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INVOLVEMENT OF PHOSPHOLIPASE A_2 IN THE REGULATION OF [^{3}H]HEMICHOLINIUM-3 BINDING

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Abstract—We have examined the effects of exogenous phospholipase A_2 (PLA_2) on the sodium-dependent high-affinity choline uptake mechanism as assessed by the specific binding of [^{3}H]hemicholinium-3 ([^{3}H]HCh-3). Incubation of striatal synaptic membranes with bee venom PLA_2 resulted in a concentration-dependent increase in the specific binding of [^{3}H]HCh-3. The effect of PLA_2 on [^{3}H]HCh-3 binding was inhibited by quinacrine, a PLA_2 inhibitor, and by removal of calcium. Scatchard analysis revealed that the observed changes in binding reflected a 2-fold increase in both the capacity and affinity of [^{3}H]HCh-3 for its binding site. Choline and *N*-butylcholine inhibited the specific binding of [^{3}H]HCh-3 in both control and PLA_2-treated membranes with similar potency. When a low concentration of PLA_2 was incubated with the striatal synaptosomes, a small but significant increase in high-affinity [^{3}H]choline uptake was observed. However, higher concentrations of PLA_2, which further increased the specific binding of [^{3}H]HCh-3, caused a reduction of [^{3}H]choline uptake, apparently due to disruption of synaptosomal integrity by PLA_2. Finally, potassium depolarization- and PLA_2-induced increases in specific [^{3}H]HCh-3 binding were not additive. These results suggest a possible role for endogenous PLA_2 in the calcium-dependent regulation of sodium-dependent high-affinity choline uptake.

The sodium-dependent high-affinity choline uptake (SDHACU)† system is a rate-limiting step for acetylcholine (ACh) synthesis in cholinergic neurons [1–4]. Accumulating evidence indicates that changes in the velocity of SDHACU correlate with the antecedent activity of cholinergic neurons [5, 6]. Membrane depolarization and increases in intraneuronal calcium appear to be two important elements in the activation of SDHACU [5, 6]. Nevertheless, the proximal molecular mechanisms regulating SDHACU remain to be determined.

Hemicholinium-3 (HCh-3) is a potent, specific and competitive inhibitor of SDHACU. [^{3}H]HCh-3 binds to a site or sites that are associated with the SDHACU carrier as suggested by similarities in regional distribution, ionic dependency, pharmacological selectivity and kinetic characteristics [7, 8]. A close correlation between decreases in choline acetyltransferase activity and [^{3}H]HCh-3 binding has been demonstrated following lesions of the septo-hippocampal pathway or the basal forebrain-cortical pathway [8, 9]. Moreover, it has been demonstrated that *in vivo* and *in vitro* pharmacological treatments shown previously to affect ACh turnover and the velocity of SDHACU produce equivalent changes in the capacity of [^{3}H]HCh-3 binding [10, 11]. Accordingly, it has become possible to examine SDHACU regulatory mechanisms in membrane preparations utilizing the specific binding of [^{3}H]HCh-3.

Previously, we reported the solubilization of a [^{3}H]HCh-3 binding site as a first step in the purification of the choline carrier [12]. Attempting to characterize the biochemical nature of both synaptic membranes and the solubilized [^{3}H]HCh-3 binding site with proteolytic and lipolytic enzymes, we observed that incubation of synaptic membranes, but not solubilized preparations, with phospholipase A_2 (PLA_2; phosphatide 2-acyl-hydrolase; EC 3.1.1.4) caused a significant increase in the specific binding of [^{3}H]HCh-3. There is considerable evidence that PLA_2 can modulate neurotransmitter metabolism and signal transduction [13–17]. For example, Fletcher and Middlebrook [13] have indicated that snake venom PLA_2 inhibits choline uptake and ACh release by synaptosomes. Thus, it seems possible that the alterations in the specific binding of [^{3}H]HCh-3 by PLA_2 may reflect a mechanism for the modulation of SDHACU. Accordingly, in the present study we examined in greater detail the effects of PLA_2 on the specific binding of [^{3}H]HCh-3 and on SDHACU.

METHODS

Materials. [^{3}H]Hemicholinium-3 (126.5 and 150 Ci/mmol) and [^{3}H]choline (80 Ci/mmol) were obtained from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL) respectively. Liquid scintillation fluid (Ready-Solv-HP/b) was purchased from Beckman (Fullerton, CA). PLA_2 (from bee venom, specific activity: 1050 units/mg protein; purity: 92%), quinacrine hydrochloride, polyethylenimine, glycylglycine, hemicholinium-3, and choline chloride were obtained from Sigma (St.

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† Abbreviations: SDHACU, sodium-dependent high-affinity choline uptake; HCh-3, hemicholinium-3; PLA_2, phospholipase A_2; ACh, acetylcholine; and LDH, lactate dehydrogenase.

Louis, MO). Glass fiber filters (No. 32) were acquired from Schleicher & Schuell (Keene, NH). *N*-Butylcholine was provided by Dr. Michael Kuhar. All other reagents were standard commercial products of analytical grade.

Preparation of synaptosomes and synaptic membranes. Male Sprague–Dawley rats weighing 150–200 g were decapitated, and the brains were removed quickly. Striata were carefully dissected free from surrounding tissues and 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 *g* for 10 min. The pellet (P_1) was resuspended and recentrifuged at 1000 *g* for 10 min. The combined supernatant fractions were centrifuged at 20,000 *g* for 20 min. The resulting pellet (P_2) was resuspended in an oxygenated Krebs bicarbonate buffer containing (mM) 120 NaCl, 4.2 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 2.6 $MgCl_2$, 1.3 $CaCl_2$ and 10 dextrose (pH 7.4) and centrifuged at 20,000 *g* for 20 min. For [^{3}H]choline uptake experiments, this pellet was resuspended at a protein concentration of 2 mg/ml in Krebs buffer and used directly. Alternately, synaptic membranes were obtained by osmotic lysis of this P_2 pellet. Briefly, the P_2 pellet was resuspended in 20 vol. of ice-cold distilled water and dispersed with a Brinkman PT-10 Polytron at setting 5 for 10 sec. This homogenate was centrifuged at 8000 *g* for 20 min. The resulting supernatant fraction and buffy coat were collected, centrifuged at 20,000 *g* for 20 min, washed once, and resuspended in 50 mM glycylglycine buffer (pH 7.8) containing 200 mM NaCl at a protein concentration of 1 mg/ml.

Treatment of synaptosomes with PLA_2 was performed at 25° for 30 min in Krebs buffer (pH 7.4). The PLA_2 activity used in all experiments refers to PLA_2 activity measured under optimal conditions at pH 8.9 as reported by the supplier (Sigma). The incubation was terminated by ice-cooling, followed by centrifugation at 20,000 *g* for 20 min at 4°. This pellet was resuspended in Krebs buffer and used for high-affinity [^{3}H]choline uptake. Alternatively, in some experiments, the supernatant fraction was used for determination of lactate dehydrogenase (LDH) activity. Treatment of synaptic membranes with PLA_2 was performed at 25° for 30 min in 50 mM glycylglycine buffer (pH 7.8) containing 200 mM NaCl and 5 mM $CaCl_2$. In some experiments, calcium was replaced by 1 mM ethyleneglycol-bis(amino-ethylether)tetra acetate (EGTA). After treatment, synaptic membranes were washed once and resuspended in 50 mM glycylglycine buffer containing 200 mM NaCl. Assessment of [^{3}H]HCh-3 binding in PLA_2-treated membranes produced equivalent results either with or without the post-incubation wash.

Slice preparation and incubation. Rat striatal slices were prepared and incubated as described previously [11]. Dissected striata were cut into 300 μm sections with a MacIlwain tissue chopper and immediately placed into an ice-cold oxygenated Krebs buffer. Tissue slices from four rats were pooled and used immediately for each experiment. Twelve slices were placed into individual glass vials containing 10 ml of oxygenated Krebs buffer and then incubated for 20 min while bubbling continually with a 95% O_2/5% CO_2 gas mixture. After the initial incubation, the medium was replaced with 10 ml of fresh buffer containing 4.2 mM or 40 mM KCl and the incubation was continued for an additional 20 min. Iso-osmolarity of potassium-enriched buffers was effected by equimolar reduction of NaCl. Incubations were terminated by separating slices from the incubation medium by filtration, after which the slices were immediately chilled in 20 vol. of ice-cold 50 mM glycylglycine buffer (pH 7.8) containing 200 mM NaCl and then were immediately sonicated. Crude membranes obtained by sonication were centrifuged at 20,000 *g* for 20 min. The resulting pellets were washed once prior to treatment with PLA_2 as described above.

[^{3}H]*HCh-3 binding.* [^{3}H]HCh-3 binding was assayed as described previously [8, 11]. In brief, a 50-μl sample of the membranes was incubated for 30 min at 25° in 50 mM glycylglycine buffer (pH 7.8) containing 200 mM NaCl and 0.6 to 20 nM [^{3}H]HCh-3 in a final volume of 0.1 ml. Non-specific binding was defined as binding in the presence of 1 μM HCh-3. Incubation was terminated by vacuum-filtration using a Brandel Cell Harvester onto the glass fiber filters which had been treated previously with 0.3% (v/v) polyethylenimine solution. Radioactivity was determined by liquid scintillation spectrometry at a counting efficiency of 50–55%.

[^{3}H]*Choline uptake.* High-affinity choline uptake was assayed as described previously [11]. In brief, synaptosomes were preincubated for 5 min at 37° in Krebs buffer. Uptake was initiated by addition of [^{3}H]choline to produce a final concentration of 40 nM (final specific activity = 8 Ci/mmol) in a volume of 1.0 ml. At the end of the 4-min incubation, uptake was terminated by the addition of 2 ml of ice-cold Krebs buffer, followed by rapid vacuum-filtration onto glass fiber filters. Non-specific uptake was defined as uptake that occurred at 4° or in the presence of 10 nM HCh-3.

LDH activity. LDH activity released from synaptosomes during incubation was assayed as described previously by Schnaar *et al.* [18]. A diluted supernatant fraction (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 recorded continuously using a Beckman DU spectrophotometer. Total synaptosomal LDH activity was determined after solubilization with Triton X-100 (0.5%). Protein content was determined according to the method of Lowry *et al.* [19] utilizing bovine serum albumin as a standard.

Statistical analyses. The IC_{50} values were calculated by Hill plot analysis. K_i values were calculated from the Cheng–Prusoff equation [20]. Statistical significance was assessed by Student's two-tailed *t*-test. Results are expressed as the mean ± SEM from at least three separate experiments, each performed in triplicate or quadruplicate.

RESULTS

Stimulation by PLA_2 of [^{3}H]HCh-3 binding to synaptic membranes. Treatment of rat striatal synaptic membranes for 30 min with purified PLA_2

Table 1. Effects of PLA_2 on [^{3}H]HCh-3 binding and [^{3}H]choline uptake

Treatment	Concn (units/ml)	[^{3}H]HCh-3 binding (fmol/mg protein)	[^{3}H]Choline uptake (pmol/4 min/mg protein)	LDH activity in supernatant (% of total activity)
Control		193 ± 19	6.80 ± 0.26	10 ± 1
PLA_2	0.03	237 ± 19*	7.65 ± 0.37*	11 ± 1
	0.11	352 ± 63†	6.13 ± 0.66	ND
	0.56	510 ± 47‡	0.50 ± 0.05‡	21 ± 2†
	2.80	516 ± 47‡		
	11.2	439 ± 5‡		

Rat striatal synaptic membranes and synaptosomes were incubated either with or without (control) PLA_2 in the presence of 5 mM $CaCl_2$ for 30 min at 25°, followed by centrifugation at 20,000 *g* for 20 min. Synaptic membranes and synaptosomes were then assayed for [^{3}H]HCh-3 binding and [^{3}H]choline uptake, respectively, as described in Methods. Final concentrations of [^{3}H]HCh-3 and [^{3}H]choline were 10 and 40 nM respectively. A supernatant of the synaptosomal fraction (0.5 ml) was used for determination of LDH activity. Total LDH activity (121 ± 12 nmol/mg protein/min) was determined after solubilization with 0.5% Triton X-100. Each value represents the mean ± SEM of three to five separate experiments performed in quadruplicate.

ND: not determined.

*–‡ Significantly different from control (no PLA_2): *$P < 0.05$, †$P < 0.01$ and ‡$P < 0.001$.

produced a significant, concentration-dependent increase in the specific binding of [^{3}H]HCh-3 (Table 1), whereas non-specific binding was not affected. Maximum stimulation was achieved at a concentration of 0.56 units/ml. Similarly, incubation of synaptosomes with PLA_2 resulted in an identical increase in the specific [^{3}H]HCh-3 binding observed in synaptic membranes. The specific binding of [^{3}H]HCh-3 in control membranes was not affected significantly by treatment with up to 10 μM quinacrine, an inhibitor of PLA_2, after subsequent washing, although quinacrine directly inhibited [^{3}H]HCh-3 binding (IC_{50} = 4.9 μM, data not shown) as well as [^{3}H]choline uptake (IC_{50} = 6 μM [21]). On the other hand, the PLA_2 stimulation of [^{3}H]HCh-3 binding was dose-dependently inhibited by co-incubation with quinacrine, demonstrating an IC_{50} of 4.5 μM (Fig. 1). Finally, the stimulatory effect of PLA_2 upon [^{3}H]HCh-3 binding was nearly completely abolished by the addition of 1 mM EGTA (Fig. 2), although a small but significant effect was observed even in the absence of calcium.

Under equilibrium binding conditions, the specific binding of [^{3}H]HCh-3 in PLA_2-treated membranes increased in a saturable manner, whereas non-specific binding increased linearly with increasing concentrations of [^{3}H]HCh-3 (data not shown). Scatchard and Hill plot analyses of saturation isotherms performed using PLA_2-treated membranes demonstrated a single class of non-interacting sites with an

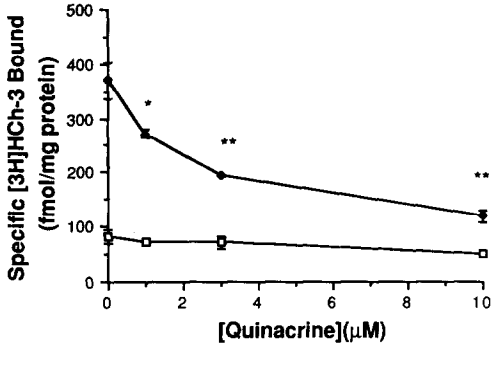

Fig. 1. Effect of quinacrine on PLA_2-induced increase in specific [^{3}H]HCh-3 binding. Rat striatal synaptic membranes were incubated with (◆) or without (□) PLA_2 (1 unit/ml) in the presence of 5 mM $CaCl_2$ and quinacrine for 30 min at 25°, followed by centrifugation at 20,000 *g* for 20 min. Synaptic membranes were then assayed for specific [^{3}H]HCh-3 (2.5 nM) binding as described in Methods. Each point represents the mean ± SEM of three separate experiments performed in quadruplicate. Key: (*) $P < 0.05$, and (**) $P < 0.001$ vs PLA_2 without quinacrine.

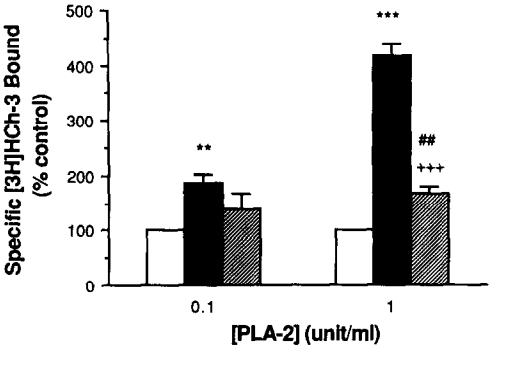

Fig. 2. Calcium-dependent effect of PLA_2 on [^{3}H]HCh-3 binding. Rat striatal synaptic membranes were incubated with the indicated concentrations of PLA_2 for 30 min at 25° in the presence or absence of $CaCl_2$ as described in Methods. After washing, synaptic membranes were assayed for [^{3}H]HCh-3 (2.5 nM) binding. Specific binding values in untreated- and PLA_2 (0.1 and 1 unit/ml)-treated membranes were 85 ± 4, 159 ± 10 and 368 ± 8 fmol/mg protein respectively. Neither $CaCl_2$ nor EGTA alone had any effects on [^{3}H]HCh-3 binding. Each value represents the mean ± SEM of three separate experiments performed in quadruplicate. Key: (□) control, (■) PLA_2 with 5 mM $CaCl_2$, and (▨) PLA_2 with 1 mM EGTA; (**) $P < 0.01$, (***) $P < 0.001$ vs control (no PLA_2, 5 mM $CaCl_2$), (##) $P < 0.01$ vs control (no PLA_2, 1 mM EGTA), and (+++) $P < 0.001$ vs 1 unit/ml PLA_2 with 5 mM $CaCl_2$.

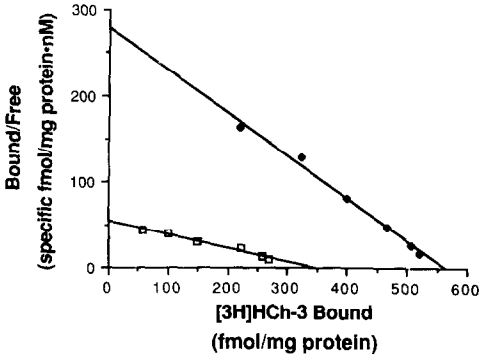

Fig. 3. Scatchard plot of specific [^{3}H]HCh-3 binding in PLA_2-treated membranes. Rat striatal synaptic membranes were treated with PLA_2 (11.2 units/ml) in the presence of 5 mM $CaCl_2$ for 30 min at 25°, followed by centrifugation at 20,000 g for 20 min. Saturation isotherms were then generated utilizing 0.65 to 20 nM [^{3}H]HCh-3. Untreated control (□) and PLA_2-treated (◆) membranes are represented by linear regression lines of the transformed data. This figure represents the typical result from three separate experiments.

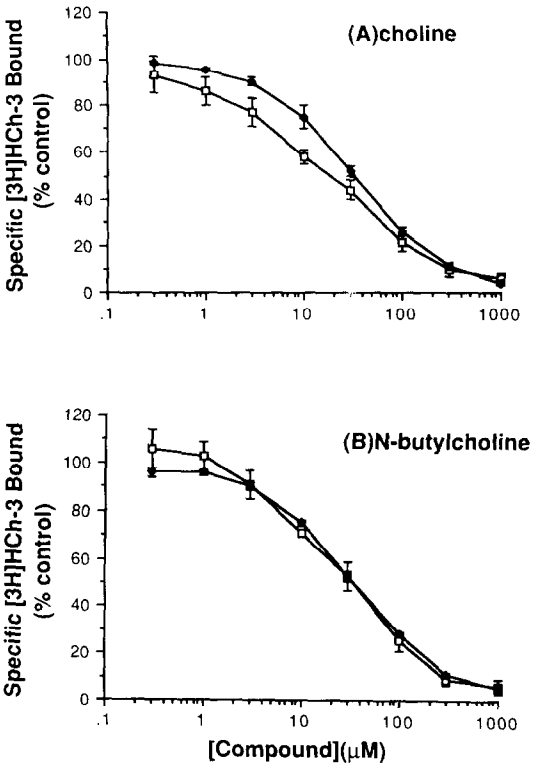

Fig. 4. Inhibition of specific [^{3}H]HCh-3 binding by choline (A) and N-butylcholine (B) to PLA_2-treated membranes. Rat striatal synaptic membranes were treated with PLA_2 (11.2 units/ml) in the presence of 5 mM $CaCl_2$ for 30 min at 25°, followed by centrifugation at 20,000 g for 20 min. Specific [^{3}H]HCl-3 (2.5 nM) binding was then assayed in aliquots of both untreated (open symbols) and PLA_2-treated (closed symbols) membranes in the presence of the indicated concentrations of each compound, and the results were expressed as percent of control (no drug) binding. Specific binding of [^{3}H]HCh-3 in untreated and PLA_2-treated membranes was 93 ± 9 and 362 ± 76 fmol/mg protein respectively. Each point represents the mean ± SEM of three separate experiments performed in triplicate.

apparent dissociation constant (K_D) of 1.8 ± 0.2 nM and a B_{max} of 520 ± 17 fmol/mg protein, while the K_D and B_{max} values measured in control membranes were 5.4 ± 0.5 nM and 286 ± 13 fmol/mg protein respectively (Fig. 3). The pharmacological characteristics of the [^{3}H]HCh-3 binding site in PLA_2-treated membranes were compared to those of untreated membranes. As shown in Fig. 4, choline and N-butylcholine displaced specific [^{3}H]HCh-3 binding in both untreated and PLA_2-treated membranes in a concentration-dependent manner. Choline inhibited [^{3}H]HCh-3 binding in untreated and PLA_2-treated membranes with IC_{50} values of 19.2 ± 4.8 and 32.8 ± 4.7 μM, respectively, while the IC_{50} values for N-butylcholine were 29.2 ± 5.5 and 33.6 ± 2.8 μM respectively. The K_i values for either choline or N-butylcholine were not changed significantly by PLA_2 treatment (choline: untreated = 13.3 ± 3.3, PLA_2-treated = 15.9 ± 3.6 μM; N-butylcholine: untreated = 20.3 ± 3.8, PLA_2-treated = 16.1 ± 2.9 μM).

High-affinity choline uptake in PLA_2-treated synaptosomes. Alterations in the specific binding of [^{3}H]HCh-3 were compared with the changes in SDHACU. When a low concentrations of PLA_2 (0.03 units/ml) was incubated with striatal synaptosomes, a small (13%) but significant increase in high-affinity [^{3}H]choline uptake was observed (Table 1). The stimulation of specific [^{3}H]choline uptake was unchanged when cold HCh-3 was used to define non-specific uptake (data not shown), suggesting that the stimulation by PLA_2 on [^{3}H]choline uptake was due to an HCh-3-sensitive process and not to a general increase in membrane permeability. However, higher concentrations of PLA_2 resulted in an apparent dose-dependent reduction of [^{3}H]choline uptake.

Since one possible explanation for this biphasic effect is that higher concentrations of PLA_2 disrupt synaptosomal integrity, we assessed the effect of PLA_2 treatment on [^{3}H]choline accumulated by striatal synaptosomes. As shown in Fig. 5, incubation of PLA_2 (final 0.56 units/ml) with synaptosomes resulted in a time-dependent loss of radiolabel from the synaptosomes. In contrast, although addition of 10 nM HCh-3, a potent inhibitor of SDHACU, completely inhibited further uptake, it did not lead to a net loss of labeled choline. Thus, the loss of accumulated [^{3}H]choline associated with PLA_2 treatment was not due simply to inhibition of uptake as this loss did not occur with HCh-3 treatment. In addition, we assessed synaptosomal integrity by measuring the release of LDH activity from synaptosomes during treatment with PLA_2 (Table 1). Although the low concentration of PLA_2 (0.03 units/ml) did not cause a significant release of LDH from the synaptosomal suspension as compared to untreated synaptosomes, treatment with a higher concentration of PLA_2 resulted in a significant release of LDH activity from synaptosomes.

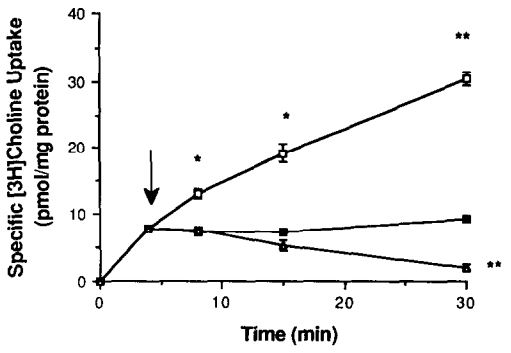

Fig. 5. Effect of PLA_2 treatment on [^{3}H]choline accumulation by striatal synaptosomes. Rat striatal P_2 fraction was prepared as described in Methods. After a 5-min preincubation at 37° in Krebs buffer, [^{3}H]choline (final specific activity = 8 Ci/mmol) was added to yield a final concentration of 40 nM (time point 0). After a 4-min incubation, unlabeled HCh-3 (■; final concentration = 10 nM), PLA_2 (△; final concentration = 0.56 units/ml) or vehicle (□; normal Krebs) was added (as indicated by the arrow), and the incubations were continued for the indicated times, after which total specific accumulated radioactivity was determined. Each value represents the mean ± SEM of three separate experiments performed in quadruplicate. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs HCh-3.

Potassium depolarization and [^{3}H]HCh-3 binding. If PLA_2 plays a physiologic role in modulating [^{3}H]HCh-3 binding sites, then its effects should not be additive with the augmentation produced by neuronal depolarization. Accordingly, rat striatal slices were depolarized by 40 mM potassium, and then membranes from both control and stimulated slices were treated with PLA_2. As shown in Table 2, potassium depolarization of striatal slices significantly increased the specific binding of [^{3}H]HCh-3 by 80% when compared to control slices, whereas PLA_2 treatment of the synaptic membranes increased specific binding of [^{3}H]HCh-3 by 220%. However, there was no significant difference between the absolute binding in membranes obtained from control slices or those subjected to potassium depolarization. Thus, the activation of binding produced by depolarization was not additive with that caused by PLA_2 treatment.

Table 2. Effect of PLA_2 treatment on potassium depolarization-induced increase in [^{3}H]HCh-3 binding in striatal slices

Incubation condition	Specific [^{3}H]HCh-3 binding (fmol/mg protein)	
	Untreated	PLA_2-treated
Control	219 ± 27	701 ± 29†
40 mM KCl	399 ± 33*	759 ± 22†

Rat striatal slices were preincubated for 20 min in Krebs buffer, followed by an additional 20-min incubation in either control (4.2 mM KCl) or 40 mM KCl containing buffer. Membrane preparations from incubated slices were then incubated either without (control) or with PLA_2 (1 unit/ml) in the presence of 5 mM $CaCl_2$ for 30 min at 25°, followed by centrifugation at 20,000 g for 20 min. Membrane preparations were then assayed for specific [^{3}H]HCh-3 (10 nM) binding as described in Methods. Each valuc represents mean ± SEM of five to six observations in three separate experiments.

* $P < 0.01$ vs control (4.2 mM KCl).

† $P < 0.01$ vs untreated membranes.

DISCUSSION

The results of this study indicate that SDHACU can be modulated by treatment with exogenous PLA_2 as demonstrated by changes in both specific [^{3}H]HCh-3 binding and [^{3}H]choline uptake. Based upon the concentration-dependent changes in [^{3}H]HCh-3 binding, the nearly absolute calcium requirement for PLA_2-induced changes, and quinacrine sensitivity, it appears that PLA_2 produces an augmentation of [^{3}H]HCh-3 binding in synaptic membranes via its catalytic action on membrane phospholipids. As revealed by the kinetic analyses, PLA_2 stimulation of specific [^{3}H]HCh-3 binding resulted from an increase in both the apparent number and affinity of binding sites for [^{3}H]HCh-3. On the other hand, K_i values of choline and its analog, *N*-butylcholine, were not changed after PLA_2 treatment. Although it is unclear why PLA_2 treatment affected the affinity of [^{3}H]HCh-3 for the carrier site, the increase in B_{max} appears to be the more significant effect since the affinity of choline, the presumed endogenous ligand for the SDHACU system, was not affected. We have demonstrated previously that alterations of cholinergic neuronal activity by various means, both *in vivo* and *in vitro*, result in changes in the B_{max} of [^{3}H]HCh-3 binding [10, 11]. Similarly, changes in the velocity of SDHACU resulting from alterations in cholinergic neuronal activity *in vivo* and *in vitro* have also been reported to reflect changes in the V_{max} rather than K_T (concentration of substrate equivalent to one-half of the V_{max}) [5, 6]. Thus, the increases in [^{3}H]HCh-3 binding induced by PLA_2 treatment, in which the B_{max} of [^{3}H]HCh-3 binding was increased but with no change in affinity for choline, resemble those found under physiological conditions.

A low concentration of PLA_2 caused a small but significant increase in high-affinity [^{3}H]choline uptake, consistent with the evidence that [^{3}H]HCh-3 binding is associated with the SDHACU carrier. The stimulatory effect of PLA_2 on [^{3}H]choline uptake was not due to a non-specific increase in membrane permeability since the [^{3}H]choline uptake could be inhibited by the specific inhibitor of SDHACU, HCh-3. On the other hand, higher concentrations of PLA_2 actually reduced [^{3}H]choline uptake in agreement with previous findings [13, 22], indicating that an apparent dissociation between [^{3}H]HCh-3 binding and [^{3}H]choline uptake can be observed after PLA_2 treatment. However, [^{3}H]choline uptake is not simply dependent upon the number of carrier sites, but also requires a structurally intact synaptosome capable of maintaining the ion gradient responsible for driving transport [3, 4]. Several observations indicate that the higher PLA_2 concentration disrupts the structural and meta-

bolic integrity of the synaptosomes. First, the high concentration of PLA_2 not only abolished uptake of [^{3}H]choline but resulted in its net loss from the synaptosomes. The specific uptake inhibitor, HCh-3, did not cause this latter effect. Second, the high concentration of PLA_2 caused leakage of LDH from the synaptosome. Since LDH is a large molecule, its leakage suggests a substantial disruption of synaptosomal membrane integrity. Third, we have observed that the high concentration of PLA_2 inhibits synaptosomal Na^+/K^+-ATPase, activity, which is shown to be dependent upon membrane phospholipids [23]. Since the Na^+ and K^+ gradients across the synaptosomal membrane drive the choline carrier [4], this effect would also compromise the transport of [^{3}H]choline.

Concerning the possible mechanisms by which PLA_2 treatment modulates SDHACU, alterations of membrane phospholipid environment may be involved since detergent treatment, which is known to modify lipid/protein interactions, also increases the specific binding of [^{3}H]HCh-3 [12]. Alternately, the end products of PLA_2 catalysis, such as fatty acids and lysophosphatides, may be responsible for the observed PLA_2 effects. For example, fatty acids such as arachidonic acid, have been shown recently to inhibit choline uptake in rat cortical synaptosomes [24], while the K_m of choline uptake in cultured human Y79 retinoblastoma cells is decreased by polyunsaturated fatty acids [25].

Although the molecular mechanisms underlying the regulation of SDHACU are unknown, it has been suggested that the activation of high-affinity choline uptake is a consequence of ACh release during depolarization, either directly by an undetermined mechanism, or indirectly via reduced cytoplasmic ACh concentration and subsequent disinhibition of the choline carrier [1, 3, 4]. However, the results of our experiments indicate that the choline carrier may be directly activated by PLA_2, as evidenced by changes in specific [^{3}H]HCh-3 binding, even in the absence of cytoplasmic components or an intact synaptosomal compartment, suggesting that release of ACh is not an absolute requirement for the activation of SDHACU under these conditions.

Murrin and Kuhar [6] and other investigators [26] have presented data which support a role for calcium in SDHACU activation *in vitro*. We have also observed a significant role of calcium in the regulation of [^{3}H]HCh-3 binding *in vitro* [27]. Interestingly, calcium is required for the activation of several intracellular enzymes, including PLA_2 [14, 28]. Endogenous PLA_2 activity has been shown to be enriched in both synaptosomal plasma membranes [29] and vesicles [15]. It also has been shown that synaptosomal PLA_2 activity is increased by depolarization in a calcium-dependent manner [14, 28]. Moreover, we observed that quinacrine and *p*-bromophenacyl bromide, inhibitors of PLA_2, can antagonize the potassium depolarization-induced stimulation of [^{3}H]HCh-3 binding in rat brain slices (unpublished observations).

In light of the present results, we propose a regulatory role for PLA_2 in SDHACU. Accordingly, depolarization of striatal slices causes an increase in intracellular calcium concentration which then activates PLA_2, resulting in the unmasking of occult carrier sites to which [^{3}H]HCh-3 binds and by which SDHACU is activated. Notably, PLA_2 treatment and detergent solubilization [12], either of which affects protein lipid interrelations, yield the maximum number of specific binding sites for [^{3}H]HCh-3, thereby demonstrating the existence of occult choline carrier sites that are not detectable under basal conditions. Pharmacologic treatments *in vivo* that increase cholinergic neuronal activity and depolarization *in vitro* produce comparable increases in SDHACU and the number of binding sites for [^{3}H]HCh-3. The fact that PLA_2 activation of [^{3}H]HCh-3 binding is not additive with the effects of prior depolarization-induced increase of [^{3}H]HCh-3 binding suggests that the consequences of PLA_2 activation involve the same final common pathway. Nevertheless, several other mechanisms have been proposed to regulate SDHACU, including the inhibition by cytoplasmic ACh [1, 3, 4], interconversion between high- and low-affinity binding sites of [^{3}H]HCh-3 binding [30] and the activation of adenylate cyclase/protein kinase C systems [31]. What remains to be determined is whether these other regulatory mechanisms are funneled through PLA_2 activation or represent independent processes for modulating SDHACU since carrier site number is only one of several processes that could regulate the velocity of choline transport.

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