# Neurotoxins Specific for the Sodium Channel Stimulate Calcium Entry into Neuroblastoma Cells†

Y. Jacques, C. Frelin, P. Vigne, G. Romey, M. Parjari, and M. Lazdunski*

ABSTRACT: Veratridine and one polypeptide toxin ($ATX_{II}$) extracted from the sea anemone *Anemonia sulcata* are known to be specific toxins for the fast $Na^+$ channels and to stimulate the rate of $^{22}Na^+$ entry into neuroblastoma cells. Their action is inhibited by tetrodotoxin (Jacques et al., 1978). It is shown in this work that veratridine and $ATX_{II}$ also increase the rate of $^{45}Ca^{2+}$ entry into neuroblastoma cells and that this effect is suppressed by tetrodotoxin. Dose–response curves for the effects of veratridine, the sea anemone toxin, and tetrodotoxin on $^{22}Na^+$ flux on one hand and on $^{45}Ca^{2+}$ flux on the other are superimposable. Scorpion toxin and batrachotoxin have effects similar to those of $ATX_{II}$ and veratridine. The toxin-induced $Ca^{2+}$ permeability is dependent on the external concentrations of both $Ca^{2+}$ and $Na^+$. The half-maximal effect of $Ca^{2+}$ is observed at 2 mM, a concentration very similar to the dissociation constant (1.3 mM) for the interaction between $Ca^{2+}$ and the $Na^+$ channel as measured by competition between $Ca^{2+}$ and a radiolabeled tetrodotoxin analogue. The toxin-induced increase in the rate of $^{45}Ca^{2+}$ influx is not seen in the absence of external $Na^+$. Conversely, the saturation of the $Ca^{2+}$ site hardly alters the toxin-induced increase in the rate of $^{22}Na^+$ influx. At 140 mM $Na^+$ and 1.8 mM $Ca^{2+}$ and in the presence of 133 mM veratridine and 13 $\mu$M $ATX_{II}$, the ratio between $Ca^{2+}$ and $Na^+$ fluxes which are inhibitable by tetrodotoxin is $J_{Ca^{2+}}/J_{Na^+} = 0.02$. The demonstration has been made by using depolarizing agents such as $K^+$ or $CCCP^+$, drugs which accumulate $Na^+$ inside of the cell such as monensin or ouabain, and voltage-clamp techniques which have shown that $ATX_{II}$ does not alter $Ca^{2+}$ channels and that veratridine blocks $Ca^{2+}$ channels, that the increase in rate of $^{45}Ca^{2+}$ influx provoked by veratridine and/or $ATX_{II}$ is not due to an indirect effect of the toxins on a slow $Ca^{2+}$ channel or on a $Na^+$–$Ca^{2+}$ exchange system. The most probable route for this toxin-stimulated $Ca^{2+}$ influx is the $Na^+$ channel itself.

Three main types of channels allowing a transmembranar $Ca^{2+}$ transport have been described in the excitable membrane of squid giant axons (Baker, 1972, 1977): (i) a rapid tetrodotoxin-inhibitable entry of $Ca^{2+}$ which develops in parallel with the time course of $Na^+$ activation–inactivation and suggests a flow of $Ca^{2+}$ through fast $Na^+$ channels (Baker et al., 1971), (ii) a calcium entry through a slow $Ca^{2+}$ channel (this slow $Ca^{2+}$ channel is insensitive to blockers of the $Na^+$ channel like TTX[1] or of the $K^+$ channel such as tetraethylammonium; it is inhibited by $Mn^{2+}$, $Co^{2+}$, or $Ni^{2+}$ ions as well as by the $Ca^{2+}$ channel inhibitors verapamil and $D_{600}$ (Baker, 1977; Hagiwara, 1975), and (iii) a sodium–calcium exchange system that is insensitive to TTX and is inhibited by lanthanum (Baker et al., 1969).

All these calcium transport systems are now known to exist in the membrane of a variety of excitable cells of neuronal and muscular origin (Rougier et al., 1969; Blaustein & Ector, 1976; Moolenaar & Spector, 1979; Stallcup, 1979; Romey et al., 1980).

It has been shown in recent years that toxins specific for the fast $Na^+$ channel are very useful tools for analyzing the structural as well as the functional properties of the channel (Lazdunski et al., 1980a,b; Catterall, 1980). These toxins belong to two main classes. Tetrodotoxin and saxitoxin block $Na^+$ flux through the electrically or chemically opened form of the $Na^+$ channel. Other toxins are specific for the gating system of the $Na^+$ channel; they change the kinetics of the opening (activation) and closing (inactivation) steps of the channel. Molecules like veratridine, batrachotoxin, or grayanotoxin cause a persistent activation of the $Na^+$ channel and, as a result, produce a depolarization of excitable membranes having this type of channels (Ulbricht, 1969; Khodorov & Revenko, 1979). Polypeptide toxins like scorpion or sea anemone toxins (Romey et al., 1975, 1976; Bergman et al., 1976; Okamoto et al., 1977) or insecticides like pyrethroids (Narahashi, 1971) slow down the inactivation of the fast $Na^+$ channel.

Ion flux experiments with neuroblastoma cells in culture have shown that the gating system toxins not only stimulate $^{22}Na^+$ entry through the $Na^+$ channel (Jacques et al., 1978, 1980a) but also stimulate $^{42}K^+$ efflux through the same channel structure (Jacques et al., 1980b). We present in this paper the effects of these toxins on $^{45}Ca^{2+}$ uptake by N1E 115 neuroblastoma cells.

## Materials and Methods

*Materials.* Veratridine was obtained from Aldrich, bovine serum albumin and ouabain were from Sigma Chemical Co., tetrodotoxin was from Sankyo, $^{22}Na^+$, $^{45}Ca^{2+}$, and L-[$^3$H]-leucine were from the C.E.A. (Saclay, France). Dulbecco-modified Eagle's medium and fetal calf serum were purchased from Gibco. The sea anemone $toxin_{II}$ was purified according to Beress et al. (1975) with minor modifications. Scorpion toxin II from *Androctonus australis Hector* was purified according to Miranda et al. (1970). Batrachotoxin was a kind gift from Dr. J. Daly. Carbonyl cyanide *m*-chlorophenylhydrazone ($CCCP^+$) was kindly given to us by Professor G. Ailhaud, and monensin was a generous gift of the Eli Lilly

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[1] Abbreviations used: $ATX_{II}$, sea anemone $toxin_{II}$; TTX, tetrodotoxin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Tris, tris(hydroxymethyl)aminoethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ($Na^+$,$K^+$)ATPase, sodium- and potassium-activated adenosinetriphosphatase.

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Co. [$^3$H]en-TTX (27 Ci/mmol) was prepared according to Chicheportiche et al. (1980).

*Cell Cultures.* Cell from N1E 115 neuroblastoma clone were grown as previously described (Jacques et al., 1978). They were cultured in 35-mm Petri dishes or 24-well Costar tissue culture clusters and allowed to differentiate in a medium containing 1.5% dimethyl sulfoxide and 1% fetal calf serum. Cells were labeled with L-[$^3$H]leucine (0.2 $\mu$Ci/mL) 24 h before the experiments, so that protein recovery in the experiments can be measured from $^3$H counts (Jacques et al., 1978). Chick embryo cardiac cells in monolayer cultures were prepared from 10-day-old chick embryos as described previously (Romey et al., 1980).

*Uptake Measurements.* All uptake experiments were carried out at 37 °C. Cells were preincubated for 20 min in a $Na^+$-free medium which consisted of 25 mM Hepes–Tris, pH 7.4, 140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 0.1 mg/mL bovine serum albumin, and neurotoxins. The incubation in $Na^+$-free medium was necessary to prevent depolarization during incubation with veratridine or its mixture with $ATX_{II}$. The $^{22}Na^+$ or $^{45}Ca^{2+}$ uptake was then initiated by incubating the cells in a medium containing 25 mM Hepes–Tris, pH 7.4, 140 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 0.1 mg/mL bovine serum albumin and labeled with 0.7 $\mu$Ci/mL of $^{22}$NaCl or 10 $\mu$Ci/mL $^{45}CaCl_2$. This medium also contained 0.5 mM ouabain ($Na^+$ uptake experiments) and the concentrations of neurotoxins used in the preincubation. Unless otherwise indicated, the uptake period was 1 min ($Na^+$ uptake experiments) or 4 min ($Ca^{2+}$ uptake experiments). At the end of the uptake experiments, cells were washed 4 times with a medium consisting of 25 mM Tris-HCl, pH 7.4, 145.4 mM choline chloride, 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$. Determinations of the initial rates of $^{45}Ca^{2+}$ or $^{22}Na^+$ uptake were carried out as previously described (Jacques et al., 1978).

In experiments where the external sodium concentration was varied, $Na^+$ was replaced by cholinium ions so that $[Na^+]$ + [cholinium$^+$] = 140 mM. Proteins were determined according to Hartree (1972) by using bovine serum albumin as standard. Computing procedure were performed by using a Wang 2200 calculator as previously described (Jacques et al., 1978).

*[$^3$H]en-TTX Binding Experiments.* Culture plates were rinsed twice with an ice-cold 20 mM Tris buffer containing 0.25 M sucrose and 1 mM EDTA at pH 7.5, resuspended into 20 mM Tris buffer containing 0.25 M sucrose, and homogenized with a Polytron apparatus (Brinkmann Instruments) at setting 5 by using three 5-s bursts separated by 30-s pauses.

Binding assays were carried out as follows: 0.4 mg of protein of homogenate was equilibrated in 1 mL of the standard incubation medium (20 mM Tris buffer containing 200 mM choline chloride at pH 7.5) containing varying amounts of [$^3$H]en-TTX in the absence (total binding) or in the presence (nonspecific binding) of 5 $\mu$M unlabeled TTX. After 20 min at 4 °C, each incubation was stopped by filtering (in less than 10 s) two aliquots of 0.4 mL through prewetted GF/B glass-fiber filters (Whatman) positioned over a vacuum Millipore filter flask apparatus. Filters were rinsed twice with 5 mL of an ice-cold 20 mM Tris buffer at pH 7.5 containing 200 mM choline chloride. Aliquots of 0.1 mL were taken in parallel to determine the total radioligand concentration. [$^3$H]en-TTX which remained bound to membranes on the filters was extracted by vigorous shaking with 8 mL of Picofluor 30 (Packard) as scintillator. Free ligand concentration was obtained by subtracting the amount of [$^3$H]en-TTX bound to the membranes from the total radioligand concentration.

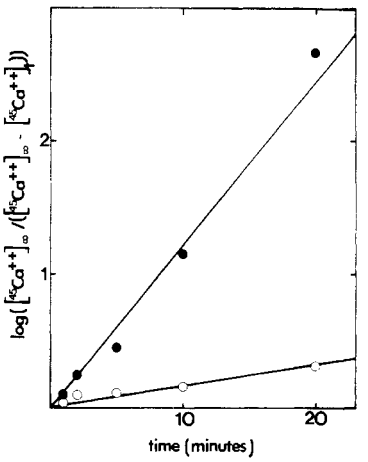


FIGURE 1: Stimulating effect of neurotoxins on the kinetics of $^{45}Ca^{2+}$ uptake by N1E 115 neuroblastoma cells. Experiments were performed in the absence of neurotoxins (○) or in the presence of 13 $\mu$M $ATX_{II}$ plus 133 $\mu$M veratridine (●). Kinetics were analyzed as described by Jacques et al. (1978) for $^{22}Na^+$ influx experiments and by using $[^{45}Ca^{2+}]_\infty$ = 4.6 nmol min$^{-1}$ (mg of protein)$^{-1}$.

Specific [$^3$H]en-TTX binding was defined as the difference between the bound radioactivity determined in the absence and in the presence of 5 $\mu$M unlabeled TTX.

In competition experiments, $Ca^{2+}$ was present in the incubation medium before the addition of 0.8 nM [$^3$H]en-TTX. When the $Ca^{2+}$ concentration was increased, choline chloride concentration was adjusted so that $[Ca^{2+}]$ + [choline] = 200 mM. The inhibition constant for $Ca^{2+}$ was calculated from

$$K_I = \frac{IC_{50}}{1 + \frac{[[^3H]\text{en-TTX}]_f}{K_D}}$$

where $IC_{50}$ was the calcium concentration that inhibits 50% of specific [3H]en-TTX binding, $[[^3H]\text{en-TTX}]_f$ was the free concentration of the radioactive ligand at the half-dissociation, and $K_D$ was the equilibrium dissociation constant determined under identical ionic strength conditions.

*Voltage-Clamp Experiments.* In voltage-clamp measurements, culture dishes containing N1E 115 neuroblastoma cells were directly used after the culture medium was replaced by a $Na^+$-free solution containing 25 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5.4 mM KCl, 25 mM Hepes–Tris, 5 mM glucose, and 25 mM tetraethylammonium buffered at pH 7.4 and adjusted to an osmotic pressure of 305 mosmol with choline chloride. Tetraethylammonium was used to block the delayed outward $K^+$ current which partially masked the $Ca^{2+}$ current. Voltage-clamp experiments were performed by using a new suction pipette method (Kostyuk & Krishtal, 1977; Horn & Brodwick, 1980; Lee et al., 1978). The technique described by Lee et al. (1978) for snail neurons was adapted to neuroblastoma cells. The internal solution was 10 mM $NaH_2PO_4$, 1 mM $MgCl_2$, and 115 mM glutamic acid adjusted to pH 7.1 with KOH and to an osmotic pressure of 305 mosmol with sucrose. The ionic currents were displayed on a storage oscilloscope (Tektronix 5103 N).

## Results

Veratridine, $ATX_{II}$, or a mixture of the two toxins accelerate $^{45}Ca^{2+}$ entry into neuroblastoma cells. Figure 1 shows the time course of $^{45}Ca^{2+}$ accumulation by N1E 115 neuroblastoma cells. Addition of a mixture of 13 $\mu$M $ATX_{II}$ and 133 $\mu$M veratridine increased the initial rate of $^{45}Ca^{2+}$ uptake from 0.2 to 1.1 nmol min$^{-1}$ (mg of protein)$^{-1}$.

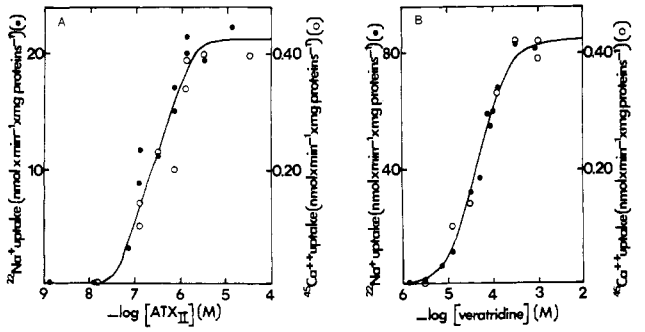


FIGURE 2: Activation by $ATX_{II}$ and veratridine of $^{45}Ca^{2+}$ (●) and $^{22}Na^+$ (○) uptake. The initial rates of cation uptake were determined as described under Materials and Methods. (Panel A) $ATX_{II}$ dose–response curves; (panel B) veratridine dose–response curves. The TTX-insensitive basal rates of $^{45}Ca^{2+}$ or $^{22}Na^+$ uptake were subtracted.

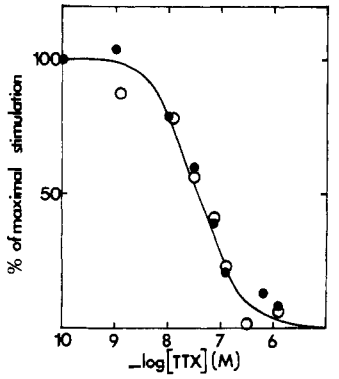


FIGURE 3: Inhibition by TTX of toxin-stimulated $^{45}Ca^{2+}$ (●) and $^{22}Na^+$ (○) uptake by neuroblastoma cells. Experiments were performed in the presence of 13 μM $ATX_{II}$ plus 133 μM veratridine and varying concentrations of TTX. The initial rates of cation uptake were determined as described under Materials and Methods.

Dose–response curves for the effects of veratridine and $ATX_{II}$ on the kinetics of $^{45}Ca^{2+}$ uptake and $^{22}Na^+$ uptake are compared in Figure 2. Saturating concentrations of $ATX_{II}$ or of veratridine increased the initial rate of $^{45}Ca^{2+}$ uptake by a factor of about 3. The half-maximum effects are observed at concentrations of 0.4 ($ATX_{II}$) and 40 μM (veratridine) and are similar for $^{45}Ca^{2+}$ and $^{22}Na^+$ uptakes.

A combination of 13 μM $ATX_{II}$ and 133 μM veratridine increased the initial rate of $^{45}Ca^{2+}$ influx by a factor of 5.5, confirming the already established fact that the polypeptide toxin and the alkaloid toxin bind to different receptor sites (Jacques et al., 1978; Vincent et al., 1980). Figure 3 shows the concentration dependence of the inhibition by TTX of the increase in the rates of $^{45}Ca^{2+}$ and $^{22}Na^+$ uptakes produced by a mixture of $ATX_{II}$ and veratridine. Half-maximum inhibition is observed at 30 nM TTX. Concentrations of TTX as high as 10 μM do not change the basal rate of $^{45}Ca^{2+}$ uptake.

The most active scorpion toxin from *Androctonus australis Hector*, toxin II, at a concentration of 0.1 μM increased the initial rate of $^{45}Ca^{2+}$ uptake from 0.2 to 1.5 nmol min$^{-1}$ (mg of protein)$^{-1}$. The increase observed with batrachotoxin (1 μM) was from 0.2 to 0.8 nmol min$^{-1}$ (mg of protein)$^{-1}$. Scorpion toxin and batrachotoxin stimulated $^{45}Ca^{2+}$ influx was also inhibited by 1 μM TTX.

Catterall & Beress (1978) failed to observe a significant stimulation of $^{22}Na^+$ uptake with $ATX_{II}$ alone whereas this effect was repetitively observed in this laboratory (Jacques et al., 1978). The reason of this discrepancy may be due to a difference in neuroblastoma cell lines used in the different laboratories. It is possible that the N 18 cell line used by

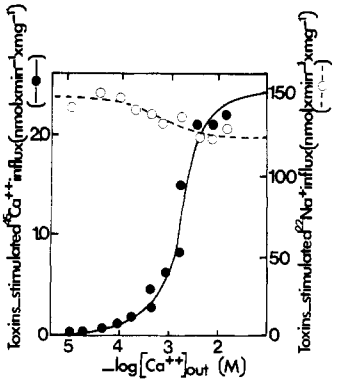


FIGURE 4: Effect of extracellular $Ca^{2+}$ concentration on toxin-activated $^{45}Ca^{2+}$ uptake (●) and $^{22}Na^+$ uptake (○). The initial rates of cation uptake were determined in the presence of 13 μM $ATX_{II}$ plus 133 μM veratridine as described under Materials and Methods. The TTX-insensitive basal rates of cation uptake were subtracted. Exact values of low external $Ca^{2+}$ concentrations were checked by atomic absorption. The unstimulated $^{45}Ca^{2+}$ flux has also been studied. It also varies with the extracellular $Ca^{2+}$ concentration. It represents 50%, 45%, and 44% of the total uptake at 0.1, 1, and 10 mM external calcium, respectively.

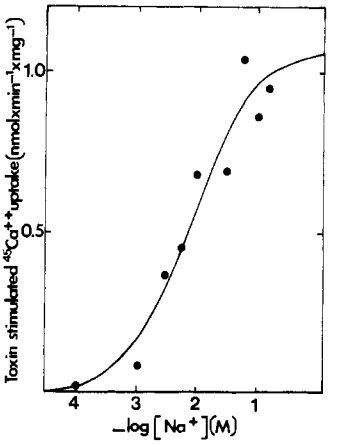


FIGURE 5: Influence of external $Na^+$ concentration on toxin-activated $^{45}Ca^{2+}$ uptake. Neuroblastoma cells were incubated in the presence of 13 μM $ATX_{II}$, 133 μM veratridine, and varying concentrations of $Na^+$ and choline, so that [$Na^+$] + [Cholinium$^+$] = 140 mM, and the initial rates of $^{45}Ca^{2+}$ uptake were determined as described under Materials and Methods. The TTX-insensitive basal rates of $^{45}Ca^{2+}$ uptake were determined for each $Na^+$ concentration and subtracted.

Catterall & Beress (1978) has much fewer channels that the N1E 115 cell line used by us after dimethyl sulfoxide induced differentiation.

The rate of toxin-activated $^{45}Ca^{2+}$ uptake increases with the extracellular calcium concentration (Figure 4). The half-maximum effect is observed at 2 mM external calcium. The saturation of the $Ca^{2+}$ site has little effect on the rate of $^{22}Na^+$ influx through toxin-activated $Na^+$ channels (Figure 4). Figure 5 shows that the toxin-stimulated uptake of $^{45}Ca^{2+}$ is dependent on extracellular $Na^+$ concentration. The half-maximum effect of $Na^+$ ions is observed at 9 mM. The Hill coefficient is close to 1. Replacement of $Na^+$ in the external medium by $Li^+$, $K^+$, $Rb^+$, guanidinium, or ammonium (140 mM) fails to allow toxin stimulation of $^{45}Ca^{2+}$ uptake.

Figure 6 shows the results of binding experiments in which increasing concentrations of [$^3$H]en-TTX are added to a fixed concentration of neuroblastoma cell homogenate either in the presence (nonspecific binding) or in the absence (total binding) of a large excess of unlabeled TTX. Figure 6A (inset) shows that [$^3$H]en-TTX specifically binds to neuroblastoma cells in a saturable fashion. The Scatchard plot (Figure 6A) dem-

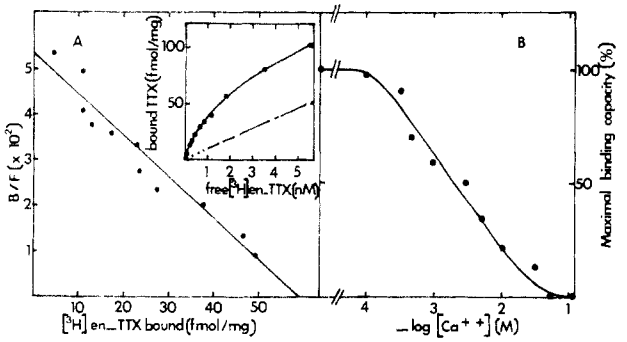


FIGURE 6: Binding properties of [$^3$H]en-TTX to neuroblastoma cell homogenates. (Inset) Plot of the total binding (●) and nonspecific binding (○) of [$^3$H]en-TTX as a function of the free ligand concentration. (Panel A) Scatchard plot of the data. (Panel B) Competition between [$^3$H]en-TTX and $Ca^{2+}$ ions. Experimental conditions as described under Materials and Methods.

onstrates the presence of a single class of TTX binding sites with high affinity. The dissociation constant of the [$^3$H]en-TTX–receptor complex is 1.2 nM and the maximal binding capacity is 58 fmol/mg proteins. Increasing concentrations of calcium prevent the association of [$^3$H]en-TTX to its receptor (Figure 6B) as previously described in cardiac membranes (Lombet et al., 1981). The $IC_{50}$ value is 2.3 mM, and the calculated dissociation constant of the $Ca^{2+}$–receptor complex is 1.3 mM. The binding of calcium ions to the TTX receptor is noncooperative (Hill coefficient close to 1) as found in cardiac membranes (Lombet et al., 1981).

A series of experiments have been carried out to try to understand the mechanism by which toxins that are specific for the $Na^+$ channel promote $^{45}Ca^{2+}$ uptake by neuroblastoma cells. $ATX_{II}$ and veratridine tend to stabilize either transiently for $ATX_{II}$ or permanently for veratridine an open form of the $Na^+$ channel (Ulbricht, 1969; Romey et al., 1976), and as a result, these toxins stimulate $Na^+$ uptake by neuroblastoma cells and provoke intracellular $Na^+$ accumulation (Jacques et al., 1978). One possibility is that this intracellular $Na^+$ accumulation may be indirectly responsible for the stimulation of $^{45}Ca^{2+}$ uptake. For that reason, control experiments have been performed with monensin and ouabain. Monensin has no effect on the fast $Na^+$ channel. The molecule is known to catalyze an electrically neutral exchange of internal $H^+$ for external $Na^+$ in neuroblastoma–glioma hybrid cells (Lichtshtein et al., 1979) and to increase the rate of $^{22}Na^+$ uptake in N1E 115 neuroblastoma cells (Jacques et al., 1980b).

The effect of 100 $\mu$M monensin in stimulating $^{22}Na^+$ uptake is similar to the effect of 100 $\mu$M veratridine (Table I). However, in contrast to veratridine or $ATX_{II}$, monensin does not promote $^{45}Ca^{2+}$ uptake (Table I). The same experiment was repeated by using chick embryonic heart cells instead of neuroblastoma cells. Chick embryonic cardiac cells in monolayer cultures are known to have an active $Na^+$–$Ca^{2+}$ exchange system which is entirely responsible for the coupling between excitation and contraction (Romey et al., 1980). With these cells, monensin increases both the rates of $^{22}Na^+$ and of $^{45}Ca^{2+}$ uptake (Table I).

Ouabain is well-known to have an important influence on the toxin-stimulated $^{22}Na^+$ uptake by neuroblastoma cells treated with veratridine and/or with $ATX_{II}$ (Jacques et al., 1978). This effect of ouabain is due to the fact that the drug blocks the efflux through the ($Na^+$,$K^+$)ATPase of $^{22}Na^+$ which has entered through the toxin-treated $Na^+$ channel. Table I shows that the increased rate of $^{22}Na^+$ uptake provoked by 0.5 mM ouabain is not accompanied by an increased rate of $^{45}Ca^{2+}$ uptake. In chick embryonic cardiac cells, ouabain (0.5 mM) increased both the rates of $^{22}Na^+$ and of $^{45}Ca^{2+}$ uptake (Table I).

Table I: Drug-Activated Rates of $^{22}Na^+$ and $^{45}Ca^{2+}$ Uptake in Neuroblastoma Cells and Embryonic Chick Cardiac Cells[a]

| drug | $^{22}Na^+$ uptake [nmol $min^{-1}$ (mg of protein)$^{-1}$] | $^{45}Ca^{2+}$ uptake [nmol $min^{-1}$ (mg of protein)$^{-1}$] |
|---|---|---|
| N1E 115 | | |
| control | 5 | 0.20 |
| veratridine ($10^{-4}$ M) | 65 | 0.50 |
| monensin ($10^{-4}$ M) | 62 | 0.20 |
| ouabain (0.5 mM) | 15 | 0.20 |
| Embryonic Chick Cardiac Cells | | |
| control | 10 | 1 |
| veratridine ($10^{-4}$ M) | 80 | 25 |
| monensin ($10^{-4}$ M) | 75 | 7.5 |
| ouabain (0.5 mM) | 20 | 8 |

[a] Experimental conditions were as described under Materials and Methods.

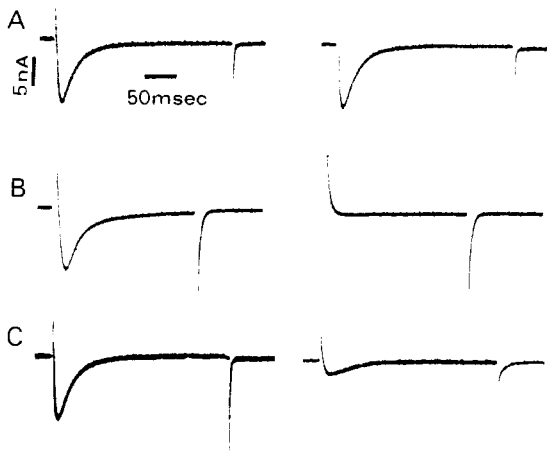


FIGURE 7: Voltage-clamp analysis of the effects of $ATX_{II}$ (A), veratridine (B), and batrachotoxin (C) on the $Ca^{2+}$ current of N1E 115 neuroblastoma cells. The $Ca^{2+}$ current observed here is the same as that described by Moolenaar & Spector (1979); it is blocked by $La^{3+}$ (2 mM), $Co^{2+}$ (10 mM), and $Mn^{2+}$ (10 mM). Depolarizing voltage pulses of 60 mV were applied from a holding potential of −70 mV. (Left) Control $Ca^{2+}$ currents. (Right) After a 20-min application of 10 $\mu$M $ATX_{II}$ (A), 300 $\mu$M veratridine (B), and 5 $\mu$M batrachotoxin (C).

Veratridine or the mixture of veratridine and $ATX_{II}$ depolarizes the plasma membrane of neuroblastoma cells in the presence of physiological $Na^+$ concentrations (Jacques et al., 1980a). It might happen that this depolarization is indirectly responsible for the stimulation of $^{45}Ca^{2+}$ uptake by the toxins. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone ($CCCP^+$) also depolarizes the membrane of neuroblastoma cells in culture (Lichtshtein et al., 1979; Jacques et al., 1980b). Depolarization can also be achieved by simply raising the extracellular $K^+$ concentration to 140 mM. Depolarization induced by 10 $\mu$M $CCCP^+$ or by 140 mM $K^+$ is without effect on the rate of $^{45}Ca^{2+}$ uptake.

Figure 7 shows voltage-clamp experiments describing the properties of the $Ca^{2+}$ channel of neuroblastoma cells in the presence of $ATX_{II}$ and of veratridine. $ATX_{II}$ (10 $\mu$M) is without effect on the $Ca^{2+}$ channel. Veratridine at a concentration of 300 $\mu$M, which is near its maximal activity on the $Na^+$ channel (see Figure 2 for veratridine-stimulated $^{22}Na^+$ influx), completely blocks the $Ca^{2+}$ channel. The same behavior was found with a mixture of veratridine (30 $\mu$M) and

$ATX_{II}$ (10 $\mu$M). Batrachotoxin (5 $\mu$M) has the same blocking effect. Therefore the stabilization of an open form of the $Na^+$ channel by alkaloid toxins is accompanied by the parallel blocking of the $Ca^{2+}$ channel.

## Discussion

Veratridine and $ATX_{II}$ which are known to be specific for the fast $Na^+$ channel and therefore stimulate $^{22}Na^+$ entry into neuroblastoma cells also accelerate the rate of $^{45}Ca^{2+}$ uptake. The ratio of $Ca^{2+}$ and $Na^+$ fluxes ($J_{Ca^{2+},max}/J_{Na^+,max}$) stimulated by 133 $\mu$M veratridine and 13 $\mu$M $ATX_{II}$ are 0.005 and 0.02, respectively. This toxin-sensitive $Ca^{2+}$ permeability of neuroblastoma cells has the following properties:

(i) The concentration dependence of toxin-stimulated $Na^+$ and $Ca^{2+}$ influx follow each other very closely (Figure 2) both for veratridine and for $ATX_{II}$. The effects of $ATX_{II}$ and veratridine are additive.

(ii) The increase in rate of $^{45}Ca^{2+}$ uptake induced by veratridine and $ATX_{II}$ is suppressed by TTX. The dose–response curve for TTX is the same for toxin-stimulated $^{22}Na^+$ influx and for toxin-stimulated $^{45}Ca^{2+}$ influx.

(iii) The $ATX_{II}$- and veratridine-stimulated $^{45}Ca^{2+}$ influxes rise with increasing external concentrations of $Ca^{2+}$. The half-maximum effect is observed at 2 mM $Ca^{2+}$.

(iv) The $Ca^{2+}$ influx induced by $ATX_{II}$ and veratridine is dependent upon the external $Na^+$ concentration.

Similarly to $ATX_{II}$ and veratridine, scorpion toxin II from *Androctonus australis Hector* and batrachotoxin also accelerate a $^{45}Ca^{2+}$ influx component that is inhibited by TTX.

At this point, the results indicate that there is a toxin-dependent $Ca^{2+}$ entry system which is coupled in some way to the functioning of the fast $Na^+$ channel. The action of veratridine and $ATX_{II}$ on a $Ca^{2+}$ entry system in neuroblastoma cells could be explained in the following three different ways:

(i) Veratridine and $ATX_{II}$ could indirectly stimulate $^{45}Ca^{2+}$ entry through a $Na^+$–$Ca^{2+}$ exchange system. Veratridine and $ATX_{II}$, because of their action on the $Na^+$ channel, provoke $Na^+$ accumulation into neuroblastoma cells. This increase in internal $Na^+$ concentration could trigger the functioning of a $Na^+$–$Ca^{2+}$ exchange system. By such a mechanism, the toxins would increase the rate of $^{45}Ca^{2+}$ uptake, and this stimulation would be inhibited by TTX. Such a mechanism is indeed known to occur in embryonic chick cardiac cells in monolayer cultures (Fosset et al., 1977; De Barry et al., 1977; Romey et al., 1980). If it were to occur in neuroblastoma cells, then $Na^+$ accumulation provoked by ouabain or monensin should also increase the rate of $^{45}Ca^{2+}$ influx into neuroblastoma cells. In fact, neither ouabain nor monensin changes the rate of $^{45}Ca^{2+}$ uptake in neuroblastoma cells whereas a drastic increase of the rate of $^{45}Ca^{2+}$ influx is found for embryonic chick cardiac cells which are known to have an active $Na^+$–$Ca^{2+}$ exchange system. In this latter case, veratridine and $ATX_{II}$ stimulate the rate of $^{45}Ca^{2+}$ entry by a factor as high as 25 for 100 $\mu$M veratridine (Fosset et al., 1977) and 12 for 10 $\mu$M $ATX_{II}$ (De Barry et al., 1977).

(ii) Veratridine and $ATX_{II}$ could indirectly stimulate $^{45}Ca^{2+}$ entry through the slow $Ca^{2+}$ channel of neuroblastoma cells. Veratridine and the mixture of veratridine and $ATX_{II}$ are known to depolarize the neuroblastoma cell membrane through their action on $Na^+$ channels (Jacques et al., 1980a). This depolarization could in turn provoke the opening of a slow $Ca^{2+}$ channel which would be normally closed at the resting potential of the cell. Here again the toxin-induced $Ca^{2+}$ permeability would be suppressed by TTX. This possibility has to be considered since voltage-clamp experiments with N1E 115 neuroblastoma have shown that slow $Ca^{2+}$ channels are present in these cells (Moolenaar & Spector, 1978).

However, this hypothesis to explain the effects of veratridine or $ATX_{II}$ on $Ca^{2+}$ influx does not seem to be verified. If veratridine and veratridine plus $ATX_{II}$ do depolarize the neuroblastoma plasma membrane from −30 mV to nearly 0 mV in the presence of 140 mM external sodium, $ATX_{II}$ by itself at concentrations lower than 1 $\mu$M has no depolarizing effect on neuroblastoma cells or on synaptosomes (Ponzio et al., 1980; Creveling et al., 1980). In addition, membrane depolarization by $CCCP^+$ or by high external $K^+$ concentrations does not stimulate the rate of $^{45}Ca^{2+}$ uptake. These observations tell us that the toxins used in this work probably do not exert their action by acting indirectly on voltage-sensitive calcium channels.

(iii) The fact that depolarization by $CCCP^+$ or by high external $K^+$ concentrations does not cause enhanced $Ca^{2+}$ uptake strongly suggests that $Ca^{2+}$ channels which are activated by depolarization rapidly inactivate, as seen in Figure 7A, and therefore cannot contribute to the observed $Ca^{2+}$ uptake. Compounds like $D_{600}$ or verapamil cannot be used unambiguously. Although they block the slow $Ca^{2+}$ channel of neuroblastoma cells at concentrations of $5 \times 10^{-5}$ to $10^{-4}$ M (G. Romey, unpublished results), they are also known to competitively block the veratridine-stimulated uptake of $^{22}Na^+$ in this range of concentration (Galper & Catterall, 1979). $Mn^{2+}$ and $Co^{2+}$ which block $Ca^{2+}$ channels (Moolenaar & Spector, 1979) at concentrations of 10 mM also antagonize veratridine in its stimulation of $^{22}Na^+$ uptake (Catterall, 1975). The best evidence that veratridine-induced $Ca^{2+}$ uptake in the absence or in the presence of $ATX_{II}$ is not due to the opening of $Ca^{2+}$ channels following depolarization is shown in Figure 7. Veratridine (300 $\mu$M) completely blocks the $Ca^{2+}$ channel in the absence and in the presence of $ATX_{II}$. The same observation was made with batrachotoxin (5 $\mu$M). In conclusion, the $ATX_{II}$-stimulated $^{45}Ca^{2+}$ influx cannot be due to a direct or an indirect effect on the $Ca^{2+}$ channel since (i) $ATX_{II}$ is without effect on the $Ca^{2+}$ channel and (ii) $ATX_{II}$ has no depolarizing effect at concentrations lower than 1 $\mu$M. Similarly the veratridine (or batrachotoxin)-stimulated $^{45}Ca^{2+}$ influx cannot be due to a depolarization-induced opening of the $Ca^{2+}$ channel since the alkaloid toxins by themselves block the $Ca^{2+}$ channel at concentrations at which they maximally increase the rates of $^{22}Na^+$ and $^{45}Ca^{2+}$ uptakes.

If one accepts that $Ca^{2+}$ enters neuroblastoma cells treated with veratridine and $ATX_{II}$ via the $Na^+$ channel, then the main properties of this $Ca^{2+}$ permeability of the $Na^+$ channel can be outlined. $Ca^{2+}$ entry through the $Na^+$ channel necessitates the saturation of a $Ca^{2+}$ site. The apparent dissociation constant of the complex formed between $Ca^{2+}$ and the $Ca^{2+}$ site can be assimilated in first approximation to the half-maximum effect of $Ca^{2+}$ as measured from Figure 4. This value is 2 mM. The $Ca^{2+}$ permeability of the $Na^+$ channel which is chemically induced by veratridine and/or $ATX_{II}$ cannot be observed when external $Na^+$ is replaced by cholinium, $K^+$, $Rb^+$, $Li^+$, ammonium, or guanidinium ions.

The saturation of the $Ca^{2+}$ site essential for the permeability to $Ca^{2+}$ of the toxin-treated $Na^+$ channel has little effect on the rate of $^{22}Na^+$ influx through the $Na^+$ channel (Figure 4). At concentrations of $Ca^{2+}$ which are saturating in Figure 4, the $Na^+$ channel conducts at the same time inside of the cell both $Na^+$ and $Ca^{2+}$ ions. The low value of the rate of the $Ca^{2+}$ flux as compared to the rate of the $Na^+$ flux probably explains that even at the highest external $Ca^{2+}$ concentrations, the flow of $Ca^{2+}$ through the $Na^+$ channel hardly decreases $Na^+$ permeability.

$Ca^{2+}$ ions prevent [$^3$H]en-TTX binding to its specific receptor (Lombet et al., 1981; Figure 6), and the dissociation constant of the $Ca^{2+}$–TTX receptor complex determined from competition experiments between $Ca^{2+}$ and a labeled TTX derivative is 1.3 mM. There is a striking similarity between this value and the value of 2 mM obtained from Figure 4.

Finally, it seems evident that toxins like veratridine or $ATX_{II}$ produce multiple effects on the permeability of a variety of important cations by acting solely at the level of the $Na^+$ channel. Clearly, their first effect is to either depolarize the target cell (veratridine) or prolong action potentials ($ATX_{II}$). However, they are also known to drastically change the specificity of the $Na^+$ channel for monovalent cations (Lazdunski et al., 1980a,b), and as a consequence, they provoke a massive efflux of internal $K^+$ through the $Na^+$ channel (Jacques et al., 1980b). This study shows that at physiological concentrations of $Ca^{2+}$ and $Na^+$ they also induce a $Ca^{2+}$ permeability.

$Ca^{2+}$ is known to be essential for a multiplicity of cellular functions. Among those are coupling processes between excitation and contraction or between excitation and secretion. Veratridine and $ATX_{II}$ are known to provoke a massive release of neurotransmitters from synaptosomes (Abita et al., 1977; Blaustein, 1975). In the case of veratridine, this releasing effect has been ascribed to the depolarizing action of the toxin which would indirectly trigger an electrical activation of a voltage-dependent $Ca^{2+}$ channel. Such an interpretation cannot be correct for $ATX_{II}$ which is now known to have no depolarizing effect on the synaptosomal membrane (Ponzio et al., 1980; Creveling et al., 1980) at the low concentrations at which it produces neurotransmitter release (Abita et al., 1977, Romey et al., 1976). Therefore it is most probable that $Ca^{2+}$ entry which releases neurotransmitters from synaptosomes treated with $ATX_{II}$ is through the $Na^+$ channel. If $Ca^{2+}$ channels in synaptosomes have the same properties as $Ca^{2+}$ channels in neuroblastoma cells, they will be blocked by veratridine concentrations that activate $Na^+$ channels. In such a case, veratridine-induced $Ca^{2+}$ entry which releases neurotransmitters from synaptosomes would also be through the $Na^+$ channel.

Finally it has been reported that veratridine and $ATX_{II}$ increase intracellular levels of cAMP and cGMP in brain tissues (Ferrendelli et al., 1973; Anhert et al., 1979). A plausible origin of the effects of these toxins (at least for $ATX_{II}$, which has no depolarizing effect) on cyclic nucleotide contents would then be their capacity to stimulate $Ca^{2+}$ entry through the $Na^+$ channel.

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# Effects of pH on Horse Liver Aldehyde Dehydrogenase: Alterations in Metal Ion Activation, Number of Functioning Active Sites, and Hydrolysis of the Acyl Intermediate†

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ABSTRACT: The reactivity of the mitochondrial (p*I* = 5) isoenzyme of horse liver aldehyde dehydrogenase was determined by studying the effects of pH on steady-state velocity, burst magnitude, and molecular weight of the enzyme in the absence and presence of $Mg^{2+}$ ions. The $Mg^{2+}$ ion activation of the steady-state velocity at pH 7.5 has been explained through a mechanism involving alteration of the tetrameric enzyme, functioning with half-of-the-sites reactivity, to a dimeric enzyme, functioning with all-of-the-sites reactivity [Takahashi, K., & Weiner, H. (1980) *J. Biol. Chem. 255*, 8206–8209]. With increasing pH, the tetrameric enzyme dissociated even in the absence of $Mg^{2+}$ ions to the more active dimeric state. The pH-dependent dissociation was goverened by proton release from a group with p$K$ = 9.5. After correcting for the increased number of functioning active sites, determined from the pre-steady-state burst, we calculated that elevated pH also caused an increase in the velocity of the rate-limiting step, hydrolysis of the acyl-enzyme intermediate. This event was governed by the ionization of two groups, with p$K$ = 7.2 and 9.5, respectively. If these groups are directly involved in the catalytic step, a mechanism involving histidine acting as a general base can be proposed for the former group. The latter group may be involved in a charge relay activation process which only occurs at elevated, nonphysiological pH. The importance of the latter is questionable, as there is only a 3-fold increase in $V_{max}$ when this group is involved in catalysis.

Both mitochondrial (p*I* = 5) and cytosolic (p*I* = 6) isozymes of horse liver aldehyde dehydrogenase (aldehyde:NAD oxidoreductase, EC 1.2.1.3) are isolated as tetramers (Feldman & Weiner, 1972; Eckfeldt et al., 1976). Each exhibits half-of-the-sites reactivity (Weiner et al., 1976; Eckfeldt & Yonetani, 1976) and have broad substrate specificity. The activity of the p*I* 5 isozyme is enhanced 2-fold by $Mg^{2+}$, $Ca^{2+}$, or $Mn^{2+}$ ions at pH 7.5 (Takahashi et al., 1980a). The p*I* 6 isozyme is inhibited by these metal ions (Weiner & Takahashi, 1981) but is activated by lanthanide ions (Venteicher et al., 1977).

The molecular basis for the $Mg^{2+}$ activation of the p*I* 5 isozyme has been explained through a mechanism relating to both a change in molecular weight and a change in the number of reacting sites. That is, in the presence of $Mg^{2+}$ ions, the tetrameric form of the enzyme which functions with half-of-the-sites reactivity is dissociated to a pair of dimers, each possessing all-of-the-sites reactivity (Takahashi & Weiner, 1980; Takahashi et al., 1980b).

We have recently extended the study of the metal ion activation to aldehyde dehydrogenases isolated from species other than horse. With both rat and beef liver aldehyde dehydrogenases, it has been found that $Mg^{2+}$ of $Ca^{2+}$ ions activate the mitochondrial enzyme but inhibit the cytosol enzyme (Weiner & Takahashi, 1981). However, when assayed at pH 9.3 rather than 7.5, it has been shown that beef liver mitochondria aldehyde dehydrogenase is actually inhibited by $Mg^{2+}$ ions but is still activated by $Ca^{2+}$ ions (Takahashi et al., 1979). The differential effect of metal stimulation has also been observed with the sheep liver mitochondrial enzyme. Dickinson has found that this enzyme is activated by $Mg^{2+}$ ions at pH 7.5 but not at pH 8.0.[1]

In this study, we investigated the effect of $Mg^{2+}$ activation of the p*I* 5 isozyme of horse liver aldehyde dehydrogenase over a wide pH range and compared it to the unactivated form. We found that the tetrameric form of the enzyme, even in the absence of $Mg^{2+}$ ions, can dissociate to the more active dimer form at higher pHs. We also determined the effect of pH on the rate-limiting deacylation step (Weiner et al., 1976) during the enzyme-catalyzed process.

## Materials and Methods

Propionaldehyde and NAD were purchased from Eastman Organic Chemicals and P-L Biochemicals, respectively. $MgCl_2$ (analytical reagent) was the product of Mallinckrodt Chemical

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