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Inhibition of Na^+, K^+-ATPase Activity by Phospholipase A_2 and Several Lysophospholipids: Possible Role of Phospholipase A_2 in Noradrenaline Release from Cerebral Cortical Synaptosomes

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Abstract—*p*-Bromophenacyl bromide (PBPB), quinacrine and indomethacin, which inhibit phospholipase A_2 (PLA_2; EC 3.1.1.4) activity in several tissues, caused a dose-dependent inhibition of prelabelled [^{3}H]noradrenaline ([^{3}H]NA) release evoked by high concentrations of K^+ from rat cerebral cortical synaptosomes. Release of prelabelled [^{3}H]NA was caused by natural lysophosphatidic acid (LPA; $10^{-6}-10^{-5}$ g mL^{-1}) and lysophosphatidylcholine (LPC; $10^{-6}-10^{-5}$ g mL^{-1}) and synthetic LPA (6×10^{-6}, 2×10^{-5} M) and LPC (6×10^{-6}, 2×10^{-5} M), but not by natural lysophosphatidylserine (LPS; 10^{-5} g mL^{-1}), lysophosphatidylethanolamine (LPE; 10^{-5} g mL^{-1}) and lysophosphatidylinositol (LPI; 10^{-5} g mL^{-1}). The release evoked by natural LPA and LPC could be inhibited only marginally by PBPB and quinacrine. Phosphatidic acid (PA)-specific and phosphatidylcholine (PC)-specific PLA_2 activities from rat cerebral cortical synaptosomes were stimulated in incubation medium containing high concentrations of K^+ or calcium ionophore A23187. Low concentrations of PLA_2 ($10^{-6}-10^{-8}$ g mL^{-1}, from bee venom) inhibited the synaptic membrane Na^+, K^+-ATPase activity in incubation media with intracellular levels of free Ca^{2+}. Several lysophospholipids (LPLs), metabolites of the PLA_2 type, also inhibited the synaptic membrane Na^+, K^+-ATPase activity in a dose-dependent manner. The minimum effective concentrations of natural LPA, LPC, LPS, LPI and LPE were 10^{-6}, $4{\cdot}7\times10^{-6}$, 10^{-5}, $4{\cdot}7\times10^{-5}$ and $4{\cdot}7\times10^{-5}$ g mL^{-1}, respectively. These results suggest that PLA_2 and/or its metabolites, LPLs, especially LPA and LPC, may play partial roles in the depolarization and/or release of noradrenaline through their inhibitory action on the Na^+, K^+-ATPase activity in the brain.

Hydrolysis of inositol phospholipids is now accepted as a common mechanism for transducing various extracellular signals (biologically active substances such as certain hormones, neurotransmitters, antigens and other peptides) into various cells, including those of the central nervous system (Hokin & Hokin 1953; Michell 1975; Nahorski et al 1986). Of the metabolites of inositol phospholipids, diacylglycerol (Berridge 1984; Hirasawa & Nishizuka 1985) has been shown to activate protein kinase C, which regulates several cell functions, and inositol-1,4,5-trisphosphate (Berridge 1984; Joseph et al 1984) to trigger the release of Ca^{2+} from intracellular stores.

Activation of cellular phospholipase A_2 (PLA_2; EC 3.1.1.4) is a critical step in the initiation of Ca^{2+}-dependent cell function such as excitation-secretion (Sklenovský 1976; Marone et al 1981; Bradford et al 1983; Bormann et al 1984) or excitation-contraction (Borda et al 1983; Frye & Holz 1983; Slapke 1984) coupling. PLA_2 hydrolyses intracellular phospholipids such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylinositol (PI) to the corresponding lysophospholipids (LPLs) and free fatty acids. There are the predominant precursors for prostaglandins (PGs). PLA_2 is thought to be the rate-limiting step enzyme in PG biosynthesis. In the central nervous system, however, the E series of PGs inhibit the excitation-secretion coupling (Bergström et al 1973; Bradford et al 1983). On the other hand, several LPLs, metabolites of the PLA_2 type, produce intense inflammation when applied locally (Phillips et al 1965), are vasoactive when administered intravenously (Tokumura et al 1978), induce aggregation of platelets (Gerrard et al 1979) or chemotaxis of neutrophils in-vitro (Gerrard et al 1980), and also are involved in cytotoxic effects on tumour cells (Andreesen et al 1978), virus-induced cell fusion (Parkes & Fox 1975) or various intracellular fusion processes such as exocytosis (Smith 1972; Bradford et al 1983; Mets 1986). However, the roles of the receptor-mediated activation of this enzyme and LPLs in these cell activations have not been thoroughly elucidated.

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Membrane Na^+, K^+-ATPase (EC 3.6.1.3.) has been shown to be involved in active ion transport across the cell membrane and considered to be the biochemical basis for the sodium pumping function (Skou 1965; Schwartz et al 1975). The Na^+, K^+-ATPase was shown to have a regulatory role in the release of neurotransmitters or other intracellular substances (Vizi 1977; Meyer & Cooper 1981) and to be concentrated in synaptic membranes of the central nervous system.

Thus, the present study was undertaken to determine the effects of high concentrations of K^+ and several LPLs on prelabelled [^{3}H]noradrenaline ([^{3}H]NA) release from cortical synaptosomes in the presence or absence of PLA_2 inhibitors. The effects of high concentrations of K^+ and calcium ionophore A23187 were also determined on the PLA_2 activity from the synaptosomes. Furthermore, the effects of commercially available PLA_2 (from bee venom) and several

phospholipids or LPLs on membrane Na^+, K^+-ATPase activities of rat synaptosomal membranes were determined in incubation media of various concentrations of free Ca^{2+} using ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate (EGTA), a divalent cation chelator with high affinity for Ca^{2+}. Here we describe the inhibitory action of PLA_2 and LPLs on synaptic membrane Na^+, K^+-ATPase activity in incubation media with intracellular levels of free Ca^{2+}. Our findings offer some explanations for the role of PLA_2 and/or LPLs, especially lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC), in the linkage of depolarization and/or Ca^{2+}-increasing mechanism mediating the release of [^{3}H]NA in the brain. Some of the results have been presented in a preliminary form (Nishikawa et al 1988).

Materials and Methods

Preparation of synaptosomes

Male Wistar rats, 150–250 g, were killed by exsanguination after a blow on the head. The cerebral cortex was rapidly removed and homogenized in 0·32 M sucrose containing 1·5 mM ethylenediaminetetraacetate (EDTA). The synaptosomal fraction was isolated by a slightly modified method of Barker et al (1972). The homogenates (10% w/v) were centrifuged for 15 min at 1000 *g* and the supernatant was centrifuged for 20 min at 13 500 *g* to sediment the crude mitochondrial fraction (P_2 fraction). This fraction was gently resuspended by hand in a glass-Teflon homogenizer and then applied to a discontinuous gradient of 1·2 and 0·8 M sucrose each containing 1·5 mM EDTA, and centrifuged for 90 min at 98 000 *g* in the swing-out rotor (RPS50-2-151; 6 × 5 mL) of a Hitachi 55P-7 ultracentrifuge. The materials from the interphase between 1·2 and 0·8 M sucrose were collected by suction with a syringe, diluted about ten times with 0·32 M sucrose containing 1·5 mM EDTA, and sedimented by centrifugation for 20 min at 16 000 *g*. The synaptosomal pellets were resuspended in 0·32 M sucrose and used for subsequent experiments.

Assay for [^{3}H]NA release

Preparation of [^{3}H]NA-containing rat brain synaptosomes and measurement of [^{3}H]NA release were carried out as described by Bradford & Marinetti (1982). All phospholipids were suspended in distilled water using a flash mixer and an ultrasonicator (Bransonic 22S) for 15 s immediately before use. LPA and lysophosphatidylinositol (LPI) in chloroform were evaporated to dryness under reduced pressure before the suspension. To study the effects of high K^+ and LPLs on the basal release of noradrenaline, 0·1 mL of [^{3}H]NA-containing synaptosomes (about 200 μg of synaptosomal membrane protein) was added to test tubes containing 50 mM K^+ and different natural and synthetic amounts of LPLs and then re-suspended by sonication for 15 s at 37°C in 0·4 mL of Ca^{2+}-free Hanks buffer containing 20 mM mannitol, 0·5 mM ascorbic acid, and 25 μM iproniazid. To inhibit the reuptake of the released NA, we added 2 μM imipramine to the buffer. Concentrations of natural phospholipids used in the experiments were expressed as g mL^{-1} (final concentration), since their exact molecular weights have not been decided, and synthetic phospholipid concentrations were expressed in molar concentrations. The mixtures were incubated at 37°C for 4 min, and then 1·0 mL of Ca^{2+} free buffer was added. To the depolarized samples, 1·0 mL of buffer containing 72·5 mM K^+ or a calculated dose of LPLs and 1·5 mM Ca^{2+} was added to obtain a final concentration of 50 mM K^+ or the concentrations of LPLs indicated in the "Results" and 1 mM Ca^{2+}. After incubation at 37°C for an additional 2 min, the samples were diluted with cold buffer and filtered through glass fibre filters under vacuum. The filters were washed and dried, placed in glass scintillation vials containing 7 mL of ACS cocktail (Amersham), and counted for radioactivity. The difference in counts remaining on the filters of control versus treated samples represented the amount of [^{3}H]NA released. When studying the effects of PLA_2 inhibitors on the stimulated release of [^{3}H]NA from synaptosomes, we included the agents in the Hanks buffer during the 4 min preincubation of synaptosomes to give the indicated concentrations. The mixtures were incubated at 37°C and then depolarized for 2 min by addition of high-K^+ buffer. The samples were diluted, filtered, and analysed for radioactivity. *p*-Bromophenacyl bromide (PBPB) and indomethacin were dissolved in ethanol. Ethanol, which was included in control and test samples, had a final concentration of 20 mM. This concentration of ethanol had no significant effect on [^{3}H]NA release.

Assay for PLA_2 activity

Synaptosomes were incubated at 18°C for 1 h in 6 mL of Ca^{2+}-free Hanks buffer containing 20 mM mannitol and [^{3}H]arachidonyl-PA and [^{14}C]arachidonyl-PC. Aliquots (0·3 mL) of the synaptosomes containing either [^{3}H]- or [^{14}C] phospholipids were added to 2·7 mL of Hanks buffer and incubated at 37°C for 8–12 min. Six mL of buffer containing 1·5 mM Ca^{2+} and 5, 72·5 or 110 mM K^+ was then added. This gave final concentrations of 5, 50 and 75 mM K^+ and 1 mM Ca^{2+}. After incubation at 37°C for 90 s, or the times indicated, the samples were placed on ice and centrifuged at 9000 *g* for 15 min to sediment the synaptosomes. Lipids in the supernatant were extracted by the method of Bligh & Dyer (1959). The synaptosomal pellets were washed in Ca^{2+}-free buffer, and the lipids were extracted by the method of Folch et al (1957). The lipid extracts were concentrated and chromatographed on thin-layer silica gel plates (Merck-Darmstadt silica gel 60, 0·25 mm) in a two-stage system. The plates were developed to two-thirds their height in a solvent system of chloroform–acetone–methanol–acetic acid–water (5:2:1:1:0·5, v/v), dried for 15 min, and then developed fully in hexane–isopropyl ether–acetic acid (60:40:3, v/v). This method separated authentic phospholipid and neutral lipid standards as well as PGs. Lipid standards included LPA, LPC, lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS), LPI, PA, PC, PE, phosphatidylserine (PS), PI, diglyceride, monoglyceride, arachidonic acid, PGE_2 and $PGF_{2\alpha}$. Individual lipid spots were visualized by exposure to iodine vapours. PE, PS, LPE, and LPS were detected with ninhydrin reagent. The lipid spots were scraped from the TLC plates into glass scintillation vials containing 7 mL of ACS cocktail (Amersham) and counted for radioactivity. K^+ of 50 or 75 mM and A23187 of 5 or 10 μM were included in the buffer during the 8–12 min preincubation. A23187 was dissolved in ethanol, which was included in control and test samples at a final concentration of 2 mM; this concentration

of ethanol had no significant effect on arachidonyl-PA and arachidonyl-PC specific PLA_2 activity.

Assay for Na^+, K^+-ATPase activity
Synaptic membrane fractions were prepared by osmotically rupturing the synaptosomes using distilled water and centrifuging the resultant suspension at 50 000 *g* for 1 h. About 40 $\mu g\ mL^{-1}$ (final) of synaptosomal membrane protein was used in each assay. All phospholipids were suspended in distilled water using a flash mixer and an ultrasonicator (Bransonic 22S) for 20 s immediately before use and were preincubated for 5 min at 37°C before the addition of substrate. Details of the media and conditions for the enzyme assay are essentially the same as reported previously (Nishikawa et al 1985). The medium used to estimate the total ATPase activity consisted of 115 mM Tris (pH 7·2), 2·0 mM $MgCl_2$, 6·25 mM KCl and 72·5 mM NaCl. The medium for Mg^{2+}-ATPase activity consisted of 180·5 mM Tris (pH 7·2), 2·0 mM $MgCl_2$ and 1·0 mM ouabain. The Na^+, K^+-ATPase activity was calculated by subtracting the Mg^{2+}-ATPase activity from the total ATPase activity. Under the present experimental conditions, the Na^+, K^+-ATPase activity was almost completely inhibited by addition of 1·0 mM ouabain; thus, the Na^+, K^+-ATPase activity is ouabain-sensitive. The concentration of free Ca^{2+} in the incubation media was buffered with EGTA and calculated (Ogawa 1968), assuming that K_{app} (apparent association constant) was $3{\cdot}16 \times 10^6\ M^{-1}$ at pH 7·2. In all experiments, the enzyme was preincubated for 5 min at 37°C in the absence of substrate. The pH value of the incubation medium was always confirmed to be at pH 7·2 before the start of preincubation. The reaction was terminated 6 min after the addition of disodium adenosine triphosphate (2·0 mM of metal-ion-free disodium ATP) by adding ice-cold 15% trichloacetic acid solution to the reaction test tube held in an ice bath. The amounts of protein (Lowry et al 1951) and inorganic phosphate (Taussky & Shorr 1953) in the supernatant were measured by previously established methods.

Chemicals
The following chemicals and phospholipids or lipids were used: 1-[7-^{3}H]-NA (20 Ci $mmol^{-1}$, New England Nuclear), 1-α-1-palmitoyl-2-[1-^{14}C]arachidonyl-PC (43 mCi $mmol^{-1}$, New England Nuclear), 1-α-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl-PA (60 Ci $mmol^{-1}$, New England Nuclear), 1-NA hydrochloric (Sigma), quinacrine dihydrochloride (Sigma), *p*-bromophenacyl bromide (PBPB; Sigma), disodium ATP (Sigma, prepared by phosphorylation of adenosine, grade I), Triton X-100 (Pierce Chemical Co.), sodium dodecyl sulphate (Sigma), PGE_2 (Sigma), $PGF_{2\alpha}$, PI Na salt (Avanti Polar Lipids, Inc., from bovine liver, >99%), LPI (Serdary Res. Lab. Inc., from pig liver, >99%), PA (Avanti Polar Lipids, Inc., from egg lecithin, >99%), LPA (Serdary Res. Lab. Inc., from egg lecithin, >99%), palmitoyl-LPA (Serdary Res. Lab. Inc.), oleoyl-LPA (Serdary Res. Lab. Inc.), PE (Avanti Polar Lipids, Inc., from bovine heart), LPE (Serdary Res. Lab. Inc., from pig liver, >99%), PC (Avanti Polar Lipids, Inc., from bovine brain, >99%), LPC (Serdary Res. Lab. Inc., from bovine brain, >99%), palmitoyl-LPC (Larodan Fine Chemicals AB.), oleoyl-LPC (Serdary Res. Lab. Inc.), PS (Serdary Res. Lab. Inc., from bovine brain, >99%), LPS (Serdary Res. Lab. Inc., from bovine brain, >99%), palmitoyl chloride (Sigma), oleoyl chloride (Sigma), PLA_2 (Sigma, from bee venom, EC 3.1.1.4), phospholipase C (Sigma, from *Clostridium perfringens*, Type I, EC 3.1.4.3), phospholipase D (Sigma, from cabbage, Type V, EC 3.1.4.4), and ouabain (Sigma).

Statistical analysis
Statistical analysis was performed using the independent *t*-test for comparisons. The results in the text and figures are expressed as the means ± s.e.m.

Results

Effects of several phospholipase inhibitors on [^{3}H]NA release evoked by high K^+ and several LPLs
To determine if the stimulation of PLA_2 might be related to neurotransmitter release, we studied the effects of several PLA_2 inhibitors, PBPB, quinacrine and indomethacin, on the release of prelabelled [^{3}H]NA evoked by high concentrations of K^+ from synaptosomes of cerebral cortices. As shown in Fig. 1, 10^{-4} M PBPB, 2×10^{-5} M quinacrine and 10^{-6} M indomethacin significantly inhibited the K^+-stimulated release of [^{3}H]NA. We studied further the effects of several natural and synthetic LPLs, metabolites of the PLA_2 type, on the release of prelabelled [^{3}H]NA. As shown in Fig. 2, addition of small amounts of natural LPA ($10^{-6} - 10^{-5}$ g mL^{-1}) or LPC ($10^{-6} - 10^{-5}$ g mL^{-1}) and synthetic LPA (6×10^{-6}, 2×10^{-5} M) or LPC (6×10^{-6}, 2×10^{-5} M) to [^{3}H]NA-prelabelled synaptosomes enhanced the dose-related increase in the release of neurotransmitter ^{3}H from the synaptosomes. However, 10^{-5} g mL^{-1} of natural LPS, LPE and LPI did not enhance a statistically significant release of neurotransmitter. And the stimulatory effects of natural LPA (10^{-5} g mL^{-1}) and LPC (10^{-5} g mL^{-1}) were not reduced by treatment with the PLA_2 inhibitors, PBPB (10^{-4} M) and quinacrine (2×10^{-5} M) (Fig. 3). These results suggest a relatively specific action of LPA and also of LPC on the neurotransmitter release. Natural PA, PC, PE, PS and the detergent sodium dodecyl sulphate (SDS; 10^{-4} M) at 10^{-5} g mL^{-1} hardly affected [^{3}H]NA release with the same preparation of synaptosomes (data not shown).

To examine the possible role for PGs in synaptic function and to ascertain whether they modulate neurosecretion, the effects of exogenous PGE_2 ($10^{-8} - 10^{-6}$ M) and $PGF_{2\alpha}$ ($10^{-8} - 10^{-5}$ M) on the high K^+-induced release of [^{3}H]NA from synaptosomes were determined. Preincubation with PGE_2 markedly inhibited high K^+ (50, 75 mM)-induced tritium release while $PGF_{2\alpha}$ hardly inhibited it (data not shown).

Effects of high K^+ and calcium ionophore A23187 on PLA_2 activity
The results of experiments demonstrating the stimulation of PLA_2 in the cerebral cortical synaptosomes by K^+ depolarization and by A23187 are summarized in Fig. 4. Since 1-acyl-2-[^{3}H]arachidonyl-PA and 1-acyl-2-[^{14}C]arachidonyl-PC were used as substrate tracers in these experiments, the production of [^{3}H]arachidonic acid and [^{14}C]arachidonic acid may have reflected PA-specific and PC-specific PLA_2 activities, respectively. When synaptic membrane fractions were

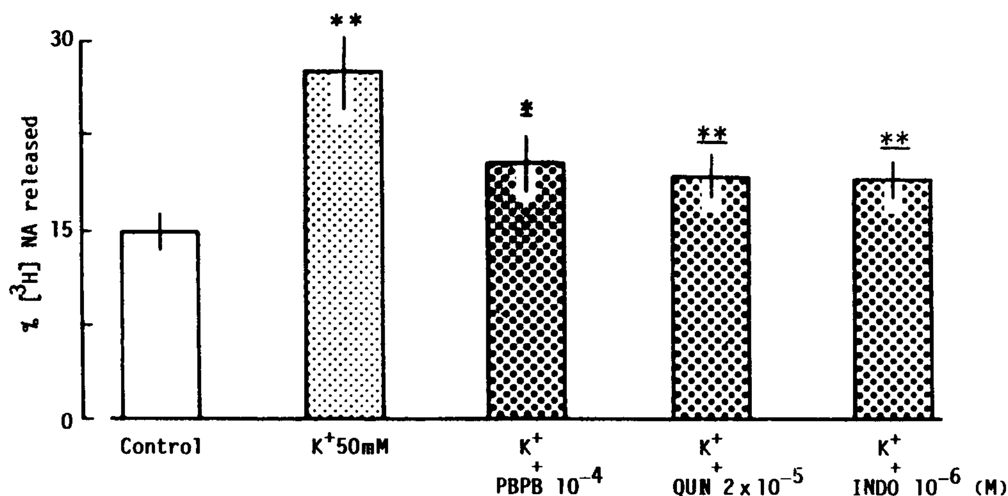

FIG. 1. Effects of PLA_2 inhibitors, PBPB, quinacrine and indomethacin, on [^{3}H]NA release from synaptosomes evoked by high K^+ concentration. All inhibitors were added to the incubation tube before the start of incubation. After 2 min, the synaptosomes were isolated and counted for radioactivity as described under "Materials and Methods". Values are expressed as a percentage release of the total synaptosomal [^{3}H]NA. The control value was 0·41 pmol mg^{-1} protein released from synaptosomes. Columns represent mean values for 6 to 7 determinations with s.e.m. as a vertical line. ** Significantly different ($P < 0.01$) from control, $\underline{*}$ significantly different ($P < 0.05$) from the response caused by 50 mM K^+ alone, $\underline{**}$ ($P < 0.01$). PBPB: *p*-bromophenacyl bromide, QUIN: quinacrine, INDO: indomethacin.

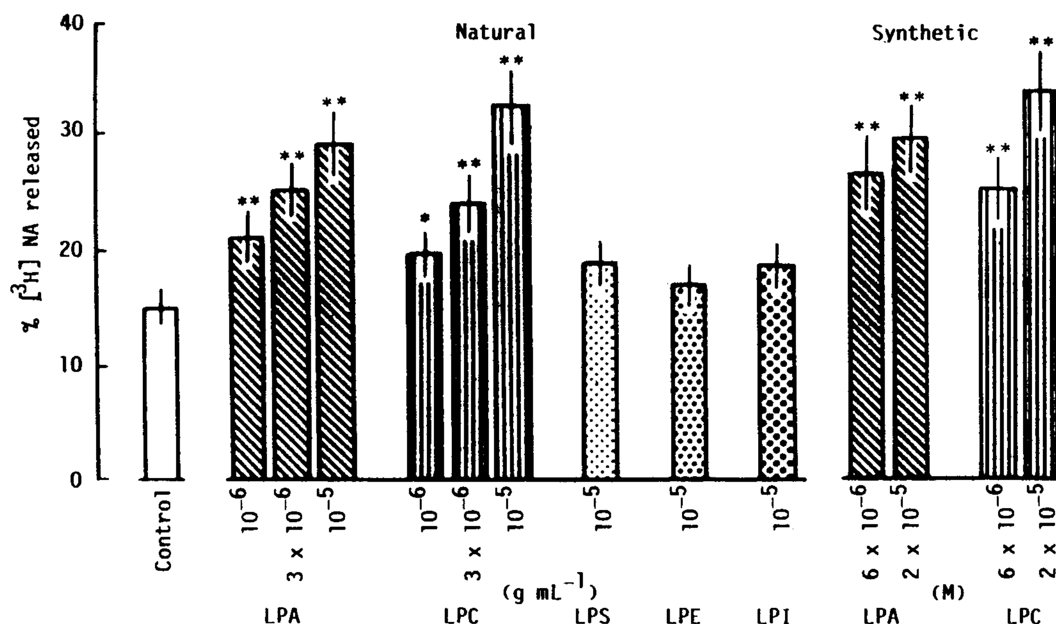

FIG. 2. Effects of several lysophospholipids on [^{3}H]NA release from synaptosomes. [^{3}H]NA-containing synaptosomes (about 200 μg synaptosomal membrane protein) were incubated at 37°C either in a low concentration (5 mM) of K^+ Hanks buffer without Ca^{2+} or in Hanks buffer containing the indicated amounts of natural and synthetic lysophospholipids and 1 mM Ca^{2+}. Columns represent mean values for 6 to 7 determinations with s.e.m. as a vertical line. * Significantly different ($P < 0.05$) from control, ** ($P < 0.01$). LPA: lysophosphatidic acid, LPC: lysophosphatidycholine, LPS: lysophosphatidylserine, LPE: lysophosphatidylethanolamine, LPI: lysophosphatidylinositol. For other abbreviations and details, see Fig. 1 and "Materials and Methods".

labelled with either [^{3}H]phospholipid or [^{14}C]phospholipid, and subsequently reisolated by Ficoll gradient centrifugation to remove unincorporated lipids, they contained 20 ± 3 or $45 \pm 8\%$ of the added labelled phospholipids, respectively. An increase in both PLA_2 activities caused by high K^+ occurred at 15 s, became maximal at 2 min and appeared to fall slightly up to 6 min. Their stimulatory effects by 75 mM K^+ were similar to those of 10 μM A23187, when examined as a function of time (data not shown). As shown in Fig. 4, PA-specific PLA_2 was stimulated by small quantities of K^+ (50 mM) and A23187 (5 μM), but PC-specific PLA_2 was not, at 2 min after stimulation. However, higher concentrations of K^+ (75 mM) and A23187 (10 μM) showed stimulatory effects on both PLA_2 activities.

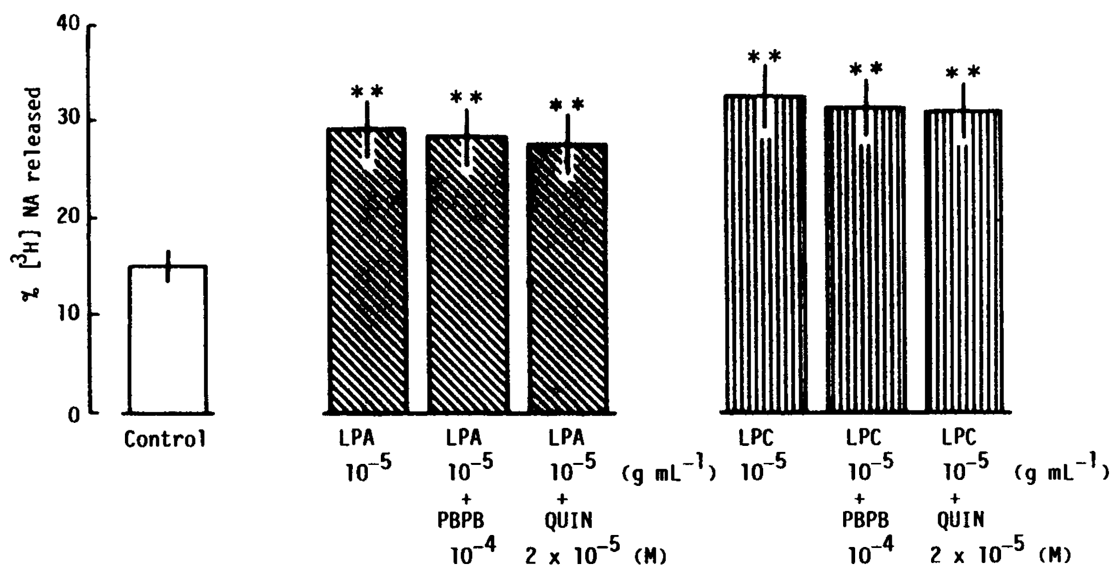

FIG. 3. Effects of PBPB and quinacrine on [^{3}H]NA release evoked by LPA and LPC. Columns represent mean values for 6 to 7 determinations with s.e.m. as a vertical line. ** Significantly different ($P<0{\cdot}01$) from control. Other details and abbreviations are given in Figs 1, 2.

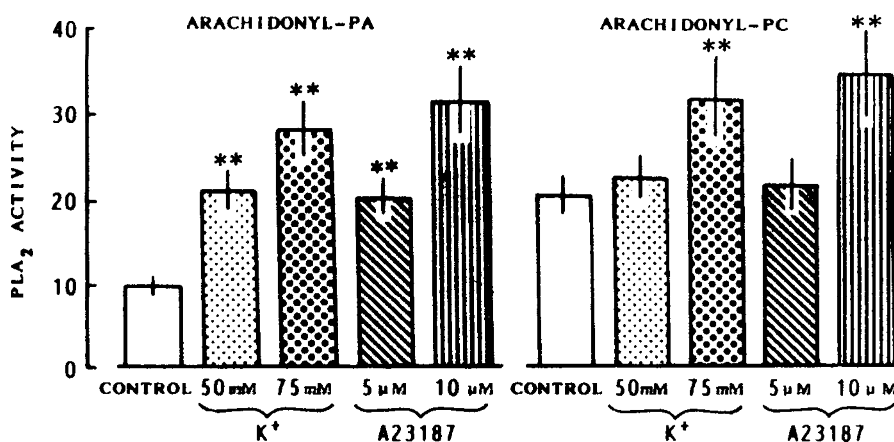

FIG. 4. Stimulation of synaptosomal PLA_2 activity by high concentrations of K^+ and calcium ionophore A23187. PLA_2 activity was expressed as pmol arachidonate formed/mg protein/2 min. Synaptosomes (about 10 mg protein) were preincubated with 2 μCi of [^{3}H]arachidonyl-PA (33·3 nmol) and 2 μCi of [^{14}C]arachidonyl-PC (46·5 nmol) in 5 mL of Hanks buffer for 60 min at 25°C. Aliquots of 0·5 mL of synaptosome (about 1 mg protein) were diluted with buffer containing 1 mM Ca^{2+} and 5 mM K^+, 50 mM K^+, 75 mM K^+, or 5 mM K^+ plus 5 μM (or 10 μM) A23187 (final concentration). After 2 min the samples were put on ice-cold test tubes and the lipids were extracted and analyzed as described in "Materials and Methods". [^{3}H]arachidonyl-PA, 0·65 nmol, and [^{14}H]arachidonyl-PC, 1·23 nmol, were incorporated per mg protein. These represent 0·26 and 0·49% of the total synaptosomal PA and PC, respectively. Thus the tracers were diluted by 385- and 204-fold by endogenous PA and PC, respectively. Columns represent mean values for 6 to 7 determinations with s.e.m. as a vertical line. For other abbreviations, see Fig. 1. * Significantly different ($P<0{\cdot}05$) from corresponding controls, ** ($P<0{\cdot}01$).

PLA_2 and several phospholipid effects on the synaptic membrane Na^+, K^+-ATPase activity

To find whether PLA_2 activation (or conversion of phospholipids into LPLs) has some role in the depolarization and/or release of neurotransmitters in the brain, commercially available PLA_2 (from bee venom) and several phospholipids or LPLs were tested for their ability to influence the Na^+, K^+-ATPase activity of cerebro-cortical synaptic membrane in the medium containing intracellular levels of free Ca^{2+}. As shown in Fig. 5, PLA_2 ($10^{-8}-10^{-6}$ g mL^{-1}) dose-dependently inhibited the Na^+, K^+-ATPase activity and this inhibition was increased with increasing free Ca^{2+} concentration within the range of $10^{-8}-4{\cdot}7\times10^{-7}$ M. However, commercially available phospholipase C ($10^{-8}-10^{-4}$) and phospholipase D ($10^{-7}-10^{-4}$) did not inhibit but slightly stimulated the Na^+, K^+-ATPase activity (data not shown).

Fig. 6 shows the effect of several natural LPLs and phospholipids on synaptosomal membrane Na^+, K^+-ATPase activity in the presence of 0·2 mM EGTA. In the presence of 0·2 mM EGTA, free Ca^{2+} concentrations in the

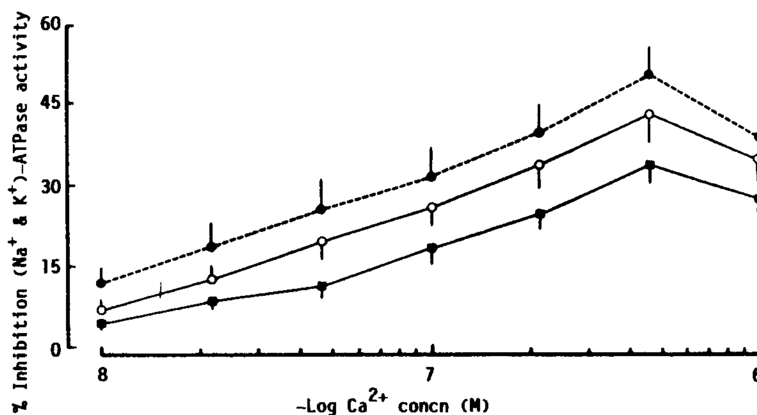

FIG. 5. PLA_2 effect on synaptosomal membrane Na^+, K^+-ATPase activity from the cerebral cortices of rats in the intracellular levels of free Ca^{2+}. Synaptosomal membranes were incubated with PLA_2 for 5 min before the addition of substrate. The Na^+, K^+-ATPase activites were expressed as μmoles inorganic phosphate formed per mg membrane protein per h. The concentrations of free Ca^{2+} were adjusted with Ca^{2+}-EGTA buffer and calculated as described in the "Materials and Methods". Each point represents the mean of 7 to 9 determinations with bar denoting s.e.m. PLA_2: phospholipase A_2 (from bee venom). (●) PLA_2 10^{-6} g mL^{-1}; (○) PLA_2 10^{-7} g mL^{-1}; (■) PLA_2 10^{-8} g mL^{-1}.

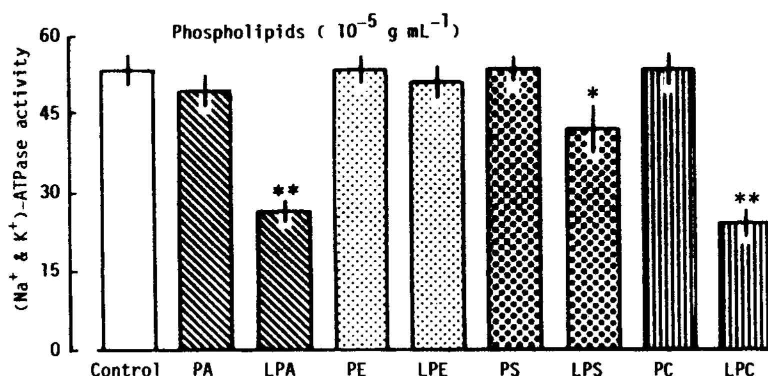

FIG. 6. LPA, LPC and other phospholipid effects in synaptosomal membrane Na^+, K^+-ATPase activity from the cerebral cortices of rats in the presence of EGTA. EGTA, final concentration 0·2 mM, was added before the start of preincubation. Total (free and bound) calcium concentration of the incubation medium (40 μg mL^{-1} H_2O synaptosomal membrane protein) was $3{\cdot}23 \pm 0{\cdot}03 \times 10^{-7}$ M and that of all the phospholipids (10^{-5} g mL^{-1}) was less than 3×10^{-8} M. In the presence of 0·2 mM EGTA, the free Ca^{2+} concentration in all the incubation media used was no more than 10^{-8} M when calculated as described in the text. Each column represents the mean of 7 to 14 determinations with bars denoting s.e.m. PA: L-α-phosphatidic acid. For abbreviations, see Fig. 2. * Significantly different ($P < 0{\cdot}05$) from control, ** ($P < 0{\cdot}01$).

incubation medium were less than 10^{-8} M free Ca^{2+}, when calculated as described in the text. Natural LPA and LPC of 10^{-5} g mL^{-1} markedly inhibited the Na^+, K^+-ATPase activity. Similar potent inhibitory action was observed with synthetic palmitoyl-LPA or oleoyl-LPA and synthetic palmitoyl-LPC or oleoyl-LPC, whereas higher concentrations of palmitoyl chloride ($10^{-5}-3\times10^{-4}$ M) and oleoyl chloride ($10^{-5}-2\times10^{-4}$ M) caused no significant effect (data not shown). The inhibitory effects of LPA and LPC were much more potent than that of LPS. With 10^{-5} g mL^{-1} of LPA, LPC and LPS, 54, 59 and 24% inhibition of Na^+, K^+-ATPase activity was observed. PA, PE, LPE, PS and PC of 10^{-5} g mL^{-1} has little or no effect on this enzyme activity, though higher concentrations of more than $4{\cdot}7\times10^{-5}$ g mL^{-1} of LPI and LPE slightly, but statistically significantly, inhibited the enzyme activity (Fig. 7). The inhibitory effects of natural and synthetic LPAs or LPCs were dose-dependent and the minimum effective concentrations of natural LPA, LPC, palmitoyl-LPA (data not shown), oleoyl-LPA (data not shown), palmitoyl-LPC (data not shown) and oleoyl-LPC (data not shown) were 10^{-6} g mL^{-1}, $4{\cdot}7\times10^{-6}$ g mL^{-1}, $2{\cdot}2\times10^{-6}$ M, $2{\cdot}3\times10^{-6}$ M, $1{\cdot}1\times10^{-5}$ M and $1{\cdot}2\times10^{-5}$ M, respectively. In the concentration range of $4{\cdot}7\times10^{-7}$ to $4{\cdot}7\times10^{-6}$ g mL^{-1}, the inhibitory action of natural LPA was more potent than that of the other natural LPLs, although, at higher concentrations ($>10^{-5}$ g mL^{-1}), the inhibitory potency of LPA was about the same as that of LPC, and at much higher concentrations ($>10^{-4}$ g mL^{-1}), LPA was less potent than LPC. PA, natural LPA, PC, LPC, PE, LPE, LPI and synthetic LPLs at 10^{-5} g mL^{-1} scarcely affected the Mg^{2+}-ATPase activity. However, all these phospholipids slightly enhanced the Mg^{2+}-ATPase activity at more than $2{\cdot}2\times10^{-5}$ g mL^{-1} (data not shown). As shown in Fig. 8, when the free Ca^{2+} concentration was less than $2{\cdot}2\times10^{-6}$ M, natural LPA and LPC (6×10^{-6} g mL^{-1}) significantly inhibited the Na^+, K^+-ATPase activity and the inhibition

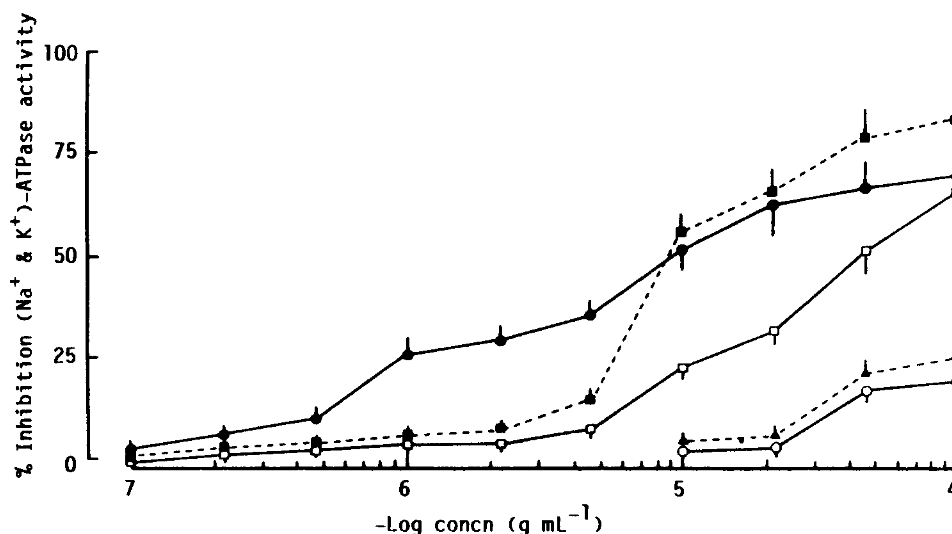

FIG. 7. Dose-response curves showing the inhibitory effects of several lysophospholipids on the Na^+, K^+-ATPase activity in the presence of EGTA (0·2 mM). The basal Na^+, K^+-ATPase activity was $44{\cdot}2 \pm 2{\cdot}4$ μmol of inorganic phosphate formed h^{-1} (mg protein)$^{-1}$. Each point represents the mean of 5 to 14 determinations with bars denoting s.e.m. For abbreviations, see Fig. 2. (●) LPA; (■) LPC; (□) LPS; (▲) LPI; (○) LPE.

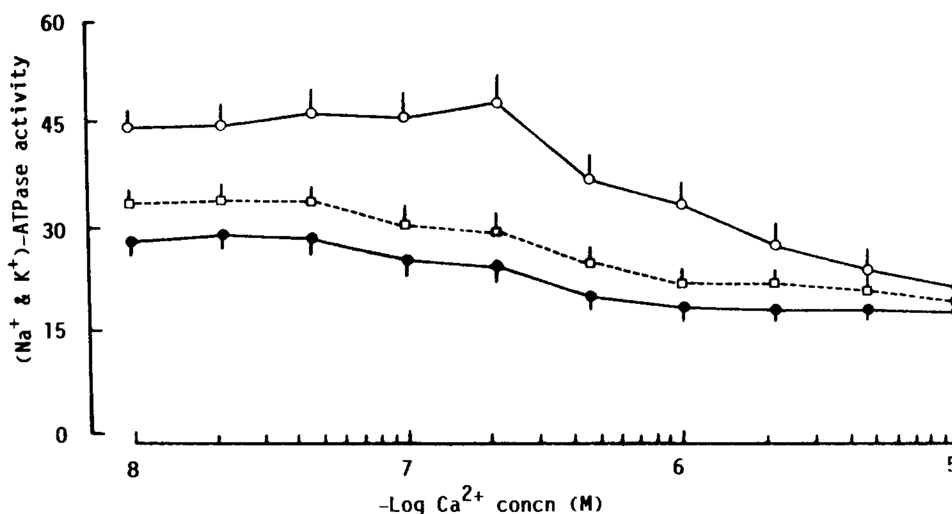

FIG. 8. Effects of LPA and LPC on synaptosomal membrane Na^+, K^+-ATPase activity at various concentrations of free Ca^{2+}. The concentrations of free Ca^{2+} were adjusted with Ca^{2+}-EGTA buffer and calculated as described in the text. Each point represents the mean of 7 to 14 determinations with bars denoting s.e.m. For abbreviations, see Fig. 7. (○) Control; (□) LPC 6×10^{-6}; (●) LPA 6×10^{-6} g mL^{-1}.

was more potent in the presence of free Ca^{2+} concentrations ranging from 10^{-7} to $4{\cdot}7 \times 10^{-7}$ M. Conversely either natural or synthetic LPLs caused a slight increase in the enzyme activity when the free Ca^{2+} concentrations was more than $4{\cdot}7 \times 10^{-5}$ M (data not shown).

Discussion

Our results showed that the release of [^{3}H]NA from rat cerebral cortical synaptosomes might be coupled to the stimulation of endogenous PLA_2 activity. Synaptosomal PA-specific or PC-specific PLA_2 activities were stimulated by high concentrations of K^+ and by A23187. The specific production of [^{3}H]LPA and [^{14}C]LPC in K^+- or A23187-stimulated synaptosomes, together with the selective effect of exogenous LPA or LPC on [^{3}H]neurotransmitter release, provide evidence that these lysolipids may be important in the secretory process, although it is not known where the added LPA and LPC are localised and if they behave exactly like the endogenous LPLs in the synaptosomal membrane. In fact, the effects of LPA and LPC on neurotransmitter release may be attributed to synaptosomal lysis. However, LPE or LPS, which have been reported to have lytic activity comparable to that of LPA or LPC (Weltzien 1979), was relatively ineffective in releasing [^{3}H]NA, indicating that a more specific action of LPA and LPC may be involved. In connection with this, stimulation of PLA_2 was shown to be necessary for antigen-stimulated release of histamine from

rat basophilic leukemia cells (McGivney et al 1981) and for potassium- or A23187-induced release of dopamine and NA from rat brain synaptosomes (Bradford et al 1983). The arachidonate metabolite tested, PGE_2, did not stimulate, but inhibited, the releasing processes.

Our result also showed that small amounts of natural and synthetic LPAs or LPCs and commercially available PLA_2 inhibited the synaptic membrane Na^+, K^+-ATPase activity from rat cerebral cortices in incubation media containing intracellular levels of free Ca^{2+} concentration. The production of LPLs, especially LPA and LPC, by PLA_2 may partly contribute to the inactivation of Na^+, K^+-ATPase activity. A rise of Na^+, K^+-ATPase and Mg^{2+}, Ca^{2+}-ATPase activities in the absence of calcium-chelating agents was observed by several investigators with PLA_2-treated preparations of sarcoplasmic reticulum (Swoboda et al 1979) and human erythrocyte membranes (Taverna & Hanahan 1980; Schmalzing & Kutschera 1982). A rise of the enzyme activity may in part be due to the chelating action of LPLs, since in the medium containing high concentrations of free Ca^{2+}, LPAs and LPCs caused a slight increase in the Na^+, K^+-ATPase activity as stated in the "Results". Membrane Na^+, K^+-ATPase activity is well known to be inhibited largely by calcium ions.

The inhibitory effect of LPC on Na^+, K^+-ATPase activity has already been found in dog and rabbit heart sarcolemma (Karli et al 1979; Pitts & Okhuysen 1984) or human erythrocyte membrane (Schmalzing & Kutschera 1982). The inhibitory actions of LPAs or LPCs may be due to some membrane-perturbing effects of the lysomoiety of the molecule. However, the precise mechanism of the inhibitory action of these LPLs on synaptic membrane Na^+, K^+-ATPase is not clear at present.

Acidic phospholipids such as PA and LPA may act as ionophores and increase the intracellular Ca^{2+} concentration (Harris et al 1981; Serhan et al 1981; Nayar et al 1984). On the other hand, it is well accepted that depolarization due to the increased intracellular Na^+ resulting from inhibition of the Na^+, K^+-ATPase activity leads to an increase of Na^+/Ca^{2+} exchange with a rise in intracellular Ca^{2+} and that neuronal depolarization sequentially activates an influx of Ca^{2+} into nerve cells (Landis & Putney 1979; Gill 1982). The Na^+, K^+-ATPase therefore may play a regulatory role in the release of neurotransmitters or other intracellular substances (Vizi 1977; Powis 1981; Meyer & Cooper 1981).

LPA or LPC has been shown to cause depolarization or to potentiate the Ca^{2+} influx in several tissues (Corr et al 1979; Tokumura et al 1982; Clarkson & Ten Eick 1983; Bradford et al 1983; Sedlis et al 1983). Recently, Mets (1986) reported that LPLs, except for LPS, might be true mediators of insulin release.

Based on these lines of findings, we suggest that in the brain tissues, LPA and/or LPC may participate in the depolarization or Ca^{2+}-increasing mechanisms mediating the release of NA through its ionophoretic action and also its inhibitory action on the membrane Na^+, K^+-ATPase activity. If this is true, LPA, LPC or PLA_2 should be able to mediate an amplification mechanism to further enhance the cell activation elicited by inositol-1,4,5-trisphosphate or diacylglycerol following receptor stimulation. Further research is needed to provide the answers.

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References

Andreesen, R., Modolell, M., Weltzien, H. U., Eibl, H., Common, H. H., Löhr, G. W., Munder, P. G. (1978) Selective destruction of human cells by alkyl-lysophospholipids. Cancer Res. 38: 3894–3899

Barker, L. A., Dowdall, M. J., Whittaker, V. P. (1972) Choline metabolism in the cerebral cortex of guinea pigs: Stable-bound acetylcholine. Biochem. J. 130: 1063–1080

Bergström, S., Farnebo, L. O., Fuxe, K. (1973) Effect of prostaglandin E_2 on central and peripheral catecholamine neurons. Eur. J. Pharmacol. 21: 362–368

Berridge, M. J. (1984) Inositol triphosphate and diacylglycerol as second messengers. Biochem. J. 345–360

Bligh, E. G., Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–917

Borda, E., Del Agostini, C. M., Peredo, H., Gimeno, M. F., Gimeno, A. L. (1983) Contractile activity of the isolated rat vas deferens and release of prostaglandin E- and F-like substances. Influences of acetylcholine and inhibitors of cyclo-oxygenase and phospholipase A_2. Arch. Int. Pharmacodyn. Ther. 263: 245–253

Bormann, B. J., Huang, C.-K., Mackin, W. M., Becker, E. L. (1984) Receptor-mediated activation of phospholipase A_2 in rabbit neutrophil plasma membrane. Proc. Natl. Acad. Sci. USA. 81: 767–770

Bradford, P. G., Marinetti, G. V. (1982) Effects of local anesthetics on phospholipid topology and dopamine uptake and release in rat brain synaptosomes. J. Memb. Biol. 67: 211–218

Bradford, P. G., Marinetti, G. V., Abood, L. G. (1983) Stimulation of phospholipase A_2 and secretion of catecholamines from brain synaptosomes by potassium and A23187. J. Neurochem. 41: 1684–1693

Clarkson, C. W., Ten Eick, R. E. (1983) On the mechanism of lysophosphatidylcholine-induced depolarization of cat ventricular myocardium. Circ. Res. 52: 543–556

Corr, P. B., Cain, M. E., Witkowski, F. X., Price, D. A., Sobel, B. E. (1979) Potential arrhythymogenic electrophysiological derangements in canine Purkinje fibers induced by lysophosphoglycerides. Ibid. 44: 822–832

Folch, J., Lees, M., Stanley, G. H. S. (1957) A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 226: 497–509

Frye, R. A., Holz, R. W. (1983) Phospholipase A_2 inhibitors block catecholamine secretion and calcium uptake in cultured bovine adrenal medullary cells. Mol. Pharmacol. 23: 547–550

Gerrard, J. M., Kindom, S. E., Petersen, D. A., Peller, J., Krantz, K. E., White, J. G. (1979) Lysophosphatidic acids: Influence on platelet aggregation and intracellular calcium flux. Am. J. Pathol. 96: 423–438

Gerrard, J. M., Clawson, C. C., White, J. G. (1980) Lysophosphatidic acids. III. Enhancement of neutrophil chemotaxis. Ibid. 100: 609–618

Gill, D. L. (1982) Receptor mediated modulation of plasma membrane calcium transport. In: Kohn L. D. (ed) Biochemistry and Biophysics: Hormone Receptors, Vol. 6, John Wiley and Sons, Chichester, New York, Brisbane, Toronto, Singapore, pp 199–236

Harris, R. A., Schmidt, J., Hitzemann, B. A., Hitzemann, R. J. (1981) Phosphatidate as a molecular link between depolarization and neurotransmitter release in the brain. Science 212: 1290–1291

Hirasawa, K., Nishizuka, Y. (1985) Phosphatidylinositol turnover in receptor mechanism and signal transduction. Ann. Rev. Pharmacol. Toxicol. 25: 147–170

Hokin, M. R., Hokin, L. E. (1953) Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. J. Biol. Chem. 203: 967–977

Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F., Williamson, J. R. (1984) Myo-inosital-1,4,5-trisphosphate. A

second messenger for the hormonal mobilization of intracellular Ca^{2+} in liver. Ibid. 259: 3077–3081

Karli, J. N., Karikas, G. A., Hatzipavlou, P. K., Levis, G. M., Moulopoulos, S. N. (1979) The inhibition of Na^+ and K^+ stimulated ATPase activity of rabbit and dog heart sarcolemma by lysophosphatidyl choline. Life Sci. 24: 1869–1876

Landis, C. A., Putney, J. W. Jr. (1979) Calcium and receptor regulation of radiosodium uptake by dispersed rat parotid acinar cells. J. Physiol. (Lond.) 297: 369–377

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Marone, G., Kagey-Sobotka, A., Lichtenstein, L. M. (1981) Possible role of phospholipase A_2 in triggering histamine secretion from human basophils in vitro. Clin. Immunol. Immunopathol. 20: 231–239

McGivney, A., Morita, Y., Crews, F., Hirata, F., Axelrod, J., Siraganian, R. (1981) Phospholipase activation in IgE-mediated and Ca^{2+} ionophore A23187-induced release of histamine from rat basophilic leukemia cells. Arch. Biochem. Biophys. 212: 572–580

Mets, S. A. (1986) Putative roles for lysophospholipids as mediators and lipoxygenase-mediated metabolites of arachidonic acid as potentiators of stimulus-secretion coupling: Dual mechanisms of p-hydroxymercuribenzoic acid-induced insulin release. J. Pharmacol. Exp. Ther. 238: 819–832

Meyer, E. M., Cooper, J. R. (1981) Correlation between Na^+, K^+-ATPase activity and acetylcholine release in rat cortical synaptosomes. J. Neurochem. 36: 467–475

Michell, R. H. (1975) Inositol phospholipids and cell surface receptor function. Biochim. Biophys. Acta 415: 81–147

Nahorski, S. R., Kendall, D. A., Batty, I. (1986) Receptors and phosphoinositide metabolism in the central nervous system. Biochem. Pharmacol. 35: 2447–2453

Nayar, R., Mayer, L. D., Hope, M. J., Cullis, P. R. (1984) Phosphatidic acid as a calcium ionophore in large unilamellar vesicle systems. Biochim. Biophys. Acta 777: 343–346

Nishikawa, T., Goto, M., Shimizu, S. (1985) Inhibitory action of phosphatidylinositol on synaptosomal Na^+, K^+-ATPase activity. Biochem. Biophys. Res. Commun. 126: 893–900

Nishikawa, T., Tomori, Y., Yamashita, S., Shimizu, S. (1988) Inhibition of synaptosomal Na^+, K^+-ATPase activity by lysophosphatidic acid: Its possible role in membrane depolarization. Jap. J. Pharmacol. 47: 143–150

Ogawa, Y. (1968) The apparent binding constant of glycoletherdiaminetetraacetic acid for calcium at neutral pH. J. Biochem., Tokyo 64: 255–257

Parkes, J. G., Fox, C. F. (1975) On the role of lysophosphatides in virus-induced cell fusion and lysis. Biochemistry 14: 3725–3729

Phillips, G. B., Bachner, P., McKey, D. G. (1965) Tissue effects of lysolecithin injected subcutaneously in mice. Proc. Soc. Exp. Biol. Med. 119: 846–850

Pitts, B. J. R., Okhuysen C. H. (1984) Effects of palmitoyl carnitine and LPC on cardiac sarcolemmal Na^+, K^+-ATPase. Am. J. Physiol. 247: H840–H846

Powis, D. A. (1981) Does Na^+, K^+-ATPase play a role in the regulation of neurotransmitter release by prejunctional α-adrenoceptors? Biochem. Pharmacol. 30: 2389–2397

Schmalzing, G., Kutschera, P. (1982) Modulation of ATPase activities of human erythrocyte membranes by free fatty acids or phospholipids A_2. J. Memb. Biol. 69: 65–76

Schwartz, A., Lindenmayer, G. E., Allen, J. C. (1975) The sodium-potassium adenosine triphosphatase: Pharmacological, physiological and biochemical aspects. Pharmacol. Rev. 27: 3–134

Sedlis, S. P., Corr, P. B., Sobel, B. E., Ahumada, G. G. (1983) Lysophosphatidyl choline potentiates Ca^{2+} accumulation in rat cardiac myocytes. Am. J. Physiol. 244: H32–H38

Serhan, C., Anderson, P., Goodman, E., Dunham, P., Weissman, G. (1981) Phosphatidate and oxidized fatty acids are calcium ionophores. Studies employing arsenazo III in liposomes. J. Biol. Chem. 256: 2736–2741

Sklenovský, A. (1976) The mechanism of effect of neuropharmacological drugs IV. The effect of phospholipase A on the release of glutamic acid in the brain tissue. Acta Univ. Palacki. Olomuc. Fac. Med. 80: 189–194

Skou, J. C. (1965) Enzymatic basis for active transport of Na^+ and K^+ across the cell membrane. Physiol. Rev. 45: 596–617

Slapke, J. (1984) Serine esterase inhibitor reduces contractions with isolated trachea and lung strips from guinea pigs induced by phospholipase A_2. Allergol. Immunopathol. Madr. 12: 289–292

Smith, A. D. (1972) Storage and secretion of hormones. Sci. Basis Med. Ann. Rev. 13: 74–102

Swoboda, G., Fritzsche, J., Hasselbach, W. (1979) Effects of phospholipase A_2 and albumin on the calcium-dependent ATPase and the lipid composition of sarcoplasmic membranes. Eur. J. Biochem. 95: 77–88

Taussky, H. H., Shorr, E. (1953) Microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. 202: 675–685

Taverna, R. D., Hanahan, D. J. (1980) Modulation of human erythrocyte Ca^{2+}/Mg^{2+} ATPase activity by phospholipase A_2 and proteases. A comparison with calmodulin. Biochem. Biophys. Res. Commun. 94: 652–659

Tokumura, A., Fukuzawa, K., Tsukatani, H. (1978) Effects of synthetic and natural lysophosphatidic acids on the arterial blood pressure of different animal species. Lipids 13: 572–574

Tokumura, A., Fukuzawa, K., Tsukatani, H. (1982) Contractile actions of lysophosphatidic acids with a chemically-defined fatty acyl groups on longitudinal muscle from guinea-pig ileum. J. Pharm. Pharmacol. 34: 514–516

Vizi, E. S. (1977) Termination of transmitter release by stimulation of sodium-potassium activated ATPase. J. Physiol. (Lond.) 267: 261–280

Weltzien, H. U. (1979) Cytolytic and membrane-perturbing properties of lysophosphatidylcholine. Biochem. Biophys. Acta 559: 259–287