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LACK OF EFFECT OF ADDED LIPOSOMES ON VERATRIDINE ACTIVATION OF $^{22}Na^+$ UPTAKE IN RAT BRAIN SYNAPTOSOMES

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Abstract—Partially purified egg yolk phospholipids in the form of both small and large unilamellar liposomes at lipid concentrations of 30 mg/ml and higher inhibited veratridine activation of sodium channel specific $^{22}Na^+$ uptake by rat brain synaptosomes. Chromatographically purified egg yolk phosphatidyl choline liposomes did not show this inhibition even at lipid concentrations up to 125 mg/ml. This demonstrates that the inhibition of veratridine-activated $^{22}Na^+$ uptake was due to an impurity in the lipid rather than an ability of the liposomes to absorb and immobilize the lipophilic veratridine molecule.

A number of substances including neurotoxins and drugs have been shown to interact with nerve sodium channels to influence their function. Many of these interactions involve and require a certain degree of lipophilicity on the part of the drug or neurotoxin molecule. Very often these substances must diffuse through the lipid bilayer phase of the membrane in order to reach their site of action, and also the binding interaction itself may involve lipophilicity. Included among these substances is the neurotoxin veratridine [1]. Previous work with the $^{22}Na^+$ uptake assay with rat brain synaptosomes has demonstrated that most of the veratridine added to a synaptosome suspension becomes associated with the membrane phase leaving only a very low concentration in the aqueous phase [2]. Furthermore, it was demonstrated that the veratridine is highly immobile in this preparation, having practically no tendency to move from one membrane vesicle to another in the preparation within a 20- to 30-min period.

Because of these considerations, it would seem logical to assume that the density of membrane particles in the suspension or more precisely the number of membrane particles per unit volume should influence the concentration–response relationship of veratridine and other lipophilic substances in this type of biological assay. In other words, for a given number of veratridine molecules in a suspension of membrane particles, it is expected that, as the amount of membrane particles per unit volume increases, the amount of veratridine required to produce the same level of response should increase. To test this hypothesis, the effects of increasing the number of membrane vesicles in the suspension were examined by increasing the concentration of synaptosomal membranes in the assay system or by using constant amounts of synaptosomal membranes and adding phosphatidyl choline liposomes.

MATERIALS AND METHODS

Veratridine was purified from veratrine (Sigma Chemical Co., St. Louis, MO) according to the method of Blount [3]. Dialyzed and lyophilized ficoll 400000, sucrose grade 1, and trizma base were obtained from Sigma. Acetone, chloroform and petroleum ether were high performance liquid chromatographic or equivalent grade. All other chemicals were reagent grade or better.

Preparation of lipids. Egg yolk phospholipids and egg yolk phosphatidyl choline were prepared according to the method of Singleton *et al.* [4] as follows: 500 g of fresh chicken egg yolks were blended with 1 liter of acetone at 25°. After standing for 1 hr, this was vacuum filtered. The solids were resuspended in 300 ml of acetone and refiltered. This was repeated two more times. The solids were then suspended in 1 liter of 95% ethanol at 25° and allowed to stand for 1 hr followed by vacuum filtration. The solids were resuspended in 500 ml of 95% ethanol at 25° and allowed to stand for 1 hr followed by vacuum filtration. The solids were discarded. The ethanol filtrates were combined and flash evaporated to dryness at 40° on a Buchler flash evaporator.

The dried ethanol extract was redissolved with petroleum ether at 25° to yield 200 ml. This was gradually poured into 1 liter of acetone at 25° with vigorous stirring. The material was then placed in the freezer at −20° for at least 2 hr, usually overnight. The solvent phase was then decanted and discarded, the solids were carefully rinsed with a small amount of acetone, and the remaining solvent was removed by flash evaporation. The material was then redissolved in petroleum ether and the acetone precipitation step was repeated. The resulting solids constituted the partially purified phospholipid fraction.

Phosphatidyl choline was isolated from the phospholipids as follows: 500 g of chromatography grade alumina (M. Woelm, Eschwege, West Germany) was dry packed into a 6.8 × 1.7 cm glass column and washed with 500 ml of methanol followed by 500 ml of chloroform. The phospholipids were dissolved in 100 ml of chloroform and applied to this column. The column was then washed with chloroform until no further material eluted.

Phosphatidyl choline (PC) was then eluted from the column with 9:1 chloroform–methanol until no further material would elute. This material was flash evaporated to yield about 30 g of PC from 500 g of egg yolk. All steps were carried out under nitrogen atmosphere using N_2 equilibrated solvents. The lipids were redissolved in chloroform, dried into glass ampules followed by overnight evacuation at 25°, and stored sealed under vacuum at −20° until used.

Preparation of liposomes. Small unilamellar liposomes were prepared by high intensity sonication according to the method described by Bangham *et al.* [5] as follows: 0.5 g of lipid was suspended in 5 ml of standard buffer (0.32 M sucrose, 10 mM Tris–HCl, 1 mM KN_3, pH 7.4) by prolonged vortexing. This was followed by sonication at 35° in a 25-ml beaker with an immersed one-half inch probe at a power setting of 2.5 using a heat system W-370 sonicator-cell disrupter. Sonication consisted of 10 cycles of 2 min on and 1 min off with the sample beaker immersed in a temperature-regulated water bath. All steps were carried out under nitrogen atmosphere using N_2 equilibrated buffer. These liposomes were used without further processing.

Large unilamellar liposomes were prepared according to the reverse phase evaporation method of Szoka and Papahadjopoulos [6]. Lipid (0.5 g) was dissolved in 30 ml of ether followed by addition of 10 ml of standard buffer. This was sonicated in a rotating round bottom flask in a Bransonic bath type sonicator for 1 hr at 0°. The uniform emulsion which was produced was flash evaporated at 25° until foaming stopped followed by flash evaporating at 45° for 1 hr. All steps prior to flash evaporation were performed under N_2 atmosphere using N_2 equilibrated buffer. Following flash evaporation, the liposome suspension was diluted 2-fold with 20% ficoll

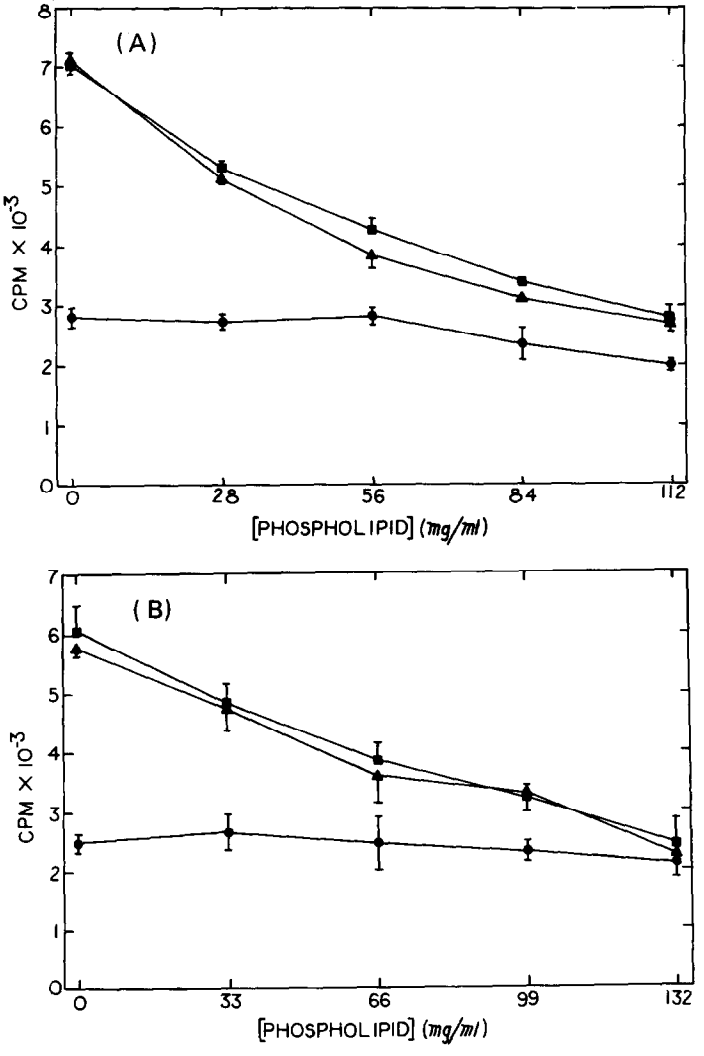

Fig. 1. Influence of small (A) and large (B) unilamellar liposomes, prepared from partially purified egg yolk phospholipid, on $^{22}Na^+$ uptake of brain synaptosomes. Key: (●) control, (▲) 5 μM veratridine, and (■) 50 μM veratridine. Each data point represents the mean ±S.D. for three independent determinations. The abscissa represents the final concentration of lipid, in mg/ml, added as unilamellar liposomes. Each assay received synaptosomes equivalent to approximately 4.2 μmoles of lipid phosphate while the liposomes contained approximately 1.25 μmoles of lipid phosphate per mg lipid.

in standard buffer, placed in a 35-ml centrifuge tube, overlayered with 10 ml of standard buffer, and centrifuged at 20,000 *g* for 30 min. The washed, concentrated liposomes were collected at the air–water interface.

Determination of lipid phosphate. Total organic phosphate content of liposomes, lipid samples, and synaptosomal membranes was determined according to the method of Ames [7]. Total organic phosphate was assumed to represent only lipid phosphate.

Preparation of synaptosomes and $^{22}Na^+$ flux assay. Synaptosomes were prepared and assayed for veratridine-stimulated $^{22}Na^+$ uptake as previously described [8].

RESULTS

Liposomes prepared from partially purified phospholipids produced inhibition of veratridine-stimulated $^{22}Na^+$ uptake in a concentration-dependent manner as shown in Fig. 1A and B, for small and large unilamellar liposomes. A 10-fold increase in veratridine concentration had little effect on this inhibition. Liposomes prepared from chromatographically isolated phosphatidyl choline showed substantially reduced inhibition of veratridine-stimulated $^{22}Na^+$ uptake (Fig. 2, A and B).

Other than a small inhibition of maximal uptake, large unilamellar liposomes prepared from phosphatidyl choline had no influence on the time course

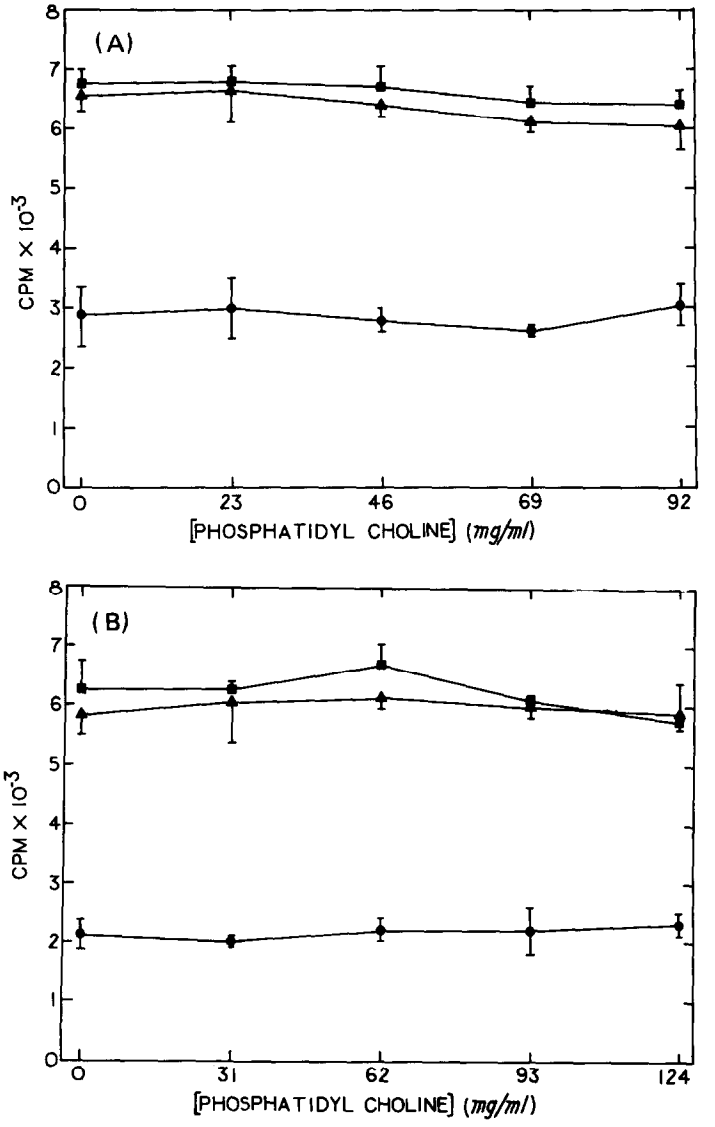

Fig. 2. Influence of small (A) and large (B) unilamellar liposomes, prepared from chromatographically purified egg yolk phosphatidyl choline, on $^{22}Na^+$ uptake of brain synaptosomes. Key: (●) control, (▲) 5 μM veratridine and (■) 50 μM veratridine. Each data point represents the mean ±S.D. for three independent determinations. The abscissa represents the final concentration of lipid, in mg/ml, added as unilamellar liposomes. Each assay received synaptosomes equivalent to approximately 4.2 μmoles of lipid phosphate while the liposomes contained approximately 1.25 μmoles of lipid phosphate per mg lipid.

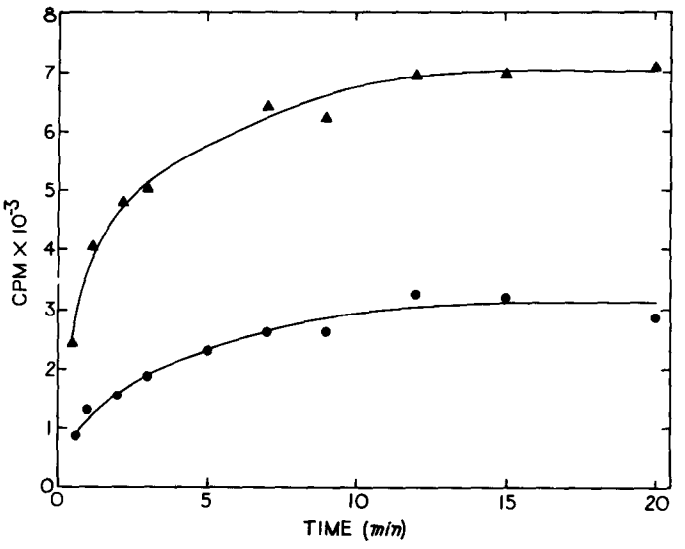

Fig. 3. Time course of $^{22}Na^+$ uptake for synaptosomes mixed with large unilamellar liposomes, prepared from chromatographically purified egg yolk phosphatidyl choline. The ratio of brain membrane lipid phosphate to liposome lipid phosphate was 12 to 1. Key: (●) control, and (▲) 5 μM veratridine. Each data point represents the means ±S.D. from three independent determinations.

or on the concentration–effect relationship for veratridine stimulation of $^{22}Na^+$ uptake (Figs. 3 and 4). These results indicate that the inhibition observed with liposomes is due to some contaminant in the lipid which can be removed by purification and is not due to partitioning of veratridine into the added lipid material reducing its effective concentration for activation of sodium channels.

This is further supported by the previously reported observation that maximal sodium uptake is a linear function of synaptosome concentration in the suspension [2] and that the position of the veratridine dose curve is unaffected by synaptosome concentration in the assay.

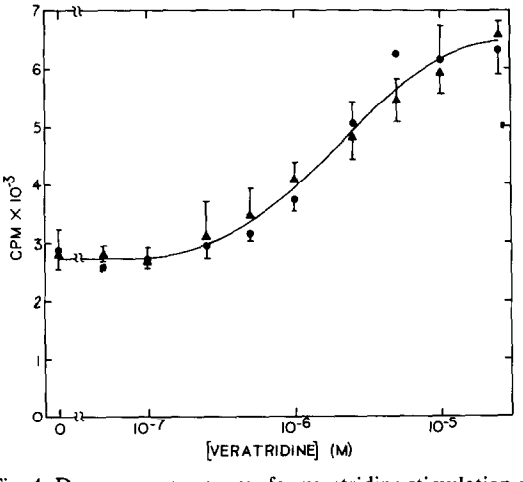

Fig. 4. Dose–response curves for veratridine stimulation of $^{22}Na^+$ uptake. Key: (●) synaptosomes, and (▲) synaptosomes plus large unilamellar liposomes prepared from chromatographically purified egg yolk phosphatidyl choline. The ratio of synaptosomal lipid phosphate to liposome lipid phosphate in the mixed membranes was 1 to 12. Each data point represents the mean ± S.D. from three independent determinations.

Analysis of liposome fusion with synaptosomes. If the added liposomes fuse with the brain synaptosomes to any measurable degree, the interpretation of the results would be different. To examine this possibility, large unilamellar liposomes were prepared with a highly fluorescent terbium dipicolinate complex sequestered inside. These liposomes were washed and transferred to standard buffer as described in Materials and Methods. Then, carefully measured aliquots of the liposomes and the brain synaptosome preparation were mixed and incubated with one another under the same conditions as the assay procedure. The mixture was then applied to the top of a discontinuous density gradient consisting of 5-ml layers of 1%, 3%, 10%, and 15% ficoll in standard buffer in a 35-ml centrifuge tube. Following centrifugation at 0° for 1 hr at 100,000 *g* in a swinging-bucket rotor, there was a dense white band at the air–water interface which contained no detectable protein by the assay of Lowry *et al.* [9]. In addition, there were bands at the 1–3%, 3–10% and 10–15% interfaces which accounted for all of the protein loaded as synaptosomes. All of the fluorescence, loaded as liposomes, was accounted for in the band at the air–water interface; no detectable fluorescence was found in the denser zones. These results demonstrate that there was essentially no fusion of the liposomes with synaptosomes.

Reversibility of veratridine activation of synaptosomes. Another important consideration in the interpretation of liposome action on veratridine stimulation of $^{22}Na^+$ uptake is whether veratridine action on sodium channels is reversible. To assess this, four 1-ml aliquots of a brain synaptosome suspension were treated as described below. Aliquots 1 and 2 received 10 μl of 2 mM veratridine in ethanol; aliquots 3 and 4 received 10 μl of ethanol. These

were incubated at 25° for 30 min after which aliquots 1 and 3 were placed in ice and aliquots 2 and 4 were diluted to 30 ml with 5B and centrifuged at 4° for 30 min at 20,000 *g*. The pellets were resuspended by homogenization with a glass–Teflon homogenizer in ice-cold standard buffer to yield 1 ml each. Triplicate $^{22}Na^+$ uptake assays were then performed on each aliquot. The $^{22}Na^+$ uptake values for aliquots 2, 3, and 4 were the same at approximately 2200 cpm which corresponded to the unstimulated uptake. Fraction 1 showed an uptake of approximately 5900 cpm corresponding to veratridine-stimulated uptake. These results demonstrate that the veratridine was washed out of aliquot 2, indicating complete reversibility.

DISCUSSION

Veratridine partitions into brain synaptosomal membranes in a relatively unbalanced fashion as demonstrated by the observation reported previously [2] that supernatant fractions from 10 mM veratridine-treated membrane suspensions will not stimulate $^{22}Na^+$ uptake when used to resuspend equal quantities of pelleted, untreated synaptosomes. Furthermore, the pelleted, veratridine-treated synaptosomes still show stimulated $^{22}Na^+$ uptake when resuspended in buffer. It can be argued that veratridine causes irreversible changes in these membranes which persist whether veratridine remains associated or not. However, the washout experiment described in Results demonstrates that veratridine action was fully reversible although probably slowly. The $^{22}Na^+$ uptake assay employed in this work is a pseudoequilibrium uptake assay where data points are generally taken after 10 min of incubation time with $^{22}Na^+$. This allows time for the membranes to reach the plateau level of uptake as seen in the time course (Fig. 3). This plateau level of uptake was veratridine concentration dependent as demonstrated by the veratridine dose–response curve in Fig. 4 and as previously reported [2]. The plateau levels of $^{22}Na^+$ uptake for subsaturating levels of veratridine do not show any convergence at $^{22}Na^+$ incubation times of upwards of 20 min. This phenomenon has been explained as being due to a very low mobility of veratridine in the assay medium, i.e. veratridine partitions into the lipid phase of the particular synaptosomal vesicle, activates the sodium channel or channels in that vesicle, but cannot readily leave that vesicle and activate others. Otherwise, the plateau levels of $^{22}Na^+$ uptake for subsaturating veratridine concentrations would converge toward the maximal uptake level at longer incubation times with $^{22}Na^+$. Therefore, since the measurement is the equilibrium level of uptake and not the rate of uptake and since uptake levels do not converge towards maximum uptake at extended uptake times, it should only be necessary to activate, on the average, a single sodium channel per vesicle to achieve the saturation level of $^{22}Na^+$ uptake. Subsaturating levels of veratridine would represent insufficient veratridine to activate, on the average, at least one sodium channel per vesicle.

It follows that, if the above described model for $^{22}Na^+$ uptake is correct, increasing the concentration of membrane vesicles or adding relatively inert vesicles in the form of liposomes should decrease the effective concentration of veratridine in any single vesicle and thus shift the veratridine dose–effect relationship toward higher veratridine concentration. This was not found to be the case. The only effect of added liposomes was to produce a slight inhibition of uptake, probably due to some impurity which was removable upon further purification of the lipid used for liposome preparation.

It is possible that liposome to synaptosome ratios on the order of 10/1 were insufficient to produce significant alterations in veratridine concentration available to the sodium channel. Much higher ratios, on the order of 100/1, or larger, might be expected to produce some effect. The synaptosome concentration could be reduced perhaps 10-fold within the constraints of the assay method to increase this ratio, but it is anticipated that the concentration of lipid and the number of lipid vesicles per unit volume of suspension are the more important variables. The experiments reported here were performed in the extreme situation considering viscosity and liposome packing, at nearly the physical limit of liposome concentration.

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