Omega-Conotoxin Blockade of Calcium Currents in Cultured Neonatal Rat Cardiomyocytes: Different Action on EGTA-Modified Calcium Channels

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Abstract. Calcium currents from neonatal rat ventricular heart muscle cells grown in primary culture were examined using the „whole-cell" voltage clamp technique. An inward current characterized by large amplitude and slow inactivation decay was induced when the extracellular Ca^{2+} concentration was reduced by EGTA. This current was suppressed by extracellular Na^+ removal, or by calcium antagonists, and increased by epinephrine and BAY K 8644. These findings suggest that this current is carried by sodium ions through Ca channels. Both Ca and Na currents through calcium channels were irreversibly blocked by omega-conotoxin. Complete blockade developed 10—15 minutes after the toxin introduction in the extracellular solution. Blockade of Na currents through calcium channels was characterized by a transient increase of current amplitude without any changes in its kinetics and voltage-dependent properties. Structural differences between calcium channels in rat and guinea-pig and frog cardiomyocytes were suggested.

Key words: Ca current — Ventricular myocytes — Omega-conotoxin

Introduction

Calcium channels are involved in the control of many biological functions including excitability, transmitter release, contraction, metabolism and gene expression. In cardiac muscle transmembrane calcium fluxes via voltage-operated calcium channels determine the plateau phase of the cardiac action potential, and underlie the spontaneous activity of cardiac pacemaker cells (Reuter 1979; 1984; Noble and Noble 1984; Trautwein and Pelzer 1985). Calcium channels also play a crucial role in coupling membrane excitation to cellular responses; in cardiac muscle Ca^{2+} influx through calcium channels

induces a rise of cytoplasmic free calcium, triggering cell contraction and regulating cellular excitability via Ca^{2+}-dependent membrane channels (such as Ca^{2+}_{in}-operated potassium channels (Schwartz and Passow 1983; Callewaert et al. 1986) and Ca^{2+}-dependent non-selective cationic channels I_{TI} — (Gigant and Cohen 1988; Partridge and Swandulla 1988)).

Separation, identification and characterization of a variety of excitable membrane channels has been achieved largely through the use of specific neurotoxins (such as tetrodotoxin, saxitoxin, batrachotoxin, etc. — see Narahashi 1974; 1986; Conn 1983; Hille 1984 for review); for long, no specific probe was available for calcium channel. Only recently some natural toxins which have a pronounced effect on calcium transmembrane movement have been found. One of these toxins — omega-conotoxin (CgTx), twenty seven amino-acid peptide from the venom of Conus Geographus marine snail (Feldman et al. 1987; Ahmad and Miljanich 1988; Tu 1987), shows a remarkabale specificity: CgTx binds to and blocks a subclass of calcium channels in neurones, but not in skeletal muscle, smooth muscle, or cardiac muscle (including single frog and guinea-pig cardiomyocytes (McCleskey et al. 1987).

In neurones CgTx also possesses a property to produce a persistent block of high-threshold calcium currents (HTI — and HTN — channels (Kostuyk et al. 1988) or N- and L-channels (Tsien et al. 1988)) but only a weak and rapidly reversible decrease of low-threshold (LTI- or T- channels correspondingly) calcium current (Fox et al. 1987a; 1987b; Glossmann and Striessing 1988).

Here we report voltage-clamp experiments demonstrating that omega-conotoxin also persistently blocks calcium channels in single neonatal rat ventricular myocytes.

Materials and Methods

Single cell isolation and culture procedure

Cells were isolated and maintained in culture using standard techniques (Lehmkhul and Sperelakis 1963; Pronchyuk 1982). Briefly, neonatal (2–4 days old) rat hearts were rapidly removed from the chest cavity of anaesthetized animals, and the atrial tissues were removed. Ventricles were rinsed in a filter-sterilized calcium-free dissociation solution containing (in mmol/l): NaCl 140; KCl 5.4; HEPES 10; glucose 10; pH — 7.4. Cell isolation was carried out by a multiple-cycle procedure (DeHaan 1967) in a trypsin (Sigma, USA 0.015 % w/v) containing dissociated solution.

Dissociated cells were plated on a flame and ultra-violet presterilized glass coverslips and maintained in a medium of the following composition: Dulbecco modified Eagle medium (DMEM, Sigma USA) + 10 % NU-Serum (Serva Feinbiochemica, FRG). By day two of culturing, sufficient single cells adhered to the cover glass so that experiments could be performed. Experiments were carried out between days 2 and 4 of culturing.

Experimental procedure

Glass coverslips with adhering cardiomyocytes were removed from the tissue-culture flasks, transferred to the experimental chamber and bathed in Tyrode solution (see below). Cells were specially chosen for recordings to minimize problems associated with spatial non-uniformities of membrane potential. If there were any signs of inadequate space clamp, the experiment was discarded.

Patch electrodes were pulled from borosilicate glass capillaries (1.5 mm in diameter) to a tip diameter of 1.5–2.0 μm. Possible errors introduced by a pipette series resistance were compensated by using dynamic series resistance control compensation. Experiments were perfomed in the „whole-cell" patch clamp configuration (Hamill et al. 1981). All experiments were carried out at room temperature (22 °C). Drugs were applied by pressure ejection from an adjacent extracellular micropipette.

Data acquisition and analysis

Voltage command pulses were generated by a mini-computer with a self-made interface. Evoked currents were measured with a patch clamp electronic setup with 1 GOhm feedback resistor. Data were recorded on FM tape, then sampled at 10 KHz with a 12 bit analog-to digital converter and stored by the computer for late off-line analysis. Data were analyzed using a software developed in our laboratory by Dr. Ya. M. Shuba (Shuba and Savtchenko 1985).

Solutions

Basic Tyrode solution contained (in mmol/l): NaCl 120; KCl 5.4; $CaCl_2$ 5.0; $MgCl_2$ 1.1; HEPES 10; pH 7.4. The sodium-free extracellular solution contained (in mmol/l): $CaCl_2$ 15; $MgCl_2$ 4; TEA-Cl 111; HEPES 10; pH 7.4; the calcium free external solution contained: NaCl 60; TEA-Cl 85; HEPES 10; EGTA 5. The pipette solution dialysing the cell interior contained (in mmol/l): NaCl 30; CsCl 90; $MgCl_2$ 4; HEPES 20; Na_2ATP 3; EGTA 10; pH 7.2.

Drugs

Tetrodotoxin (TTX) and BAY K 8644 were obtained from Calbiochem (Switzerland), nifedipine was from Sigma Chemical Co. (USA), gallopamil (D-600) was the gift of Dr. G. Trube (Hoffman La Roche, Switzerland), omega-conotoxin was kindly provided by Dr. M. C. Nowycky, Medical College of Pensylvania, USA.

Results

Calcium current

General

Depolarization pulses of 160 ms duration to membrane potentials between −50 and +30 mV (stimulation frequency 0.5 Hz) from the holding potential −100 mV evoked two inwardly directed current components. These com-

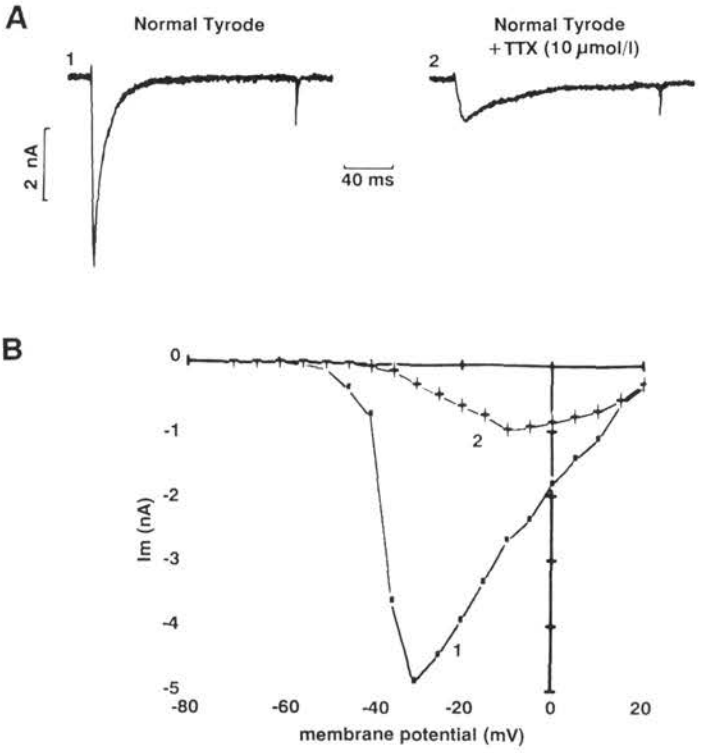

Fig.1. Separation of calcium and sodium currents in neonatal rat ventricular myocytes. *A*: (1) current elicited by depolarizing pulse for 160 ms to −30 mV from the holding potential of −80 mV in normal extracellular solution; (2) current elicited by depolarizing pulse to −10 mV from the holding potential of −80 mV after 10 μmol/l TTX administration. *B*: the I—V curves for these currents.

ponents (Fig. 1) were classified as sodium (I_{Na}) and calcium (I_{Ca}) inward currents. After tetrodotoxin administration (10 μm, a relatively high concentration which completely blocks sodium currents in cardiomyocyte membranes (Follmer et al. 1987; Antoni et al. 1988)) only calcium current remained (Figs.1, 2, left panel). Further TTX elevation in extracellular milieu produced no changes in calcium current parameters.

Calcium current could also be separated from I_{Na} by reducing the holding potential to − 40 mV (Fig. 2, middle panel) when steady-state inactivation of sodium channels is virtually complete (Brown et al. 1981; Pidoplichko and Verkhratsky 1987).

Substitution of sodium ions in extracellular solutions by impermeable cations is another way for I_{Ca} separation (Fig. 2, right panel). Characteristics of

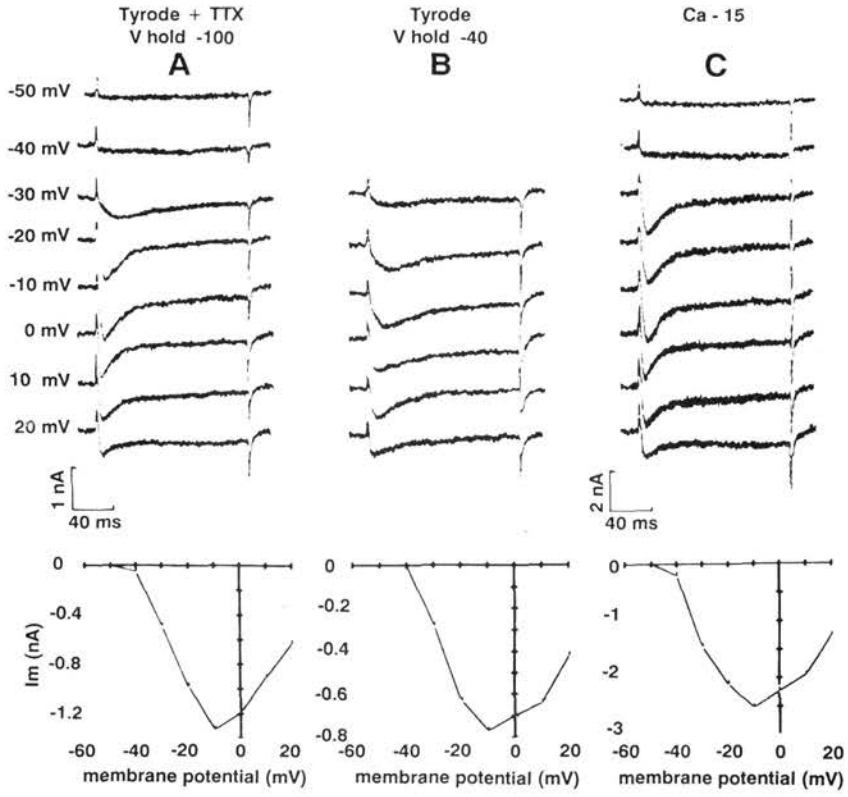

Fig. 2. Calcium currents in neonatal rat ventricular myocytes. *A*, *top*: currents elicited by depolarizing pulses to different potentials from the holding potential of −100 mV in the presence of 10 μmol/l TTX. *B*, *top*: currents from the holding potential of −40 mV in TTX-free extracellular solution. *C*, *top*: currents from the holding potential of −100 mV in Na^+-free, Ca^{2+}-containing solution. Membrane potentials are shown at the respective current traces. *Bottom*: I—V-curves for the respective currents.

calcium currents separated by the above methods were nearly identical (Fig. 2); the obtained Ca current records illustrate that there was no TTX-sensitive current contribution to I_{Ca}.

A single type of calcium channels in rat neonatal cardiomyocytes

The co-existence of multiple types of Ca^{2+}-selective channels have recently been shown in several excitable cells (Fedulova et al. 1985; Fox et al. 1987a; 1987b;

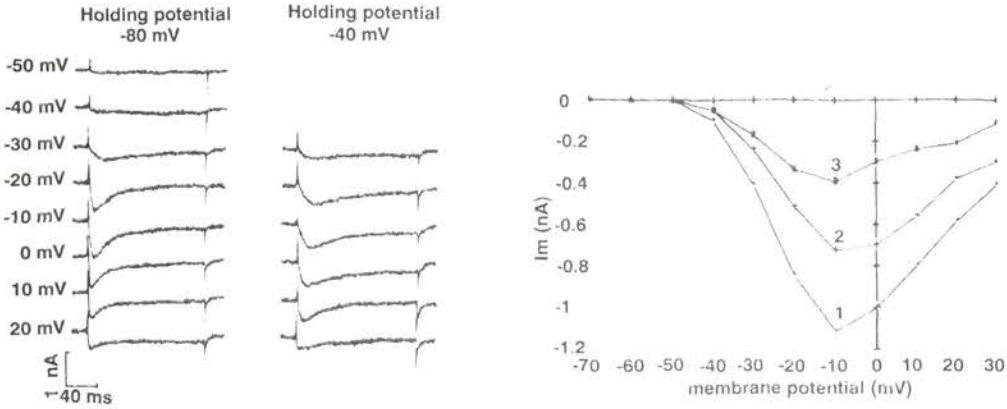

Fig. 3. Evidence for the presence of a single type of calcium current in single neonatal rat cultured cardiomyocyte. The cell was depolarized from two different holding potentials, −80 or −40 mV, to membrane potentials indicated at the respective current traces. I—V relationships for the currents shown in the right part of the picture: 1 — holding potential −80 mV; 2 — holding potential −40 mV; 3 — net difference inward current (trace 1 − trace 2).

Tsien et al. 1988; Kostyuk 1989). These current components were distinguished on the basis of their voltage and time dependences, ionic selectivity and pharmacology. Two kinds of calcium channels have been observed in different cardiac preparations: in whole-cell recordings from atrial and ventricular cells of adult dog (Bean 1985), ventricular myocytes of adult guinea-pig (Mitra and Morad 1986), frog atrial myocytes (Bonvallet 1988) and in single channel recording from adult guinea-pig cardiomyocytes (Nilius et al. 1985). The predominant component of calcium permeability of cardiomyocyte membrane activated at membrane voltages more positive than − 40 mV was labelled the „L"-type, and the second component, activated at lower membrane potential values the „T"-type (see for review Tsien et al. 1987a). Cardiac T-channels were distinguished by (1) rapid inactivation; (2) more negative activation potential range, and (3) relative insensitivity to pharmacological modulation (Tytgat et. al. 1988).

For this reason, we examined the types of calcium currents present in single neonatal rat cardiac myocytes using the conventional voltage-clamp protocol for the separation of high and low-threshold I_{Ca} components (Bean 1985).

Fig. 3 shows families of I_{Ca} currents at different holding potential levels. When the holding potential was − 40 mV as compared to − 80— − 100 mV, I_{Ca} was smaller at each testing potential. However, the current-voltage relationship of calcium current and the threshold potential of I_{Ca} were not affected by

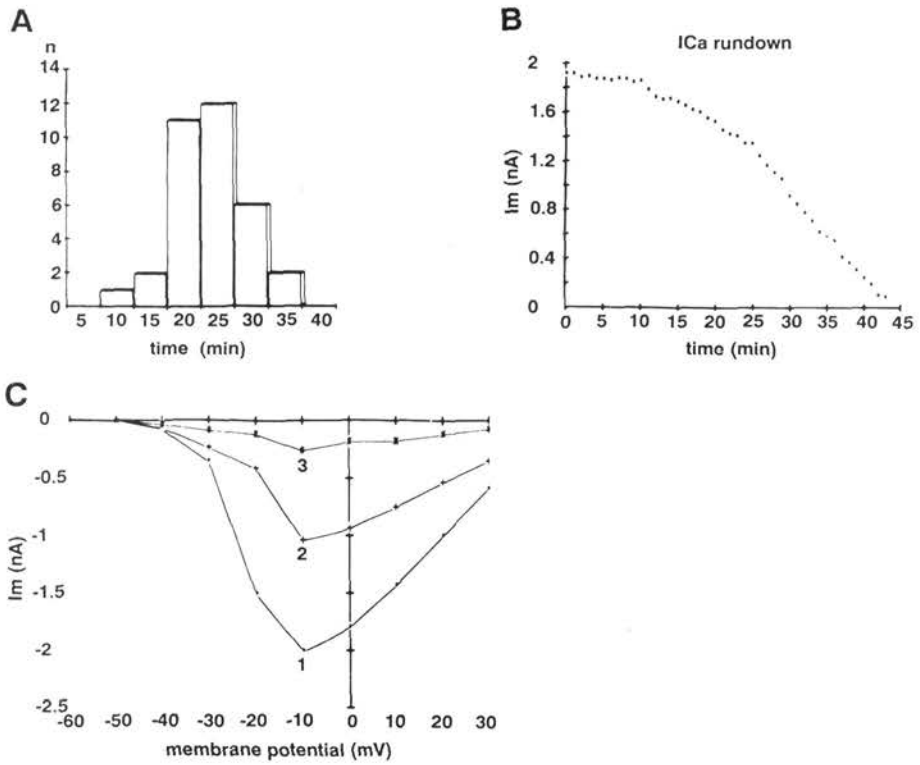

Fig. 4. Calcium current run-down during intracellular perfusion of a single neonatal rat ventricular myocyte. *A*: Histogram of the latencies since the start of the recordings to the times at which a 50% decrease of the I_{ca}. was observed. *B*: Changes in current amplitude during intracellular perfusion (testing potential −10 mV). *C*: I—V curves ofr I_{ca}. at 11(1), 27(2), and 33(3) minutes after the start of intracellular perfusion. Holding potential −80 mV.

the change in holding potential. Moreover, there was no obvious hump on the current-voltage curve which would have suggested that low-threshold calcium current was responsible for an appreciable amount of the total Ca current. The I—V curve for the difference current obtained by subtracting the current elicited at holding potential − 40 mV from the one elicited at − 80— − 100 mV had a similar shape as that of total I_{Ca}.

The obtained threshold of calcium current (− 40 mV) and its potential--dependent parameters correspond to those typical of the high-threshold calcium current. Additionally, we checked the sensitivity of the calcium current to pharmacological agents. Both gallopamil and nifedipine in micromolar con-

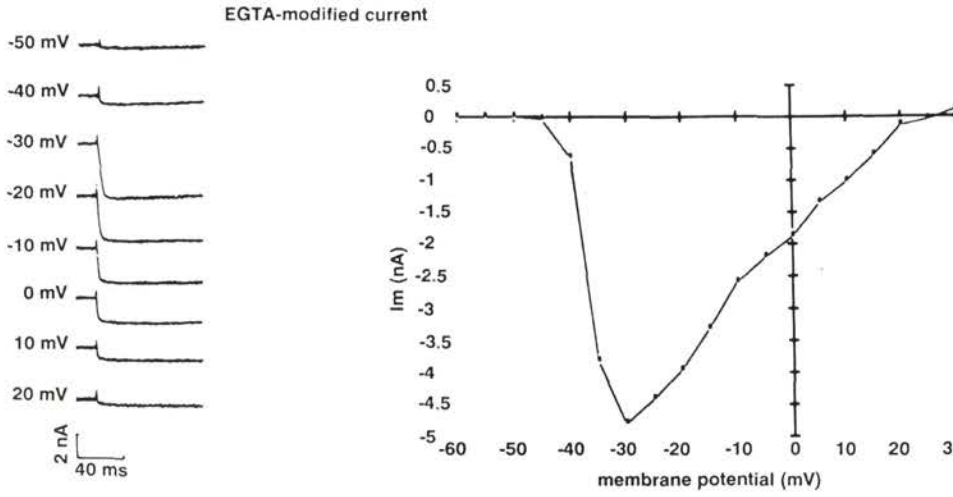

Fig. 5. Membrane currents in Ca^{2+}-free solution containing Na^+ as the major permeable cation; to prevent contamination by currents through sodium 10 μmol/l TTX channels was added to the extracellular solution. The cell was depolarized for 160 ms from holding potential of −80 mV to membrane potentials indicated at the respective current traces. I—V relationship for these currents is shown in the right part of the figure.

centrations completely abolished the calcium current; the Ca-channel agonist BAY K 8644 produced a 10—30 % increase in I_{Ca} amplitude (at the holding potential −40 mV at which BAY K 8644 acts as a calcium channel agonist (Sanguinetti et al. 1986)). The calcium current sensitivity to Ca-channel modulators also supports the idea about the presence of only one component in the calcium current in neonatal rat cardiomyocytes. Low-threshold calcium current was also reported to be absent by Argibay et al. (1988) in frog vetricular myocytes. No clear evidence about the existence of T-type calcium channels in rat cardiomyocytes was also presented by other investigators (Cohen and Lederer 1987; 1988; Field et al. 1988).

I_{Ca} rundown

It is widely believed that functional activity of calcium channels is controlled by a number of cytoplasmic metabolism-dependent processes (Kostyuk and Krishtal 1977a; Chad and Eckert 1984; Sperelakis 1985a; 1985b). Exchange of soluble components between the cytoplasm and the recording pipette during intracellular dialysis leads to the disappearance of metabolic support of calcium channels and a progressive fall of I_{Ca} amplitude. In our experiments the run-

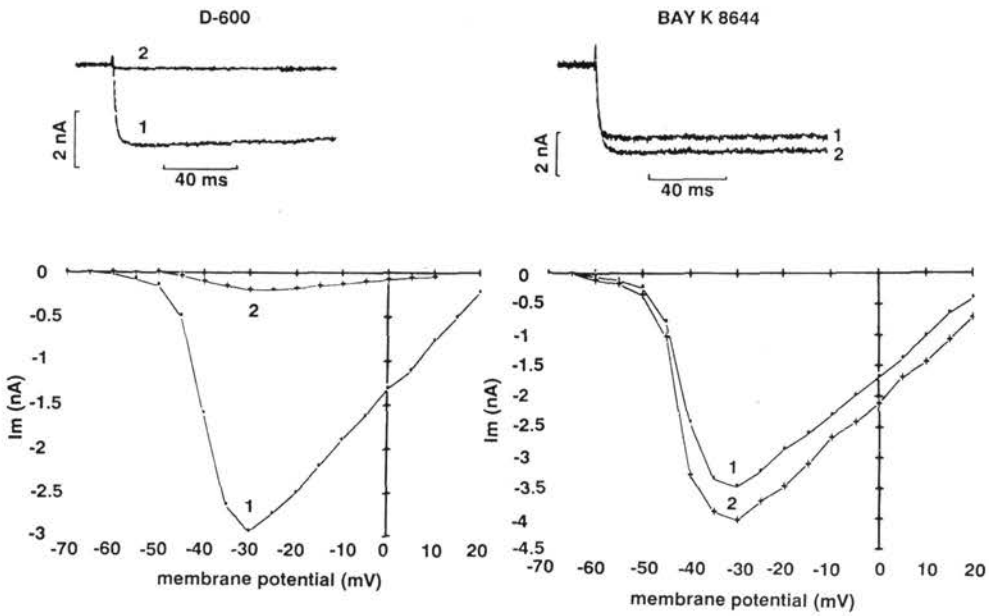

Fig. 6. Ca-channel-modulators sensitivity of Na current in low-Ca^{2+}, TTX-containing extracellular solution. The upper panels show superimposed current records before (1) and after (2) addition of 1 μmol/l D-600 (left) or 5 μmol/l BAY K 8644. Current records elicited by step depolarizations from -80 mV to -30 mV. Lower panels: Peak current levels before (1) and after (2) addition of D-600 or BAY K 8644 are plotted against the membrane potential.

down of the calcium current was almost complete within 40—50 min (Fig. 4); these data are in line with previous reports (Irisawa 1984; Belles et al. 1987).

Sodium current through calcium channels

Inward currents carried by monovalent ions through calcium channels when the external [Ca^{2+}] is reduced have been detected in a variety of excitable cells: in snail neurones (Kostyuk and Krishtal 1977b; Kostyuk 1981; Shuba 1983), frog skeletal muscle fibers (Almers et al. 1984; Almers and McCleskey 1984), and in mouse lymphocytes (Fukushima and Hagiwara 1985).

In cardiac muscle a lowering of external Ca^{2+} concentration by chelating agents such as EGTA extremely prolongs (up to several seconds) the duration of action potential (Hoffman and Suckling 1956; Goto and Abe 1964; Rougier et al. 1969; Miller and Morchen 1978). It has been shown that an inward sodium current through calcium channels underlies this action potential prolongation (Imoto et al. 1985; Matsuda 1986; Levi and DeFelice 1986; Mazzanti and

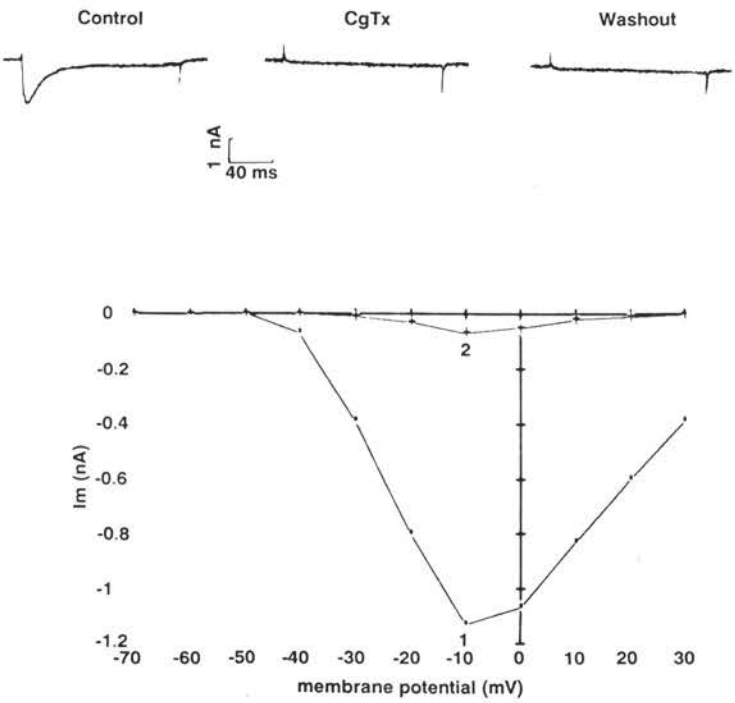

Fig. 7. Omega-conotoxin action on calcium current in the neonatal rat ventricular myocyte. Upper panel: Current records in control conditions, 15 min after CgTx administration, and 20 min after washout by toxin-free extracellular solution. Holding potential: −80 mV; testing depolarization: 10 mV. Lower panel: I—V curves for the initial I_{Ca} (1) and I_{Ca} 10 minutes after CgTx application (2).

DeFelice 1987). This kind of calcium channel modification is explained in terms of the existence of high affinity binding sites for Ca^{2+} in the calcium channel: occupation of these sites by Ca^{2+} ions determines the selective properties of the calcium channel and prevents the permeation of monovalent cations (Kostyuk et al. 1983; Hess and Tsien 1984; Tsien et al. 1987b); however, the location of this site is still a matter of discussion.

Figure 5 shows membrane currents in low-calcium solution [Ca^{2+}] < 100 nmol/l) containing Na^+ as the major permeable cation. Sodium ion flux through Ca channels produces a large inward current (approximately 3—5-fold greater in amplitude compared with the initial Ca current in the same cell) which inactivates much slowlier than the initial I_{Ca}. Calcium removal from the ex-

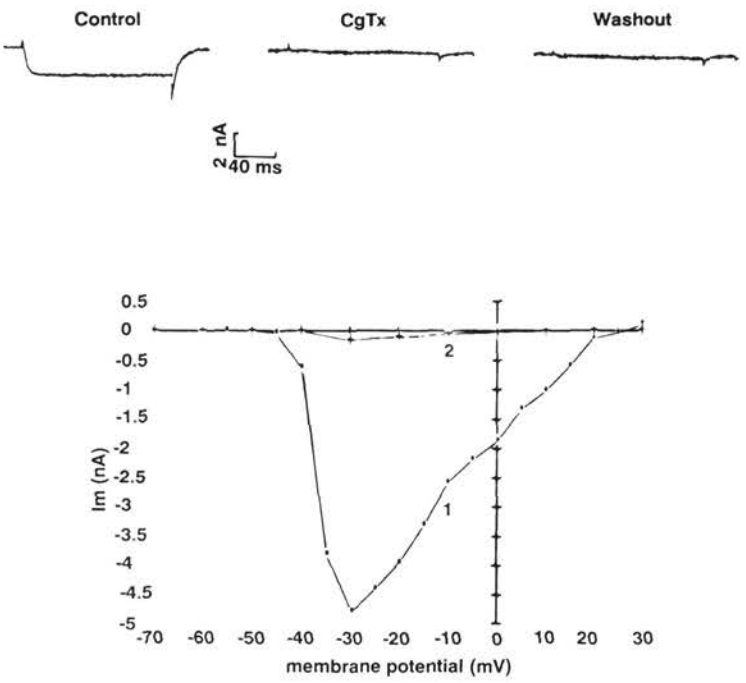

Fig. 8. Omega-conotoxin action on sodium current through calcium channels in a neonatal rat ventricular myocyte. Upper panel: Current records in control conditions. 15 min after CgTx administration, and 20 min after washout by toxin-free extracellular solution. Holding potential: −80 mV; testing depolarization: −10 mV. Lower panel: I—V curves for the initial current (1) and current 10 minutes after CgTx application (2).

tracellular solution also produced a 20 mV hyperpolarizing shift in Na^+-carried current threshold and in maximum of its I—V curve (Fig. 5).

The suggestion that in the absence of external Ca^{2+} the inward current is carried by sodium ions through calcium channels is derived from the following findings.

(1) The inward current was not affected by addition of 50 μmol/l TTX to the external solution (note that the initial TTX concentration in Ca^{2+}-free solution was 10 μmol/l to prevent I_{Na} contamination).

(2) Organic calcium channel blockers, such as gallopamil (Fig. 6*A*) and nifedipine (not shown) in micromolar concentrations, completely suppressed this current.

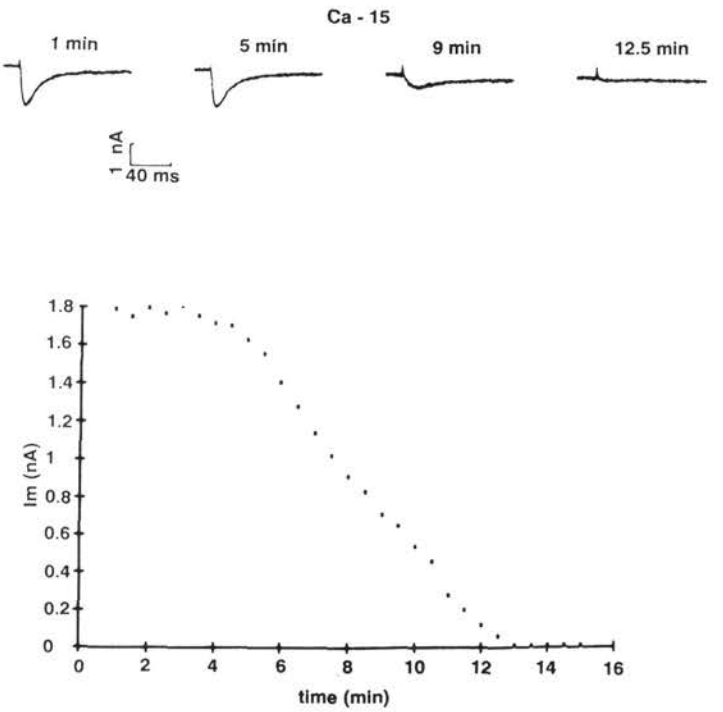

Fig. 9. Time course of Ca^{2+}-carried current blockade by omega-conotoxin. Current traces obtained at different times after CgTx application. Currents were evoked by 160 ms test pulses from holding potential of −80 mV to −10 mV. The respective plot of peak current versus time is shown below.

(3) Calcium channel agonist BAY K 8644 increased the amplitude of the recorded current (Fig. 6*B*).

(4) In the presence of isoproterenol (0.5 μmol/l) the amplitude of the inward current was increased (not shown).

(5) Replacing Na^+ in extracellular solutions by $Tris^+$ ions led to the disappearance of this current.

(6) The reversal potential of the current studied was approximately + 25 mV, i. e. close to the Nernst value predicted for the given Na_o and Na_{in} (Na_{in} = 30 mmol/l; Na_0 = 60 mmol/l; E_{Na^+} = + 20 mV).

Omega-conotoxin action on current through Ca channels

Omega-conotoxin in 10 μmol/l concentration abolished both calcium and so-

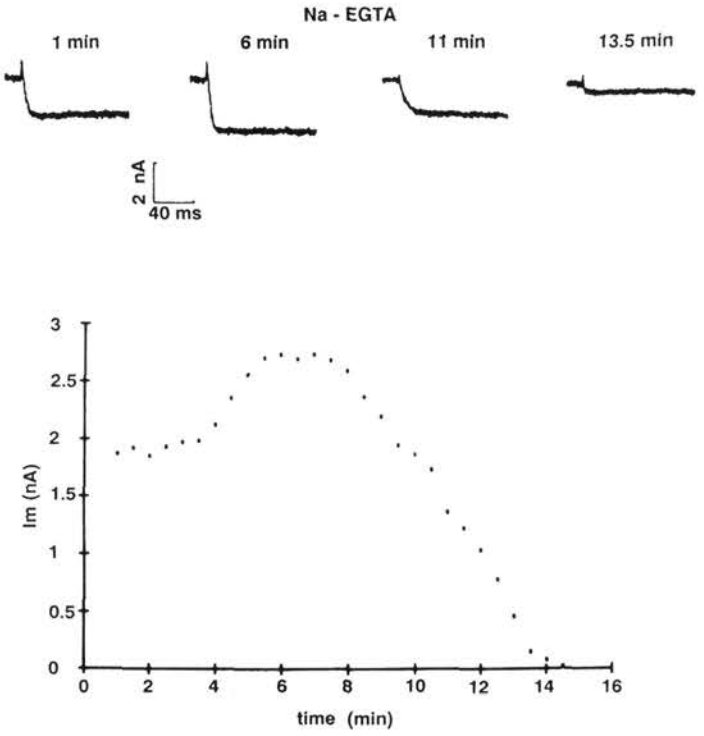

Fig. 10. Time course of Na^+-carried current blockade by omega-conotoxin. Current traces obtained at different times after CgTx application. Currents were evoked by 160 ms test pulses from holding potential of −80 mV to −10 mV. The respective plot of peak current versus time is shown below.

dium currents through calcium channels.

Fig. 7 shows Ca^{2+}-carried current traces before and after exposure to the toxin, and the current-voltage relationships obtained from the same cell in control conditions and during CgTx action; 12—15 minutes after CgTx application, the I_{Ca} was almost completely abolished at all test potentials. The washout of the toxin could not restore I_{Ca} (up to 30 minutes after the start of the washout — Fig. 7, right current trace).

Inhibition of the sodium current through calcium channels by omega--conotoxin (Fig. 8) showed the same pattern.

The time course of omega-conotoxin-dependent I_{Ca} depression is shown in Fig. 9. CgTx produces a progressive decrease of calcium-carried current amplitude. In contrast, CgTx action on sodium current through calcium channels shows two different phases: during the first 8 minutes after the toxin exposure

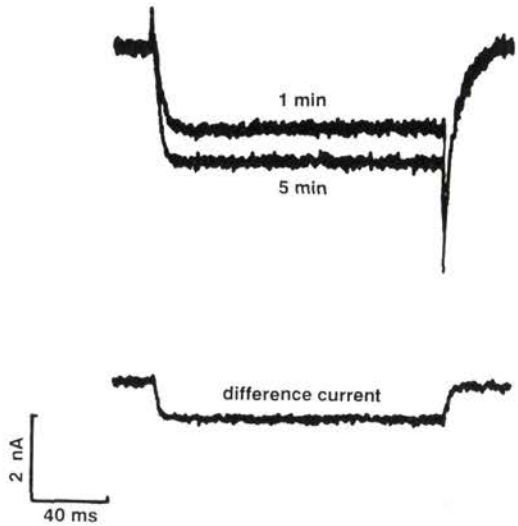

Fig. 11. The increase of Na current through calcium channels due to the omega-conotoxin action. Currents were evoked by test depolarizations from holding potentials −60 mV to −20 mV; current records 1 min, 5 min after CgTx application, and the difference current are shown.

an increase of current amplitude without changes in its potential-dependent and kinetic properties was observed (Fig. 10, 11), followed by fast suppression of the current amplitude.

Discussion

Calcium channels in neonatal rat ventricular myocytes

General characteristics of the calcium current obtained in our experiments are in good agreement with data of other authors (Cohen and Lederer 1987; 1988; Field et al. 1988). We could not find any evidence for the presence of multiple types of Ca channels in neonatal rat ventricular myocytes. Our results indicate a single type of calcium current in rat ventricular cells. This finding contradicts most published data in this field (Bean 1985; Tsien et al. 1987a; 1988;); however, a similar observation on frog ventricular cardiomyocytes was made by Argibay and co-workers (1988).

Our results also confirm the findings about the CA_o^{2+}-dependent modification of calcium channels. $[Ca^{2+}]_o$ reduction below 1 μmol/l led to the appearance of a sodium current through calcium channel. It is important to emphasize that

lowering of extracellular calcium not only caused a loss of Ca channel selectivity, but led to a significant shift in voltage-gated properties of calcium channels. This finding supports the hypothesis (Kostyuk et al. 1983) about the existence of two external Ca^{2+}-binding sites in calcium channel; these sites control both selectivity and voltage-operated properties of the calcium channel.

Secondly, extracellular Ca^{2+} removal led to a dramatic slow-down of calcium current inactivation: the inactivation decay of EGTA-induced sodium current was approximately one degree slowlier as compared with the initial calcium-carried current. These changes may be attributed to the disappearance of Ca^{2+}-mediated calcium channel inactivation (Eckert and Chad 1984; Chad and Eckert 1986). Calcium-dependent as well as voltage-dependent inactivation has been demonstrated in calf Purkinje fibers (Kass and Sanguinetti 1984; Lee et al. 1985), in single ventricular myocytes (Josephson et al. 1984; Mitchell et al. 1983) and in isolated atrial cells (Bechem and Pott 1985a; 1985b). Moreover, in contrast to our data Imoto et al. (1985) and Hardley and Hume (1987) observed two components (fast and slow) in the Na^{+}-carried current through calcium channels in single guinea-pig ventricular myocytes; the fast inactivating component of EGTA-induced current was attributed to pure voltage-dependent inactivation of calcium channels (Hardley and Hume 1987). Our data support the idea that in rat cardiomyocytes Ca^{2+}-mediated incativation plays a primary role in calcium current decay.

CgTx action on calcium channels

Our results show that CgTx produces a persistent blockade of calcium channels in the memebranes of cultured neonatal rat ventricular myocytes. These data contradict the results obtained by Tsien group (McCleskey et al. 1987); they suggested that calcium channels in cardiac muscle cell membranes are insensitive to CgTx. This insensitivity distinguishes between two subcathegories of high-treshold (L) calcium channels: L_n blocked by CgTx and L_m, resistant to this agent (Cruz et al. 1987). Our data may indicate that calcium channels in rat cardiomyocytes are structurally different in comparison with Ca channels in guinea-pig and frog cardiac muscle cells.

Secondly, we found that CgTx distinguishes between calcium and EGTA-modified calcium channels: omega-conotoxin action on sodium currents through calcium channels is characterized by a transient increase of current amplitude without any changes in current kinetics and potential-dependence. The nature of this difference is not yet clear. Interestingly, another calcium channel-related natural toxin — maitotoxin — displays a similar time course of action on calcium currents in isolated adult rat cardiac ventricular cells. The suppression of calcium current by maitotoxin is preceded by a transient increase

in Ca current amplitude (Coraboeuf et al. 1988). Similar transient increase in Ca^{2+}-carried current under CgTx action was also observed in pheochromocytoma PC12 cells (Savtchenko and Verkhratsky, unpublished observation). All these results may suggest that it is the so far unknown part of the calcium-controlled calcium channel open probability that is the main target of CgTx action. There is some evidence about a protective role of divalent cations against CgTx block development (McCleskey et al. 1987). Obviously, such an interaction between divalent cations and CgTx underlies the change of toxin action during EGTA-modification of calcium channels.

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