Phospholipase A₂ and ³H-Hemicholinium-3 Binding Sites in Rat Brain: A Potential Second-Messenger Role for Fatty Acids in the Regulation of High-Affinity Choline Uptake

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The involvement of phospholipase A₂ (PLA₂) and fatty acid release in the regulation of sodium-dependent high-affinity choline uptake in rat brain was assessed *in vitro* through the use of the specific binding of ³H-hemicholinium-3 (³H-HCh-3). Addition of arachidonic acid and other unsaturated fatty acids to rat striatal membranes *in vitro* resulted in a dose-dependent, temperature-independent activation of ³H-HCh-3 binding. Scatchard analysis revealed that these changes in binding result from a 2-fold increase in the affinity and capacity of ³H-HCh-3 binding. Saturated fatty acids, lysophospholipids, and phospholipids did not affect specific ³H-HCh-3 binding. Addition of defatted BSA to membranes, which had been treated previously with arachidonic acid, completely reversed the increase in specific ³H-HCh-3 binding. However, several inhibitors of fatty acid metabolism, including nordihydroguaiaretic acid, indomethacin, catalase, and superoxide dismutase, did not alter arachidonic acid-induced changes in ³H-HCh-3 binding, suggesting that unsaturated fatty acids, and not their metabolites, are directly responsible for the observed activation of specific ³H-HCh-3 binding. Additionally, unsaturated fatty acids dose-dependently inhibited high-affinity ³H-choline uptake in rat striatal synaptosomes, apparently due to the disruption of synaptosomal integrity. The phospholipase A₂ inhibitors quinacrine hydrochloride, trifluoperazine, and 4-bromophenacylbromide dose-dependently inhibited potassium depolarization-induced activation of specific ³H-HCh-3 binding in slices of rat brain *in vitro*. Similarly, both quinacrine and trifluoperazine inhibited the metabolism of phospholipids and the release of fatty acids evoked by either elevated KCl or calcium ionophore A23187. These results support the involvement of PLA₂ and subsequent fatty acid release in the increase of ³H-HCh-3 binding in cholinergic neurons and suggest that activation of PLA₂ may be the penultimate step in regulating the velocity of sodium-dependent choline transport.

Sodium-dependent high-affinity choline uptake (SDHACU) is considered the primary mechanism by which cholinergic neuronal terminals accumulate choline for ACh synthesis (Yamamura and Snyder, 1972, 1973). The SDHACU carrier is likely saturated at the concentration of choline present within the extracellular fluid (for reviews, see Kuhar and Murrin, 1978; Jope, 1979; Tucek, 1985). The levels of ACh within various tissues remain remarkably stable, even during prolonged periods of neuronal activity and release of ACh (Birks and MacIntosh, 1961), consistent with evidence that the alterations in the velocity of SDHACU represent the rate-limiting step in ACh synthesis (Simon and Kuhar, 1975; Jenden et al., 1976; Murrin and Kuhar, 1976; Marchbanks and Wonnacott, 1979; Vaca and Pilar, 1979). Nevertheless, the proximate mechanisms responsible for regulating SDHACU remain poorly defined.

Our laboratory and others have utilized the specific binding of ³H-hemicholinium-3 (³H-HCh-3), a potent and specific inhibitor of SDHACU (Guyenet et al., 1973), to characterize the choline carrier in brain membranes and whole tissue sections (Rainbow et al., 1984; Sandberg and Coyle, 1985; Vickroy et al., 1985a, b; Lowenstein et al., 1987; Quirion, 1987). A wide variety of pharmacological, anatomical, and biochemical evidence supports the hypothesis that ³H-HCh-3 is binding to the choline carrier (Sandberg and Coyle, 1985; Watson et al., 1985; Lowenstein and Coyle, 1986; Saltarelli et al., 1987; Chatterjee et al., 1987). Through the exploitation of ligand binding techniques, we have observed that various treatments shown previously to alter the activity of cholinergic neurons, as demonstrated by changes in SDHACU (Atweh et al., 1975; Antonelli et al., 1981), ACh turnover, and ACh release, similarly alter the *B*_{max} of ³H-HCh-3 binding (Lowenstein and Coyle, 1986; Saltarelli et al., 1987, 1988a), suggesting that changes in SDHACU are accomplished via regulation of the actual number of functional carriers within the plasma membrane.

During the course of purifying the choline carrier, we observed recently that incubation of rat striatal synaptic membranes with phospholipase A₂ (PLA₂; phosphatide 2-acyl-hydrolase; EC 3.1.1.4) isolated from bee venom caused a pronounced Ca²⁺-dependent increase in the specific binding of ³H-HCh-3 (Yamada et al., 1988a). Further characterization (Yamada et al., 1988c, 1989) of this phenomenon revealed that (1) exogenous PLA₂ dose-dependently activated specific ³H-HCh-3 binding, (2) these changes in ³H-HCh-3 binding were due to about a 2-fold increase in both the affinity and number of sites in striatal membranes, (3) incubation of rat striatal synaptosomes with low concentrations of PLA₂ activated specific ³H-choline up-

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take, (4) potassium depolarization and PLA₂-induced increases in specific ³H-HCh-3 binding were not additive, and (5) the activation of ³H-HCh-3 binding by PLA₂ was unique among sodium-dependent uptake systems, since PLA₂ inhibited the specific binding of both ³H-mazindol and ³H-desipramine. These results suggested the involvement of one of the several products of PLA₂-mediated hydrolysis of membrane phospholipids, namely, lysophosphatides and fatty acids (Van den Bosch, 1980; Irvine, 1982), or their metabolites, in the regulation of ³H-HCh-3 binding and high-affinity choline uptake, and implicated endogenous PLA₂ as a second-messenger-generating enzyme involved in the regulation of SDHACU and ACh synthesis. In the present study, we present evidence which supports this contention. A portion of this work has been presented previously in abstract form (Saltarelli et al., 1988b; Yamada et al., 1988b).

Materials and Methods

Materials. Male Sprague-Dawley rats (150–220 gm; Harlan, Walkersville, MD) were utilized in all experiments. ³H-HCh-3 (diacetate salt; specific activity = 122–166 Ci/mmol) and ³H-arachidonic acid (³H-AA; specific activity = 191 Ci/mmol) were purchased from New England Nuclear (Boston, MA). ³H-choline (specific activity = 80 Ci/mmol) was obtained from Amersham (Chicago, IL). Antibiotic A23187 (free acid), a calcium ionophore, was purchased from Calbiochem (La Jolla, CA). Liquid scintillation cocktail (Ready-Solv-HP/b) was supplied by Beckman (Fullerton, CA). Silica H plates were obtained from Analtech (Newark, DE). Triethylamine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Bonded phase aminopropyl columns (Bond Elut) were purchased from Analytichem International (Harbor City, CA). All other compounds (analytical grade) were obtained from either Sigma (St. Louis, MO) or JT Baker (Fairlawn, NJ).

Slice preparation and incubation. Rats were decapitated, and their brains were quickly removed and placed on an ice-chilled metal plate. Striata, hippocampi, or frontal cortical tissue blocks were dissected free from surrounding tissues, cut into 300 μ m sections with a McIlwain tissue chopper, and immediately placed into an ice-cold Krebs bicarbonate buffer containing (in mM): 120 NaCl, 4.2 KCl, 25 NaHCO₃, 1.2 NaH₂PO₄, 10 dextrose, 2.6 MgCl₂, and 1.3 CaCl₂ (normal Krebs) which had been recently bubbled with a 95% O₂/5% CO₂ mixture. In some experiments, CaCl₂ was omitted from the medium and replaced with 1 mM EGTA (Ca²⁺-free). Tissue slices from 3–6 animals were pooled and used immediately for each experiment.

Twelve (striatum) or 20 (hippocampus and cortex) slices were placed into individual glass vials containing 10 ml of either oxygenated normal or Ca²⁺-free Krebs buffers, pH 7.4 at 37°C, and then incubated for 20 min while bubbling continually with a 95% O₂/5% CO₂ gas mixture. After the initial incubation, the medium was replaced with 10 ml of fresh buffer [4.2 or 40 mM (KCl)], and the incubation was continued for an additional 20 min. Quinacrine hydrochloride, EGTA, A23187, and various salts were added to the incubation buffer from concentrated stock solutions as indicated. Iso-osmolarity of potassium-enriched buffers was effected by equimolar reductions of NaCl. All buffers maintained physiological pH throughout the incubations. Incubations were terminated by separating slices from the incubation media by filtration, after which the slices were immediately chilled in 40 vol of ice-cold 50 mM glycylglycine buffer containing 200 mM NaCl, pH 7.8 (GGB), sonicated for 10 sec on setting 10 with a Sonifier Cell Disruptor (Plainview, NY), and immediately centrifuged at 20,000 $\times$ g for 20 min at 4°C. The resulting pellets (crude membranes) were washed twice prior to resuspension in GGB to yield a final protein concentration in the ³H-HCh-3 binding assay between 200 and 800 μ g/ml.

Preparation of synaptosomes and synaptic membranes. Rat striata were homogenized in 20 vol of ice-cold 0.32 M sucrose with a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1000 $\times$ g for 10 min. The pellet (P₁) was resuspended and recentrifuged at 1000 $\times$ g for 10 min. The combined supernatants were centrifuged at 20,000 $\times$ g for 20 min. The resulting pellet (P₂) was resuspended in Krebs bicarbonate buffer and centrifuged at 20,000 $\times$ g for 20 min. For ³H-choline uptake experiments, this pellet was resuspended in Krebs buffer and used directly. Alternately, synaptic membranes were obtained by osmotic lysis of this P₂ pellet. Briefly, the P₂

pellet was resuspended in 20 vol of ice-cold distilled water and dispersed with a Brinkmann PT-10 Polytron at setting 5 for 10 sec. This homogenate was centrifuged at 8000 $\times$ g for 20 min. The resulting supernatant and buffy coat were collected, centrifuged at 20,000 $\times$ g for 20 min, washed once, and resuspended in GGB at a protein concentration of 1 mg/ml.

Treatment of synaptosomes with fatty acids was performed at 25°C for 30 min in Krebs buffer. Lipids and fatty acids used for uptake experiments were dissolved in absolute ethanol and added directly to synaptosomal suspensions. The incubation was terminated by ice cooling, followed by centrifugation at 20,000 $\times$ g for 20 min at 4°C. In some experiments, the supernatant was used for determination of lactate dehydrogenase (LDH) activity. The pellet was resuspended in Krebs buffer and used for high-affinity ³H-choline uptake. Lipid and fatty acid stocks (100 $\times$) used in membrane experiments were prepared by dissolving compounds in either chloroform or chloroform:methanol (1:2; vol/vol). Aliquots of these mixtures were then added to individual incubation tubes and then evaporated to dryness under a stream of nitrogen gas prior to the addition of membranes. The membrane/lipid mixtures were then sonicated for 10 sec prior to incubation. Treatment of synaptic membranes with fatty acids, lysophospholipids, phospholipids, and other drugs was performed at 25°C for 30 min in GGB containing 5 mM CaCl₂. After treatment, synaptic membranes were washed once and resuspended in GGB. Fatty acid and lipid stocks were stored under nitrogen gas at –20°C.

³H-HCh-3 binding. ³H-HCh-3 binding was performed as described previously (Sandberg and Coyle, 1985), with minor modifications (Saltarelli et al., 1987). Crude membrane homogenates were incubated at 25°C for 30 min and assayed in quadruplicate. The final incubation volume (0.1 ml) contained 10 nM ³H-HCh-3 in GGB. Incubations were terminated by vacuum filtration with a Brandel Cell Harvester (Gaithersburg, MD) over glass fiber filters (Schleicher & Schuell, #32) which had been treated previously in a 0.3% (vol/vol) aqueous solution of polyethyleneimine to reduce ³H-HCh-3 binding to the filters. The filters were then washed rapidly with 4 ml vol of ice-cold 50 mM Tris-HCl buffer containing 200 mM NaCl. Radioactivity was determined by liquid scintillation spectrometry, with a 50% counting efficiency.

Nonspecific binding was defined as binding in the presence of 1 μ M HCh-3. Specific filter paper binding was obtained by subtracting total from nonspecific binding in the absence of tissue suspension and was always <1000 dpm. Specific tissue binding was then calculated by subtracting the sum of nonspecific tissue binding plus specific filter binding from total binding, and expressed as specific femtomoles ³H-HCh-3 bound per milligram protein. Specific tissue binding amounted to 50–80% of total binding in all experiments.

Saturation isotherms were generated by measuring the amount of specific binding in the presence of varying concentrations of ³H-HCh-3 (0.65–20 nM).

³H-choline uptake. After incubation, striatal slices were homogenized in 40 vol of ice-cold 0.32 M sucrose with a glass homogenizer fitted with a Teflon pestle, and a washed P₂ preparation was obtained as described previously (Murrin and Kuhar, 1976). These pellets were resuspended in normal Krebs buffer and used for uptake studies. High-affinity ³H-choline uptake was measured as described previously (Saltarelli et al., 1987). A 0.1 ml aliquot of each synaptosomal suspension was preincubated with 0.875 ml normal Krebs buffer for 5 min at 37°C. Uptake was initiated by the addition of 0.025 ml ³H-choline (final specific activity, 8 Ci/mmol) to each tube, resulting in a final concentration of 40 nM in a total assay volume of 1.0 ml. At the end of 4.0 min, uptake was terminated by the addition of 2 ml ice-cold normal Krebs buffer to each tube, followed by immediate vacuum filtration onto glass fiber filters using a Brandel cell harvester. Filters were then washed with 12 ml ice-cold 0.9% NaCl. Radioactivity was determined by liquid scintillation spectrometry. Nonspecific uptake was defined as uptake occurring at 0°C. Specific ³H-choline uptake was calculated by subtracting nonspecific from total uptake and was expressed as picomoles/4 min/mg protein.

Determination of fatty acid turnover. Rat striatal prisms (300 $\times$ 300 μ m) prepared from 5 rats (approximately 500 mg wet weight) were incubated for 90 min at 37°C in a glass vial containing Ca²⁺-free Krebs buffers, 1 mg/ml defatted BSA, and 0.5 μ Ci/ml ³H-AA. After the labeling period, the prisms were incubated for 10 min at 37°C in 20 ml fresh Ca²⁺-free Krebs buffers containing 5 mg/ml defatted BSA, followed by centrifugation at 1000 $\times$ g for 5 min. This washing procedure was then repeated 3 times. Approximately 4% of the radioactivity present at the

Figure 1. Dose-dependent modulation of ³H-HCh-3 binding by arachidonic acid. Rat striatal synaptic membranes were prepared as described in Materials and Methods and incubated in the presence (shaded bars) or absence (solid bars) of 1 unit/ml phospholipase A₂ (PLA₂) and various concentrations (10–300 μg/ml; 30–1000 μM) of arachidonic acid for 30 min at 25°C, followed by centrifugation at 20,000 × *g* for 20 min. Synaptic membranes were then assayed for specific ³H-HCh-3 (2.5 nM) binding. Each value represents the mean ± SEM of 3 separate experiments. **p* < 0.05, ***p* < 0.01 vs. respective control (no arachidonic acid, identical enzyme treatment), +*p* < 0.05 vs 100 μg/ml arachidonic acid.

beginning of the labeling period recovered within the tissue prisms after the incubation and washing procedures. Labeled prisms were then divided (approximately 50 mg wet weight/vial) into individual vials and preincubated for 5 min at 37°C in 1 ml Ca²⁺-free Krebs buffer containing 50 μM ethylmercurithiosalicylate (thimerosal), an inhibitor of lysolecithin acyltransferase activity (Szamel and Resch, 1981), and either quinine, trifluoperazine (TFP), and/or A23187. A23187 stocks were prepared by dissolving the free acid in ethanol:dimethylsulfoxide, 1:1 (vol/vol), to yield a 100× solution, and then added directly to the incubation vials. Control vials received the same amount of vehicle. CaCl₂ and KCl were then added from concentrated stocks to the vials as indicated and the incubations continued for an additional 10 min. Incubation was terminated by cooling the vials in an ice-water bath and by adding 3 ml of chloroform:methanol (1:2) mixture. Total lipids were then extracted according to the method of Bligh and Dyer (1959).

The identity of radiolabeled phospholipids was determined by thin-layer chromatography. Aliquots of total lipids obtained from incubated slices were separated utilizing 20 × 20 cm preadsorbent channelled silica G plates (250 μm) and a mobile phase consisting of chloroform:ethanol:triethylamine:water (30:40:30:8). Radioactivity present within 15 subsections within each lane was determined by scintillation spectrometry. Nonradiolabeled phospholipid standards were run on each plate and detected by iodine vapor.

Lipid classes were separated by previously described procedures (Kalusny et al., 1985) through the use of a primary aminopropyl bonded solid-phase column (500 mg). Briefly, aminopropyl columns (prewashed with 4 ml hexane) were loaded under vacuum with the chloroform:methanol-extracted lipid mixtures obtained from the incubated slices. Neutral lipids, followed by unesterified fatty acids, and finally phospholipids were then sequentially eluted from the bonded phase column by adding solutions (4 ml) of increasing polarity to the column under vacuum: chloroform:2-propanol (2:1), followed by 2% acetic acid in diethylether, followed by methanol. Radioactivity (dpm's) present in each fraction was then directly determined by scintillation spectrometry and was expressed as a percentage of the total sum of radioactivity recovered in the 3 aminopropyl column fractions (total radioactivity recovered from each extracted lipid mixture). Direct addition of ³H-AA to the aminopropyl bonded phase column resulted in >96% recovery of total added radioactivity in the 2% diethylether fraction (fatty acid fraction).

Biochemical assays. Lactate dehydrogenase activity released from synaptosomes during incubation was assayed as previously described by Schnaar et al. (1978). A diluted supernatant (0.5 ml) was placed in

a 1 ml quartz cuvette and mixed with LDH assay buffer. The rate of change of absorbance at 340 nm was continuously recorded using a Beckman DU spectrophotometer. Total synaptosomal LDH activity was determined after solubilization with Triton X-100 (0.5%).

(Na⁺+K⁺)ATPase activity of the osmolyzed P₂ fraction was assayed according to the method of Ahmed and Thomas (1971). An aliquot of 50 μl of sample was incubated for 15 min at 37°C in a buffer containing (in mM) 20 Tris-HCl, 3 MgCl₂, 115 NaCl, 20 KCl, and 3 ATP in a final volume of 1.0 ml. Mg²⁺-ATPase activity was determined by omitting potassium from and adding 750 μM ouabain to the assay mixture. (Na⁺+K⁺)ATPase activity was defined as the difference between total and Mg²⁺-ATPase activity. Quantification of ATP hydrolysis was determined by the method of Fiske and Subbarow (1925). Protein in all experiments was determined by the method of Lowry et al. (1951) utilizing BSA as a standard.

One-way analysis of variance (ANOVA) and 2-tailed Dunnett's test were used to determine statistical significance.

Results

Activation of ³H-HCh-3 binding in membranes by fatty acids

To determine the mechanism by which PLA₂ activates ³H-HCh-3 binding, our initial experiments sought to characterize the effects of the 2 products released by the calcium-dependent action of PLA₂ upon membrane phospholipids, fatty acids, and lysophospholipids. As shown in Figure 1, the addition of AA to rat striatal membranes resulted in a dose-dependent increase of specific ³H-HCh-3 binding, attaining about a 200% increase over baseline at 100 μg/ml (330 μM). However, treatment of striatal membranes with AA resulted in a biphasic effect upon ³H-HCh-3 binding, such that 300 μg/ml (1 mM) returned specific binding to control levels. Additionally, when AA was added to striatal membranes which had been treated previously with 1 unit/ml PLA₂, a dose-dependent inhibition of specific ³H-HCh-3 binding was observed. AA similarly increased the specific binding of ³H-HCh-3 in rat striatal membranes even when the incubation was carried out at 0°C (ice-water bath), suggesting that the fatty acid itself, and not the product of a subsequent enzyme-mediated event, is responsible for the observed change in specific ³H-HCh-3 binding (data not shown).

When saturation isotherms of the specific binding of ³H-HCh-3 were performed using membranes treated with 100 μg/ml (330 μM) arachidonic acid, Scatchard plot transformation of the data revealed approximately a 2-fold increase in both the affinity (*K_d* = 1.9 ± 0.1; *p* < 0.01) and capacity (*B_{max}* = 537 ± 19; *p* < 0.01) of ³H-HCh-3 for its binding site in rat striatal membranes (Fig. 2). The Hill coefficient was not significantly different from unity (*n_H* = 0.82 ± 0.04, *p* > 0.05). These alterations in ³H-HCh-3 binding parameters mimic those changes previously described which result after incubation of striatal membranes with PLA₂ (Yamada et al., 1988c).

Table 1 demonstrates the effect of several fatty acids, lysophospholipids, and phospholipids on specific ³H-HCh-3 binding. Addition of saturated fatty acids such as palmitic (16:0), stearic (18:0), or arachidic (20:0) acids produced no significant alteration of specific ³H-HCh-3 binding when added to striatal membranes in concentrations up to 100 μg/ml. Similarly, addition of lysophospholipids, such as lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylserine, did not result in a significant alteration of specific ³H-HCh-3 binding. Likewise, addition of 100 μg/ml phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylserine (PS) had no effect on ³H-HCh-3 binding. Also, addition of these phospholipids (100 μg) to PLA₂-treated membranes did not affect ³H-HCh-3 binding (data not shown), suggesting that the activation of binding by phospholipase treatment did not result

Figure 2. Scatchard plot of specific ^3H -HCh-3 binding in arachidonic acid-treated membranes. Rat striatal synaptic membranes were treated with either 100 $\mu\text{g}/\text{ml}$ arachidonic acid (300 μM ; filled circles) or vehicle (Control; open squares) for 30 min, followed by centrifugation at $20,000 \times g$ for 20 min. Saturation isotherms were generated utilizing 0.65–20 nM ^3H -HCh-3. These data are derived from a representative experiment performed 3 times.

from the degradation of an essential membrane phospholipid constituent. However, other unsaturated fatty acids, such as oleic (18:1), linoleic (18:2), and linolenic (18:3) acids resulted in a dose-dependent increase in specific ^3H -HCh-3 binding similar to that observed with AA, though the monounsaturated fatty acid oleate was less potent. Thus, unsaturated fatty acids appear to be the only possible immediate products of PLA_2 -mediated hydrolysis of phospholipids which are capable of increasing ^3H -HCh-3 binding in striatal membranes.

Fatty acids released as a result of PLA_2 activation can be subsequently metabolized by 3 well-characterized mechanisms: (1) conversion to prostaglandins and endoperoxides by cyclooxygenase (Samuelsson, 1972; Hamberg and Samuelsson, 1974), (2) lipoxygenase-mediated conversion of fatty acids to leukotrienes (Samuelsson et al., 1987), and (3) peroxidation of unsaturated fatty acids by oxygen-free radicals (Tapel, 1973). Since second-messenger functions have been described for these fatty acid metabolites (Yoneda et al., 1985; Piomelli et al., 1987a, b; Schaad et al., 1987; Schwartz et al., 1988), we sought to determine whether one of these metabolic products might be involved in the activation of specific ^3H -HCh-3 binding observed after addition of fatty acids or following incubation with PLA_2 . As shown in Table 2, addition of either 100 μM indomethacin or nordihydroguaiaretic acid (NDGA), nonspecific inhibitors of cyclooxygenase (Shen and Winter, 1977) and lipoxygenases (Egan and Gale, 1984), respectively, failed to significantly inhibit the activation of specific ^3H -HCh-3 binding induced by either PLA_2 or AA. Similarly, the inclusion of either superoxide dismutase or catalase, scavengers of free radicals (Demopoulos et al., 1980), failed to significantly affect the activation of ^3H -HCh-3 binding induced by AA, though a small, significant inhibition by superoxide dismutase was observed during incubation with PLA_2 . However, subsequent exposure of either PLA_2 - or AA-treated membranes to 1% defatted BSA, which noncovalently binds fatty acids (Goodman, 1958), completely reversed the activation of ^3H -HCh-3 binding normally induced by either PLA_2 or AA. These studies implicate the direct action of fatty acids in the activation of ^3H -HCh-3 binding by PLA_2 .

Table 1. Effects of several fatty acids, lysophospholipids, and phospholipids on ^3H -HCh-3 binding

Treatment	Concentration ($\mu\text{g}/\text{ml}$)	^3H -HCh-3 bound (% control)
Palmitic acid (16:0)	30	114 $\pm$ 19
	100	113 $\pm$ 10
Stearic acid (18:0)	30	85 $\pm$ 5
	100	100 $\pm$ 4
Oleic acid (18:1)	30	114 $\pm$ 7
	100	187 $\pm$ 15 ^a
Linoleic acid (18:2)	30	178 $\pm$ 14 ^a
	100	323 $\pm$ 51 ^a
Linolenic acid (18:3)	30	185 $\pm$ 37 ^a
	100	338 $\pm$ 21 ^a
Arachidic acid (20:0)	30	110 $\pm$ 18
	100	102 $\pm$ 4
Arachidonic acid (20:4)	30	163 $\pm$ 15 ^a
	100	324 $\pm$ 66 ^a
lysophosphatidylcholine	30	98 $\pm$ 13
	100	87 $\pm$ 2
lysophosphatidylethanolamine	30	121 $\pm$ 17
	100	130 $\pm$ 25
lysophosphatidylserine	30	108 $\pm$ 16
	100	121 $\pm$ 20
Phosphatidylcholine	100	108 $\pm$ 10
Phosphatidylethanolamine	100	115 $\pm$ 13
Phosphatidylserine	100	106 $\pm$ 21

Rat striatal synaptic membranes were prepared as described in Materials and Methods and incubated in the presence of the indicated concentrations of fatty acids, lysophospholipids, or phospholipids for 30 min at 25°C. Membrane mixtures were then centrifuged at $20,000 \times g$ for 20 min, resuspended in 50 mM glycylglycine buffer containing 200 mM NaCl, and assayed for specific ^3H -HCh-3 (2.5 nM) binding. Each value represents the mean $\pm$ SEM of 3 separate experiments. ^3H -HCh-3 in control membranes was 59 ± 10 fmol/mg protein. Lipid and fatty acid stocks were prepared as chloroform or chloroform:methanol (1:2, vol/vol) solutions, and aliquots of these stocks were evaporated to dryness under N_2 prior to membrane addition and sonication. Molar concentrations of fatty acids range from 100 μM (arachidic acid; 30 $\mu\text{g}/\text{ml}$) to 390 μM (palmitic acid; 100 $\mu\text{g}/\text{ml}$).

^a $p < 0.01$ vs control.

Fatty acids and high-affinity choline uptake

Table 3 demonstrates the effect of several fatty acids on sodium-dependent high-affinity ^3H -choline uptake measured in a crude striatal synaptosomal preparation. Addition of polyunsaturated fatty acids, such as arachidonic, linoleic, and linolenic acids, resulted in a dose-dependent inhibition of ^3H -choline uptake, which attained statistical significance at 100 $\mu\text{g}/\text{ml}$. Alternately, saturated fatty acids, such as stearic and arachidic acids, as well as the monounsaturated fatty acid oleate, did not significantly affect high-affinity ^3H -choline uptake when added to synaptosomes at 100 $\mu\text{g}/\text{ml}$.

Since the detection of high-affinity choline uptake requires the maintenance of an intact synaptosomal compartment, membrane potential, and appropriate ionic gradients, we assessed the effect of several fatty acids on both LDH release, a cytoplasmic enzyme marker for synaptosomal integrity, and on ouabain-sensitive ($\text{Na}^+ + \text{K}^+$)ATPase activity. As shown in Table 4, incubation of striatal synaptosomes for 30 min at 25°C with the polyunsaturated fatty acid arachidonate resulted in a significant 140% increase in LDH activity released into the incubation buffers, consistent with disruption of synaptosomal integrity.

Table 2. Effects of various inhibitors on PLA₂- and arachidonate-induced increases in specific ³H-HCh-3 binding

Treatment	Conc.	³ H-HCh-3 bound (fmol/mg protein)		
		Untreated	PLA ₂	AA
Control		81 ± 12	371 ± 33	300 ± 27
Indomethacin	100 μM	84 ± 4	353 ± 28	290 ± 25
Nordihydroguaiaretic acid	100 μM	103 ± 3	340 ± 36	334 ± 28
Control		113 ± 6	511 ± 4	300 ± 27
Superoxide dismutase	10 unit/ml	110 ± 5	490 ± 6 ^a	310 ± 22
Catalase	10 unit/ml	103 ± 2	501 ± 12	279 ± 42
Control		136 ± 13	374 ± 25	300 ± 27
BSA	1%	127 ± 24	102 ± 10 ^b	148 ± 36 ^c

Rat striatal synaptic membranes were treated with either phospholipase A₂ (PLA₂; 1 unit/ml with 5 mM CaCl₂) or arachidonic acid (AA; 330 μM) in the presence of each of the indicated drugs or enzymes for 30 min at 25°C, followed by centrifugation at 20,000 × g for 20 min. Alternately, PLA₂- or AA-treated membranes were resuspended in glycylglycine buffer containing 1% defatted BSA, incubated for an additional 30 min at 4°C and centrifuged at 20,000 × g for 20 min. Synaptic membranes were then assayed for specific ³H-HCh-3 (2.5 nM) binding. Each value represents the mean ± SEM of 3 separate experiments.

^a *p* < 0.05.

^b *p* < 0.01 vs PLA₂-treated control.

^c *p* < 0.05 vs arachidonate-treated control.

However, neither the saturated nor the monounsaturated fatty acids tested significantly affected the release of LDH into the supernatant. Additionally, none of the fatty acids tested significantly affected (Na⁺+K⁺)ATPase activity (data not shown).

Phospholipid and fatty acid turnover in striatal slices

Incubation of striatal slices with 0.5 μCi/ml ³H-AA resulted in the incorporation of fatty acid into several phospholipids. When total lipids were extracted from previously incubated slices and separated by thin-layer chromatography, peaks of radioactivity

were reproducibly observed to comigrate with phosphatidylinositol and phosphatidylcholine (data not shown). Additionally, a significant proportion of the total radioactivity present within the slices either remained within the unesterified form (comigrates with AA) or within neutral lipids which form a peak of radioactivity at the front (comigrates with diacylglycerol).

Figure 3 demonstrates the quantification of radioactivity through the use of solid-phase bonded aminopropyl chromatography. When striatal slices were incubated in the presence of ³H-AA, followed by 5 min incubation with thimerosal in Ca²⁺-free Krebs buffer, 60.5 ± 0.1% of the radioactivity extracted with total lipids was contained in the phospholipid fraction, 26.3 ± 0.5% in the fatty acid fraction, and 13.4 ± 0.5% in the neutral lipid fraction. When identically treated slices were subsequently incubated in normal Krebs medium containing 50 μM thimerosal for 10 min at 37°C, a significant 17 ± 1% (*p* < 0.01) increase in radioactivity appeared in the fatty acid fraction, while a significant 9 ± 1% (*p* < 0.02) decrease in radioactivity was observed in the phospholipid fraction. No significant change in radioactivity occurred in the neutral lipid fraction.

Table 3. Dose-dependent inhibition of high-affinity choline uptake by polyunsaturated fatty acids

Treatment	Concentration (μg/ml)	Choline uptake (pmol/4 min/mg protein)
Control (1% EtOH)	—	5.27 ± 0.26
Arachidonic acid	10	5.13 ± 0.27
	30	4.73 ± 0.26
	100	2.70 ± 0.05 ^a
Control (2% EtOH)	—	5.07 ± 0.20
Linoleic acid	100	3.39 ± 0.39 ^a
Linolenic acid	100	2.75 ± 0.20 ^a
Control (6% EtOH)	—	3.95 ± 1.03
Stearic acid	100	4.66 ± 0.75
Arachidic acid	100	3.79 ± 0.18
Control (1% EtOH)	—	4.82 ± 0.17
Oleic acid	100	4.88 ± 0.37

Synaptosomes prepared from rat striatum were treated with ethanol stocks of the indicated fatty acids in Krebs buffer for 30 min at 25°C, followed by centrifugation at 20,000 × g for 20 min. The final concentration of added ethanol in treated and control conditions is indicated in parentheses. Synaptosomes were then resuspended in Krebs buffer and assayed for specific ³H-choline (40 nM) uptake. Each value represents the mean ± SEM of 3 separate experiments. Molar concentrations of fatty acids range from 30 μM (arachidonic acid; 10 μg/ml) to 350 μM (oleic acid; 100 μg/ml).

^a *p* < 0.01 vs control.

Table 4. Effect of fatty acids on synaptosomal integrity

Treatment	LDH release (% total activity)
Control (1% EtOH)	12 ± 1
Arachidonic acid	29 ± 5 ^a
Oleic acid	10 ± 3
Arachidic acid	12 ± 2

Synaptosomes prepared from rat striatum were incubated for 30 min at 25°C in normal Krebs medium containing the indicated fatty acids (100 μg/ml), followed by centrifugation at 20,000 × g for 20 min. Lactate dehydrogenase (LDH) activity present within the supernatants was then assessed and expressed as percent total activity. Total LDH activity (13.5 ± 0.3 μmol substrate/mg protein/min) was determined after solubilization of the synaptosomal aliquot in 0.5% Triton X-100. Each value represents the mean ± SEM of 3 separate experiments performed in duplicate.

^a *p* < 0.01 vs control.

Figure 3. Shift of ^3H -arachidonic acid from phospholipid to fatty acid pools by potassium depolarization. Rat striatal slices were preincubated for 90 min at 37°C in Ca^{2+} -free Krebs buffer containing $0.5 \mu\text{Ci/ml}$ ^3H -arachidonic acid and 1 mg/ml defatted BSA. After 5 min preincubation with $50 \mu\text{M}$ thimerosal, slices were either placed on ice until extraction of total lipids (Blank; solid bars) or further incubated in fresh Krebs buffer containing 1.2 mM CaCl_2 , $50 \mu\text{M}$ thimerosal, and either 4 (Normal Krebs; open bars) or 40 (40 mM KCl ; shaded bars) mM KCl as indicated. Total lipids were extracted from the groups of slices and separated into lipid classes by solid-phase chromatography. Radioactivity present within each fraction was then expressed as the percentage of total radioactivity in each sample. Each value represents the mean $\pm$ SEM of 6 observations performed in 3 separate experiments. $3.3 \pm 0.1\%$ of total radioactivity initially present in the medium was recovered in the Blank phospholipid fraction ($n = 20$). * $p < 0.01$ vs Blank; + $p < 0.01$ vs Normal Krebs.

When slices which had been previously loaded with ^3H -AA were incubated in Krebs buffer containing 40 mM KCl , a further potentiation of fatty acid release and loss of radioactivity from the phospholipid fraction was observed. As shown in Figure 3, potassium depolarization of slices for 10 min at 37°C resulted in significant 71 ± 9 and $(-103 \pm 49)\%$ changes in radioactivity occurring in the fatty acid and phospholipid fractions, respectively, as compared with radioactivity changes occurring in slices which had been incubated in normal Krebs. No significant change in radioactivity was observed in the neutral lipid fraction, and no changes in radioactivity were observed in the absence of thimerosal (data not shown).

In order to characterize further the involvement of endogenous PLA_2 in the potassium-stimulated release of fatty acids, we assessed the effects of several drugs which have been shown previously to inhibit PLA_2 activity and fatty acid release *in vitro*. When $50 \mu\text{M}$ quinacrine hydrochloride, a nonspecific inhibitor of PLA_2 -mediated fatty acid release (Blackwell and Flower, 1983; Chang et al., 1987), is included during potassium stimulation of ^3H -AA-labeled striatal slices, a significant $66 \pm 16\%$ inhibition of potassium-stimulated fatty acid release as determined by solid-phase aminopropyl chromatography was observed (Fig. 4). Similarly, inclusion of $50 \mu\text{M}$ TFP, a nonspecific inhibitor of PLA_2 activity (Wong and Cheung, 1979; Withnall et al., 1984), resulted in a similar inhibition of fatty acid release. Both quinacrine and TFP inhibited basal fatty acid release determined in normal Krebs medium [$50 \pm 15\%$ ($p < 0.01$ vs normal Krebs) and $15 \pm 7\%$ ($p > 0.05$), respectively]. Changes in phospholipid fraction radioactivity inversely paralleled changes in fatty acid fraction radioactivity (data not shown). No significant effects on fatty acid turnover in any condition were observed when calcium was omitted from the incubation medium (data not shown),

Figure 4. Stimulation of fatty acid release by potassium depolarization and A23187. Rat striatal slices were preincubated for 90 min at 37°C in low-calcium Krebs buffer containing $0.5 \mu\text{Ci/ml}$ ^3H -arachidonic acid and 1 mg/ml fatty acid free BSA. After extensive washing, slices were resuspended in fresh Ca^{2+} -free Krebs buffer containing $50 \mu\text{M}$ thimerosal and incubated for 5 min at 37°C with quinacrine hydrochloride (Quin), trifluoperazine (TFP), or vehicle (control). CaCl_2 (final $[1.2 \text{ mM}]$) and either 4 (NK) or 40 mM KCl , or $20 \mu\text{M}$ A23187 were added as indicated from concentrated stocks, and the incubation continued for an additional 10 min. A23187 was dissolved in EtOH:DMSO (1:1) and was added to incubation vials to yield a final concentration of 1% . Total lipids were extracted from the groups of slices and separated into lipid classes by solid-phase aminopropyl chromatography (Kalusny et al., 1985). Radioactivity present within each fraction was then expressed as the percentage of total radioactivity in each sample. Each value represents the mean $\pm$ SEM of 6 observations performed in 3 separate experiments. * $p < 0.05$, ** $p < 0.01$ vs NK (no drugs, 4.2 mM KCl , same vehicle), + $p < 0.05$ vs 40 mM KCl (no drugs), ** $p < 0.01$ vs NK + A23187.

consistent with the described requirement for $\text{mM} [\text{Ca}^{2+}]_{\text{ext}}$ for activation of PLA_2 (Irvine, 1982).

Fatty acid release in striatal slices could also be demonstrated after incubation with the calcium ionophore A23187. When striatal slices were incubated for 10 min at 37°C in the presence of 1.2 mM CaCl_2 , $20 \mu\text{M}$ A23187, and $50 \mu\text{M}$ thimerosal, a significant increase in the amount of radioactivity present within the fatty acid fraction was observed (Fig. 4). Co-inclusion of $50 \mu\text{M}$ quinacrine completely blocked the increase in fatty acid release induced by A23187. As with potassium depolarization, changes in phospholipid fraction radioactivity inversely paralleled changes in fatty acid fraction radioactivity (data not shown). Similarly, omission of calcium from the external bathing medium completely inhibited A23187-stimulated fatty acid release (data not shown).

Inhibition of striatal slice PLA_2 and ^3H -HCh-3 binding

In order to assess the possible involvement of endogenous PLA_2 activity in the activation of SDHACU in intact striatal slices, we determined the effects of several inhibitors of PLA_2 on the well-characterized activation of ^3H -HCh-3 binding by potassium depolarization. When slices prepared from rat striatum, hippocampus, or cortex are incubated in Krebs buffer containing 40 mM KCl , a significant increase in specific ^3H -HCh-3 binding occurs in each brain region, ranging from 80 – 100% over values obtained in control slices incubated in normal Krebs containing 4 mM KCl (Fig. 5). However, if quinacrine hydrochloride is included during both the preincubation period and during potassium depolarization with 40 mM KCl Krebs buffer, a significant dose-dependent inhibition of potassium-stimulated ^3H -HCh-3 binding was observed in all brain regions tested, with complete inhibition observed when $50 \mu\text{M}$ quinacrine was pres-

Figure 5. Dose-dependent inhibition by quinacrine of KCl-stimulated changes in ³H-HCh-3 binding in rat brain slices. Rat striatal, hippocampal, or cortical slices were incubated for 20 min at 37°C in normal Krebs buffer containing the indicated concentration of quinacrine hydrochloride. Slices were then further incubated for 20 min at 37°C in Krebs buffers containing either 4 (solid bars) or 40 (shaded bars) mM KCl and the same concentration of quinacrine. Specific ³H-HCh-3 binding was then measured in a crude washed membrane preparation obtained from the slices in each vial and expressed as the percent control (4 mM KCl, no quinacrine). Each value represents the mean $\pm$ SEM of at least 6 independent observations performed in at least 3 separate experiments. Control ³H-HCh-3 binding levels were (fmol/mg protein): striatum (170 $\pm$ 15), hippocampus (50 $\pm$ 9), and cortex (40 $\pm$ 9). Significant stimulation of specific ³H-HCh-3 binding by 40 mM KCl was observed in all regions in the absence of quinacrine ($p < 0.01$), and in the presence of 10 μ M quinacrine in striatum ($p < 0.01$) and hippocampus ($p < 0.05$). No significant stimulation of specific binding by 40 mM KCl was observed in the presence of 50 μ M (all regions) or 10 μ M (cortex) quinacrine. * $p < 0.05$, ** $p < 0.01$ vs 4 mM KCl Krebs (no quinacrine); + $p < 0.01$ vs 40 mM KCl Krebs (no quinacrine).

ent in the bathing medium. Additionally, significant inhibition of baseline ³H-HCh-3 binding was observed in the nondepolarized slices.

Several additional inhibitors of PLA₂ and fatty acid release resulted in the inhibition of K⁺-stimulated ³H-HCh-3 binding. As shown in Figure 6, incubation of striatal slices with TFP resulted in a dose-dependent inhibition of potassium-stimulated ³H-HCh-3 binding, achieving complete inhibition of stimulated binding at 50 μ M. As observed with quinacrine, TFP also reduced basal levels of ³H-HCh-3 binding in control slices, though these differences attained statistical significance only at 50 μ M. However, if striatal slices are incubated as described above, except at 0°C (ice-water bath), neither quinacrine nor TFP (10 or 50 μ M) affects greater than a 20% inhibition of specific ³H-HCh-3 binding compared with control slices (data not shown). Finally, the alkylating agent 4-BPB also inhibited potassium-stimulated ³H-HCh-3 binding in a dose-dependent manner, though it was much less potent than either quinacrine or TFP (Fig. 7).

Discussion

The major new finding of this study is that unsaturated fatty acids can directly regulate the specific binding of ³H-HCh-3 in rat brain membranes. The hydrolysis of membrane phospholipids via the activation of endogenous PLA₂ and concomitant release of fatty acids may comprise a signal transduction mechanism which utilizes fatty acids as second messengers directly involved in the regulation of high-affinity choline uptake. The current study presents data which support the involvement of such a signal transduction system as follows: (1) Incubation of rat striatal membranes with either PLA₂ or unsaturated fatty acids results in a dose-dependent increase in specific ³H-HCh-3 binding, which could be completely abolished by posttreatment incubation with defatted BSA, (2) the effect of AA is biphasic, as demonstrated by the dose-dependent inhibition of specific ³H-HCh-3 binding by AA in PLA₂-treated membranes, as well as by the direct addition of 1 mM AA to untreated membranes, (3) the alteration in the parameters of the binding of ³H-HCh-3 to rat striatal membranes observed after incubation with either PLA₂ or an unsaturated fatty acid are identical, resulting in 2- to 3-fold increases in both the affinity and capacity of specific ³H-HCh-3 binding, (4) the effects of PLA₂ and AA upon ³H-HCh-3 binding is apparently due to the direct action of the fatty acids themselves and cannot be attributed to the metabolites of fatty acids produced by 3 common metabolic pathways, namely, the conversion of fatty acids by cyclooxygenases, lipoxygenases, or oxidation, (5) the activation of specific ³H-HCh-3 binding by the products of membrane phospholipid hydrolysis is restricted to unsaturated fatty acids since lysophospholipids and saturated fatty acids have no effect on ³H-HCh-3 binding, and finally (6) treatments which stimulate or inhibit the activity of PLA₂ in intact slices, as demonstrated by the alterations in fatty acid release and phospholipid turnover, produce parallel changes in both basal and potassium-stimulated ³H-HCh-3 binding.

Although many investigators have demonstrated the release of fatty acids from neuronal primary cultures (Lazarewicz et al., 1988), transformed cells lines (Murphy et al., 1988), synaptosomes (Lazarewicz et al., 1983), and pineal glands maintained in organotypic culture (Ho and Klein, 1987), few descriptions of the release of fatty acids from brain slices exist in the literature. When we employed previously reported conditions used for the measurement of fatty acid release in cell and organ culture

Figure 6. Dose-dependent inhibition of ^3H -HCh-3 binding by trifluoperazine. Rat striatal slices were incubated for 20 min at 37°C in normal Krebs buffer containing the indicated concentration of trifluoperazine (TFP). Slices were then further incubated for 20 min at 37°C in fresh Krebs buffers containing either 4 (Nml Krebs; solid bars) or 40 (shaded bars) mM KCl and the same concentration of TFP. Specific ^3H -HCh-3 binding was then measured in quadruplicate in a crude washed membrane preparation obtained from the slices in each vial and expressed as specific fmol/mg protein. Each value represents the mean $\pm$ SEM of 4–17 observations performed in 4–13 separate experiments. Potassium-stimulated slices are significantly different from control slices (4 mM KCl; $p < 0.01$) at all TFP concentrations except 50 μM . * $p < 0.01$ vs normal Krebs (no TFP), + $p < 0.01$ vs 40 mM KCl Krebs (no TFP).

Figure 7. Dose-dependent inhibition of ^3H -HCh-3 binding by 4-bromophenacyl bromide. Slices prepared from rat striatum were incubated for 20 min at 37°C in normal Krebs buffer containing the indicated concentrations of 4-bromophenacylbromide (4-BPB). Slices were then further incubated for 20 min at 37°C in fresh Krebs buffers containing either 4.2 (solid bars) or 40 (shaded bars) mM KCl and the same concentration of 4-BPB. Specific ^3H -HCh-3 binding was then measured in quadruplicate in crude washed membrane preparations prepared from slices and expressed as the percentage control (4 mM KCl, no 4-BPB). Each value represents the mean $\pm$ SEM of 3–10 observations performed in 4–8 separate experiments. * $p < 0.01$ vs 40 mM KCl (no 4-BPB); + $p < 0.05$ vs control (4 mM KCl, no 4-BPB).

experiments, we failed to observe specific fatty acid release from intact slices. Since the amount of time utilized to label slices is by necessity brief, we assumed that our difficulties were due to differences in the relative signal to noise ratio. Therefore, we employed several techniques which would improve the detection of released fatty acid. First, calcium was omitted during the labeling of the slice phospholipids with ^3H -AA, which should inhibit the activity of PLA_2 and allow greater fatty acid incorporation, as well as lower basal levels of unesterified ^3H -AA. Second, albumin was not included in the incubation buffer during stimulation to permit complete recovery of fatty acids within the tissue slices. Finally, the reacylation of released fatty acids was inhibited during slice stimulation by the use of thimerosal, one of several ethylmercurisalicylate compounds shown previously to potently inhibit the activity of endogenous lysophospholipid acyltransferases (Szamel and Resch, 1981; Förstermann et al., 1986) without affecting the activity of several other membrane-associated enzymes, including $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ (Szamel and Resch, 1981) and PLA_2 (Gopelt-Strübe et al., 1986).

The use of low concentrations of thimerosal in these experiments can be compared with the use of lithium to inhibit myo-inositol-1-phosphatase, thereby allowing the accumulation of inositol-1-phosphate for the determination of inositol phospholipid turnover (Hallcher and Sherman, 1980; Berridge et al., 1982). The incorporation and release of fatty acids under our conditions exhibited many characteristics similar to those reported by other investigators, including preferential incorporation into PI (Barkai and Murthy, 1988) and PC, little incorporation into PS (DeGeorge et al., 1987), calcium dependency (Baba et al., 1986), high basal fatty acid levels even in the absence of potassium- or ionophore-induced stimulation (Lazarewicz et al., 1983), stimulated release by calcium ionophore A23187 and potassium depolarization (Bradford et al., 1983),

and inhibition of release by quinacrine (Murphy et al., 1988) and TFP (Watanabe et al., 1986). Though the measurement of fatty acid release provided a direct, well-characterized mechanism by which to follow the efficacy of the various drug treatments used in these experiments, the use of slices precludes the determination of the relative contribution of presynaptic cholinergic terminals to the overall total fatty acid release.

Inhibition of neurotransmitter high-affinity uptake can result from the disruption of several processes which are distinct from an effect upon the transporter molecule per se, such as perturbation of synaptosomal integrity (Yamada et al., 1988c), inhibition of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ (Ahmed and Thomas, 1971; Chan et al., 1983), and disruption of electrochemical gradients and transmembrane electric potentials required for sodium-dependent transport (Holz and Coyle, 1974; Simon and Kuhar, 1976; Troeger et al., 1984). In the present study, we observed that incubation of rat striatal synaptosomes with polyunsaturated fatty acids resulted in a dose-dependent inhibition of ^3H -choline uptake, as has been reported recently by Boksa et al. (1988). Additionally, we observed that only those fatty acids (polyunsaturated) which were capable of disrupting the synaptosomal compartment, as evidenced by the release of LDH into the supernatant, were capable of inhibiting specific ^3H -choline uptake. Given the large size of the LDH molecule (140 kDa), the level of disruption for the ion gradients critical for sodium-dependent transport is likely grossly underestimated by the use of this cytoplasmic marker. The potent inhibition of ^3H -choline uptake likely results from the significant disruption of synaptosomal integrity induced by unsaturated fatty acids.

Unsaturated fatty acids may induce synaptosomal swelling and subsequent disruption by several mechanisms. The fluidity of biological membranes is altered by changes in the fatty acid content, an effect which is dependent upon both the chain length and the degree of unsaturation (Usher et al., 1978). These changes

in membrane fluidity affect the function of many membrane-localized enzymes, including (Na⁺+K⁺)ATPase (Swann, 1984). Inhibition of (Na⁺+K⁺)ATPase by fatty acids would allow unchecked sodium influx and subsequent osmotic swelling and rupture. Additionally, changes in membrane fluidity may directly allow increased leakage of sodium and subsequent osmotic rupture (Schramm et al., 1967).

An important criticism of these studies concerns the physiological relevance of the effects of unsaturated fatty acids on the choline carrier in the presence of significant disruption resulting in the inhibition of high-affinity choline uptake. Several possible explanations exist. First of all, it has been widely recognized that synaptosomes behave more like organelles in solution than intact tissue (Adam-Vizi and Marchbanks, 1983), and thus the degree of disruption observed in the presence of unsaturated fatty acids in these studies may not occur *in vivo*. Additionally, the *in vitro* application of fatty acids differs in many ways from the PLA₂-mediated release which occurs in intact tissue. (1) Tissue levels of fatty acids are maintained at very low levels through the action of acyltransferases, and thus only transiently increase during stimulation, an effect which is not possible to replicate *in vitro*. (2) The release of fatty acids during stimulation of PLA₂ could occur into a sequestered compartment. The local fatty acid concentration present in the membrane would likely vary depending on the distance from the activated enzyme in a time-dependent manner. Spatial organization of enzymes and target molecules within the membrane could represent a molecular basis for sequestration. (3) Other products produced as a result of phospholipid hydrolysis and the subsequent metabolism of fatty acids could play some role in reducing the effects of unsaturated fatty acids on membrane integrity.

Three inhibitors of PLA₂—quinacrine, 4-BPB, and TFP—prevented the K⁺-depolarization-induced increase in ³H-HCh-3 binding in striatal slices, suggesting that the regulation of the choline carrier in slices is dependent upon the activity of PLA₂ and subsequent fatty acid release. Since basal ³H-HCh-3 binding and ³H-AA release were also reduced by TFP and quinacrine, a similar role for PLA₂ and fatty acid release is implicated in the maintenance of resting carrier activity. Nevertheless, these inhibitors lack the specificity required for unequivocally confirming a role for endogenous PLA₂ in the activation of ³H-HCh-3 binding in intact slices. Quinacrine has previously been demonstrated to inhibit the secretion of several different neurotransmitters and hormones, including evoked catecholamine release from brain synaptosomes (Bradford et al., 1983) and adrenal medullary cells (Wada et al., 1983), as well as histamine release from rat basophilic leukemia cells (McGivney et al., 1981). Similarly, 4-BPB is an alkylating agent with potential nonspecific effects (Best et al., 1984; Kyger and Franson, 1984). Trifluoperazine, an antipsychotic with demonstrated effects on the inhibition of dopamine receptors, binding of acidic phospholipids (Watanabe et al., 1986), antagonism of voltage-dependent calcium channels (Greenberg et al., 1987), and inhibition of calmodulin (Levin and Weiss, 1979), is equally nonspecific for the inhibition of PLA₂. However, the minimal inhibition of specific ³H-HCh-3 binding observed when slices are incubated with either quinacrine or TFP at 0°C supports the hypothesis that these agents alter ³H-HCh-3 binding levels via inhibition of a distinct regulatory process, and not by inhibition of the interaction of ³H-HCh-3 with its binding site.

Joep and Johnson (1986) provided evidence that inhibition

of ACh release by quinacrine may result from either a direct, competitive inhibition of choline uptake by quinacrine or a noncompetitive inhibition of ACh transport into synaptic vesicles in manner similar to vesamicol (AH5183). Since some studies have suggested that ACh release is directly coupled to the activation of SDHACU (Mulder et al., 1974; Vaca and Pilar, 1979), one might hypothesize that the inhibition of potassium-stimulated ³H-HCh-3 binding by quinacrine is indirect. However, Murrin and colleagues (1977) have demonstrated that depolarization-activated ³H-choline uptake can be observed when ACh release is completely inhibited, suggesting that a release-coupled mechanism for SDHACU activation is not involved in all conditions.

The importance of PLA₂ and fatty acid release as a signal-transduction mechanism has recently been discussed (Burgoyne et al., 1987). Furthermore, unsaturated fatty acids have been shown to act as second messengers in several studies, including the direct activation of protein kinase C by oleic (Murakami et al., 1986) and AAs (McPhail et al., 1984), the direct potentiation of *myo*-inositol 1,4,5-triphosphate-mediated calcium release in dissociated pancreatic islet cells by AA (Wolf et al., 1986), and as a possible inducer of long-term potentiation (Linden et al., 1987; Lynch et al., 1988).

One important discrepancy concerns the effect of oleic acid in these studies. This monounsaturated fatty acid significantly elevated specific ³H-HCh-3 binding in membranes similar to other polyunsaturated fatty acids. However, in contrast to the polyunsaturated fatty acids, oleic acid neither activated nor inhibited synaptosomal ³H-choline uptake at the effective concentrations of oleic acid employed in these studies. Two possible explanations for this phenomenon exist. Since the activation of specific ³H-HCh-3 binding by oleic acid supports the activation of the SDHACU carrier, ³H-choline uptake in oleic acid-treated synaptosomes might be partially inhibited by another indirect mechanism. Our data indicate that oleic acid affects neither synaptosomal integrity nor (Na⁺+K⁺)ATPase activity. Whether oleic acid is depolarizing the synaptosomes and partially inhibiting sodium-dependent uptake in the presence of an activated carrier in these studies, as demonstrated by Troeger et al. (1984), remains to be determined.

Alternately, our data point to the possible existence of an undetermined process subsequent to the unmasking of occluded carrier sites, which are required for the increase in the velocity of choline transport. Though substantial data exist which support a direct correlation between the activation of ³H-HCh-3 binding, activation of ³H-choline uptake, and the activity of cholinergic neurons in a number of different *in vivo* and *in vitro* experimental paradigms, it appears that the activation of ³H-HCh-3 binding is a necessary, yet insufficient, event for the activation of ³H-choline uptake. We hypothesize that through the activation of PLA₂ and the subsequent release of unsaturated fatty acids, previously occult choline carriers are recruited, thereby resulting in an increase in specific ³H-HCh-3 binding. The subsequent modification of these previously recruited carriers by a separate mechanism may be further required for the functional increase in SDHACU *in vitro*.

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