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Effects of Neurotoxins (Veratridine, Sea Anemone Toxin, Tetrodotoxin) on Transmitter Accumulation and Release by Nerve Terminals in Vitro†

Jean-Pierre Abita,‡ Robert Chicheportiche, Hugues Schweitz, and Michel Lazdunski*

ABSTRACT: Two of the three toxic compounds used in this work, veratridine and the sea anemone toxin, provoke neurotransmitter release from synaptosomes; the third one, tetrodotoxin, prevents the action of both veratridine and the sea anemone toxin. The half-maximum effects of veratridine and sea anemone toxin actions on synaptosomes are $K_{0.5}$ = 10 and 0.02 μM, respectively. Although veratridine and the sea anemone toxin similarly provoke neurotransmitter release, they act on different receptor structures in the membrane. Tetrodotoxin antagonizes the effects of both veratridine and the sea anemone toxin. The half-maximum inhibitory concentration of tetrodotoxin is $K_{0.5}$ = 4 nM for veratridine and 7.9 nM for ATX_{II}. It is very similar to the dissociation constant measured from direct binding experiments with the radioactive toxin. The analysis of this antagonistic action offers an easy in vitro assay for tetrodotoxin interaction with its receptor.

Several neurotoxins now appear to be potentially important tools in studying the mechanism of action potential generation in axons. This group of toxins includes (i) the alkaloids veratridine and batrachotoxin which cause firing and depolarization of nerves by activating the action potential Na^+ ionophore (Albuquerque et al., 1971; Narahashi, 1974; Ulbricht, 1969), (ii) scorpion neurotoxin which markedly slows down sodium inactivation (i.e., the closing of the Na^+ channel) and also alters the steady-state potassium current (i.e., the opening of the K^+ channel) (Romey et al., 1975; Narahashi et al., 1972; Koppenhöffer and Schmidt, 1968), (iii) sea anemone neurotoxin which selectively prevents the closing of the Na^+ channel (Romey et al., 1976a,b), and (iv) tetrodotoxin, the most widely used of all these neurotoxins, which is a specific inhibitor of the action potential Na^+ ionophore (Evans, 1972; Narahashi, 1974).

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Electrophysiological analysis is the usual route to study the interactions between these toxins and the axonal membrane. Another approach has been taken very recently which consists in determining the activity of the action potential Na^+ ionophore from measurements of Na^+ uptake by populations of electrically exitable neuroblastoma cells (Catterall and Nirenberg, 1973; Catterall, 1975).

We analyze in this paper the effects of some of these toxins (veratridine, the sea anemone toxin, tetrodotoxin) on neurotransmitter accumulation and release by rat brain synaptosomes.

Materials and Methods

Preparation of Synaptosomes. Adult male Sprague-Dawley rats (150–200 g) were killed by decapitation. Their brains were rapidly removed and homogenized in 10 vol of ice-cold 0.32 M sucrose, buffered at pH 7.4 with 5 mM Tris-Cl, in a Thomas glass homogenizer fitted with a Teflon pestle (10 passes at 700 rpm). The homogenate was centrifuged at 1200*g* for 5 min and the precipitate was discarded. The supernatant fluid was centrifuged at 23 000*g* for 20 min. The resulting pellet was resuspended in 1 mL of a solution of 0.32 M sucrose in 5 mM Tris-Cl at pH 7.4 and layered on a discontinuous sucrose gradient buffered by 5 mM Tris-Cl at pH 7.4 and consisting of three 10-mL layers of 1.2, 1, and 0.8 M sucrose, respectively. Centrifugation was carried out at 83 000*g* during 60 min in a SW 27 swinging buckets rotor in a Beckman LS 50 refrigerated ultracentrifuge. After centrifugation, the synaptosomes which had accumulated at the interface between the 1 and 1.2 M sucrose layers were collected and kept at 0 °C.

γAbu[1] *Influx Measurements.* The filters which were used in γAbu transport measurements (Millipore EHWP 02500 and Sartorius SM 11106, 0.45 μm pore size) were kept in 1% bovine serum albumin for 2 h and then washed twice with 5 mL of the standard incubation buffer before use. This incubation buffer consisted of 140 mM NaCl, 5 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 20 mM Tris-Cl at pH 7.4. Bovine serum albumin at a concentration of 0.1% was added to the incubation buffer in experiments involving ATX_{II}. Synaptosomes (70–180 μg/mL) were suspended in 1 mL of this medium after addition of 0.1 mg of the γAbu-transaminase inhibitor, sodium dipropylacetate (Godin et al., 1969), to prevent γAbu transformation. Influx measurements were carried out at 22 ± 0.1 °C. The influx reaction was initiated by addition of [^{3}H]γAbu (54 μM). Aliquots of 0.1 to 0.15 mL were taken from the incubation medium at appropriate times and synaptosomes loaded with [^{3}H]γAbu were isolated by filtration. Filters were then washed twice with 5 mL of the incubation buffer at 22 °C and placed in counting vials containing 10 mL of Bray's solution which totally dissolves them. Radioactivity measurements were carried out in Packard 3390 and 2450 liquid scintillation spectrometers. The efficiency of radioactivity measurements was routinely determined by the automatic external standard.

Blanks were obtained by passing the same incubation medium containing appropriate concentrations of [^{3}H]γAbu, but lacking synaptosomes, through the same filters. The radioactivity which remains on the filter under these conditions is subtracted from the radioactivity obtained in the experiment involving synaptosomes. The radioactivity in the blank never exceeds 5% of the total radioactivity measured for loaded synaptosomes.

[1] Abbreviations used are: γAbu, γ-aminobutyric acid; TTX, tetrodotoxin; ATX_{II}, sea anemone neurotoxin.

γAbu Efflux Measurements. Synaptosomes were preloaded with 54 μM [^{3}H]γAbu by incubation at 22 °C for 15 min under conditions described in the preceding section. The synaptosome suspension was then diluted 10 times in the usual incubation medium (see previous section on influx) to trigger γAbu efflux. To measure toxin action, the dilution is carried out in the presence of the toxin. Efflux kinetics are followed by taking aliquots at different times after the dilution. The [^{3}H]γAbu remaining in the synaptosomes was determined by the filtration technique described in the previous section.

Another technique has also been used for measuring efflux. In this technique, synaptosomes which have first been preloaded with [^{3}H]γAbu during 15 min, as previously described, are separated from external γAbu by centrifugation at 20 000*g* for 10 min at 1 °C. The pellet containing the synaptosomes is then washed twice with 5 mL of ice-cold sucrose (0.32 M) buffered with 5 mM Tris-Cl at pH 7.4 and finally resuspended in the same sucrose solution. This suspension is kept at 0 °C. For efflux measurements, 0.2 mL of this synaptosomal suspension preloaded with [^{3}H]γAbu was added to 0.8 mL of the standard incubation medium, free of γAbu, at 22 °C. The kinetics of γAbu efflux were then followed using the filtration technique. The two techniques give identical results.

Glutamate Influx and Efflux Measurements. Experimental data concerning glutamate were obtained using techniques identical with those previously described for γAbu.

Synaptic Membrane Preparation. Synaptic membranes were prepared by two different methods. In the first method derived from that described by Whittaker (1965) the synaptosomes suspension (15 mg of protein/mL in 1 M sucrose) was diluted in water (threefold dilution) and centrifuged 30 min at 100 000*g*. The pellet, called P_3, was submitted to an osmotic shock by suspension in water at 1 °C (20 mL for 1 mL of initial synaptosome suspension) for 1 h under stirring and then centrifuged 30 min at 100 000*g*. The new pellet, called P_4, was resuspended in water and then layered in a discontinuous sucrose gradient buffered by 5 mM Tris-Cl at pH 7.4 and consisting of three 10-mL layers of 1.2, 1, and 0.8 M sucrose, respectively. Centrifugation was carried out at 83 000*g* for 1 h in a SW 27 swinging buckets rotor in a Beckman LS 50 ultracentrifuge refrigerated at 4 °C. After centrifugation, the fraction at the 0.8–1 M sucrose interface, called M_1, was collected and kept at 0 °C. The density of the fraction corresponds to that of synaptic membranes (Whittaker and Barker, 1972; Morgan et al., 1971). Unfortunately, this first method does not totally exclude contamination of the synaptic membrane fraction by myelinated axons (Morgan et al., 1973). For that reason, we have also used a second technique in which we included a step to eliminate myelinated axons before the hypotonic shock. This step is derived from the methods of preparation of myelin-free axons axolemna described by De Vries et al. (1972). It consists of a washing of synaptosomes in a medium containing 0.85 M sucrose, 0.15 M NaCl, and 50 mM KH_2PO_4 at pH 6.0 followed by centrifugation for 10 min at 85 000*g*. This technique readily separates myelinated axons which contaminate the synaptosomal fraction from synaptosomes. Myelinated axons float at the surface of the solution after the centrifugation, whereas synaptosomes are in the pellet (P_3'). This pellet was then used to prepare synaptic membranes as described in the first technique. The membrane fraction obtained at the end at the 0.8–1 M sucrose interface was called M_1'.

Binding Assays. Equilibrium dialysis measurements of [^{3}H]TTX[1] binding to its receptor were performed as previously described (Balerna et al., 1975) in a 10-cell device with 200-μL

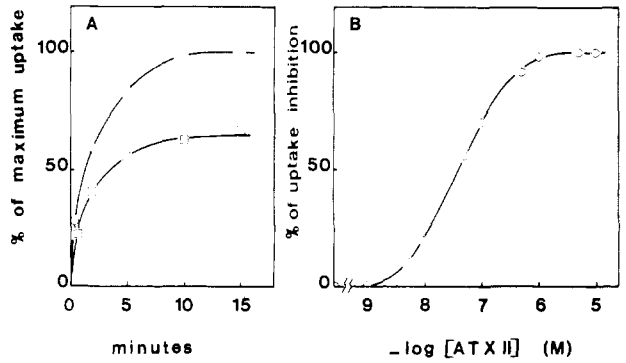

FIGURE 1: The influence of ATX_{II} upon [^{3}H]γAbu uptake into rat brain synaptosomes. (A) Kinetics of [^{3}H]γAbu uptake. Synaptosomes (180 μg of protein/mL) were preincubated at pH 7.4, 22 °C, for 30 min in the standard incubation medium before adding 54 μM [^{3}H]γAbu: (○) preincubation without toxin; (□) preincubation with 1 μM ATX_{II}. With the different synaptosomal preparations used in that work the maximum uptake in the absence of toxin was 2.2–2.7 nmol of [^{3}H]γAbu/mg of protein. (B) ATX_{II} concentration dependence of maximum [^{3}H]γAbu uptake (plateau value in Figure 1A) into synaptosomes.

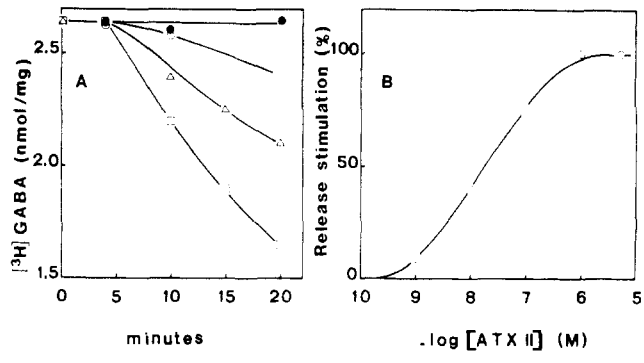

FIGURE 2: (A) Effect of ATX_{II} on the kinetics of [^{3}H]γAbu release from preloaded synaptosomes: (○) control; efflux measured in the presence of (△) 10 nM and (□) 3 μM ATX_{II}; (●) efflux measured in the presence of 3 μM ATX_{II} plus 1 μM TTX. (B) ATX_{II} concentration dependence of the stimulation of [^{3}H]γAbu release. Experimental points were obtained at pH 7.4, 22 °C, after a release time of 20 min; 0% of release stimulation corresponds to the control measured in the absence of any toxin; 100% corresponds to the maximal stimulation which can be obtained at a high concentration of ATX_{II}. Throughout the figures the abbreviation GABA is used for γAbu.

compartments equipped with SM 11 535 Sartorius membranes. Specific binding of [^{3}H]TTX was obtained by subtracting from determinations of radioactivity bound to synaptosomes or synaptic membranes at a series of [^{3}H]TTX concentrations the values obtained by conducting the same series of experiments in the presence of both [^{3}H]TTX (at various concentrations) and unlabeled TTX at a final concentration of 10 μM. [^{3}H]TTX not displaced by unlabeled TTX was considered to be bound nonspecifically.

Chemicals. [^{3}H]γAbu (10 Ci/mmol) and [^{3}H]glutamic acid (10 Ci/mmol) were obtained from New England Nuclear Corporation. Nonradioactive γAbu and glutamic acid were obtained from Sigma. Tetrodotoxin was obtained from Sankyo. [^{3}H]Tetrodotoxin was obtained as previously described (Balerna et al., 1975) with a specific radioactivity of 1 Ci/mmol. ATX_{II},[1] the most abundant of the three neurotoxic polypeptides present in *Anemonia sulcata*, was purified according to Berêss et al. (1975).

Results

γAbu Accumulation and Release in Rat Brain Synaptosomes in the Presence of ATX_{II}. Synaptosomes have been particularly useful in studying the localization, synthesis, uptake, metabolism, and, recently, the release of neurotransmitters. Figure 1A shows the kinetics of [^{3}H]γAbu uptake by rat brain synaptosomes. Incubation of ATX_{II} with synaptosomes for 30 min decreases the maximum uptake of transmitter by about 40%.

Figure 1B shows the toxin concentration dependence of the inhibition of γAbu uptake. The results indicate that the toxin at low concentrations prevents neurotransmitter accumulation. The value of the half-maximal effect of ATX_{II} derived from these results is $K_{0.5} = 0.04$ μM.

The ATX_{II} effect upon γAbu uptake by synaptosomes is reversible. The demonstration of this result was made as follows: synaptosomes were first incubated at 22 °C, pH 7.4, with concentrations of ATX_{II} between 30 nM and 3 μM during 30 min; after this time they were isolated by centrifugation as described under Materials and Methods and carefully washed twice with 5 mL of ice-cold sucrose solution (0.32 M) buffered with 5 mM Tris-Cl at pH 7.4. Since each washing was followed by centrifugation at 20 000*g* for 10 min, the total washing period was about 30 min long. After washing, the synaptosomes were resuspended in 0.32 M sucrose buffered at pH 7.4, brought to 22 °C, and incubated with [^{3}H]γAbu under standard conditions to measure neurotransmitter uptake. The kinetics of uptake of [^{3}H]γAbu by this preparation of synaptosomes which has been initially incubated with ATX_{II} were compared to kinetics of uptake of [^{3}H]γAbu by synaptosomes which have undergone exactly the same manipulations except that during the first 30-min period they were left without ATX_{II}. The plateau value giving maximal [^{3}H]γAbu uptake between 10 and 15 min (see Figure 1A) is identical for the control and for the synaptosomal preparation which has been initially incubated with the toxin. This experiment demonstrates that dissociation of ATX_{II} from its receptor occurs in a medium free of toxin after 30 min at 1 °C (washing period) plus 15 min at 22 °C (period of incubation with [^{3}H]-γAbu).

The ATX_{II} effect upon γAbu efflux from synaptosomes is presented in Figure 2A. The data clearly indicate that the toxin stimulates γAbu release. This stimulation is suppressed when synaptosomes are treated by ATX_{II} in the presence of TTX. The ATX_{II} concentration dependence of the release of γAbu is presented in Figure 2B. The dose–response curve indicates a half-maximal effect at 0.02 μM, nearly identical with the one evaluated from Figure 1B. Lactate dehydrogenase is a classical marker of cytoplasm occluded within synaptosomes (Fonnum, 1968). This enzyme is completely released after a 30-min treatment of synaptosomes at 22 °C with 1% of the detergent Triton X100 well known to destroy plasma membranes. The amount of lactate dehydrogenase released by 1 μM ATX_{II} after a 30-min treatment with the toxin at 22 °C is less than 1% of the amount released by Triton X100. It is identical with the release of lactate dehydrogenase observed in the control in the absence of Triton X100 or ATX_{II}. Thus, ATX_{II} does not appear to cause any synaptosome disruption. In parallel with its effect on neurotransmitter release, ATX_{II} provokes a stimulation of Ca^{2+} uptake by synaptosomes. ATX_{II} at a concentration of 3 μM stimulates the rate of $^{45}Ca^{2+}$ entry (measured according to Blaustein, 1975) by a factor of 1.7 at 25 °C.

The electrophysiological approach has clearly shown that Mg^{2+} and Mn^{2+} inhibit the Ca^{2+}-dependent release of neurotransmitter at neuromuscular junctions and at the squid giant synapse (Miledi, 1973; Narahashi, 1974). Therefore, it

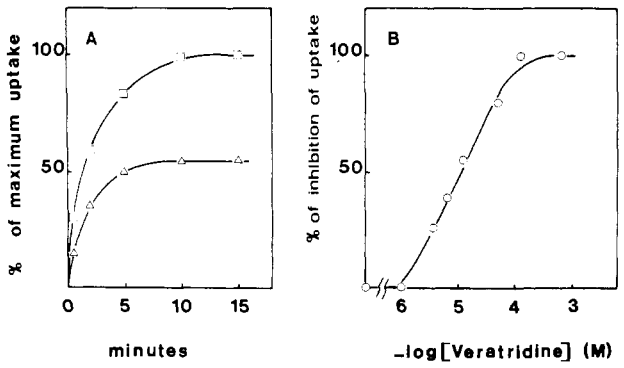

FIGURE 3: The influence of veratridine upon [^{3}H]γAbu uptake into rat brain synaptosomes. (A) Kinetics of [^{3}H]γAbu uptake. Conditions are the same as in Figure 1; (△) preincubation in 0.5 mM veratridine. (B) Veratridine concentration dependence of maximum [^{3}H]γAbu uptake into synaptosomes; pH 7.4, 22 °C.

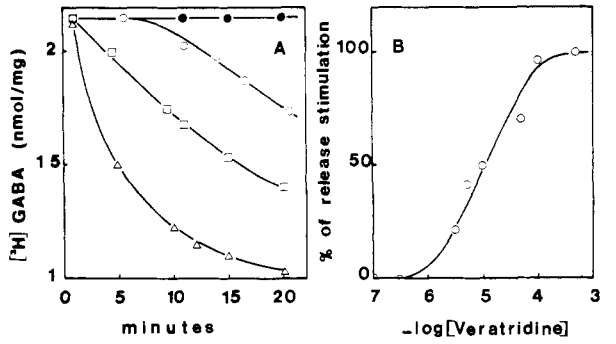

FIGURE 4: (A) Effects of veratridine on the kinetics of [^{3}H]γAbu release from preloaded synaptosomes: (○) control; efflux measured in the presence of (□) 5 and (△) 300 μM veratridine and (●) in the presence of 300 μM veratridine plus 1 μM TTX. (B) Veratridine concentration dependence of the stimulation of [^{3}H]γAbu release. Experimental points were obtained at pH 7.4, 22 °C, after a release time of 10 min; 0% of release stimulation corresponds to the control measured in the absence of any toxin; 100% corresponds to the maximal stimulation which can be obtained at a high concentration of veratridine.

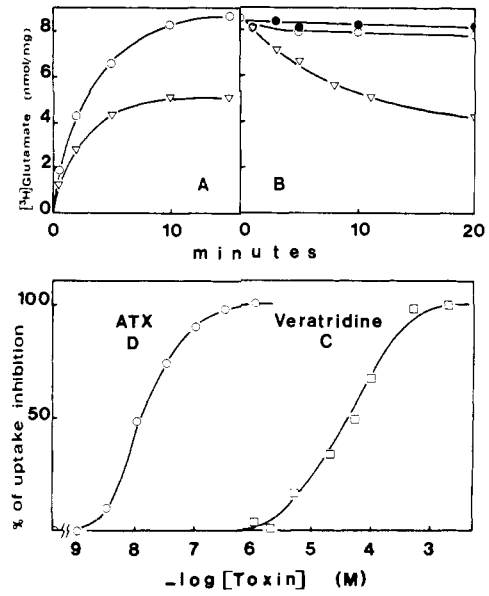

FIGURE 5: The effects of veratridine and ATX_{II} upon synaptosomal uptake and release of [^{3}H]glutamate. (A) Influence of veratridine upon [^{3}H]glutamate uptake: (○) no veratridine; (▽) 300 μM veratridine. Synaptosomes were preincubated 5 min with veratridine before adding [^{3}H]glutamate (72 μM). (B) Influence of veratridine upon [^{3}H]glutamate efflux from preloaded synaptosomes: (○) control; efflux measured in the presence of (▽) 300 μM veratridine and (●) 300 μM veratridine plus 3 μM TTX. (C) Veratridine concentration dependence of maximum [^{3}H]glutamate uptake into synaptosomes: [^{3}H]glutamate concentration, 72 μM; incubation time with veratridine, 5 min. (D) ATX_{II} concentration dependence of maximum [^{3}H]glutamate uptake into synaptosomes; incubation time with ATX_{II}, 30 min. All experiments were carried out at pH 7.4, 22 °C. ATX_{II} decreases the maximum [^{3}H]glutamate uptake by 40%.

is not surprising that these two divalent cations have been found to inhibit γAbu release stimulated by ATX_{II}. Mg^{2+} or Mn^{2+} (15 mM) inhibit the releasing effect induced by 1 μM ATX_{II} (for a 15-min period) by 51 and 80%, respectively.

γAbu Accumulation and Release in Rat Brain Synaptosomes in the Presence of Veratridine. Blaustein et al. (1972) were the first to show that veratridine at a concentration of 75 μM stimulates norepinephrine release from rat brain synaptosomes and that the action of veratridine was antagonized by 0.2 μM TTX. Figure 3A shows that, similarly to ATX_{II}, veratridine decreases the maximum uptake of γAbu by about 50%. Figure 3B shows the toxin concentration dependence of the inhibition of γAbu uptake. The value of the half-maximal effect of veratridine derived from these results is $K_{0.5}$ = 10 μM.

The veratridine effect upon γAbu efflux from the synaptosomes is presented in Figure 4A. Similarly to ATX_{II}, the toxin stimulates γAbu release. The dose–response curve of the veratridine-induced stimulation indicates that the effect is half-maximal at 10 μM, a value identical with the one found in Figure 3B. Under conditions where ATX_{II} effect on synaptosomes is reversible (see preceding section), we find that the veratridine effect on synaptosomes is irreversible. However, veratridine, similarly to ATX_{II}, fails to provoke the release of lactate dehydrogenase, the classical marker of cytoplasm occluded within synaptosomes and in consequence does not appear to cause any synaptosome disruption. Veratridine similarly to ATX_{II} also accelerates Ca^{2+} uptake by synaptosomes. At a concentration of 0.3 mM, it increases the initial rate of $^{45}Ca^{2+}$ entry by a factor of 2.7.

Redburn et al. (1976) have recently shown that neurotransmitter release stimulated by veratridine has properties similar to release stimulated by K^+ or the Ca^{2+} ionophore A 23187. Release is Ca^{2+} dependent and inhibited by Mn^{2+} (10 mM) as in the case of neuromuscular junctions and the squid giant synapse (Miledi, 1973; Narahashi, 1974). An inhibition of veratridine stimulated transmitter release by Mn^{2+}, similar to that observed for ATX_{II}-stimulated release, has also been observed in this work.

Glutamate Accumulation and Release in Rat Brain Synaptosomes in the Presence of Veratridine and ATX_{II}. Glutamate is another neurotransmitter of the central nervous system (Logan and Snyder, 1972; Bennett et al., 1973). Figure 5A shows that veratridine decreases the maximum uptake of [^{3}H]glutamate by about 40%. Veratridine also stimulates neurotransmitter release from synaptosomes preloaded with [^{3}H]glutamate (Figure 5B). A very similar situation is found when veratridine is replaced by ATX_{II}. The toxin concentration dependences of the inhibitions of [^{3}H]glutamate uptake by veratridine and ATX_{II} are represented in Figure 5C. The dose–response curves are nearly identical with those obtained with [^{3}H]γAbu instead of [^{3}H]glutamate in Figures 1B and 3B.

Suppression of ATX_{II} and Veratridine Effects by Tetrodotoxin; [^{3}H]Tetrodotoxin Binding to Synaptosomes and Synaptic Membranes. In contrast to veratridine or ATX_{II},

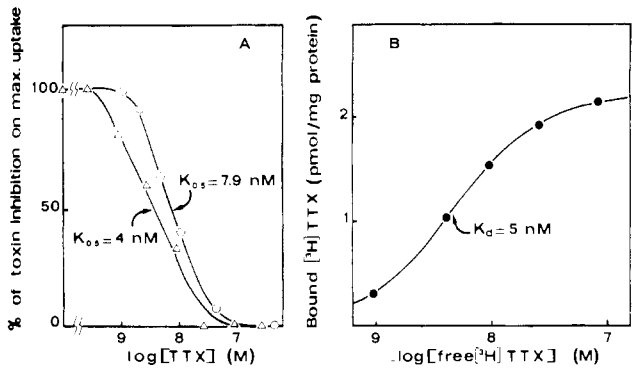

FIGURE 6: (A) Reversion by TTX of the inhibition by ATX_{II} and veratridine of [^{3}H]γAbu uptake by rat brain synaptosomes. Synaptosomes were preincubated at 22 °C, pH 7.4, with (△) ATX_{II}, 30 nM, for 30 min or with (○) veratridine, 12.5 μM, for 5 min in the presence of different concentrations of TTX before starting γAbu uptake measurements. (B) Equilibrium dialysis titration of the TTX receptor in rat brain synaptosomes at pH 7.4, 27 °C. Titrations were carried out in the absence of ATX_{II} or veratridine. The experimental data presented here are corrected for unspecific binding as described in Materials and Methods. In the range of [^{3}H]TTX concentrations in which we have worked, unspecific binding was always below 10% of the total binding. The experimental points have been fitted according to Gache et al. (1976) by a curve which indicates a dissociation constant of 5 nM for the TTX-receptor complex and a Hill coefficient of 1.1 (i.e., a single family of TTX binding sites).

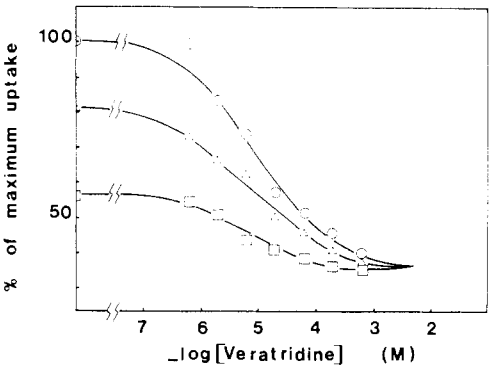

FIGURE 7: Effect of ATX_{II} upon the veratridine concentration dependence of maximum [^{3}H]γAbu uptake by rat brain synaptosomes. Synaptosomes were preincubated for 15 min at pH 7.4, 22 °C, in the presence of increasing concentrations of veratridine and of different concentrations of ATX_{II}: (○) no ATX_{II}; (△) 10 nM ATX_{II}; (□) 1 μM ATX_{II}. [^{3}H]γAbu uptake experiments were carried out with a concentration of 60 μM γAbu in the incubation buffer containing 0.1% serum albumin. The plateau value for maximal uptake was measured after 12 and 14 min of uptake.

TABLE I: [^{3}H]TTX Binding to Synaptosomes and Synaptic Membranes.[a]

Fraction		Bound [^{3}H]TTX (pmol/mg of Protein)
Crude synaptosomes		1.7 ± 0.3
Washed synaptosomes	P_3	1.8 ± 0.3
	P_3'	2.2 ± 0.4
Synaptic membranes	M_I	3.3 ± 0.4
	M_I'	3.4 ± 0.4

[a] The binding was performed in a buffer free of calcium and containing 10 mM EGTA and 40 nM [^{3}H]TTX, at 4 °C overnight. The concentration of membrane protein was between 0.5 and 2 mg/mL. Each value of bound [^{3}H]TTX represents the average of 4 different determinations.

TTX by itself has no effect on γAbu uptake by synaptosomes measured during periods of 15 min as in Figures 1A and 3A. Results in Figures 2A and 4A show that TTX inhibits the effects of ATX_{II} and veratridine on γAbu release from loaded synaptosomes. TTX also antagonizes the effects of ATX_{II} and veratridine on γAbu uptake by synaptosomes. The dose–response curves of the TTX-induced prevention of ATX_{II} and veratridine inhibition of synaptosomal accumulation of γAbu are presented in Figure 6A. The $K_{0.5}$ values for TTX are 4 and 7.9 nM in the presence of 30 nM ATX_{II} and 12.5 μM veratridine, respectively. [^{3}H]TTX binding to rat brain synaptosomes is presented in Figure 6B. Synaptosomes have a binding capacity of 2.2 pmol/mg of protein; the apparent dissociation constant of the tetrodotoxin–receptor complex is 5 nM. Comparative values of capacities of [^{3}H]TTX binding for synaptosomes and for synaptic membranes are given in Table I. Under our experimental conditions we observed an enrichment by a factor of about 2 on passing from synaptosomes to synaptic membranes. The binding that we observe is specific to synaptosomes and not to contaminations of the synaptosomal preparation by myelinated axons or axonal membranes. The evidence which lead to this conclusion is: (i) the [^{3}H]TTX binding is increased when passing from synaptosomes to synaptic membranes (Table I) although we use at that step a technique to eliminate myelinated axons; (ii) the maximal [^{3}H]TTX binding capacity of purified membranes from rat brain myelinated axons is only 3 pmol/mg of protein (De Vries et al., submitted for publication), a value hardly higher than that found for synaptosomes and lower than that found with synaptic membranes (Table I). This sole observation would be sufficient to exclude that [^{3}H]TTX binding to synaptosomes and synaptic membranes is due to a contamination of preparations by axonal membranes.

Cumulative Effects of Veratridine and ATX_{II}. Figure 7 shows the dose–response curves for veratridine in the presence of different concentrations of ATX_{II}. It is quite clear that the effects of the two toxins are additive and that the $K_{0.5}$ value for the veratridine effect is independent of the ATX_{II} concentration. It is 10 μM between 0 and 1 μM ATX_{II}.

Discussion

Electrophysiological studies by Katz and Miledi (Katz and Miledi, 1970, 1971; Miledi, 1973) on the squid giant synapse provide convincing evidence that transmitter release is controlled by a depolarization-dependent calcium permeability increase at the presynaptic terminals. Synaptosomes fulfill all of the criteria for stimulus–secretion coupling defined from the electrophysiological approach. Recent in vitro studies with synaptosomes have indeed confirmed that depolarization provokes the entry of calcium ions and the release of transmitter (Blaustein et al., 1973; Blaustein, 1975).

Two of the three toxic compounds used in that work, veratridine and the sea anemone toxin, provoke neurotransmitter release from synaptosomes; the third one, tetrodotoxin, prevents the action of veratridine and ATX_{II}.

Veratridine depolarizes excitable membranes by inducing a persistent activation of the Na^+ ionophore (it opens the Na^+ channel and prevents its closing) (Ohta et al., 1973; Ulbricht, 1969; Narahashi, 1974). The physicochemical properties of the binding of veratridine to nerve membranes have been studied until now by electrophysiology on squid and crayfish giant axons (Ohta et al., 1973; Ulbricht, 1969; Narahashi, 1974) and by measurements of Na^+ uptake by electrically excitable neuroblastoma cells (Catterall, 1975). The half-

maximum effect for veratridine action on synaptosomes is $K_{0.5}$ = 10 μM as compared to $K_{0.5}$ values of 30 and 40 μM obtained for axonal membranes of the squid giant axon (Ohta et al., 1973; Narahashi, 1974) and for the excitable membrane of neuroblastoma cells (Catterall, 1975), respectively.

The sea anemone toxin displays two kinds of effects on axons: (i) the toxin selectively affects the closing (inactivation) of the Na^+ channel by slowing it down considerably; it hardly alters the opening mechanism of the Na^+ channel or the steady-state potassium conductance; (ii) besides its effect on the action potential, the sea anemone toxin displays a veratridine-like depolarizing action. This latter effect, similar to the veratridine effect on axons (Ulbricht, 1969), is prevented at high Ca^{2+} concentrations. TTX antagonizes all axonal effects of ATX_{II}: the effect on the action potential and the induced increase in resting nerve membrane permeability to sodium ions (Romey et al., 1976a,b).

There are two possible interpretations for ATX_{II} stimulation of neurotransmitter release: (i) ATX_{II} increases Ca^{2+} permeability by direct interaction with the calcium influx system without affecting the resting membrane potential; (ii) ATX_{II} increases Ca^{2+} permeability, i.e. neurotransmitter release, by simply depolarizing the presynaptic membrane in a way similar to veratridine. Considering that one of the actions of ATX_{II} on the axonal membrane is a veratridine-like action, we presently favor the second interpretation. The action of ATX_{II} on synaptosomes appears to be very similar to that displayed by another protein neurotoxin, the scorpion neurotoxin (Romey et al., 1976a,b).

Although veratridine and ATX_{II} both similarly provoke neurotransmitter release from rat brain synaptosomes, they apparently bind to different receptor structures in the membrane. Figure 7 has shown that the effects of the two toxins are additive and that the shape of dose–response curves as well as the value of $K_{0.5}$ for veratridine are unaffected by the presence of ATX_{II} even in large concentrations.

ATX_{II} and veratridine also apparently have different sites of action in the axonal membrane. The ATX_{II} receptor is situated on the external face of the axonal membrane (Romey et al., 1976a,b), whereas veratridine appears to act from inside the nerve membrane (Narahashi, 1974).

The importance of protein neurotoxins in the study of molecular aspects of nerve conduction and transmission is beautifully illustrated by the wide use which has been made of postsynaptic toxins, snake neurotoxins. These neurotoxins are now essential tools for the identification, for the localization, and for the isolation of the acetylcholine receptor (for reviews see Changeux, 1976; Raftery et al., 1976; Vogel and Daniels, 1976; Barnard and Dolly, 1976). This success is due to three factors: (i) the toxin action is very specific; (ii) the snake toxin associates very tightly with its receptor; (iii) the fact that the toxin is a protein permits radioactive labeling (in a variety of ways) essential for receptor purification and histochemical techniques; it also permits the use of cross-linkers to irreversibly graft the toxin to its membrane receptor; finally, it allows the easy preparation of an affinity column for the purification of the acetylcholine receptor. The sea anemone toxin might have in the future an importance among presynaptic toxins similar to that of snake neurotoxins among the postsynaptic toxins.

Tetrodotoxin is well known to be without effect on the release of transmitters from nerve terminals (Narahashi, 1974; Evans, 1972). TTX, however, antagonizes release of neurotransmitter stimulated by veratridine and ATX_{II}. The reversion of the ATX_{II} and veratridine effects offers an easy in vitro assay for TTX action. This assay necessitates neither an electrophysiological equipment nor the use of neuroblastoma cells in cultures for $^{22}Na^+$ uptake experiments.

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Association Products and Conformations of Salt-Dissociated and Acid-Extracted Histones. A Two-Phase Procedure for Isolating Salt-Dissociated Histones†

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ABSTRACT: We present an extremely rapid and efficient method for the separation of salt-dissociated histones from DNA in which the macromolecular components of chicken erythrocyte chromatin are partitioned in a two-phase system of the water-soluble, nonionic polymers, poly(ethylene glycol) and dextran. We have compared the association products and conformations of salt-dissociated histones purified with the two-phase procedure and histones that had been extracted with 0.4 M H_2SO_4. In the gel chromatography system of D. R. van der Westhuyzen and C. von Holt [(1971), *FEBS Lett. 14,* 333-337] the association products of salt-dissociated and acid-extracted histones are indistinguishable. Furthermore, the circular dichroism spectra of histones prepared with the two methods are identical within experimental error. These results indicate that histones extracted with sulfuric acid can adopt conformations at least very similar to those of salt-dissociated histones. In addition, we have found that the circular dichroism properties of total erythrocyte histones are the same in 2 M NaCl as those of these histones bound to DNA in chromatin in 1 mM Tris-Cl (pH 7.5). This result and the studies of Weintraub et al. [Weintraub, H., Palter, K., and Van Lente, F. (1975), *Cell 6,* 85-110] on the patterns of tryptic digest products of histones strongly suggest that in 2 M NaCl the histones exist in conformations very similar to their conformations when bound to DNA. The concept of native histone conformations is discussed in light of our results.

Proteins are usually isolated with considerable care to avoid denaturation, but this precaution has until recently been ignored in the purification of histones, perhaps because they lack any readily monitored activity that can be used to assess whether they are in their native conformation. In the absence of a clear cut probe of conformational integrity and therefore of the ability to determine if the native state has been regained after probable denaturation under harsh isolation conditions, the safest approach is to avoid overt denaturing conditions in the isolation procedure. A widely used method for the isolation of histones that was developed with the expressed intent of avoiding denaturing conditions is that of van der Westhuyzen and von Holt (1971), who used 3 M NaCl and protamines to displace histones from DNA rather than the much harsher standard conditions of relatively concentrated mineral acids. Their procedure and the gel chromatography system they introduced to fractionate the isolated histones have had a major impact on studies of histones in recent years, especially after Kornberg and Thomas (1974) demonstrated that specific oligomers of histones are obtained in the gel chromatography separation.

Further study of histone association reactions will require substantial quantities of histones that either have been isolated without exposure to denaturing conditions or have been demonstrated to have returned to their native conformations after an isolation that used denaturing conditions. We present here an extremely efficient new procedure for isolating salt-dissociated histones that is considerably faster and more convenient than previously published methods. We have used histones prepared by this procedure for two types of studies. First, by examining association products and circular dichroism properties, we have demonstrated that acid-extracted histones can very likely assume the same conformations as salt-dissociated histones. Secondly, we have found that in 2 M NaCl histones in solution have the same helix content as histones that are bound to DNA in chromatin. This provides quantitative corroborative support for the suggestion of Weintraub et al. (1975) that in 2 M NaCl histones adopt the same conformations as histones that are bound to DNA.

Materials and Methods

Chicken blood (with heparin) was purchased from Pel-Freeze Biologics. Poly(ethylene glycol) 6000 was a product of Union Carbide and Dextran 500 was obtained from Sigma.

All preparative and chromatographic steps were carried out at 4 °C.

Preparation of Chromatin. Chicken blood was suspended in 10 vol of 0.15 M NaCl and centrifuged at 200*g* for 10 min. After removing the layer of white cells with an aspirator, the

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