

*Determination of phospholipid hydrolysis*

For the determination of tissue phospholipid hydrolysis, lipids were extracted from brain synaptosomes with chloroform:methanol mixtures [9] and washed [10], and phospholipids were separated by two-dimensional TLC [11]. The individual phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin, were detected with iodine vapor and ninhydrin spray, and the phosphorus content of each spot was measured [12]. The percent phospholipid hydrolysis was calculated by using the following methods: (1) dividing the phosphorus content of the lysophospholipid spot by the total phosphorus content of the lyso plus the parent phospholipid and multiplying by 100; or (2) determining the expected amounts of each phospholipid in the experimental plates using sphingomyelin (not hydrolyzed by $PLA_2$) as an internal standard as follows: from the phospholipid values in untreated control synaptosomes the ratio of each individual phospholipid to sphingomyelin is calculated. From this ratio, and the values of sphingomyelin in $PLA_2$-treated synaptosomes, it is possible to calculate the amount of each phospholipid expected if there were no hydrolysis. By relating the actual amount on the plate to the expected amount, percent hydrolysis can be calculated.

*Preparation of synaptosomal lipid mixed micelles*

Lipids were extracted from synaptosomes with chloroform:methanol mixtures and washed as described above; the extract was evaporated to dryness under $N_2$. The lipids were suspended in Tris–sucrose buffer in the presence of Triton X-100 at a molar ratio of Triton:phospholipid 2:1. Mixed micelles were dispersed by a brief sonication and supplemented with $Ca^{2+}$ and EDTA as described in the legend to Table 7. They were then used on the same day as substrates for $PLA_2$ enzymes.

*Determinations of synaptosomal plasma membrane integrity*

*Osmotic activity.* Synaptosomes (0.5 mg protein/mL) were suspended in physiological buffer of the following millimolar concentrations: 119.7 NaCl, 23.4 $NaHCO_3$, 0.36 $MgSO_4$, 3.15 KCl, 0.45 $NaH_2PO_4$, 2.97 urea and 25 $CaCl_2$ (pH 7.4) with or without EDTA and incubated with $PLA_2$ toxins and enzymes at 37°. Following treatment, the synaptosomal suspension was adjusted to 0.25 mg protein/mL ($O.D._{450} \simeq 1$) and 67 $\mu$L of saturated NaCl solution was added to 0.5 mL of synaptosomal suspensions resulting in a 5-fold increase in osmolarity (from 300 mOsM to 1500 mOsM) and in a shrinkage of the synaptosomes. Osmotic behavior of the synaptosomes was then monitored using a light-scattering technique where an increase in absorbance at 450 nm is correlated with shrinkage of the synaptosomes. Osmotic activity was determined within 1.5 min following incubation. The percent increase in absorbance was calculated using the formula: $(A_{15} - A_0) \times 100$, where $A_0$ = the initial absorbance of the synaptosomal suspension prior to NaCl addition and $A_{15}$ = the absorbance recorded 15 sec after NaCl addition (when maximum shrinkage of synaptosomes was observed).

*Leakage of LDH.* Samples were removed from the incubation mixtures of synaptosomal suspensions with $PLA_2$ enzymes and toxins. LDH activity was assayed before centrifugation and in the supernatant following sedimentation of synaptosomes at 15,000 *g* for 15 min. LDH activity was assayed spectrophotometrically using pyruvate as substrate [13] in a UVIKON 860 spectrophotometer.

*Acetylcholine (ACh) release.* Synaptosomal suspensions (1 mg protein/mL) were preloaded with [$^3$H]choline (0.5 $\mu$M final concentration, 80 Ci/mmol) for 20 min at 37°.

After centrifugation and washing with Krebs–Tris buffer containing physostigmine but no choline, synaptosomes were incubated with 5 nM $PLA_2$ toxins or *N. nigricollis* $PLA_2$ for 15 or 30 min at 37° in the presence of (A) 2.5 mM $Ca^{2+}$ (normal incubation conditions), (B) 10 mM EDTA or (3) 10 mM EDTA + 50 mM $Ca^{2+}$. At the end of incubation, synaptosomal suspensions were put on ice and centrifuged. The pellets were discarded and supernatants were extracted to measure [$^3$H]ACh [14, 15]. The assay is based on phosphorylation of [$^3$H]choline by choline kinase in the presence of ATP and separation of the resulting [$^3$H]phosphorylcholine from [$^3$H]ACh by liquid cation exchange with 2% tetraphenylboron in butyronitrile.

## RESULTS

The extent of hydrolysis of individual phospholipids in synaptosomes treated with $PLA_2$ enzymes and toxins either in the presence of EDTA and excess $Ca^{2+}$, or in the presence of $Ca^{2+}$ only, is shown in Table 1. In the presence of EDTA plus excess $Ca^{2+}$, hydrolysis by *N. n. atra* $PLA_2$ of all synaptosomal phospholipids but especially of PC was decreased appreciably compared with the level of hydrolysis in the absence of EDTA. Similarly, the presence of EDTA plus $Ca^{2+}$ also antagonized the enzymatic activity of *N. nigricollis* $PLA_2$, resulting in a decreased hydrolysis of PC and no hydrolysis of PI.

The neurotoxins with $PLA_2$ activity ($\beta$-BuTx and notexin) caused hardly any hydrolysis when incubated with synaptosomes in the presence of EDTA·$Ca^{2+}$ although they were active in the presence of $Ca^{2+}$ only (Table 1). We therefore chose one of the neurotoxins, $\beta$-BuTX, to examine further the inhibition of phospholipid hydrolysis by EDTA·$Ca^{2+}$. The data in Table 2 show that, in

Table 1. Inhibition of phospholipid hydrolysis in synaptosomes by EDTA·$Ca^{2+}$

| $PLA_2$ (50 nM) | EDTA | Hydrolysis (%) PC | PE | PS | PI |
|---|---|---|---|---|---|
| *N. n. atra* | − | 40 ± 1.5 (3) | 45.7 ± 1.6 (3) | 35 ± 5.1 (3) | 34.7 ± 2.5 (3) |
| | + | 6.2 ± 0.3 (8) | 26 ± 3.0 (8) | 13 ± 2.3 (8) | 0 (8) |
| *N. nigricollis* | − | 26 ± 1.2 (3) | 24.3 ± 1:6 (3) | 17 ± 2.5 (3) | 10.3 ± 6.8 (3) |
| | + | 8.4 ± 0.5 (8) | 25.5 ± 4.4 (6) | 26.4 ± 3.6 (8) | 0 (8) |
| β-BuTx | − | 24.6 ± 0.6 (3) | 14.3 ± 3.5 (3) | 17 ± 0.6 (3) | 0 (3) |
| | + | 1.3 ± 0.9 (6) | 0.3 ± 0.3 (6) | 0 (6) | 0 (6) |
| Notexin | − | 14.3 ± 0.3 (3) | 20 ± 2.0 (3) | 21.7 ± 3.4 (3) | 4.7 ± 4.7 (3) |
| | + | 1.1 ± 0.9 (9) | 1.9 ± 0.9 (9) | 0.9 ± 0.9 (8) | 0 (8) |

Rat brain synaptosomes were suspended at 2.5 mg protein/mL in Tris–sucrose buffer containing either 5 mM $Ca^{2+}$ (EDTA−) or 25 mM $Ca^{2+}$ plus 10 mM EDTA (EDTA+). Incubation with enzymes and toxins was at 37° for 10 min. Control synaptosomes contained, per 100 μg phospholipid, 5.7 μg sphingomyelin, 40.2 μg PC, 3.6 μg PI, 12.6 μg PS and 37.8 μg PE. Results are means ± SD for N indicated in parentheses.

Table 2. $Ca^{2+}$ requirement for synaptosomal phospholipid hydrolysis by β-BuTX

| $Ca^{2+}$ (mM) | Hydrolysis (%) PC | PE | PS | PI |
|---|---|---|---|---|
| None | 24, 22 | 15, 18 | 12, 13 | 0, 0 |
| 5 | 44, 49 | 23, 23 | 11, 21 | 0, 0 |
| 10 | 43, 46 | 23, 25 | 20, 16 | 20, 0 |
| 15 | 41, 45 | 17, 20 | 16, 14 | 11, 0 |
| 20 | 34 | 18 | 15 | 0 |
| 25 | 27, 34 | 26, 22 | 15, 17 | 10, 0 |

Synaptosomes suspended in Tris–sucrose buffer and supplemented with the indicated amounts of $Ca^{2+}$ were incubated with 500 nM β-BuTX for 10 min at 37°. Phospholipid distribution in control synaptosomes is given in the legend of Table 1. Results are individual experiments.

Table 3. Inhibition of β-BuTX-induced phospholipid hydrolysis in synaptosomes by EDTA·$Ca^{2+}$ at various $Ca^{2+}$ concentrations

| $Ca^{2+}$ (mM) | Hydrolysis (%) PC | PE | PS | PI |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 25 | 0, 1, 0 | 0, 3, 11 | 0, 0, 0 | 0, 0, 0 |
| 30 | 0 | 0 | 0 | 0 |
| 35 | 0 | 7 | 0 | 0 |
| 40 | 0, 0, 0 | 20, 2, 10 | 0, 0, 0 | 0, 0, 0 |
| 45 | 0 | 17 | 0 | 0 |
| 55 | 0, 0 | 9, 6 | 5, 3 | 13, 26 |
| 70 | 0, 0 | 8, 14 | 23, 29 | 14, 0 |
| 85 | 0, 0 | 3, 3 | 34, 50 | 40, 26 |
| 100 | 0, 0 | 3, 2 | 70, 34 | 0, 0 |

Synaptosomes suspended in Tris–sucrose–10 mM EDTA were supplemented with the indicated amounts of $Ca^{2+}$ and incubated with 500 nM β-BuTX for 10 min at 37°. Phospholipid distribution in control synaptosomes is given in the legend of Table 1. Results are individual experiments.

the absence of EDTA, hydrolysis of synaptosomal phospholipids by β-BuTX was optimal with $Ca^{2+}$ concentrations ranging from 5 to 15 mM; it declined slightly at higher concentrations. It also appears that in the synaptosomal preparation there was sufficient endogenous $Ca^{2+}$ to support a limited phospholipid hydrolysis when $Ca^{2+}$ was not added (Table 2). In the presence of EDTA, however, there was an inhibition of hydrolysis which was not relieved even by a large excess of $Ca^{2+}$. Table 3 shows that the inhibition of hydrolysis induced by 10 mM EDTA was essentially maintained in the presence of increasing $Ca^{2+}$ concentrations, up to 100 mM. Some hydrolysis of PS and PI appeared at a very high $Ca^{2+}$ concentration, although the values were inconsistent.

The question was asked whether the effect of EDTA·$Ca^{2+}$ on phospholipid hydrolysis in synaptosomes depended on the order of addition of EDTA, $Ca^{2+}$ and enzyme. This possibility was checked by preincubating synaptosomes with EDTA for various time intervals before the addition of $Ca^{2+}$ and enzyme. It was found (Table 4) that preincubation with EDTA was not required; whenever EDTA was present during incubation, the hydrolysis by *N. n. atra* $PLA_2$ of most phospholipids, mainly of PC, was decreased and hydrolysis by β-BuTX was abolished. This was regardless of whether EDTA was added before incubation with $PLA_2$ and $Ca^{2+}$, added together with $Ca^{2+}$, or after preincubation with $Ca^{2+}$ (latter not shown).

Synaptosomes incubated in the presence of EDTA·$Ca^{2+}$ appear to be more resistant to $PLA_2$. The possibility that EDTA·$Ca^{2+}$ functions as an inhibitor of proteolysis, thus stabilizing the synaptosomes during the incubation, was checked further. This, however, was ruled out since the protein patterns obtained on SDS–PAGE from synaptosomes incubated in 10 mM $Ca^{2+}$ or in 25 mM $Ca^{2+}$ + 10 mM EDTA were identical (data not shown).

The inhibitory effect of EDTA·$Ca^{2+}$ on synaptosomal phospholipid hydrolysis by 500 nM β-BuTX was dependent on the EDTA concentration. At a constant $Ca^{2+}$ concentration of 25 mM, the

Table 4. Effect of time of preincubation with EDTA on inhibition of phospholipid hydrolysis

| Preincubation | Incubation | Hydrolysis (%) PC | PE | PS | PI |
|---|---|---|---|---|---|
| $Ca^{2+}$, 5 min | *N. n. atra* | 42 | 49 | 33 | 0 |
| | β-BuTX | 32 | 23 | 18 | 0 |
| EDTA, 0 min | $Ca^{2+}$ + *N. n. atra* | 8 | 30 | 12 | 22 |
| | $Ca^{2+}$ + β-BuTX | 0 | 0 | 0 | 0 |
| EDTA, 5 min | $Ca^{2+}$ + *N. n. atra* | 9 | 32 | 17 | 0 |
| | $Ca^{2+}$ + β-BuTX | 0 | 0 | 0 | 0 |
| EDTA 10 min | $Ca^{2+}$ + *N. n. atra* | 9 | 47 | 10 | 0 |
| | $Ca^{2+}$ + β-BuTX | 0 | 0 | 0 | 0 |
| EDTA + $Ca^{2+}$, 5 min | *N. n. atra* | 11 | 35 | 35 | 0 |
| | β-BuTX | 0 | 0 | 0 | 0 |

Synaptosomes suspended in Tris–sucrose were supplemented, as indicated, with 10 mM EDTA and 25 mM $Ca^{2+}$. *N. n. atra* $PLA_2$ was 50 nM and β-BuTx was 500 nM. Preincubation was at 37° for the times indicated, and incubation was for 10 min at 37°. Phospholipid distribution in control synaptosomes is given in the legend of Table 1.

Table 5. Inhibition of β-BuTX-induced hydrolysis in synaptosomes by EDTA·$Ca^{2+}$ at various EDTA concentrations

| EDTA (mM) | Hydrolysis (%) PC | PE | PS | PI |
|---|---|---|---|---|
| 0 | 23, 26 | 32, 32 | 27, 20 | 21, 13 |
| 1 | 24, 24 | 21, 30 | 25 | 9, 23 |
| 2 | 17, 17 | 33, 25 | 26, 28 | 25, 21 |
| 3 | 4, 4 | 12, 20 | 29 | 44, 33 |
| 4 | 2, 3 | 3, 11 | 9, 12 | 23, 34 |
| 6 | 2, 2 | 5, 16 | 35, 10 | 23, 26 |
| 8 | 0, 0 | 7, 10 | 22, 25 | 0, 0 |
| 10 | 0, 3 | 2, 6 | 7, 7 | 0, 0 |

Synaptosomes suspended in Tris–sucrose–25 mM $Ca^{2+}$ were supplemented with EDTA in the amounts indicated and incubated with 500 nM β-BuTX for 10 min at 37°. Phospholipid distribution in control synaptosomes is given in the legend of Table 1. Results are individual experiments.

inhibition by EDTA increased from 3 to 10 mM. Interestingly, the hydrolysis of individual phospholipids was inhibited at different EDTA concentrations, i.e. PC and PE at lower EDTA concentrations than PS and PI (Table 5). Maximal inhibition of hydrolysis for all phospholipid species was reached at 10 mM EDTA (Table 5). Essentially similar effects were obtained upon replacing EDTA with ethyleneglycolbis(aminoethylether)tetraacetate (EGTA) (data not shown).

The demonstration of an inhibitory action of EDTA·$Ca^{2+}$ on synaptosomal phospholipid hydrolysis prompted the question of whether other effects of $PLA_2$ such as effects on synaptosomal osmotic activity, on LDH leakage or on ACh release would also be altered by the presence of EDTA·$Ca^{2+}$. The synaptosomal osmotic activity reflected their intactness as measured by shrinkage upon addition of hypertonic NaCl solution to the medium [16]. $PLA_2$ enzymes and neurotoxins decrease or abolish this osmotic behavior, i.e. render the synaptosomes permeable to NaCl. We found that the decreases in osmotic activity induced by either 50 mM *N. n. atra* $PLA_2$ or 50 mM β-BuTX were similar whether tested in the presence of 25 mM $Ca^{2+}$ alone or 25 mM $Ca^{2+}$ + 10 mM EDTA (Fig. 1). EDTA alone blocks the effect of the $PLA_2$ toxins and enzymes [1].

Treatment of synaptosomes with $PLA_2$ enzymes and neurotoxins promotes the release of ACh [17, 18] as well as leakage of LDH [1]. The increase in ACh release induced by 5 nM β-BuTx, 5 nM notexin and 5 nM *N. nigricollis* $PLA_2$ in a $Ca^{2+}$-containing medium, was decreased markedly or abolished in the presence of 10 mM EDTA–50 mM $Ca^{2+}$ (Table 6). Experiments concerned with the leakage of LDH from synaptosomes, induced by $PLA_2$ enzymes in the presence of EDTA·$Ca^{2+}$, were difficult to interpret. While synaptosomes incubated at 37° in Tris–sucrose and 5 mM $Ca^{2+}$ had stable LDH values, in the presence of 10 mM EDTA + 25 mM $Ca^{2+}$ the total LDH activity in synaptosomes was reduced drastically during incubation. This unexpected result suggested that EDTA·$Ca^{2+}$ may directly inhibit the LDH activity. We confirmed this possibility by incubating commercial LDH in medium supplemented with 10 mM EDTA + 25 mM $Ca^{2+}$ or with either 10 mM EDTA or 5 mM $Ca^{2+}$ separately. Indeed, while the commercial enzyme was quite stable in the medium containing either $Ca^{2+}$ or EDTA, full activity being retained after 50 min at 37°, the samples incubated in EDTA·$Ca^{2+}$ retained only 6% activity. Full details concerning the inhibition of LDH from commercial sources and from red cell lysates by EDTA·$Ca^{2+}$ are the object of a separate paper [19].

The inhibition of phospholipid hydrolysis first observed with synaptosomes as substrate occurred also in the absence of a biological membrane. This was shown by preparing a synaptosomal total lipid

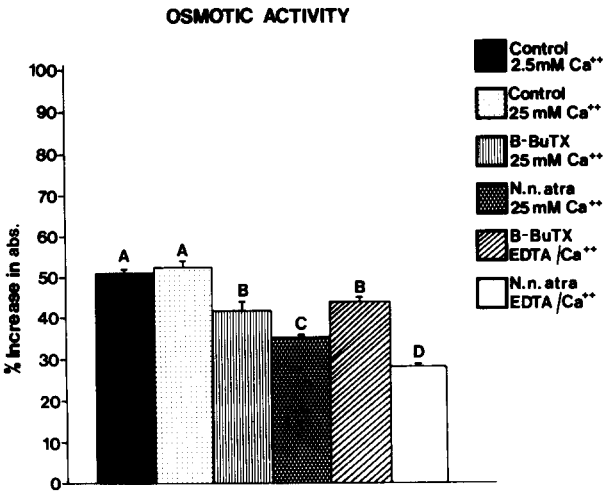


Fig. 1. Osmotic activity of synaptosomes in the presence of phospholipases and EDTA·$Ca^{2+}$. Synaptosomes were incubated with *N. n. atra* or β-BuTx (50 nM) for 40 min at 37°, in the presence of either 25 mM $Ca^{2+}$ or 10 mM EDTA plus 25 mM $Ca^{2+}$. Aliquots of synaptosomal suspensions (0.25 mg protein/mL, $O.D._{450} \simeq 1.0$) were used for osmotic activity determinations. Osmotic activity is expressed as percent increase in absorbance (shrinkage) in response to a hyperosmotic environment. Data are presented as means ± SE, N = 4. Means with the same letter are not significantly different ($P > 0.05$).

Table 6. Inhibition by EDTA·$Ca^{2+}$ of phospholipase-induced ACh release

| | Acetylcholine release (%) | | |
|---|---|---|---|
| 5 nM | A<br>2.5 mM $Ca^{2+}$ | B<br>10 mM EDTA | C<br>10 mM EDTA + 50 mM $Ca^{2+}$ |
| β-BuTX | 212 ± 11 | 85 ± 5 | 111 ± 14 |
| Notexin | 259 ± 16 | 98 ± 16 | 124 ± 7 |
| *N. nigricollis* $PLA_2$ | 185 ± 6 | 113 ± 7 | 90 ± 3 |
| | N = 7–10 | N = 6–10 | N = 3–4 |

Results are ACh release expressed as a percentage of the release from untreated control synaptosomes in conditions A, B, and C. The actual values for ACh released from controls were (for a 30-min incubation): A, 120,000 ± 6,000; B, 135,000 ± 31,000; and C, 126,000 ± 12,000 cpm/mg protein. Results are means ± SD for the indicated ranges of N.

extract which, dispersed with Triton X-100, was used as a substrate for *N. nigricollis* and *N. n. atra* $PLA_2$. The activity of both phospholipases was inhibited in the presence of EDTA·$Ca^{2+}$ (Table 7).

## DISCUSSION

EDTA is a ubiquitous addition during the preparation of subcellular particles, its "stabilizing" effect being due to inhibition of proteolytic and lipolytic enzymes by chelation of $Ca^{2+}$ and other bivalent metals. In addition, its $Ca^{2+}$-chelating property makes it a widely used inhibitor of $PLA_2$ activity which is $Ca^{2+}$ dependent. It is quite common to reverse this inhibition by an excess of $Ca^{2+}$.

Our data show, for the first time, that EDTA can be a $PLA_2$ inhibitor, even in the presence of excess

Table 7. Inhibition of phospholipase-induced hydrolysis by EDTA·$Ca^{2+}$ in synaptosomal lipid extracts

| $PLA_2$ (50 nM) | EDTA | Hydrolysis (%) PC | PE | PS | PI |
|---|---|---|---|---|---|
| *N. n. atra* | − | 33 ± 1 | 80 ± 1 | 89 ± 4 | 44 ± 10 |
| | + | 4 ± 0.3 | 19 ± 3 | 22 ± 4 | 24 ± 4 |
| *N. nigricollis* | − | 39 ± 1 | 90 ± 2 | 94 ± 1 | 48 ± 10 |
| | + | 6 ± 0.5 | 29 ± 3 | 47 ± 5 | 20 ± 13 |

A synaptosomal total lipid extract containing 30 μmol phospholipid was dispersed in 20 mL of Tris–sucrose buffer containing 60 μmol of Triton X-100. For incubation with enzymes the dispersion was supplemented with either 5 mM $Ca^{2+}$ or with 25 mM $Ca^{2+}$ plus 10 mM EDTA (EDTA+). Incubations were at 37° for 10 min. Phospholipid distribution in control synaptosomal lipid extracts is given in the legend of Table 1. Results are means ± SD for N = 4.

$Ca^{2+}$, provided that EDTA is at a sufficiently high concentration. In our system the inhibition induced by 10 mM EDTA could not be reversed even when the $Ca^{2+}$ concentration was increased to 10-fold that of EDTA.

We observed the $Ca^{2+}$-irreversible effect of EDTA on the hydrolysis of synaptosomal phospholipids by two snake venom $PLA_2$ enzymes (*N. n. atra* and *N. nigricollis*) and by two neurotoxins with $PLA_2$ activity (notexin and β-BuTx). An inhibitory effect of EDTA that could not be reversed by $Ca^{2+}$ has been described by Zucker and Grant [20] and by Zucker *et al.* [21] and Pidard *et al.* [22]. In their experiments, EDTA inhibited platelet aggregation following a short incubation at 37°, and subsequent addition of $Ca^{2+}$ failed to restore this property. The irreversible loss of platelet aggregability was attributed to an effect of EDTA on two surface glycoproteins which, in $Ca^{2+}$-deprived platelets, diffuse too far apart in the membrane to be able to recombine when $Ca^{2+}$ is restored [21]. The inhibitory effect of EDTA·$Ca^{2+}$ observed by us bears no similarity to the effect on platelet aggregation. First, the inhibition of $PLA_2$ was not dependent on the order in which EDTA and $Ca^{2+}$ were added and, second, the effect was not membranal, inhibition of the $PLA_2$ occurring also with dispersed phospholipid-Triton micelles as substrate.

Inhibitors of $PLA_2$ may exert their effects either by inducing a change in the conformation of the substrate or by acting directly on the enzyme. As mentioned above, EDTA·$Ca^{2+}$ inhibited $PLA_2$ hydrolysis of phospholipids in synaptosomes as well as in mixed micelles of synaptosomal phospholipids with Triton X-100. This suggests an inhibition which is independent of the state of organization of the substrate. Furthermore, the observation that a different enzyme (LDH) acting on soluble substrate was also inhibited in the presence of EDTA·$Ca^{2+}$ supports the assumption that inhibition was exerted on the enzyme and not on the substrate.

The experiments with LDH allowed us to demonstrate that the inhibitor is a complex of EDTA·$Ca^{2+}$ and not EDTA alone. Obviously, this could not be demonstrated directly in experiments with $PLA_2$ which is a $Ca^{2+}$-dependent enzyme and, therefore, all tests were performed in conditions of excess $Ca^{2+}$. Based on our data, we postulate that EDTA has a dual inhibitory activity on $PLA_2$. The first, occurring at relatively low EDTA concentrations, which in our experimental conditions is under 3 mM, is the well-known inhibition due to $Ca^{2+}$ depletion and is reversed by $Ca^{2+}$. The other, that has not been described so far, is the inhibition by the EDTA·$Ca^{2+}$ complex formed at higher EDTA concentration and which is not relieved by excess $Ca^{2+}$. We interpret the difference between the reversible inhibition at low EDTA concentration and the irreversible one at high EDTA concentration (Table 5) as deriving from the respective amounts of EDTA·$Ca^{2+}$ inhibitor formed. Thus, in our experimental conditions, the amount of complex formed at 1 mM EDTA·25 mM $Ca^{2+}$ was not inhibitory, whereas the amount formed at 10 mM EDTA·25 mM $Ca^{2+}$ gave full inhibition.

EDTA and other chelators have been reported to induce conformational changes in metallo-proteins. Thus, binding of EDTA or of an EDTA·$Ca^{2+}$ complex to β-lactalbumin induces a shift from A to N conformer [23]. Moreover, EDTA has been shown to bring about slow irreversible spectral changes in oxidized cytochrome *c* even in the presence of excess $Ca^{2+}$ [24]. A similar conformational change may be the source of the $PLA_2$ and LDH inactivation reported by us. Although, based on the LDH inhibition, we favor the hypothesis that the inhibitor is an EDTA·$Ca^{2+}$ complex [19], a direct effect of EDTA *per se* on the enzyme conformation cannot be excluded. To elucidate this point, experiments in which spectral responses of $PLA_2$ to EDTA, $Ca^{2+}$ and EDTA·$Ca^{2+}$ have been undertaken.

Inhibitory concentrations of EDTA·$Ca^{2+}$ may be reached in routine experimental conditions and may have important consequences when $PLA_2$ are used to investigate membrane structure and function. Thus, synaptosomes treated with $PLA_2$ in inhibitory conditions appear to have a decreased phospholipid availability and they also release less acetylcholine. Of the functions we tested, only the synaptosomal osmotic activity was not affected by the EDTA·$Ca^{2+}$ inhibitor. Since the osmotic activity is a very sensitive test of permeability to small solutes, it seems that the damage done by phospholipid hydrolysis, although decreased by enzyme inhibition, is still sufficient to affect the passage of salt. Obviously, the inactivation of LDH, a common indicator of cellular integrity, by EDTA·$Ca^{2+}$ may also lead to erroneous conclusions.

The mechanism of enzyme inhibition by an EDTA·$Ca^{2+}$ complex is not yet known. The fact that both $PLA_2$ and LDH were inhibited points to a non-specific mode of action. Work on the LDH inhibition by EDTA·$Ca^{2+}$ is in progress.

*Acknowledgements*—This work was supported by a U.S. Public Health Service Grant (ROINS 14521) awarded to P. R. We thank Dr. C-C. Yang from the Institute of Molecular Biology, National Tsing Hua University, Hsinchu, Taiwan, for the purification of $PLA_2$ enzymes and toxins.

## REFERENCES

1. Yates SL, Burns M, Condrea E, Ghassemi A, Shina R and Rosenberg P, Phospholipid hydrolysis and loss of membrane integrity following treatment of rat brain synaptosomes with β-bungarotoxin, notexin, and *Naja naja atra* and *Naja nigricollis* phospholipase $A_2$. *Toxicon* **28**: 939–951, 1990.
2. Yates SL, Levine L and Rosenberg P, Leukotriene and prostaglandin production in rat brain synaptosomes treated with phospholipase $A_2$ neurotoxins and enzymes. *Prostaglandins* **39**: 425–438, 1990.
3. Yang C-C and King K, Chemical modification of the histidine residue in basic phospholipase $A_2$ from the venom of *Naja nigricollis*. *Biochim Biophys Acta* **614**: 373–388, 1980.
4. Yang C-C, King K and Sun TP, Chemical modifications of lysine and histidine residues in phospholipase $A_2$ from the venom of *Naja naja atra* (Taiwan cobra). *Toxicon* **19**: 645–659, 1981.
5. Karlsson E, Eaker D and Ryden L, Purification of a presynaptic neurotoxin from the venom of the Australian tiger snake *Notechis scutatus scutatus*. *Toxicon* **10**: 405–413, 1972.

6. Yang C-C and Chang LS, Tryptophan modification of phospholipase $A_2$ enzymes and presynaptic neurotoxins from snake venoms. *J Protein Chem* **3**: 195–213, 1984.
7. Booth RFG and Clark JB, A rapid method for the preparation of relatively pure metabolically competent synaptosomes from rat brain. *Biochem J* **176**: 365–370, 1978.
8. Markwell MAK, Haas SM, Bieber LL and Tolbert NE, A modification of the Lowry procedure to simplify protein determinations in membrane and lipoprotein samples. *Analyt Biochem* **87**: 206–210, 1978.
9. Marinetti GV, Albrecht M, Ford T and Stotz E, Analysis of human plasma phosphatides by paper chromatography. *Biochim Biophys Acta* **36**: 4–13, 1959.
10. Folch J, Lees M and Sloane Stanley GH, A simple method for the purification of total lipids from animal tissue. *J Biol Chem* **226**: 497–509, 1957.
11. Condrea E, Rosenberg P and Dettbarn WD, Demonstration of phospholipid splitting as the factor responsible for increasing permeability and block of axonal conduction induced by snake venom. 1. Study on lobster axons. *Biochim Biophys Acta* **135**: 669–681, 1967.
12. Bartlett GR, Phosphorus assay in column chromatography. *J Biol Chem* **234**: 466–468, 1959.
13. Johnson MK, The intracellular distribution of glycolytic and other enzymes in rat brain homogenates and mitocondrial preparations. *Biochem J* **77**: 610–618, 1960.
14. Boska P and Collier B, Acetylation of homocholine by rat brain: Subcellular distribution of acetylhomocholine and studies on the ability of homocholine to serve as substrate for choline acetyltransferase *in situ* and *in vitro*. *J Neurochem* **34**: 1470–1482, 1980.
15. Goldberg AM and McCaman RE, The determination of picomole amounts of acetylcholine in mammalian brain. *J Neurochem* **20**: 1–5, 1973.
16. Michaelson DM and Raferty MA, Purified acetylcholine receptor: Its reconstruction to a chemically excitable membrane. *Proc Natl Acad Sci USA* **71**: 4768–4772, 1974.
17. Sen J and Cooper JR, Effect of $\beta$-bungarotoxin on the release of acetylcholine from brain synaptosomal preparations. *Biochem Pharmacol* **24**: 2107–2112, 1975.
18. Shabo-Shina R and Bdolah A, Interactions of the neurotoxic complex from the venom of false hornet viper (*Pseudocerastes fieldi*) with rat striatal synaptosomes. *Toxicon* **25**: 253–266, 1987.
19. Shina R and Condrea E, Inhibition of lactate dehydrogenase by an EDTA·$Ca^{2+}$ complex. *Clin Chim Acta* **189**: 231–236, 1990.
20. Zucker MB and Grant RA, Non-reversible loss of platelet aggregability induced by calcium deprivation. *Blood* **52**: 505–511, 1978.
21. Zucker MB, Varon D, Masiello NC and Karpatkin S, The combining ability of glycoproteins IIb, IIIa and $Ca^{2+}$ in EDTA-treated nonaggregable platelets. *Thromb Haemost* **50**: 848–851, 1983.
22. Pidard D, Didry D, Konicki TJ and Norden AT, Temperature-dependent effects of EDTA on the membrane glycoprotein IIb–IIIa complex and platelet aggregability. *Blood* **67**: 604–611, 1986.
23. Kronman MJ and Bratcher SC, An experimental artifact in the use of chelating metal ion buffers. *J Biol Chem* **258**: 5707–5709, 1983.
24. Konstantinov A, Vygodina T, Popova E, Berka V and Musatov A, Spectral shifts of cytochrome *c* oxidase induced by complexons. *FEBS Lett* **245**: 39–42, 1989.