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STIMULATION OF SODIUM AND CALCIUM UPTAKE BY SCORPION TOXIN IN CHICK EMBRYO HEART CELLS

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SUMMARY

Scorpion toxins, the basic miniproteins of scorpion venom, stimulated the passive uptake of Na^+ and Ca^{2+} in chick embryo heart cells. Half-maximum stimulation was obtained for 20–30 nM Na^+ and 40–50 nM Ca^{2+}. Scorpion toxin-activated Na^+ and Ca^{2+} uptakes were fully inhibited by tetrodotoxin, a specific inhibitor of the action potential Na^+ ionophore in excitable membranes. Half-maximum inhibition was obtained with the same concentration of tetrodotoxin (10 nM) for both Na^+ and Ca^{2+}. Scorpion toxin-stimulated Ca^{2+} uptake was dependent on extracellular Na^+ concentration and was not inhibited by Ca^{2+} channel blocking drugs which are inactive on heart cell action potential. Thus, in heart cells scorpion toxin affects the passive Ca^{2+} transport, which is coupled to passive Na^+ ionphore. Other results suggest that (1) tetrodotoxin and scorpion toxin bind to different sites of the sarcolemma and (2) binding of scorpion toxin to its specific sites may unmask latent tetrodotoxin – sensitive fast channels.

INTRODUCTION

Embryonic heart cells in culture provide the possibility of studying cardiac muscle in a nerve-free environment. They maintain autorhythmic activity and many of their electrophysiological characteristics are similar to those of cells in intact embryonic or adult hearts [1–3]. Electro-optical systems [4–5] can be used to monitor rate, amplitude and rhythm of heart cell contraction and to study the effect of physiological stimuli and drugs on single cells.

The neurotoxic miniproteins contained in scorpion venoms [6–7] provoke an increase in cell beat frequency and a decrease in the amplitude of contraction at low concentrations (1–10 nM), and fibrillation and contracture at higher concentration (1 μM) [4–5]. Electrophysiological investigations have shown that total scorpion venom [8–9] produces a persistent depolarization of nerve axons. In the cray-

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

fish and lobster axonal membrane, purified scorpion toxins selectively affect the closing of the Na^+ channel and the opening of the K^+ channel [10]. Up to now their effect on embryonic heart cell action potential has not been determined and related to their effect on contraction. However, evidence was recently given [11] that veratridine-dependent Na^+ uptake provided a means of assaying action potential Na^+ ionophore activity in neuroblastoma and muscle cell lines. In this communication, we report on the effect of scorpion neurotoxin on embryonic heart cell $^{22}Na^+$ and $^{45}Ca^{2+}$ permeabilities.

MATERIALS AND METHODS

Chemicals

Ouabain, protoveratrine B and D,L-propranolol · HCl were obtained from Sigma (St. Louis, Mo., USA), 5-methoxy-(α-isopropyl-α(*N*-methyl-*N*-homoveratryl)-γ-aminopropyl)-3,4-dimethoxyphenylacetonitrile hydrochloride (D 600) from Knoll (West Germany), crude trypsin from Institut Pasteur (Paris, France), fetal calf serum from Sorga (Paris, France) and Eagle's minimal essential medium and NCTC 109 media from Eurobio (Paris, France). Tetrodotoxin was the generous gift of Professor Cheymol and toxin II of *Androctonus australis* Hector (mol. wt. 7249) was purified according to Miranda et al. [7]. $^{22}NaCl$ (0.09 Ci/mol) and [^{14}C]sucrose (10 Ci/mol) were purchased from the Radiochemical Centre (Amersham, England) and $^{45}CaCl_2$ (40 mCi/mg) from the Commissariat à l'Energie Atomique (Saclay, France).

Trypsinization procedure and cell incubation

Cells were isolated from the hearts of chick embryo aged 11 days, according to the method previously described [4, 5]. Cells ($1 \cdot 10^6$–$1.5 \cdot 10^6$ per ml) were suspended in NCTC 109 medium [12] containing 20 % fetal calf serum, penicillin G (200 units/ml) and streptomycin sulfate (50 μg/ml), and 2 ml of cell suspension were seeded in plastic Petri dishes (diameter 35 mm). Incubation was at 35 °C in 95 % air/5 % CO_2.

Measurement of $^{22}Na^+$ and $^{45}Ca^{2+}$ uptake

Uptake of labeled ions was measured after three days of culture without medium change. The culture medium was removed and replaced by assay medium (90 % Eagle's minimal essential medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2, and 10 % fetal calf serum) 15 min before addition of $^{22}Na^+$ or $^{45}Ca^{2+}$. Assay medium contained 145 mM Na^+ and 1.8 mM Ca^{2+}. At time 0, the medium was aspirated and replaced by fresh assay medium (1.5 ml per dish) containing 1–2 μCi/ml of $^{22}NaCl$ or 0.5 μCi/ml of $^{45}CaCl_2$ and appropriate drugs as described in the text and legends of figures. Incubations were carried out in Petri dishes partially immersed in a water bath maintained at 35 °C and using media pre-equilibrated at this temperature. Uptake measurements were terminated at timed intervals by aspirating off the radioactive assay medium and washing the cell layer three times as rapidly as possible with wash medium (Earle salts pH 7.2 containing 20 mM HEPES) at room temperature. The total washing time was about 45 s. The washed cells were removed by incubation at room temperature with 0.1 M

NaOH (1 ml/dish) and a further wash with 0.5 ml 0.1 M NaOH. Radioactivity of $^{22}Na^+$ was determined in a gamma spectrometer and that of $^{45}Ca^{2+}$ in a liquid scintillation spectrometer after addition of 12 ml Instagel Packard to the samples.

Protein was determined by the method of Lowry et al. [13] and the results expressed as nmol of $^{22}Na^+$ or $^{45}Ca^{2+}$ per mg protein. All experimental points are the means of closely agreeing duplicates. Measurement of the intracellular volume was based on the estimation of the total volume (difference between wet and dry weights) and the extracellular volume using [^{14}C]sucrose as the label. The difference between total and extracellular volumes gave the intracellular volume.

RESULTS

$^{22}Na^+$ uptake

In the absence of ouabain, $^{22}Na^+$ uptake by chick embryo heart cells was low, consisting of an exchange of external $^{22}Na^+$ with internal Na^+ (Fig. 1). Equilibrium was obtained in 40–60 min. In the presence of ouabain (0.5 mM) which inhibits the ($Na^+ + K^+$)-activated ATPase, uptake of $^{22}Na^+$ increased by a factor of 9–10 (Fig. 1). The time course of $^{22}Na^+$ uptake was approximated by an exponential curve with a half-life of 60 min. Estimation of intracellular volume suggested that the $^{22}Na^+$ equilibrated completely with intracellular volume. The measured intracellular volume was 4.0±0.5 μl per mg of cell protein. With an intracellular $^{22}Na^+$ concentration of 600 nmol/mg (Fig. 1) we obtained a final $^{22}Na^+$ concentration of 150±17 mM, which can be compared to the extracellular concentration of 145 mM.

The dose-response curve of the effect of ouabain on $^{22}Na^+$ uptake at 60 min is shown in Fig. 2. Stimulation of uptake was half-maximal at a concentration of 4 μM and complete at 0.2 mM. These results are in full agreement with the inhibition of chick embryo ($Na^+ + K^+$)-dependent ATPase by ouabain [14], which showed half-maximum activity inhibition at a concentration of 3 μM ouabain and complete inhibition at 0.1 mM. In subsequent experiments an ouabain concentration of 0.5 mM was used.

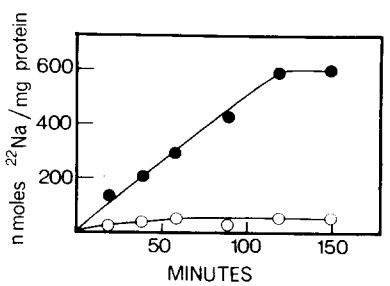


Fig. 1. Time-course of the effect of ouabain on $^{22}Na^+$ uptake by chick embryo heart cells in culture (see Methods for experimental conditions). ○, control without ouabain; ●, 0.5 mM ouabain.

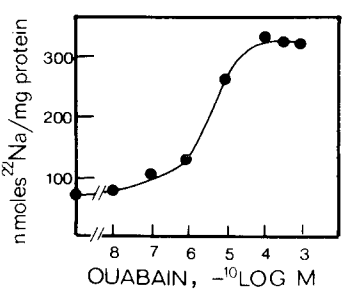


Fig. 2. Effect of ouabain concentration on $^{22}Na^+$ uptake by chick embryo heart cells. Uptake of $^{22}Na^+$ was measured for 60 min at 35 °C in the presence of increasing concentrations of ouabain.

Effect of scorpion toxin

When toxin II of *Androctonus australis* Hector (7 μg/ml) was added at zero time in the presence of 0.5 mM ouabain the rate of $^{22}Na^+$ uptake was markedly increased, while maximum incorporation was not modified (Fig. 3). Assuming that the decay of the $^{22}Na^+$ concentration gradient across the cell membrane is described by a single exponential, the following equation relating the intracellular $^{22}Na^+$ concentration to time has been derived [11]:

$$\ln \frac{[^{22}Na^+]^{\infty}_{in}}{[^{22}Na^+]^{\infty}_{in} - [^{22}Na^+]_{in}} = kt = \frac{\ln 2}{t_{\frac{1}{2}}}(t) \qquad (1)$$

where $[^{22}Na^+]^{\infty}_{in}$ is the intracellular $^{22}Na^+$ concentration at equilibrium and $t_{\frac{1}{2}}$ the half time for equilibration, in min.

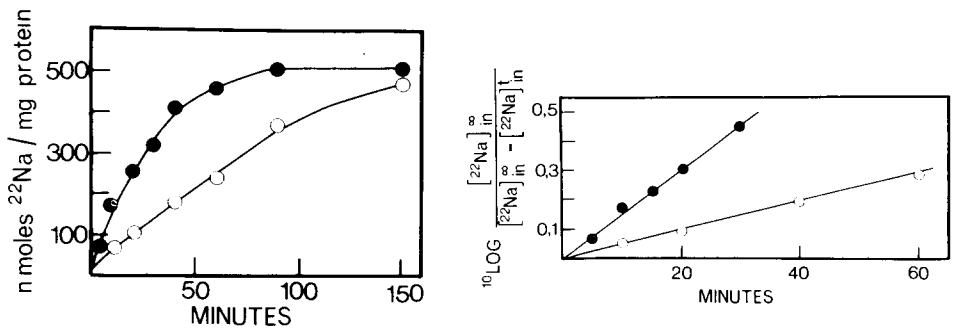


Fig. 3. Stimulation of $^{22}Na^+$ uptake by toxin II of *Androctonus australis*. Uptake of $^{22}Na^+$ was estimated for the indicated times at 35 °C in the presence of 0.5 mM ouabain (○) or 0.5 mM ouabain and 1 μM scorpion toxin (●).

Fig. 4. Time-course of stimulation of $^{22}Na^+$ uptake by scorpion toxin. The data of Fig. 3 are plotted according to equation 1 (see text) using $^{22}Na^+{}^{\infty}_{in}$ = 500 nmol/mg cell protein.

As shown in Fig. 4, the kinetics of $^{22}Na^+$ incorporation in the presence or in the absence of scorpion toxin is first order. Stimulation of the rate of $^{22}Na^+$ uptake by the toxin calculated from half-time for equilibration was 1.5–3 according to cell

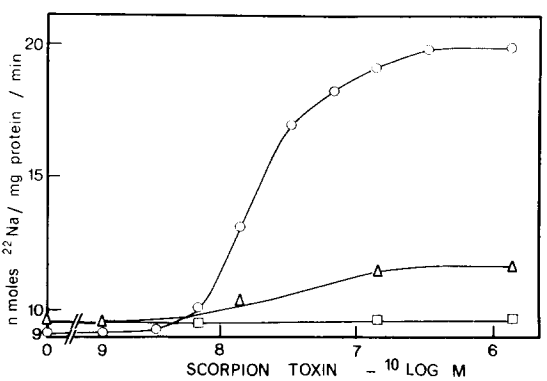


Fig. 5. Dose-response curve for the stimulating effect of scorpion toxin on $^{22}Na^+$ uptake and its inhibition by tetrodotoxin. Uptake was measured at 35 °C for 15 min in the presence of 0.5 mM ouabain (○) and 0.5 mM ouabain plus 10 nM (△) or 1 μM (□) tetrodotoxin.

preparations. The same effect was obtained with the alkaloid protoveratrine B and was maximum at a concentration of 0.1 mM (not illustrated). The increase of Na^+ uptake by the cells at 15 min of incubation depends on the dose of toxin, half-maximum effect being obtained for a concentration of 20–30 nM (Fig. 5).

Effect of tetrodotoxin and other compounds

Tetrodotoxin, which specifically blocks fast Na^+ channels of excitable membranes [15–16], completely inhibited the increased rate of $^{22}Na^+$ uptake provoked by scorpion toxin (Fig. 6). Half-maximum inhibition was obtained at a concentration of 10 nM and complete inhibition at 100 nM (Fig. 7). Tetrodotoxin effect was the same, whether added 15 min before or after scorpion toxin addition.

In the presence of 10 nM tetrodotoxin, the dose-response curve of scorpion toxin on $^{22}Na^+$ uptake was not displaced, but the maximum effect was markedly decreased (Fig. 5), thus favoring an inhibiting effect of the non-competitive type.

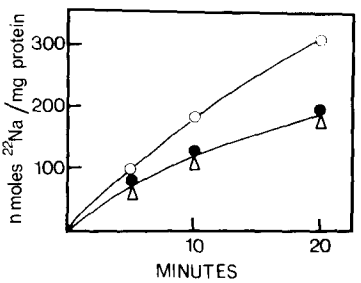


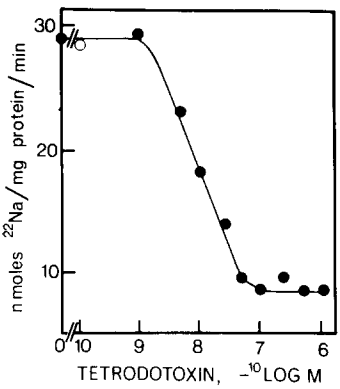
Fig. 6. Time-course of inhibition by tetrodotoxin of scorpion toxin-stimulated $^{22}Na^+$ uptake. Uptake was measured at 35 °C in the presence of 0.5 mM ouabain (●), 0.5 mM ouabain and 1 μM scorpion toxin (○) and 0.5 mM ouabain, 1 μM scorpion toxin and 1 μM tetrodotoxin (△).

TABLE I

EFFECT OF SEVERAL DRUGS ON $^{22}Na^+$ UPTAKE BY CHICK EMBRYO HEART CELLS

Uptake was measured for 15 min at 35 °C. Results are means ± S.D. of 3 determinations.

Drug	Na^+ uptake (nmol ^{22}Na/mg protein)
None	11.8 ± 1.4
0.5 mM ouabain	159.1 ± 6.0
0.5 mM ouabain + 1 μM scorpion toxin	264.3 ± 0.4
0.5 mM ouabain + 0.1 mM protoveratrine B	314.2 ± 17.1
0.5 mM ouabain + 1 μM scorpion toxin + 1 mM D,L-propranolol	152.9 ± 4.5
0.5 mM ouabain + 1 μM scorpion toxin + 1 μM tetrodotoxin	159.5 ± 14.7
0.5 mM ouabain + 1 μM scorpion toxin + 1 μM D 600	277.8 ± 3.5



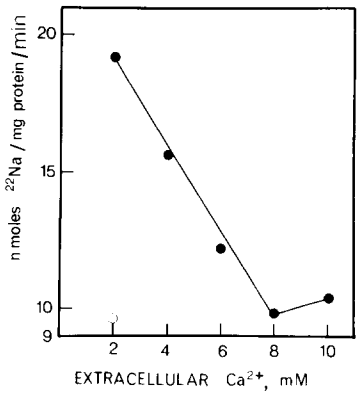


Fig. 7. Dose-response curve of the inhibition by tetrodotoxin of scorpion toxin-stimulated $^{22}Na^+$ uptake. Uptake was measured for 15 min at 35 °C in the presence of 0.5 mM ouabain, 1 μM scorpion toxin and increasing concentrations of tetrodotoxin.

Fig. 8. Effect of extracellular Ca^{2+} concentration on scorpion toxin-dependent $^{22}Na^+$ uptake. Uptake was estimated for 15 min at 35 °C in the presence of 0.5 mM ouabain (○) and 0.5 mM ouabain plus 1 μM scorpion toxin (●).

At a concentration of 1 μM, the adrenoreceptor agonist isoproterenol and the β-adrenoreceptor blocking agent D,L-propranol had no effect on the increased $^{22}Na^+$ uptake provoked by scorpion toxin. This indicates that the β-adrenoreceptors present in chick embryo heart cells [4] are not involved in the mechanism of action of scorpion toxin. However, 0.1 mM D,L-propranol completely inhibited the effect of scorpion toxin (Table I). This effect is probably related to the well known local anesthetic property of the drug, which occurs at high concentrations. Drugs such as D 600 can inhibit excitation-contraction coupling in a specific way, probably by blocking special Ca^{2+} channels in the cardiac muscle fibre membranes without affecting the action potential [17]. At concentrations up to 1 μM, D 600 was ineffective in inhibiting the effect of scorpion toxin on $^{22}Na^+$ uptake (Table I). Voltage clamp experiments performed on myelinated nerve fibres showed that Ca^{2+} abolished the incomplete Na^+ inactivation which is typical for nodes of Ranvier treated with scorpion toxin [18]. Increasing extracellular Ca^{2+} concentration also inhibited the stimulating effect of scorpion toxin on $^{22}Na^+$ uptake, full inhibition being obtained at a concentration of about 8 mM (Fig. 8).

$^{45}Ca^{2+}$ uptake

In the absence of ouabain, uptake of $^{45}Ca^{2+}$ was low (Fig. 9), reaching an intracellular concentration of about 1 mM after 2 h incubation. In the presence of scorpion toxin, $^{45}Ca^{2+}$ uptake increased by a factor of 2. As shown in Fig. 10 ouabain greatly stimulated $^{45}Ca^{2+}$ uptake. Intracellular Ca^{2+} concentration reached a maximum (22–25 mM) at 60 min and then decreased up to 2 h. Dose-response curves for ouabain showed that the stimulation was half-maximal at a concentration of 60 μM and complete at 0.25 mM (Fig. 11).

In the presence of 0.5 mM ouabain, scorpion toxin markedly enhanced the rate of $^{45}Ca^{2+}$ uptake (Fig. 9). Maximum uptake occurred between 10 and 20 min and then slowly decreased up to 2 h. The increase of $^{45}Ca^{2+}$ uptake by heart cells

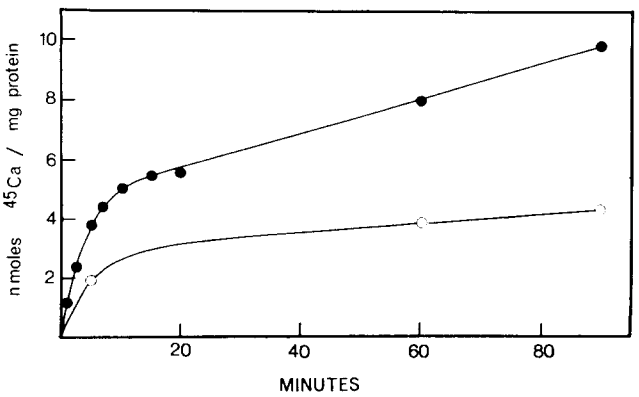


Fig. 9. Time-course of effect of scorpion toxin on $^{45}Ca^{2+}$ uptake. ○, no toxin; ●, 1 μM scorpion toxin.

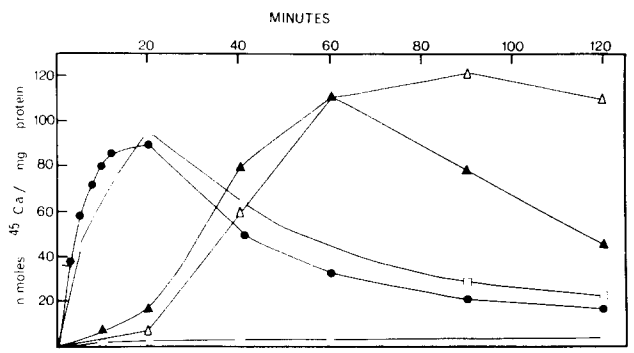
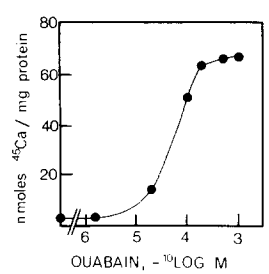


Fig. 10. Time-course of stimulation of $^{45}Ca^{2+}$ uptake by ouabain and scorpion toxin and the effect of tetrodotoxin and D 600. $^{45}Ca^{2+}$ uptake was estimated at timed intervals in the presence of: no additions (○), 0.5 mM ouabain (▲), 0.5 mM ouabain and 1 μM scorpion toxin (●), 0.5 mM ouabain, 1 μM scorpion toxin and 1 μM tetrodotoxin (△), 0.5 mM ouabain, 1 μM scorpion toxin and 1 μM D 600 (□).



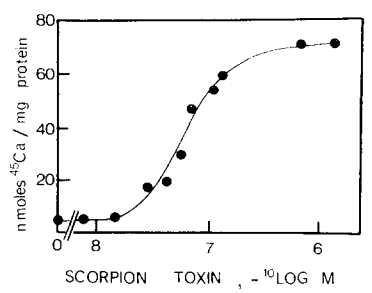
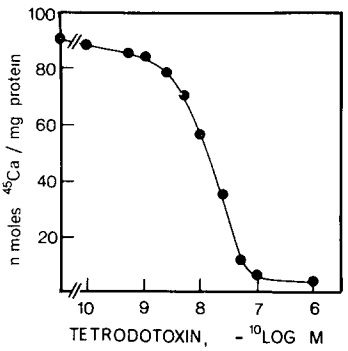


Fig. 11. Effect of ouabain concentration on $^{45}Ca^{2+}$ uptake. Uptake was measured at 35 °C for 60 min.

Fig. 12. Dose-response curve of the effect of scorpion toxin on $^{45}Ca^{2+}$ uptake. Uptake measured for 10 min at 35 °C in the presence of 0.5 mM ouabain.



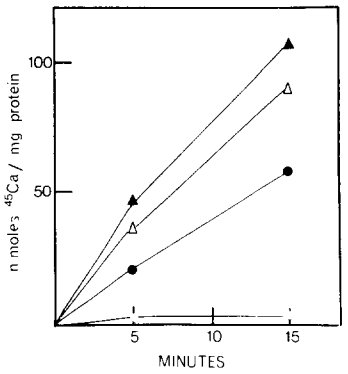


Fig. 13. Dose-response curve of the inhibition by tetrodotoxin of scorpion toxin-stimulated $^{45}Ca^{2+}$ uptake. Uptake measured for 10 min at 35 °C in the presence of 0.5 mM ouabain and 1 μM scorpion toxin.

Fig. 14. Effect of extracellular Na^+ concentration on scorpion toxin-stimulated $^{45}Ca^{2+}$ uptake. Uptake was measured for 5 and 15 min at 35 °C in the presence of 0.5 mM ouabain an 1 μM scorpion toxin, and extracellular Na^+ concentration of zero (○), 50 mM (●), 100 mM (△) and 140 mM (▲). In the standard incubation medium NaCl was replaced by choline chloride to attain at a final concentration of choline or choline plus Na^+ of 140 mM.

depended on the dose of toxin, half-maximal effect being obtained at 40–50 nM (Fig. 12) as compared to 20–30 nM for the same effect on $^{22}Na^+$ uptake (Fig. 5). Interestingly, the dose-response curve of inhibition by tetrodotoxin of the increased $^{45}Ca^{2+}$ uptake due to scorpion toxin (Fig. 13) is almost superimposable on the curve obtained in the same conditions for $^{22}Na^+$ uptake, half-maximum inhibition being obtained at a concentration of 10 nM. This suggests that the Ca^{2+} channel sensitive to scorpion toxin is closely coupled to the tetrodotoxin-inhibited Na^+ channel.

Indeed, when Na^+ is replaced by choline in the assay medium, the increased $^{45}Ca^{2+}$ uptake provoked by scorpion toxin declined as extracellular Na^+ decreased and was abolished at zero Na^+ concentration (Fig. 14).

DISCUSSION

In heart muscle the action potential consists of (1) a rapid phase of depolarization associated with a large rapid inward Na^+ current, (2) a relatively slow inward Ca^{2+} current which contributes to the slow repolarization of the membrane during the plateau phase of the action potential and (3) an outward K^+ current associated with the complete repolarization of the membrane. It has been suggested that the rapid inward Na^+ current is probably responsible for depolarizing the membrane to a level at which the inward Ca^{2+} current is readily activated. The embryonic heart cells in monolayer cultures show the electrical and mechanical properties characteristic of the cardiac muscle of embryonic or adult hearts [18]. The most rapid rate of tension development occurs during the early rising phase of the action potential. The main increase in the force of contraction develops in the potential range when the inward Na^+ current is small and the Ca^{2+} inward current is large. We have previously shown [4] that the effect of scorpion toxin on chick embryo heart cells was probably related to its effect on ionic permeabilities of the membrane. Recent

studies demonstrated that $^{22}Na^+$ uptake measurement correlated well with the veratridine-activated action potential of neuroblastoma and muscle cell lines [11]. That increase in Na^+ permeability due to veratridine reflected ion activity of the action potential Na^+ ionophore was derived from inhibition of the veratridine effect by low concentrations of tetrodotoxin and the absence of response to veratridine of clonal cell lines defective in Na^+ ionophore activity.

In our studies, scorpion toxin at low concentration was shown to increase markedly the rate of passive uptake of $^{22}Na^+$ in chick embryo heart cells. Protoveratrine B had the same effect but at much higher concentrations. This effect of the scorpion toxin at the postsynaptic level is similar to its presynaptic effect on nerve. Indeed, on the giant axons of crayfish and lobster nerves [10], pure scorpion toxin selectively affects the time course of Na^+ inactivation and drastically decreases the steady-state K^+ current.

Scorpion toxin activation of Na^+ uptake in heart cells is inhibited by tetrodotoxin at concentrations compatible with the association constant of the latter to other excitable membranes such as axonal membranes [19–22]. The non-competitive character of the inhibition by tetrodotoxin of scorpion toxin effect on Na^+ uptake indicates that the two toxins act on different sites of the plasma membrane.

Three kinds of evidence show that scorpion toxin-dependent increase in $^{45}Ca^{2+}$ uptake is coupled to passive Na^+ permeability. (1) The increase in both $^{22}Na^+$ and $^{45}Ca^{2+}$ uptakes are inhibited by the same concentration of tetrodotoxin, half-maximum inhibition being obtained at 10 nM. (2) The amplitude of scorpion toxin effect on $^{45}Ca^{2+}$ permeability depends on external Na^+ concentration and is completely abolished when Na^+ is absent from the medium. (3) No inhibition was observed with D 600, a drug which blocks Ca^{2+} channels in heart, without affecting the action potential of cardiac muscle [17].

It is therefore clear that chick embryo heart cells contain at least two types of Ca^{2+} channel, (1) one which is coupled to the passive Na^+ action potential ionophore and which is activated by scorpion toxin and (2) another which is inactivated by D 600, inactivation by the latter being reversed by adrenorceptor stimulants (see ref. 17).

From the kinetics of scorpion toxin effect on $^{22}Na^+$ and $^{45}Ca^{2+}$ uptake (Figs. 3 and 10), it appears that maximum increase in Ca^{2+} occurs when Na^+ uptake is still low; this correlates well with previous observations [23–24] that, during the onset of the mechanical response of heart, the main increase in the force of contraction develops in the potential range when the inward Na^+ current is small and the Ca^{2+} inward current is large.

In scorpion toxin- and ouabain-treated heart cells, a maximum increase in $^{45}Ca^{2+}$ uptake is observed at 20 min followed by a decrease up to 120 min. In the heart, cellular exchange of Ca^{2+} is complex, resulting from transmembrane Ca^{2+} permeability and binding to subcellular structures, among which the sarcoplasmic reticulum is especially involved. It is probable that the decrease in Ca^{2+} accumulation observed after the peak value is related to the enhanced intracellular Na^+ concentration due to ouabain and ouabain plus scorpion toxin treatment. Indeed, (1) decreasing extracellular Na^+ delays the decrease in intracellular calcium uptake (unpublished results) and (2) scorpion toxin which stimulates Na^+ uptake also speeds the decrease of intracellular Ca^{2+}. Biochemical studies have shown that Ca^{2+} and

Na^+ compete for Ca^{2+} sarcotubular binding sites [25]. It is therefore probable that increasing intracellular Na^+ concentration decreases Ca^{2+} storage in the sarcoplasmic reticulum and stimulated Ca^{2+} efflux from cell to medium.

Electrophysiological studies [26] on intact chick heart have shown that the Na^+ channels involved in the inward current during the action potential change during development, from tetrodotoxin-insensitive slow Na^+ channels in the young heart up to 6 days to tetrodotoxin-sensitive fast Na^+ channels in the older hearts (from day 6 to hatching). However, the latter revert when the cells are cultured. In our cells obtained from 11-day-old chick embryos, tetrodotoxin had no effect on cell contraction [4], in agreement with the absence of effect on the action potential [27–28], but inhibited the increase in Na^+ and Ca^{2+} uptake provoked by scorpion toxin. Since scorpion toxin and tetrodotoxin act on different sites, it is possible that binding of scorpion toxin to its specific sites unmasks latent tetrodotoxin sites. This possibility is rendered likely by the observation of McLean and Sperelakis [29], who showed that the loss of tetrodotoxin-sensitive fast channels in chick embryo heart cells is rapid, independent of protein synthesis and prevented or reversed by culturing in elevated extracellular K^+. Studies on the binding of tetrodotoxin and scorpion toxin to cultured heart cells or their derived plasma membranes, combined to electrophysiological investigations, should help to clarify the mechanism of this phenomenon.

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