Two Different Tetrodotoxin-Separable Inward Sodium Currents in the Membrane of Isolated Cardiomyocytes

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Abstract. Isolated ventricular myocytes of 3 to 5 weeks old rats were studied under conditions of intracellular perfusion and voltage clamp. The existence of two inward sodium currents with different TTX-sensitivities and different properties was shown. The fast sodium current was more sensitive to TTX (K_d about 8×10^{-8} mol/l). The block of the slow sodium current by TTX was less specific (K_d about 7×10^{-6} mol/l). There was an about four fold difference in the inactivation time constants between these currents. The maximum on the I—V curve of the slow sodium current was shifted along the voltage axis by about 15 mV in the positive direction as compared with that of the fast sodium current. A slow current carried by calcium ions was observed in sodium-free solution. The kinetics and TTX-sensitivity of this current were similar to those of the slow sodium current. The amplitude of this current was 15 to 20 times lower as compared with the slow sodium current observed in Na-containing solution with 10^{-6} mol/l TTX (a concentration which completely blocked the fast sodium current). It is suggested that the slow voltage-gated TTX-sensitive channels described are not highly selective and pass both sodium and calcium ions.

Key words: Cardiomyocytes — Tetrodotoxin — Sodium-current — Calcium current

Introduction

The capacity of tetrodotoxin to selectively block sodium channels has been widely used to separate ionic currents and to analyse the molecular mechanisms of ionic channels functioning (Hille 1970; Narahashi 1974; Ritchie and Rogart 1977). It is generally accepted that sodium current (I_{Na}) in the membrane of heart muscle cells is much less sensitive to TTX than I_{Na} in nerve tissue or in skeletal muscle (Caterall and Coppersmith 1981; Lazdunsky and Renaud 1982). The sensitivity of cardiac tissue to tetrodotoxin is different in various species: e.g., in the membrane of mammalian Purkinje fibres, an effective block of sodium channels is observed at

TTX concentrations exceeding 10^{-5} mol/l (Dudel et al. 1967); 3×10^{-8} mol/l TTX reduced 10-fold the peak magnitude of I_{Na} of single bullfrog atrial myocytes (Hume and Giles 1983); a high sensitivity to TTX ($K_{0.5}$ about 10^{-9} mol/l) has also been found in the chick embryonic heart (Iijima and Pappano 1979).

TTX action on sodium permeability ($\bar{g}_{Na}$) of the myocardial cell membrane has been mostly studied on multicellular preparations. In some studies, the effects of the toxin on $\bar{g}_{Na}$ were estimated from maximal increase rates in action potential ($\dot{V}_{max}$) following the application of TTX (Baer et al. 1976; Reuter et al. 1978; Inoue and Pappano 1984). Based on these data it was suggested that the action of TTX on sodium current in rat papillary muscle is potential-dependent (Baer et al. 1976), in contrast to data obtained on nerve and skeletal muscle tissues. In addition, it was shown that changes in $\dot{V}_{max}$ could not be a reliable criterion for the estimation of changes in $\bar{g}_{Na}$ (Cohen and Strichartz 1977; Strichartz and Cohen 1978; Cohen et al. 1984). The voltage clamp technique which allows direct measurements of transmembrane current changes secondary to variations in the external TTX concentration, has provided the most suitable conditions for the investigation of the action of TTX on $\bar{g}_{Na}$. This technique was used in experiments on heart muscle fragments (Dudel et al. 1967; Bean et al. 1980; Cohen et al. 1981) and showed that the blocking action of TTX is independent of membrane potential. A 50% sodium current block was reached with about 10^{-6} mol/l TTX in the external solution. However, the application of the voltage clamp technique to multicellular preparations meets a number of limitations: errors due to space clamp nonuniformity, the existence of diffusion barriers etc. (Attwell and Cohen 1977; Baumgarten and Isenberg 1977; Beeler and McGuigan 1978; Attwell et al. 1979). The development of methods of Ca^{2+}-tolerant single cardiac cells isolation (for reviews see Dow et al. 1981 a, b; Farmer et al. 1983; Trube 1983) and the use of single cardiomyocytes for electrophysiological studies allowed to overcome problems with the recording of ionic currents in multicellular preparations. Moreover, isolated cardiac cells also allowed the application of the most advanced electrophysiological techniques (the intracellular perfusion method and patch clamp) to study transmembrane ionic permeability. Single cell investigations initiated a flow of new facts in cardiac electrophysiology (see the reviews by Fozzard 1984; Irisawa 1984; Noble 1984). Yet the action of TTX on the membrane currents in isolated cardiomyocytes has been studied by few authors only (Undrovinas et al. 1980; Brown et al. 1981). The question concerning the number of TTX receptor types in the cardiac cell membrane remains still open. Some authors postulated the existence of two populations of sodium channels with different TTX-sensitivities (Coraboeuf et al. 1979; TenEik et al. 1984); in other studies (Cohen et al. 1981; Brown et al. 1981) the existence of only one TTX receptor was postulated. The present work was aimed at studying changes in ionic currents in cardiomyocytes

induced by external application of TTX in concentrations ranging from 10^{-9} to 10^{-4} mol/l.

Materials and Methods

Cell isolation

Experiments were carried out on cells obtained from the left ventricles of rat hearts. The age of rats varied from 3 to 5 weeks. The "chunk method" (see Isenberg and Klokner 1982) was used to isolate cardiomyocytes; it consisted in treating the tissue with a mixture of collagenase (0.3 U/mg, Serva, F. R. G.) and papain (Loba Chemie, Austria). After the isolation the cardiomyocytes were kept in culture medium (MEM, Serva F. R. G.) supplemented with 20% of bovine serum.

Intracellular pefusion and voltage clamp

Intracellular perfusion under voltage clamp conditions was performed using a plastic micropipette (Kostyuk et al. 1981, 1984). A simplified scheme of the set up is shown in Fig. 1. The diameter of the perfusing pore varied from 5 to 7 μm the thickness of the pipette tip wall was about 5 μm. The inward current amplitudes varied from 3 to 20 nA depending on the cell size. Large currents resulted in a poor potential control (a steeper initial part of the I—V curve). Small cells with comparatively small currents were therefore used in our experiments. No special R_s compensation arrangements were used. Both the pore and the cell size were adjusted to obtain currents of several nA in order to diminish the potential error below 3 mV (the maximum R_s value of the system with a perfused cell was about 600 KΩ; R_s values were estimated either after puncturing the perfused cell with a blunt glass microelectrode or after destroying a part of the membrane by gentle touching the cell with the tubing). R_s values estimated in this way were close to those obtained from measurements of values of the capacitance charge transient on perfused cells. The capacitance charge transient could be fitted with one exponential with time constants ranging from 60 to 70 μs (estimated in the bandwidth range of 0—10 kHz).

The appearance or disappearance of outward sodium current following changing the intracellular perfusion tubing from intracellular Tris(HF) to intracellular solution containing 50 mmol/l Na^+ and vice versa, indicated the completion of the replacement of the intracellular environment by artificial intracellular solution. The complete substitution took 2 to 5 min. The quality of the replacement of the intracellular ionic content was estimated from the reversal potential upon varying $[Na^+]_o$ from 50 to 150 mmol/l (the intracellular solution contained 50 or 25 mmol/l Na^+). The experimentally obtained values of the reversal potential were in good agreement with the theoretical values for Na^+-electrode for the given $[Na^+]_i$ and $[Na^+]_o$ concentrations.

The cells were perfused intracellularly with potassium-free solutions to eliminate potassium currents. Fluorine was the major intracellular anion. The inward current records were not contaminated by the current through calcium channels. It is known that, at a holding potential of −50 mV, the Ca^{2+} current becomes separated from other currents due to the steady-state inactivation of the latters. In our experimental conditions, no current was observed in Na-free external solution at $V_h = -50$ mV; this was probably due to the absence of cyclic nucleotides in the intracellular perfusion solution and to the possible effect of the fluoride perfusion (Kostyuk et al. 1975).

High input resistance (R_{in} values ranging from 600 MΩ to 1 GΩ) was obtained using fluorine as the major intracellular anion, and by lowering the osmolality of intracellular solutions by 25% as compared to that of extracellular solutions; it should be noted that such an osmotic pressure gradient resulted in an about two fold slowing down of the kinetics of inward currents (Krishtal et al. 1983). Due to high input resistance values no correction for leakage was performed. The average duration of the "working

condition" of a single perfused cardiomyocyte was 1 to 1.5 hour. All measurements were performed at 18—20 °C.

Exchange of extracellular solutions

External solutions were substituted using the fast hydrodynamic application technique (Pidoplichko 1983). The application system included three interchangeable rotating disc cassettes (each containing 12 chambers with a volume of one milliliter each). The tip of a plastic micropipette with the perfused cell was introduced through a side opening into a plastic tube (diameter 1 mm) (Fig. 1). One end of the tube

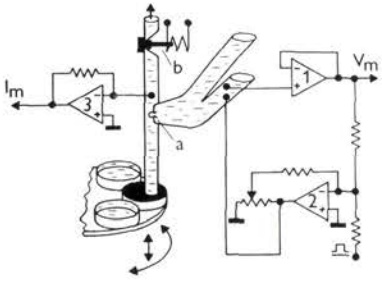

Fig. 1. A schematic representation of the set-up for intracellular perfusion with a fast hydrodynamic exchange of extracellular solutions. Possible movements of the rotating disc cassette with 12 one-milliliter chambers are indicated by arrows (only a part of the cassette with three chambers is shown). The solution to be changed is marked by black. Constant suction at the outlet of the vertical tube. A plastic pipette with the cell is introduced into the tube through a side opening. *a* — the perfused cell in a plastic micropipette; *b* — electromagnetic valve. The conventional voltage clamp circuit consisted from: 1 — input unity gain amplifier; 2 — voltage clamp amplifier; 3 — virtual ground amplifier.

was placed into one of the 12 chambers of the rotating cassette; the other end (through a normally closed electromagnetic valve) was connected to the hermetized vessel. Negative pressure was constantly maintained in the vessel. To apply the desired solution, the electromagnetic valve was opened for about 100 ms and the solution was sucked into the tube bathing the cell. The duration of the solution exchange procedure was estimated in test experiments from the disappearence of the inward sodium current during the application of Na-free solution (test pulses were delivered with a frequency of 50 Hz). The inward sodium current completely disappeared within about 50 ms after the application of the Na-free solution. Under our experimental conditions, the application of about 40 µl of the solution were sufficient to replace the previous solution in 50 ms. The application system used allowed to test 35 external solutions with different TTX concentrations on one cell within 10—15 min (36 chambers in 3 cassettes, one chamber being filled with a TTX-free solution). It is worth mentioning that smaller cells tolerated the procedure better.

Recording technique

Experiments were carried out in a semi-automatic mode using a microcomputer driven multichannel analyser NTA-1024 (EMG, Hungary). Ionic current traces were stored in a digital form on a magnetic tape and were analysed off-line. The routine recordings were performed in a frequency range between 0 and 2 kHz; higher frequencies were filtered out using an active low-pass filter. To improve the signal-to-noise ratio the averaging procedure was applied in some experiments.

Solutions

The extracellular solution contained (mmol/l): NaOH 160; $Ca(NO_3)_2$ 3.6; pH 7.4 was adjusted with methanesulphonic acid (Koch-Light LTD., U. K.). The extracellular solution was chloride-free to ensure the absence of a postulated TTX-sensitive chloride current (Pidoplichko and Verkhratsky 1984; Verkhratsky and Pidoplichko 1985). $Tris^+$ cations were used as a substitute for Na^+.

Intracellular solutions contained (mmol/l):

1) Tris(HF) 135; pH 7.2; 2) Tris(HF) 110; NaF 25; pH 7.2; 3) Tris(HF) 85; NaF 50; pH 7.2.

(Solutions 2 and 3 were used to check the quality of the intracellular perfusion; see *Intracellular perfusion and voltage clamp*).

All solutions were prepared using deionised water (17 MΩ/cm).

Tetrodotoxin (Serva, F.R.G.; Sankyo, Japan) was dissolved in deionized water to obtain a 1×10^{-2} mol/l stock solution. Several basic solutions with TTX concentrations ranging from 10^{-3} to 10^{-7} mol/l were prepared from this stock solution. To obtain the required TTX concentration in the one-milliliter-volume application chambers, the necessary amounts of basic solutions were added directly into the chambers.

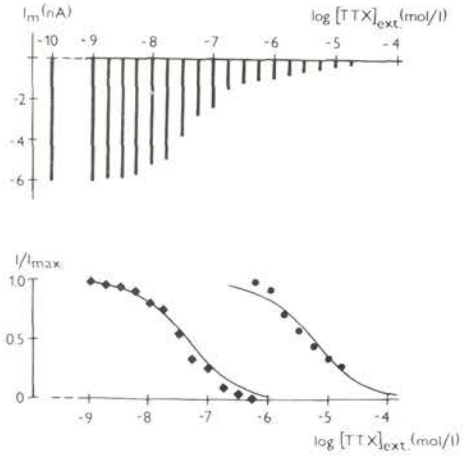

Fig. 2. The dependence of the peak values of the inward current through the membrane of isolated perfused cardiomyocytes on TTX concentration in the external solution. The vertical lines in the upper part of the Figure correspond to peak current amplitudes. The vertical line on the extreme left: the amplitude of the current in the initial TTX-free solution. Abscissa: external TTX concentrations (logarithmic scale). In the lower part of the Figure the corresponding one-to-one binding curves for the blocking action of TTX (normalized) are shown. $K_{d1} = 5 \times 10^{-8}$ mol/l; $K_{d2} = 6 \times 10^{-6}$ mol/l. Experiment on one cardiomyocyte; $V_h = -120$ mV; $V_t = -20$ mV.

Results

Following the disruption of a part of the cardiomyocyte membrane sucked into the perfusing pore and the completion of the subsequent intracellular perfusion with Tris fluoride, 60 ms long depolarizing pulses from a holding potential (V_h) level of -120 mV were applied in second intervals. The level of $V_h = -120$ mV was chosen to ensure the activation of all possible components of membrane permeability. The maximum of the I—V curve for the inward current in TTX-free solution was around $V_t = -35 \; -40$ mV (Fig. 3 *B*). The effect of TTX on inward currents was estimated from changes in their maximum amplitude at a fixed V_t at different TTX concentrations in the external solutions. One example of these experiments is shown in Fig. 2. Peak current amplitudes were recorded from the

same cell during successive applications of increasing TTX concentrations. The testing potential was -30 mV. Changes in the inward current amplitude are illustrated in the upper part of Fig. 2 (identical scale). Starting with external TTX concentrations higher than 5×10^{-9} — 10^{-8} mol/l a progressive block of the inward current was observed. A complete block was obtained at a TTX concentration of about 10^{-4} mol/l. The plot of the concentration dependence of the inward current amplitude showed a bend in the region of TTX concentrations between 7×10^{-7} and 2×10^{-6} mol/l (Fig. 2), suggesting either a complex nature of the toxin binding to the channel, or the existence of two populations of channels with different TTX-sensitivities. The latter possibility seemed more probable and the blocking action of TTX on inward currents was fitted by one-to-one binding curves with $K_{d1} = (7 \pm 3) \times 10^{-8}$ mol/l and $K_{d2} = (8 \pm 3) \times 10^{-6}$ mol/l ($n = 7$). The curves for one cell are shown in the lower part of Fig. 2.

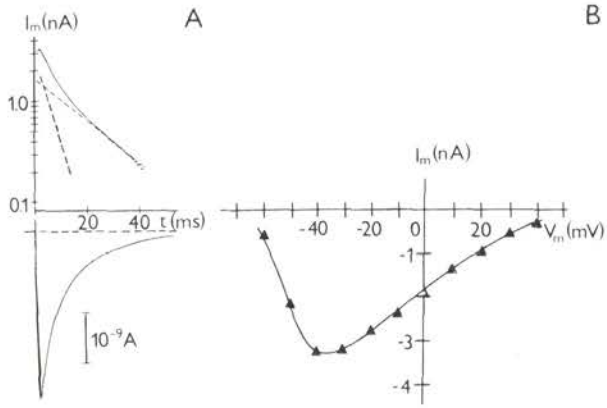

Fig. 3 *A*: Record of the initial inward current in TTX-free solution. Current trace shown in a semilogarithmic scale (upper part). The current inactivation could be fitted by the sum of two exponentials (estimated in experiments on the same cell) with $\tau_1 = 4.6$ ms; $\tau_2 = 21$ ms. $V_h = -120$ mV; $V_t = -20$ mV. *B*: I—V curve for the current shown in *A*. $V_h = -120$ mV.

In addition to the effect on the current amplitude, TTX also affected the current kinetics. The inactivation of the current in the initial TTX-free solution could be fitted by two exponentials with time constants $\tau_1 = 4.6$ ms and $\tau_2 = 21$ ms (for $V_t = -20$ mV; Fig. 3 *A*). After the application of the solutions containing TTX concentrations higher than 10^{-6} mol/l the amplitude of the current diminished and its maximum was delayed. The inactivation of this latter current could be well fitted by one exponential with a time constant of about 21 ms (Fig. 4 *A*). This current component could be fully blocked by 10^{-4} mol/l TTX. Digital subtraction of the current obtained in the solution with 10^{-6} mol/l TTX from the initial current revealed a fast current component. The inactivation of this difference current could be fitted by one exponential with $\tau_{in} = 4.6$ ms (Fig. 5 *A*).

TTX-separated inward current components showed different potential dependences: the maximum of the I—V curve for the fast component was around −40 mV (Fig. 5 *B*); the maximum of the I—V curve obtained in solutions containing more than 10^{-6} mol/l TTX was between −25 and −20 mV (Fig. 4 *B*).

When Tris ions were substituted for sodium ions in the external saline (only Ca^{2+} ions were left as permeable ions) an inward current of a low amplitude was observed. Its inactivation process could be fitted by one exponential with a time constant of about 22 ms. The I—V curve maximum was between −25 to −20 mV (Fig. 6 *B*). The blocking action of TTX on this current in Na—Mg—K—Cl-free

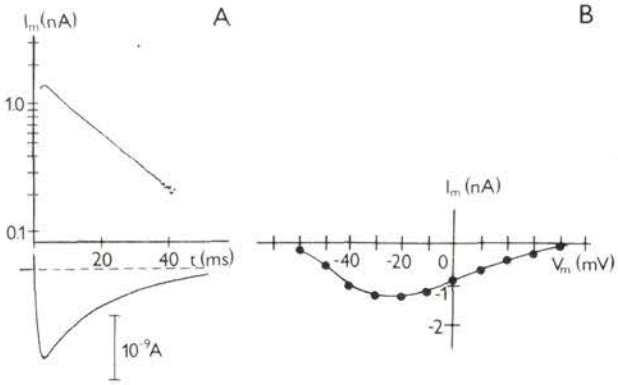

Fig. 4. *A*: Inward current in an external solution containing TTX 10^{-6} mol/l. Current in semilogarithmic representation (upper part). The inactivation process could be fitted by one exponential with $\tau = 21$ ms. $V_h = -120$ mV; $V_t = -20$ mV. *B*: I—V curve for currents in a solution with 10^{-6} mol/l TTX. $V_h = -120$ mV.

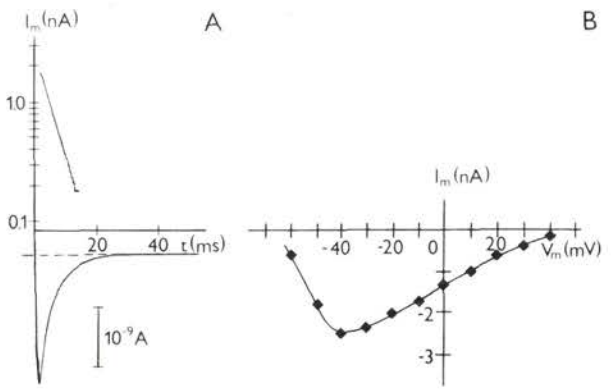

Fig. 5. *A*: The fast fraction of the inward current obtained in 10^{-6} mol/l TTX-containing solution from the initial current trace. Semilogarithmic representation of the difference current (upper part). $\tau = 4.6$ ms; $V_h = -120$ mV; $V_t = -20$ mV. *B*: I—V curve of the fast difference current. $V_h = -120$ mV.

solution could be fitted by a one-to-one binding curve (Fig. 7). The average $K_d = (7 \pm 2) \times 10^{-6}$ mol/l TTX (for 5 cells). The current observed in the solution containing only Ca ions (nonpermeable ions substituted for other ions) showed several similarities with the current observed in normal sodium-containing solution with TTX concentrations higher than 10^{-6} mol/l. It should be noted that the amplitude of the TTX-sensitive current component carried by Ca^{2+} ions was about 15 times lower than that of the current recorded in the Na-containing solution with 10^{-6} mol/l TTX.

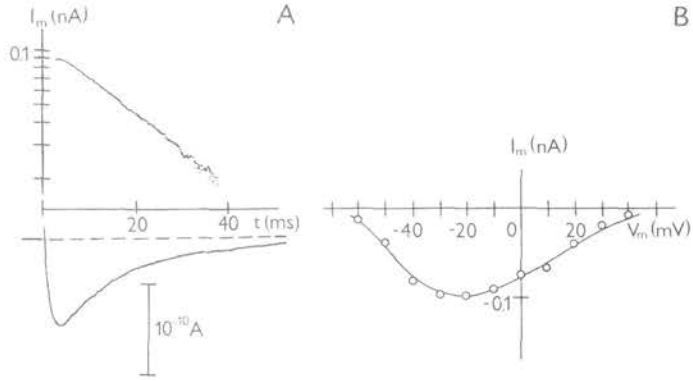

Fig. 6. *A*: The inward current in a solution containing Ca^{2+} ions (as the only permeable ions). Semilogarithmic representation of the current (upper part). $\tau = 22$ ms. $V_h = -120$ mV; $V_t = -20$ mV. *B*: I—V curve for the Ca^{2+} current as shown in *A*. $V_h = -120$ mV.

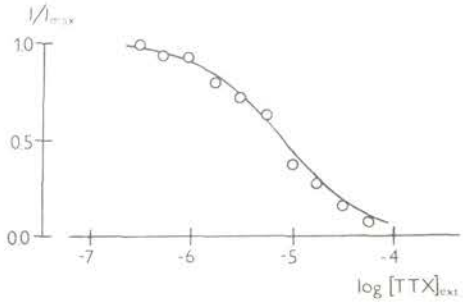

Fig. 7. One-to-one binding curve ($K_d = 7 \times 10^{-6}$ mol/l) representing the blocking action of TTX on inward current carried by Ca^{2+} ions (normalized). Ordinate: the ratio of the current at a given TTX concentration to the current in TTX-free solution. Abscissa: external TTX concentrations (logarithmic scale). $V_h = -120$ mV; $V_t = -20$ mV.

Discussion

Although the involvement of sodium ions in the process of the generation of action potential could already be suggested from the first experiments with interacellular microelectrodes (Weidmann 1956; Hoffmann and Cranefield 1960) sodium con-

ductance of the cardiac cell membrane is still far from being completely characterised and remains a subject of experimental investigations. The analysis of sodium current in multicellular preparations (Deck and Trautwein 1964; Dudel and Rüdel 1970) meets with considerable difficulties, mainly due to the unsufficient time resolution capacity of voltage clamp records (in the experiments on multicellular preparations the capacitance charge transient lasted for several ms: Fozzard 1966; Freygang and Trautwein 1977). The introduction of isolated myocardial cells in electrophysiological studies allowed an adequate recording of sodium currents (Lee et al. 1979; Undrovinas et al. 1980; Brown et al. 1981; Bodewei et al. 1982). However data on electrically gated sodium permeability are rather contradictory as far as the description of I_{Na} features in concerned. For example Brown et al. (1981) reported the maximum of I_{Na} I—V curve to be around -20 mV; the data presented by Undrovinas (1984) give the position of the I—V curve maximum on the voltage axis in the region of about -35 mV. Single sodium channel analysis (Grant et al. 1983) could not help clearing the problem either: these investigations were carried out in the "cell attached" mode; consequently the value of V_t represents the sum of the cell resting potential and the command potential. The actual value of V_t thus remains the subject of considerable contradictions.

The problem concerning the number of I_{Na} components in the cardiac cell membrane is far from being resolved. It was shown (Brown et al. 1981; Yatani et al. 1984) that the sodium current inactivation is a two-exponential process, with an approximately 4-fold difference in the time constants; this may suggest the existence of two kinds of sodium channels with different inactivation properties also.

The data obtained in the present study on membranes of isolated cardiomyocytes suggested the existence of inward sodium current components with different sensitivities to TTX. Two I_{Na} components could be distinguished in their kinetic properties (a 4-fold difference in inactivation time constants) and voltage dependence (a difference of about 15 mV in the position of the I—V curves maxima). The faster (and more TTX-sensitive) component disappeared in Na-free solution; this suggested that it was highly selective for Na^+ and was therefore labelled as fast sodium current (I^f_{Na}). The other (more slow) component persisted in Na-free solution (Ca^{2+} ions were left as the only permeable ions) and its amplitude diminished about 15 times in Ca^{2+}-containing Na^+-free solution. The inactivation kinetics, voltage dependence and TTX-sensitivity were the same as in Na^+-containing solution. This can be explained on the assumption that the channels passing this current are not highly selective and pass both sodium and calcium ions. This current is therefore not purely sodium in nature and was labelled as slow sodium-calcium current ($I^s_{Na,Ca}$). (It should be noted that Na^+—Ca^{2+}-free solution was used for intracellular perfusion in our experiments; i.e. the concentration

gradient for the above ions tended to approach infinity. At the same time, when sodium ions were removed from the extracellular solution and the quantity of charge carriers diminished by about 22 times the current carried solely by Ca^{2+} ions also diminished by 15 to 20 times. This suggested approximately equal selectivities to Na^+ and Ca^{2+} of this channel; the permeability ratio approached 1. This problem needs further detailed investigation).

In connection with the observed capacity of the $I^s_{Na,Ca}$-channels to pass Ca^{2+} ions, another point has to be discussed. It has been shown from studies of Ca^{2+}-dependent slow inward current (I_{si}) on multicellular cardiac preparations that I_{si} can be carried by both Ca^{2+} and Na^+ ions (see reviews by Trautwein 1973; Reuter 1979; McDonald 1982; Keung and Aronson 1983). On the other hand, investigations of the selective properties of Ca^{2+} channels in isolated myocardial cells (Lee and Tsien 1984; Matsuda and Noma 1984) showed an extremely high selectivity of Ca^{2+} channels. It can be supposed that the fraction of I_{si} carried by Na^+ ions reported in earlier experiments may have been a results of $I^s_{Na,Ca}$ channels functioning. Similar speculations can certainly only concern data on I_{si} separated from other currents by TTX (TTX concentrations of abour 10^{-5} mol/l were used for this purpose; these concentrations are insufficient to induce a complete block of $I^s_{Na,Ca}$).

The experimental results presented herein suggest the existence in the membrane of adult rat cardiomyocytes of two sodium current components differing from each other by their TTX sensitivities and other principal features. TTX could serve as a pharmacological tool to separate these currents.

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