Molecular Mechanism of the Cardiotoxic Action of a Polypeptide Neurotoxin from Sea Anemone on Cultured Embryonic Cardiac Cells†

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ABSTRACT: The polypeptide neurotoxin, ATX_{II}, extracted from the sea anemone *Anemonia sulcata* considerably and selectively stimulates $^{45}Ca^{2+}$ uptake by embryonic cardiac cells in culture (maximum stimulation, 12-fold). The half-maximum effect of the toxin was observed at a concentration of 0.5 μM under our experimental conditions. ATX_{II} also alters $^{22}Na^{+}$ uptake in the same range of toxin concentration, but the stimulation (of the order of 1.5) obtained with this cation is much less than that observed for the Ca^{2+} uptake. ATX_{II} does not affect the rates of efflux of $^{22}Na^{+}$, $^{45}Ca^{2+}$, and $^{86}Rb^{+}$. The properties of the $^{45}Ca^{2+}$ entry system opened (or maintained in an open state) by ATX_{II} can be summarized as follows. (a) The apparent dissociation constant for the Ca^{2+}–$^{45}Ca^{2+}$ entry system interaction $K_{Ca^{2+}}$ is 0.3 mM. High concentrations of Ca^{2+} (>1 mM) inhibit $^{45}Ca^{2+}$ entry through the system opened by ATX_{II}. Sr^{2+} is a good substitute for Ca^{2+}. ATX_{II}-stimulated $^{45}Ca^{2+}$ and $^{85}Sr^{2+}$ fluxes have very similar properties. (b) Mn^{2+}, Co^{2+}, Ni^{2+}, or La^{3+} block the $^{45}Ca^{2+}$ influx stimulated by ATX_{II}. These cations bind to the open form of the calcium entry system stabilized by ATX_{II} with very similar affinities ($K(Me^{2+}$ or $Me^{3+}) = 0.6$ mM under our experimental conditions). (c) Verapamil and D600, two compounds well known for their antiarrythmic actions, inhibit $^{45}Ca^{2+}$ entry stimulated by ATX_{II}. The half-maximum inhibitory effect of both drugs under our experimental conditions was observed at 5 μM. (d) Anesthetics block $^{45}Ca^{2+}$ entry through the permeation system opened or maintained opened by ATX_{II} (the order of efficiency being tetracain > etidocain > lidocain). (e) ATX_{II}-stimulated $^{45}Ca^{2+}$ influx is dependent upon the external Na^{+} concentration. Li^{+} cannot replace Na^{+}. (f) Tetrodotoxin, a selective blocker of fast Na^{+} channels, inhibits Na^{+} entry stimulated by ATX_{II}; it also inhibits $^{45}Ca^{2+}$ entry stimulated by ATX_{II} (apparent dissociation constant of the complex = 1.4 nM). This work, carried out with embryonic cardiac cells, presents data useful to a better understanding of the molecular aspects of the maturation of Na^{+} and Ca^{2+} entry systems in the developing cardiac cell membrane.

Cultured embryonic ventricular heart cells provide a useful experimental system to study the properties of permeability systems for Na^{+}, Ca^{2+}, and K^{+} in the cardiac membrane (Fayet et al., 1974; Sperelakis et al., 1975; Couraud et al., 1976; Fosset et al., 1977).

A series of protein neurotoxins has recently been isolated from the sea anemone *Anemonia sulcata*. One of them, ATX_{II},[1] has a molecular weight of 4948 corresponding to a polypeptide chain reticulated by three disulfide bridges (Wunderer et al., 1976). This neurotoxin has been described as a useful tool for the analysis of the molecular aspects of nerve conduction (Romey and Lazdunski, 1975; Romey et al., 1976; Bergman et al., 1976) and of the coupling between excitation and secretion in synapses (Abita et al., 1977). Extracts of the sea anemone *Calliactis polypus* and *Anemonia sulcata* have been described to have cardiotoxic effects (Huang and Mir, 1972; Alsen, 1975; Alsen et al., 1976). Moreover, electrophysiological work carried out in this laboratory has shown that ATX_{II} at very low concentration considerably prolongs the action potential of chick embryo cardiac cells in culture (G. Romey, to be published). In the present work we have investigated the effects of the protein neurotoxin ATX_{II} on the properties of Na^{+}, Ca^{2+}, and K^{+} permeabilities of the cardiac cell membrane. Results have shown that the neurotoxin selectively stimulates the $^{45}Ca^{2+}$ entry in embryonic cardiac cells and is thus a very useful agent for the analysis of the properties of the $^{45}Ca^{2+}$ entry system.

Materials and Methods

Cell Cultures. Hearts were obtained under sterile conditions from 10-day-old chick embryos. Heart cells were then isolated by trypsinization according to Fayet et al. (1974). Cells were seeded in 35-mm diameter petri dishes (Nunclon Plastics Denmark) at a concentration of $1.5–2 \times 10^{6}$ cells/mL. Cells were grown in a mixture of Eagle's MEM and NCTC medium (75:25) supplemented with 5% fetal calf serum containing 200 units/mL of penicillin and 50 μg/mL of streptomycin, according to Samuel et al. (1976). Cultures were maintained at 37 °C in a water-saturated atmosphere of air/CO_2 (95:5). The growth medium was replaced after 2 days by a fresh medium. This last medium was supplemented with 0.2 μCi/mL of [^{3}H]leucine 24 h prior to use in transport experiments to allow use of ^{3}H counts per minute as a measure of protein in the experiments.

Myoblast cells from 11-day-old chick embryo thighs were grown according to Fiszman and Fuchs (1975) and used for transport experiments after differentiation to myotubes on day 9 of culture.

Measurements of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ Uptakes. Standard measurements of rates of uptake of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ into cells in monolayer culture were carried out at 37.0 ± 0.2 °C. Before each assay, the culture medium was removed by suction and immediately replaced by a medium for standard influx kinetic measurements (1.5 mL per dish). This standard medium

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[1] Abbreviations used are: TTX, tetrodotoxine; ATX_{II}, sea anemone toxin fraction II; verapamil, α-isopropyl α-(*N*-methyl-*N*-homoveratryl)-γ-aminopropyl-3,4-dimethoxyphenylacetonitrile hydrochloride; D600, methoxy derivative of verapamil; (Na^{+},K^{+})-ATPase, sodium–potassium activated adenosine triphosphatase; MEM, minimum essential medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; Tris, tris(hydroxymethyl)aminomethane.

consists of a 25 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonate] buffer adjusted to pH 7.4 with tris(hydroxymethyl)aminomethane (Tris) and containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 0.1 mg/mL bovine serum albumin. Cells were then equilibrated for 5 min with the assay medium. Effectors of $^{22}Na^+$ and Ca^{2+} transports were usually added 1 min before starting the cation uptake experiment. Under certain circumstances the time of incubation was longer; it is then indicated in the figure legends. The uptake experiment was started by addition of assay medium containing 2 μCi/mL of $^{22}Na^+$ or 0.5 μCi/mL of $^{45}Ca^{2+}$. Uptake was determined by removing the radioactive assay medium and washing three times at 20 °C with 1.5 mL of a wash medium consisting of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$ in a 25 mM Tris buffer at pH 7.4. The washing procedure lasted less than 20 s, a time sufficient to remove extracellular radioactive ion without loss of intracellular radioactive ion. Washed cells were then suspended in 0.1 N NaOH and radioactivity was measured in a scintillation counter using Insta-gel and a Packard B 2450 scintillation spectrometer. In each series of experiments, a "zero time" assay was carried out by adding radioactive assay medium to the cultures and immediately (in less than 10 s) washing the cells by the standard procedure. The zero time value was subtracted from every uptake measurement. Uptake rates in nmol min^{-1} mg^{-1} of cell protein were calculated from the measurements of $^{22}Na^+$ and $^{45}Ca^{2+}$ taken up and from determinations of cell protein by the method of Hartree (1972). Values were corrected for slightly variable protein recovery during the assay and washing procedures on the basis of [^{3}H]leucine radioactivity recovered in each sample.

Each experimental point in a series of experiments to obtain a given curve in this paper has been done in duplicate. Moreover, it has been checked that all curves presented in the paper were fully reproducible using different proparations of cultured cardiac cells at an interval of several months.

Purification of ATX_{II}. The purification of toxins from the sea anemone *Anemonia sulcata* was carried out according to Béress et al. (1975) with minor variations, and utilizing 15 kg of anemone as starting material. Four neurotoxic polypeptides were obtained by this method instead of three described by Béress et al. (1975). Fresh stock solutions of ATX_{II} were prepared every day at a concentration of 1 mM toxin in 1% serum albumin.

Chemicals. Chemicals were obtained from the following sources: bovine serum albumin fraction V, ouabain, lidocaine, and tetracaine from Sigma; D600 and verapamil from Knoll Pharmaceuticals; tetrodotoxin (free of citrate) from Sankyo; $^{45}CaCl_2$, $^{22}NaCl$, $^{85}SrCl_2$, and [^{3}H]leucine from the Centre de l'Energie Atomique (Saclay); $^{86}RbCl$ from Amersham; lyophilized proteolytic enzymes from the Pasteur Institute; Eagle's minimal essential medium (MEM) from Eurobio; and the NCTC medium and fetal calf serum from Gibco.

Computing Procedure. In each figure of this paper describing concentration dependence of kinetic parameters, the curve presented is the theoretical curve giving the best fit with the experimental data. The search for the best fit was carried out according to Atkins (1973) using a Wang 2200 calculator.

Results

ATX_{II} Stimulation of $^{45}Ca^{2+}$ and $^{22}Na^+$ Uptake by Embryonic Cardiac Cells in Culture. Figure 1, inset a and main figure, shows that ATX_{II} greatly stimulates $^{45}Ca^{2+}$ uptake by cardiac cells in culture. The dose-response curve of this ATX_{II} effect shows that the stimulation occurs between 0.1 and approximately 3 μM ATX_{II}, the maximal stimulation being about 12-fold. The half-maximum effect ($K_{0.5}(ATX_{II})$) is observed at 0.5 μM (Figure 1). Inset b shows that ATX_{II} stimulation of $^{45}Ca^{2+}$ uptake in the presence of K^+ concentration of 5.4 mM is observed only in the presence of ouabain (which by itself does not stimulate uptake (Fosset et al., 1977)). The dose-response curve for the ouabain dependence of the ATX_{II} effect indicates a $K_{0.5}$(ouabain) of 44 μM in good agreement with determinations of other workers for the affinity of ouabain for the cardiac cells in culture (Couraud et al., 1976). Ouabain is not necessary at low K^+ concentrations. The K^+ concentration dependence of ATX_{II} stimulation in the absence of ouabain is presented in Figure 1, inset c. $K_{0.5}(K^+)$ was found to have a value of 0.4 mM, which is in the range of values reported for the K^+ site of (Na^+, K^+)-ATPases in other systems (Gache et al., 1976). ATX_{II} associates very rapidly with cardiac cells. We have found the same stimulation factor for $^{45}Ca^{2+}$ influx after preincubations of the cells with the toxin for periods ranging from 30 s to 20 min. Dissociation of the toxic compound from the heart cell membrane was followed by $^{45}Ca^{2+}$ uptake experiments. Cardiac cells were preincubated in a standard medium with 10 μM ATX_{II} and 0.5 mM ouabain. ATX_{II} was then removed by washing with a standard medium containing 0.5 mM ouabain for 0.5, 2, 5, and 10 min. Finally cells were assayed without ATX_{II} for $^{45}Ca^{2+}$ uptake as described in Materials and Methods. Full reversal of the ATX_{II} effect was already completed at the shortest washing

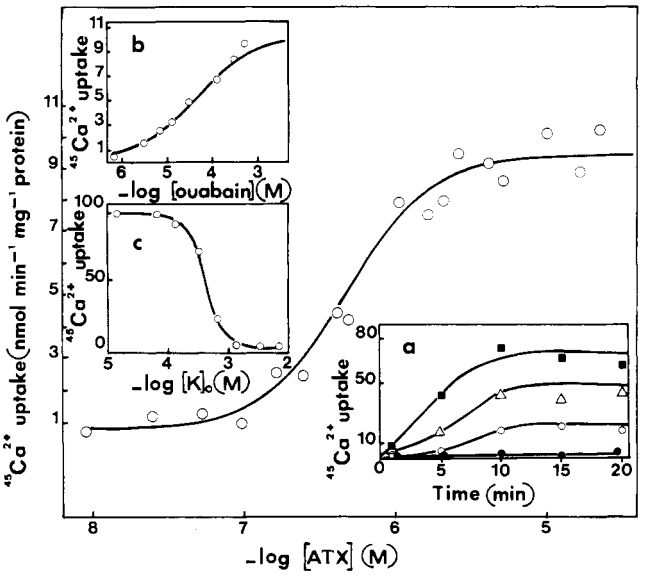

FIGURE 1: Activation of $^{45}Ca^{2+}$ uptake by ATX_{II}. The initial rate of $^{45}Ca^{2+}$ uptake was measured as described under Materials and Methods after a period of uptake of 5 min. Experiments were carried out in the presence of 0.5 mM ouabain. The initial rate of $^{45}Ca^{2+}$ uptake in the absence of ATX_{II} and in the presence of 0.5 mM ouabain is 0.8 nmol min^{-1} (mg of protein)$^{-1}$, i.e. 0.1 pmol cm^{-2} s^{-1}. (Inset a) Kinetics of $^{45}Ca^{2+}$ uptake without ATX_{II} (●) and in the presence of 0.15 μM (○), 0.5 μM (△), and 1.5 μM (■) ATX_{II}. Ordinates are expressed in nmol (mg of protein^{-1}). (Inset b) Influence of increasing ouabain concentrations upon ATX_{II}-stimulated $^{45}Ca^{2+}$ uptake. The ATX_{II} concentration was 10 μM. Ordinates are expressed in nmol (mg of protein)$^{-1}$. (Inset c) Influence of increasing external concentrations of K^+ upon ATX_{II} stimulated $^{45}Ca^{2+}$ uptake. Cells were first washed twice and then incubated 5 min with the standard medium devoid in this case of K^+. After this time of 5 min, adequate K^+ concentrations and 10 μM ATX_{II} were added to the K^+ free medium. After another time lapse of 30 s $^{45}Ca^{2+}$ uptake experiments were started by the addition of $^{45}Ca^{2+}$ to the medium. Ordinates are expressed in percent of the maximal stimulation obtained in the presence of 10 μM ATX_{II}. All experiments were carried out at 37 °C.

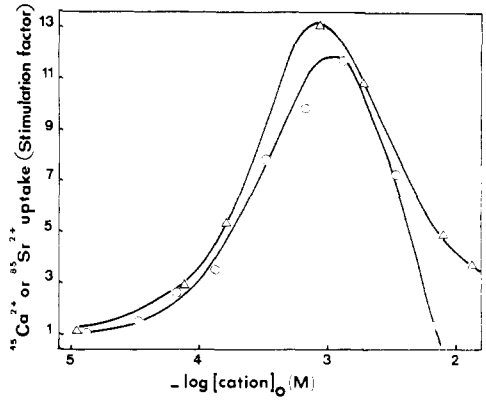

FIGURE 2: The effect of extracellular Ca^{2+} and Sr^{2+} concentrations upon $^{45}Ca^{2+}$ and $^{85}Sr^{2+}$ uptakes stimulated by ATX_{II}. Cells were first washed twice and then preincubated 5 min with the standard medium deprived of Ca^{2+}. After this 5-min incubation, adequate Ca^{2+} or Sr^{2+} concentrations, 10 μM ATX_{II}, and 0.5 mM ouabain were added to the Ca^{2+} free medium. After another time lapse of 30 s Ca^{2+} or Sr^{2+} uptakes were started by addition of $^{45}Ca^{2+}$ or of $^{85}Sr^{2+}$ to the medium: (○) $^{45}Ca^{2+}$ uptake; (△) $^{85}Sr^{2+}$ uptake. All experiments were carried out at 37 °C. The stimulation factor was calculated as the ratio, at each Ca^{2+} (or Sr^{2+}) concentration of uptakes of $^{45}Ca^{2+}$ (or $^{85}Sr^{2+}$) measured in the presence to that recovered in the absence of 10 μM ATX_{II}.

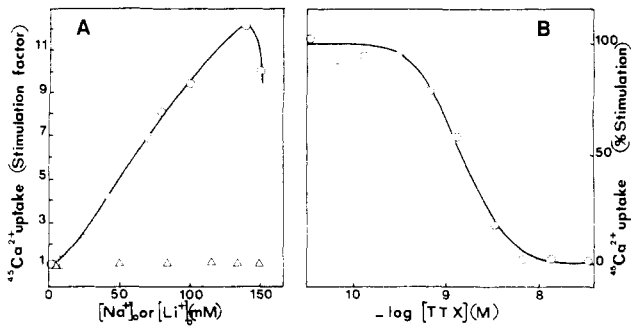

FIGURE 3: (A) The effect of extracellular Na^+ or Li^+ concentrations upon $^{45}Ca^{2+}$ uptake stimulated by ATX_{II}. Cultured cardiac cells were first washed twice with the standard medium in which NaCl (140 mM) was replaced by choline chloride (140 mM). Fluxes of $^{45}Ca^{2+}$ were then measured in the presence of different Na^+ concentrations. Isotonicity was maintained by replacing Na^+ by cholinium (the sum of concentrations of NaCl and choline chloride is maintained at 140 mM). Experiments were carried out in ouabain (0.5 mM). Na^+ replacement by Li^+ fails to stimulate $^{45}Ca^{2+}$ uptake (△). Experiments were carried out at 37 °C and ATX_{II} is 10 μM. (B) Tetrodotoxin inhibition of ATX_{II}-dependent $^{45}Ca^{2+}$ uptake. In these experiments TTX was added simultaneously with ATX_{II} to the cell culture. Fluxes measurements were carried out in the presence of 0.5 mM ouabain and 10 μM ATX_{II} at 37 °C. It has been checked that the order of introduction of ATX_{II} and TTX does not affect the experimental results.

period of 0.8 min indicating a fast rate of dissociation of the toxin from its receptor.

ATX_{II} also stimulates $^{22}Na^+$ uptake. The stimulation occurs in the same range of toxin concentrations as that for $^{45}Ca^{2+}$ uptake (Figure 1). At concentrations below 0.1 μM of ATX_{II} no stimulation was observed. The maximal stimulation is observed at concentrations between 3 and 10 μM of toxin; the maximal stimulation of $^{22}Na^+$ influx is of the order of 1.5. This stimulation factor of 1.5 is much lower than that observed for $^{45}Ca^{2+}$ uptake stimulation (a factor of 12).

ATX_{II} (10 μM) was found to stimulate neither $^{22}Na^+$ nor $^{45}Ca^{2+}$ entry in chick embryo myotubes, in contrast to veratridine which stimulated $^{22}Na^+$ entry by a factor of 3 (Fosset et al., 1977).

Cells with a nonexcitable membrane do not present an ATX_{II} activation of $^{45}Ca^{2+}$ (or $^{22}Na^+$) uptake. It was observed, for example, that $^{45}Ca^{2+}$ (and $^{22}Na^+$) influxes into a culture of primary chick embryo fibroblasts and cell line swiss 3T3 are insensitive to ATX_{II}.

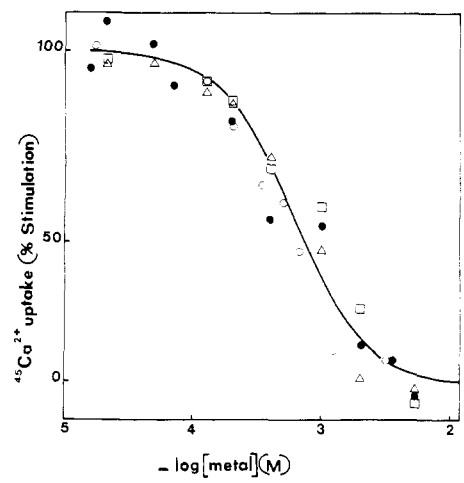

FIGURE 4: *Inhibition* of ATX_{II}-dependent $^{45}Ca^{2+}$ uptake by Ni^{2+}, Co^{2+}, Mn^{2+}, and La^{3+}. Experiments with Ni^{2+} (□), Co^{2+} (●), and Mn^{2+} (△) were carried out in the standard medium at the usual pH of 7.4. The effect of La^{3+} (○) was measured at pH 7.0 for solubility reasons; ouabain, 0.5 mM; ATX_{II}, 10 μM; 37 °C.

Cultured cardiac cells treated with 0.3 mM dinitrophenol to deplete the intracellular store of ATP from 8 mM down to 80 μM after 1-h incubation are still markedly activated (a factor of 6) by ATX_{II} for $^{45}Ca^{2+}$ influx.

Dependence of ATX_{II}-Stimulated $^{45}Ca^{2+}$ and $^{85}Sr^{2+}$ Uptakes upon the Cation Concentration. Figure 2 shows the variation of $^{45}Ca^{2+}$ uptake stimulated by ATX_{II} with increasing concentrations of Ca^{2+}. Sr^{2+} can replace Ca^{2+} as the entering cation and then the $^{85}Sr^{2+}$ uptake stimulated by ATX_{II} is dependent on the Sr^{2+} concentration as also shown in Figure 2. Profiles obtained with $^{45}Ca^{2+}$ and $^{85}Sr^{2+}$ uptakes are very similar. The rate of $^{45}Ca^{2+}$ or $^{85}Sr^{2+}$ influx increases with the divalent cation concentration between 10 μM and approximately 1 mM Ca^{2+} or Sr^{2+}. At higher Ca^{2+} or Sr^{2+} concentration one observes an inhibition by the cations of their own transport through the plasma membrane.

The rising phase of the curves corresponds to the progressive saturation of the divalent cation channel or carrier by Ca^{2+} or Sr^{2+}. The apparent dissociation constants for these interactions computed from this part of the curve according to Atkins (1973) are 0.3 mM for Ca^{2+} and for Sr^{2+}.

Dependence of ATX_{II}-Stimulated $^{45}Ca^{2+}$ Uptake upon External Na^+ Concentration. Figure 3A shows that ATX_{II} stimulation of Ca^{2+} uptake is strongly dependent upon external Na^+ concentration. The half-maximal effect of Na^+ is observed at a concentration of 65 mM. Replacement of Na^+ in the external medium by Li^+ prevents ATX_{II} stimulation of $^{45}Ca^{2+}$ uptake.

Inhibition of the ATX_{II}-Stimulated $^{45}Ca^{2+}$ and $^{22}Na^+$ Uptakes by TTX. Figure 3B shows the inhibition of ATX_{II}-stimulated $^{45}Ca^{2+}$ uptake by TTX. The dose-response curve indicates a half-maximum effect of TTX, $K_{0.5}$(TTX), at 1.4 nM. This value can be taken in a first approximation as the apparent dissociation constant for the TTX–receptor complex. $K_{0.5}$(TTX) is independent of the ATX_{II} concentration used to provoke the opening of the $^{45}Ca^{2+}$ entry system.

Tetrodotoxin at a concentration of 0.1 μM also completely inhibits the ATX_{II}-stimulated Na^+ influx.

Inhibition of ATX_{II}-Stimulated $^{45}Ca^{2+}$ Uptake by Co^{2+}, Mn^{2+}, Ni^{2+}, and La^{3+}. Co^{2+}, Mn^{2+}, Ni^{2+}, and La^{3+} inhibit

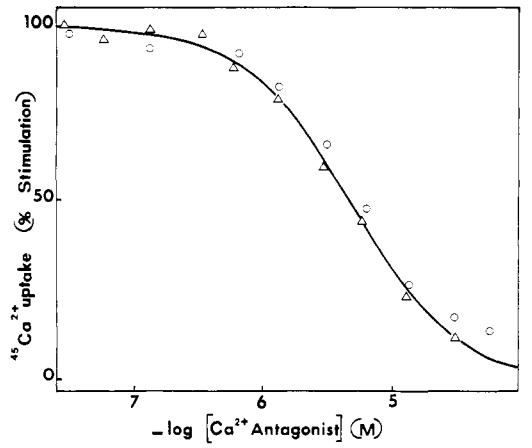

FIGURE 5: Inhibition of ATX_{II}-dependent $^{45}Ca^{2+}$ uptake by verapamil and D600. Verapamil (△) and D600 (○) inhibitions of Ca^{2+} uptake. Experiments were carried out in the presence of 0.5 mM ouabain and 10 μM ATX_{II} at 37 °C.

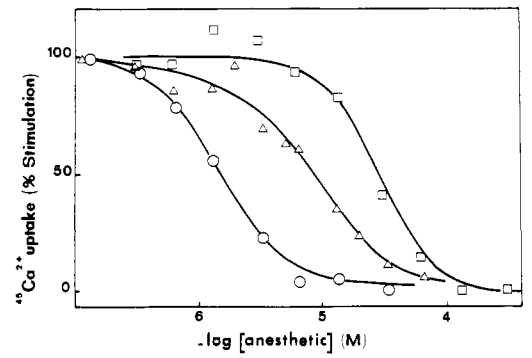

FIGURE 6: Inhibition of ATX_{II}-dependent $^{45}Ca^{2+}$ uptake by local anesthetics. Tetracain (○), etidocain (△), and lidocain (□) inhibition of $^{45}Ca^{2+}$ uptake. Experiments were carried out in the presence of 0.5 mM ouabain and 10 μM ATX_{II} at 37 °C.

the ATX_{II}-stimulated $^{45}Ca^{2+}$ uptake. The dose-response curve for this inhibition effect is presented in Figure 4. The half-maximum inhibition effect is observed at the same concentration for all these cations: $K_{0.5}(Me^{2+}$ or $Me^{3+}) = 0.6$ mM.

Inhibition of ATX_{II}-Stimulated $^{45}Ca^{2+}$ Uptake by D600 and Verapamil. Figure 5 shows the inhibition effect of verapamil and of its methoxy derivative D600 upon ATX_{II}-stimulated $^{45}Ca^{2+}$ uptake. Both compounds completely inhibit $^{45}Ca^{2+}$ uptake. Furthermore, the dose-response curves obtained with these compounds indicate that they are active in exactly the same concentration range. The half-maximum effect ($K_{0.5}$(verapamil or D600)) is observed at 5 μM.

Effect of Anesthetics upon ATX_{II} Stimulation of $^{45}Ca^{2+}$ Uptake. Anesthetics are known to block action potentials in nerve and muscle (Narahashi, 1974; Seeman, 1972). They are known also to block the functioning of the ionic channels in heart muscle (Kohlhardt et al., 1972; Hagiwara and Nakajima, 1966). Figure 6 shows the inhibition of ATX_{II}-stimulated $^{45}Ca^{2+}$ uptake by three different local anesthetics: tetracain, etidocain, and lidocain. The dose-response curves for these inhibition effects give $K_{0.5}$ values (half-maximum effects) of 1.5, 9, and 30 μM for tetracain, etidocain, and lidocain, respectively.

Temperature Dependence of ATX_{II}-Stimulated $^{45}Ca^{2+}$ Uptake. Figure 7 shows the drastic effect of temperature variations in the vicinity of 25 to 45 °C upon ATX_{II}-stimulated $^{45}Ca^{2+}$ uptake. No effect is observed below 25 °C while a

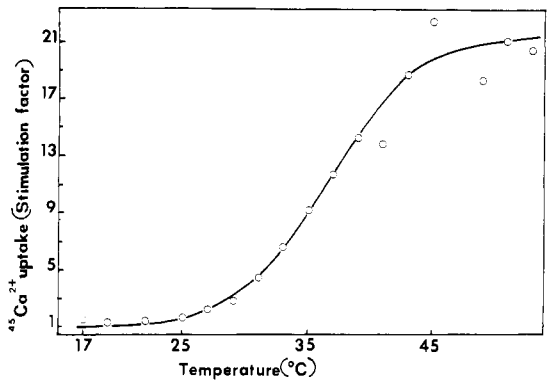

FIGURE 7: Influence of temperature on ATX_{II}-dependent $^{45}Ca^{2+}$ uptake. Experiments were carried out in the presence of 0.5 mM ouabain and 10 μM ATX_{II}.

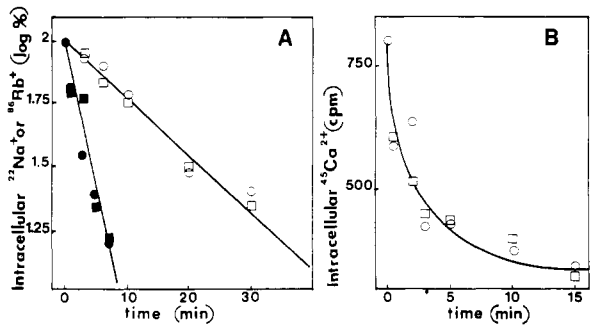

FIGURE 8: Kinetics of $^{22}Na^+$, $^{86}Rb^+$, and $^{45}Ca^{2+}$ efflux from embryonic cardiac cells in culture. Cells were preloaded overnight in the incubator using the complete culture medium containing 3 μCi/mL $^{45}Ca^{2+}$, 10 μCi/mL $^{22}Na^+$, or 0.1 μCi/mL $^{86}Rb^+$. Immediately before starting measurements of efflux kinetics, cardiac cells were washed twice (in less than 10 s) with the standard medium at 37 °C. Efflux kinetics were measured at 37 °C in the same medium containing 0.5 mM ouabain. (A) Semilog plots of: (●) basic $^{22}Na^+$ efflux; (■) $^{22}Na^+$ efflux measured in the presence of 10 μM ATX_{II}; (○) basic ^{86}Rb efflux; (□) ^{86}Rb efflux measured in the presence of 10 μM ATX_{II}. (B) Ca^{2+} efflux measured in the absence (○) and in the presence (□) of 10 μM ATX_{II}.

drastic change in $^{45}Ca^{2+}$ uptake stimulation is observed between 25 and 45 °C. The temperature effect on ATX_{II}-stimulated $^{45}Ca^{2+}$ uptake is mainly due to the fact that the affinity of the toxin for its receptor in the membrane decreases enormously with decreasing temperatures. $K_{0.5}(ATX_{II})$ is 0.5 μM at 37 °C (Figure 1); it is about 50 μM at 27 °C.

Analysis of $^{22}Na^+$, $^{86}Rb^{2+}$, and $^{45}Ca^{2+}$ Effluxes from Cultured Myocytes in the Absence and Presence of ATX_{II}. This series of experiments was undertaken to analyze the effect of the toxin upon the rate of efflux of monovalent and divalent cations in the presence of ouabain. Since Rb^+ is known to be a very effective substituent for K^+ in the K^+ channel (Fosset et al., 1977) and because radioactive Rb^+ is easier to handle than radioactive K^+, experiments were carried out with $^{86}Rb^+$. $^{22}Na^+$ and $^{86}Rb^+$ effluxes follow first-order kinetics corresponding to rate constants of 0.26 min^{-1} ($t_{1/2}$ = 2.7 min) for $^{22}Na^+$ efflux and 0.052 min^{-1} ($t_{1/2}$ = 13 min) for ^{86}Rb efflux (Figure 8A). The kinetics of efflux of $^{22}Na^+$ and $^{86}Rb^+$ are unaffected by the presence of ATX_{II}. The kinetics of $^{45}Ca^{2+}$ efflux are more complex and do not follow simple first-order kinetics (Figure 8B). ATX_{II} does not affect the $^{45}Ca^{2+}$ efflux rate.

Discussion

The rising phase of the action potential of the adult cardiac cell can be resolved into a "fast" and a "slow" component each

of which has been associated with separate inward currents by voltage-clamp analyses. The fast component is due to a current of sodium ions flowing through a selective sodium channel (Rougier et al., 1968) which is blocked by tetrodotoxin, the specific inhibitor of fast Na^+ channels in a variety of excitable cells (Narahashi, 1974). The later slow component, on the other hand, is due to a current of Ca^{2+} and sometimes Na^+ ions (Rougier et al., 1969; Beeler and Reuter, 1970; Vitek and Trautwein, 1971) flowing through the membrane by a different conductance mechanism which is blocked by substances such as Mn^{2+} (Ochi, 1970), La^{3+} (Sanborn and Langer, 1970), and verapamil or D600 (Kohlhardt et al., 1973; McLean et al., 1974). The slow inward current increases or maintains the depolarization and contributes to the characteristic plateau phase in the action potential of the cardiac cell (Rougier et al., 1969). The rapid repolarizing phase of the adult cardiac action potential is due to an outward current of K^+ ions (Trautwein, 1973; Noble, 1975). Since this work deals with embryonic chick heart cells it is worthwhile to briefly summarize the present knowledge concerning the electrophysiological properties of embryonic chick hearts (Sperelakis et al., 1975). Cells in young hearts (2–4 days in ovo) lack fast Na^+ channels. Their action potential is generated by kinetically slow Na^+ channels which are insensitive to TTX. TTX sensitivity appears on about day 5 marking the initial development of fast Na^+ channels. After day 8, TTX completely abolishes cell excitability; slow Na^+ channels have given way to fast channels. When cells are dissociated from ventricles of old (11 to 16 day) embryonic hearts and placed in monolayer culture, they tend to assemble in contacts and to contract synchronously as a whole. However, the electrical activity of these cells resembles that of cells in young embryonic heart (2 days) rather than that of the mature ventricle from which they were initially obtained. The cells display slow Na^+ channels and have lost their TTX sensitivity.

ATX_{II} has no effect on $^{86}Rb^+$ efflux and since Rb^+ is well known to flow easily through the K^+ channel (Pappano and Sperelakis, 1969) it can be concluded that ATX_{II} does not interact with the opening system of this channel. Moreover, ATX_{II} does not alter the rates of $^{22}Na^+$ or $^{45}Ca^{2+}$ efflux. The sea anemone toxin used in this work stimulates, although very moderately (a factor of 1.5), $^{22}Na^+$ influx into cultured cardiac cells. This stimulation is prevented by TTX. These properties are similar to those found for veratridine (Fosset et al., 1977), a well-known modifier of Na^+ permeability in excitable cells (Narahashi, 1974). On the other hand, ATX_{II} opens, or maintains in an open state, a $^{45}Ca^{2+}$ entry system exhibiting the following properties: (i) verapamil and D600, two compounds well known for their antiarrythmic actions, inhibit the $^{45}Ca^{2+}$ entry stimulated by ATX_{II}; (ii) cations such as Mn^{2+}, Co^{2+}, Ni^{2+}, or La^{3+} also block the $^{45}Ca^{2+}$ influx stimulated by ATX_{II}.

The detailed physicochemical properties of this $^{45}Ca^{2+}$ entry system, as defined in this work, are the following.

(i) The apparent dissociation constant of the Ca^{2+}–$^{45}Ca^{2+}$ entry system interaction is $K_{Ca^{2+}} = 0.3$ mM. High concentrations of Ca^{2+} (>1.8 mM) inhibit Ca^{2+} entry through the system opened by ATX_{II}. This effect is either due to inhibition of the Ca^{2+} entry system by excess Ca^{2+} (a phenomenon similar to enzyme inhibition by excess substrate) or to a prevention of ATX_{II} binding to the cardiac cell membrane by high Ca^{2+} concentrations. It is not presently possible to choose between these two hypotheses. Sr^{2+} can flow through the $^{45}Ca^{2+}$ entry system opened by ATX_{II}. The affinity of the $^{45}Ca^{2+}$ entry system for Sr^{2+} is similar to that found for Ca^{2+} ($K_{Sr^{2+}} = 0.3$ mM). High Sr^{2+} concentrations inhibit $^{85}Sr^{2+}$ entry just as high Ca^{2+} concentrations inhibited $^{45}Ca^{2+}$ entry.

(ii) The apparent affinities of cationic blockers of the Ca^{2+} channel, Co^{2+}, Mn^{2+}, Ni^{2+}, and La^{3+}, for the Ca^{2+} entry system can be easily measured with this system of cell in culture. Co^{2+}, Mn^{2+}, Ni^{2+}, and La^{3+} bind to the open form of the calcium entry system stabilized by ATX_{II} with very similar affinities. The apparent dissociation constant of the complex formed between these cations and the $^{45}Ca^{2+}$ entry system is 0.6 mM under our experimental conditions. Concentrations of Mn^{2+} of the order of 2 mM are used by electrophysiologists to block slow Ca^{2+} channels. The observation that different types of cations bind with very similar affinities with the same receptor site is not very astonishing for enzymologists. For example, Mg^{2+} and Co^{2+} bind with the same affinity to the Mg^{2+} site of the kidney alkaline phosphatase (Cathala et al., 1975) and Co^{2+} and Ni^{2+} bind with the same affinity to the Mg^{2+} site of the axonal (Na^+,K^+)-ATPase (Gache et al., 1976).

(iii) Antiarrythmic drugs, D600 and verapamil, have the same apparent affinity for the Ca^{2+} entry system machinery: $K_{D600} = K_{verapamil} = 5\ \mu M$ under our experimental conditions. Concentrations of the order of 1–100 μM routinely used by electrophysiologists are large enough to completely saturate the drug binding site.

(iv) The $^{45}Ca^{2+}$ entry system opened by ATX_{II} can be blocked by local anesthetics. The order of efficiency of the anesthetics used in this work is tetracain > etidocain > lidocain. Tetracain binds tightly to its receptor structure: $K_{tetracain} = 1.5\ \mu M$.

The pharmacological interest of ATX_{II} having been underlined, it is worthwhile to discuss some of the peculiarities of its mode of action. ATX_{II} action upon the $^{45}Ca^{2+}$ entry system has been observed in this work under conditions in which (i) the (Na^+,K^+)-ATPase is blocked either by ouabain or by low external K^+ concentrations, and in which (ii) the external Na^+ concentration is high enough, Li^+ being unable to replace Na^+. These observations taken together with the fact that ATX_{II} stimulates both $^{22}Na^+$ and $^{45}Ca^{2+}$ uptakes suggest that ATX_{II} action on Ca^{2+} entry necessitates the intracellular accumulation of Na^+. The most surprising characteristic of ATX_{II} action is that it opens a $^{45}Ca^{2+}$ entry system which is blocked by TTX as well as by D600, verapamil, Mn^{2+}, and other di- or trivalent cations. This is at first sight a very paradoxical pharmacological observation. TTX is well known to be a very selective blocker of fast Na^+ channels (Narahashi, 1974) and not of Ca^{2+} entry in excitable cells.

However, if ATX_{II} action on Ca^{2+} entry does indeed necessitate the intracellular accumulation of Na^+, the inhibition of $^{45}Ca^{2+}$ uptake by TTX becomes easier to understand. TTX will block $^{22}Na^+$ entry stimulated by ATX_{II} and will in consequence also considerably decrease $^{45}Ca^{2+}$ uptake stimulated by ATX_{II}.

Cardiac cells in culture provide a convenient system to study the differentiation of cationic channels. As previously mentioned, the electrophysiological approach indicates that monolayer cultures of embryonic cardiac cells have a slow Na^+ channel which is insensitive to TTX or verapamil (Sperelakis et al., 1975), two properties shown by adult cardiac cells.

In spite of the fact that embryonic cardiac cells in culture lack a fast Na^+ channel as well as TTX sensitivity, the work presented here shows that the receptor of TTX, which constitutes an important component of the fast Na^+ channel machinery, is present in the cell membrane. The apparent dissociation constant for the TTX–cardiac membrane receptor complex found in this work is 1.4 nM. It is very similar to dis-

sociation constants found with nerves (Narahashi, 1974), axonal membranes (Balerna et al., 1975), and neuroblastoma cells (Catterall and Nirenberg, 1973; Catterall, 1975). Components of fast Na^+ channels, although not yet functional, have been shown to be present in the membrane of embryonic cardiac cells in culture by experiments with the alkaloid veratridine (Sperelakis and Pappano, 1969; Fosset et al., 1977) with the polypeptide neurotoxin extracted from scorpion venom (Couraud et al., 1976) and also in this work with the sea anemone toxin. These three toxic compounds open or maintain in an open state a Na^+ entry system in cultured embryonic cardiac cells just as they maintain open the fast Na^+ channels of nerves (Ulbricht, 1969; Romey et al., 1975) and neuroblastoma cells (Catterall and Nirenberg, 1973; Catterall, 1976; Y. Jacques, M. Fosset, and M. Lazdunski, manuscript in preparation). Once opened by veratridine (Sperelakis and Pappano, 1969; Fosset et al., 1977) by the scorpion neurotoxin (Couraud et al., 1976) or by the sea anemone toxin, the Na^+ entry system can be blocked by tetrodotoxin as expected for a fast Na^+ channel.

In conclusion it seems that the sea anemone toxin ATX_{II} might be an extremely useful pharmacological agent to analyze the molecular mechanism of $^{45}Ca^{2+}$ entry in cardiac cells and to follow the differentiation of the Ca^{2+} entry system during development.

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