A whole-cell patch clamp study of ionic currents in single atrial cells of the tropical toad Bufo marinus.

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A WHOLE-CELL PATCH CLAMP STUDY OF IONIC CURRENTS IN SINGLE ATRIAL CELLS OF THE TROPICAL TOAD *Bufo marinus*

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ABSTRACT

A simple enzymatic dissociation method is described to obtain isolated cells from the atrium of adult toad *Bufo marinus*. The electrical activity of single cells were recorded with the whole-cell patch clamp technique. Action potential characteristics and Na, K, and Ca currents are compared to those measured on uni- or multicellular cardiac preparations from other adult amphibians.

ESTUDIO DE LAS CORRIENTES IONICAS EN CELULAS CARDIACAS AISLADAS DEL SAPO *Bufo marinus*

RESUMEN

Se describe un método simple de disociación enzimática para obtener células aisladas de la aurícula del sapo *Bufo marinus*. La actividad eléctrica de las células aisladas se registró con la técnica del *patch clamp* de célula entera. Las características del potencial de acción y de las corrientes de Na, K y Ca se comparan con aquellas medidas en preparaciones de otros anfibios adultos.

INTRODUCTION

Electrical properties of isolated cells from cardiac tissue have been extensively studied over the past ten years. The use of intracellular microelectrodes has first demonstrated the effectiveness of the various dissociation procedures to maintain intact most of the ionic channels and transport processes of the sarcolemmal membrane. But it was not until the introduction of the patch clamp technique by Hamill *et al.*,[7] that it became possible to clearly identify and analyse, at single channel or whole-cell level, the ionic currents responsible for the electrical activity of the cell.

A large number of reports have described procedures for the dissociation of myocytes from neonatal or adult mammalian hearts (for review, see refs. 3 and 4). However, only few studies have dealt with dissociation procedures on amphibian hearts. Tarr and Trank[16] and Hume and Giles[9] have dissociated cells from adult frog hearts using both mechanical and enzymatic dispersion methods, while Arrio-Dupont and De Nay[1] and Fischmeister and Hartzell[6] have used an enzymatic perfusion method to obtain isolated cells from adult frog ventricle.

In the present study, we have used a very simple enzymatic dissociation procedure to obtain rod-shaped, Ca-tolerant myocytes from the atrium of the tropical toad *Bufo marinus*. This is the first study on cardiac isolated cells from this amphibian species. The electrical properties of the single cells were recorded with the whole-cell patch clamp technique using a single low-resistance pipette filled with an "intracellular" medium.[5-6] Action potential characteristics and Na, Ca and K currents from the *Bufo marinus* atrial cells resemble those recorded in various species of *Rana*.

MATERIAL AND METHODS

Solutions and drugs:

Normal Ringer solution contained (mM): NaCl, 88.4; KCl, 2.5; $NaHCO_3$, 24; NaH_2PO_4, 0.6; $MgCl_2$, 1.8; $CaCl_2$, 1.8; glucose, 5. Ca-free Ringer was prepared by simply omitting $CaCl_2$ from normal Ringer. Dissociation medium # 2 was composed of: Ca-free Ringer + 5 mM creatine + 10 µl/ml minimum essential medium "EAGLE" solution (Gibco, USA) + 1 mg/ml fatty acid-free BSA (Sigma, USA) + 100 IU/ml penicillin and 100 µg/ml streptomycin. Dissociation medium # 1 was composed of dissociation medium # 2 + 0.4 mg/ml trypsin (Sigma, type III) + 3 mg/ml collagenase (Boehringer Mannheim, F.R.G., # 103 586). Storage medium was composed of normal Ringer + 5 mM sodium pyruvate + 100 IU/ml penicillin + 100 µg/ml streptomycin. The pH of these solution was maintained at 7.4 by gassing with 95% O_2, 5% CO_2. Two experimental Ringer solutions were used, ES#1 and ES#2. ES#1 contained (mM): NaCl, 112; KCl, 2.5; $MgCl_2$, 1.8; $CaCl_2$, 1.8; glucose, 5; sodium pyruvate, 5; HEPES, 10; it was properly triteated to pH = 7.4 with KOH. The ES#2 differed from ES#1 by replacing all KCl by 20 mM

CsCl, this solution was triteated with NaOH. Tetrodotoxin (Sankyo, Japan) was added when required at 6×10^{-7} M. Two different electrode solutions were used, MIS#1 and MIS#2. MIS#1 contained (mM): KCl, 120; EGTA, 5; $MgCl_2$, 4; Na_2CP, 5; Na_2ATP, 3; HEPES, 10; pH = 7.1 (triteated with KOH). MIS#2 differed from MIS#1 by replacing all KCl by the same amount of CsCl, and was triteated with NaOH.

Preparations:

The dissociation procedure was inspired from Arrio-Dupont and De Nay[1] and Fischmeister and Hartzell.[6] Briefly, the heart was removed from the toad and washed in normal Ringer solution. The atrium was dissected, opened and the tissue separating the two atrial chambers was removed. This preparation was then placed in Ca-free Ringer and transferred, after 10 min, to 5 ml of dissociation medium # 1, where it was gently stirred under a gassed atmosphere at 30 °C. After 30 to 50 min of enzymatic dissociation, single cells were present in the medium. The atrium was then placed in 5 ml of dissociation medium # 2 containing no collagenase or trypsin, where it was gently stirred under gassed atmosphere for 30 min. After that time, non dissociated tissue was discarded and 1 ml of the final suspension was aliquoted into ordinary (non sticky) Petri dishes (Falcon, USA) and diluted with 3 ml of storage medium. The cell containing dishes were stored in a sterile gassed atmosphere for 1 to 32 hours prior to experiments.

Experimental setup:

We used a similar setup to that described by Fischmeister *et al.*[5] and Fischmeister and Hartzell.[6] Briefly, the cell-containing dishes were placed on the stage of an inverted-optics microscope (Nikon, Japan, model Diaphot) at room temperature (22 to 25 °C). Patch electrodes (1.5 to 3 Mohms) were prepared from glass micropipettes (Drummond, USA, 100 µl "microcaps"), filled with MIS#1 or MIS#2 solutions, and used with an extracellular patch clamp system (Biologic, France, model RK 300). The liquid junction potentials between the interior of the electrode and the bat were compensated. After the electrodes was successfully sealed to an unattached cell, the patch was disrupted by mouth suction for whole-cell recording.[7] The cell could be positionned at the extremity of one of four micro-capillaries (I.D. 300 µm) independently perfused by gravity (20 µl/min) allowing rapid changes (< 5 sec) in the extracellular medium.

Data analysis:

Current and voltage recordings were displayed on a digitizing oscilloscope (Tektronix, USA, model 5223) and all measurements were made from photographs taken from the screen (Nihon Kohden Kogyo, Japan, model PC-2A). Currents were low-pass filtered through 1 KHz or 3 KHz 5-pole Tchebicheff-response filters provided on the patch clamp system.

RESULTS

Figure 1A shows a micrograph of a typical myocyte isolated from toad atrium with a patch-electrode sealed on its external membrane. Cell length ranged from 150 to 300 µm and maximal diameter was 3 to 8 µm. Although the cells were quiescent, action potentials developed when a depolarizing current was injected inside the cell with the patch electrode in whole-cell current clamp configuration. Figure 1B shows such an action potential obtained on a cell bathed in normal Ringer solution with a patch electrode containing MIS#1. Action potential characteristics were determined under these conditions in 6 cells: maximal action potential amplitude was 115 ± 9 mV, action potential duration (at 90% repolarization) was 481 ± 70 ms, maximal diastolic potential (i.e. resting potential) was −87 ± 6 mV (mean ± s.d.). Ca action potentials were observed when the cellular membrane was first depolarized to −50 mV holding potential under voltage clamp conditions and the system was switched back to current clamp conditions (figure 1C). Due to the inactivation of the fast inward Na current by the predepolarization, Ca action potentials were slower, with a lower maximal amplitude and a more pronounced plateau phase.

The ionic currents responsible for the development of the toad atrial action potential were studied under voltage clamp conditions. The cell interior was maintained at a constant holding potential (−80 mV or −50 mV) while the external bath was connected to 0 mV signal ground potential. From this holding potential, the cell was polarized every 4 sec, during 200 or 400 ms, to various depolarizing or hyperpolarizing levels.

Fast Na inward currents were observed upon depolarizations above −50 mV from −80 mV holding potential (figure 2B). Time to peak decreased as the membrane potential became more depolarized (figure 2A) and was in the order of 1 ms at −10 mV. Maximal current was observed approximately at this potential as shown on the peak current-voltage relationship (figure 2B). No fast inward current was observed above + 40 mV which corresponds approximately to the equilibrium potential for Na ions with the ionic Na concentrations used in the extracellular and intracellular solutions (see Methods). Maximal amplitude of the fast Na current could reach 1 to 2 nA per cell. This current was completely blocked by 6×10^{-7} M extracellular tetrodotoxin (TTX), while incomplete blockade was observed at half concentration.

When the fast Na inward current was blocked with TTX or inactivated by a depolarizing holding potential at −50 mV, a slower Ca inward current was observed. A current record obtained after TTX treatment is shown in the inset of figure 3. The peak inward current in this case had a much smaller amplitude than the Na current. Maximal peak inward Ca current occured near 0 mV and reached 50 to 150 pA per cell. At this potential, the current peaked in 5 to 10 ms and then completely inactivated in less than 200 ms (see inset figure 3).

The peak current-voltage relationship of figure 3 (fill circles), shows that no Ca current was activated below −40 mV depolarization and that reversal potential for that current was near + 30 mV.

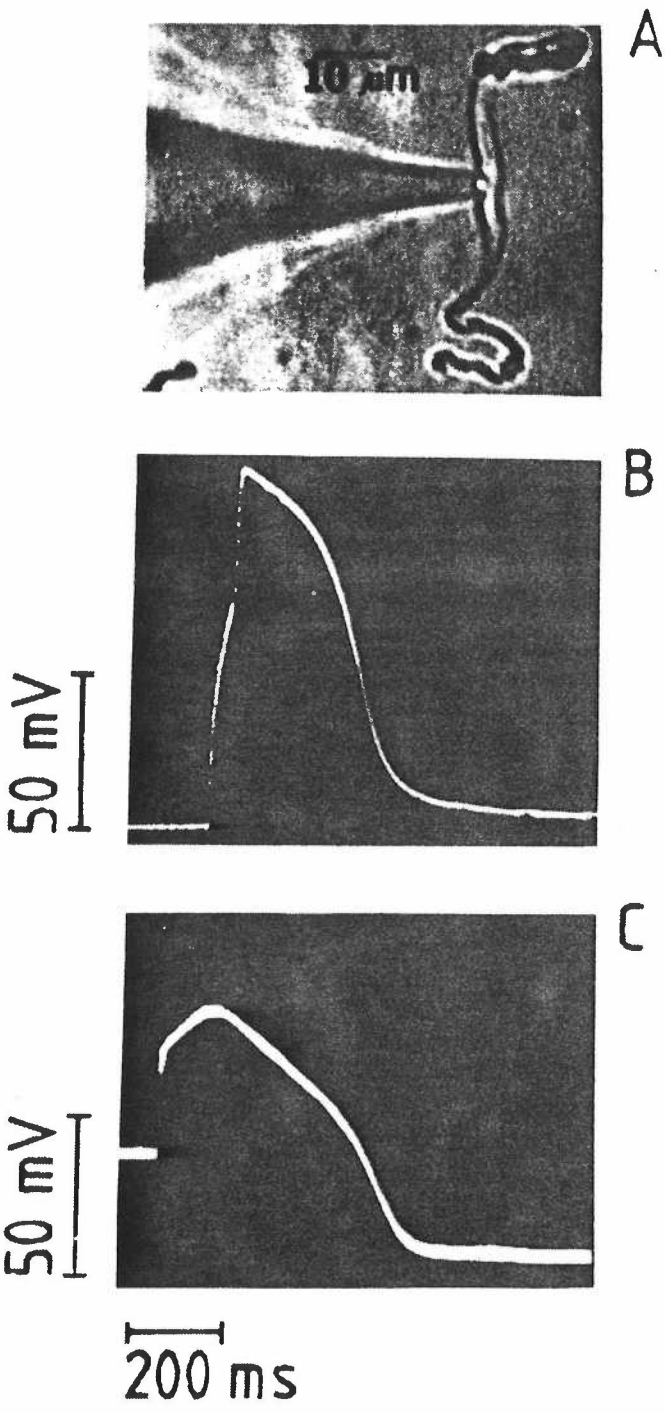

Figure 1. (A) Micrograph of a cardiac cell isolated from toad atrium and a patch electrode sealed to its membrane. (B) Action potential obtained in a cell in normal Ringer solution. The resting potential was −80 mV. (C) Action potential obtained in a cell depolarized to −50 mV. 200 ms bar applies to B and C.

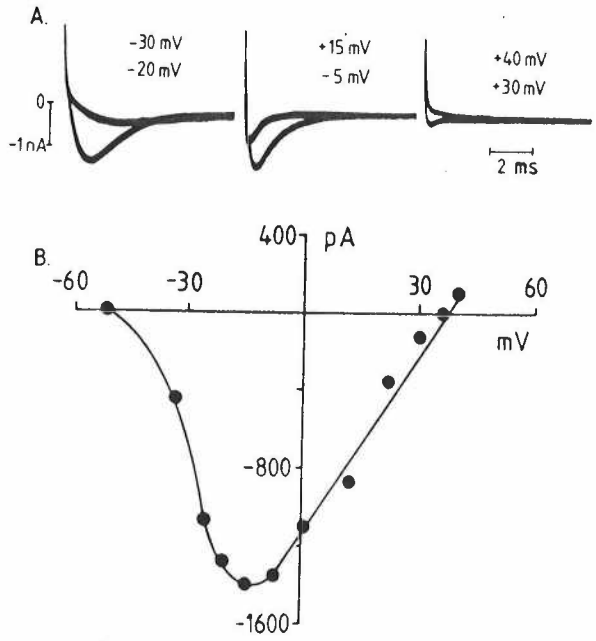

Figure 2. Sodium currents in single cells from toad atrium. (A) Records of sodium currents obtained with different depolarizations from a holding potential of −80 mV. Actual values of membrane potential are given for each record. (B) Peak current-voltage relationship obtained in the same cell. Curve fitted by eye.

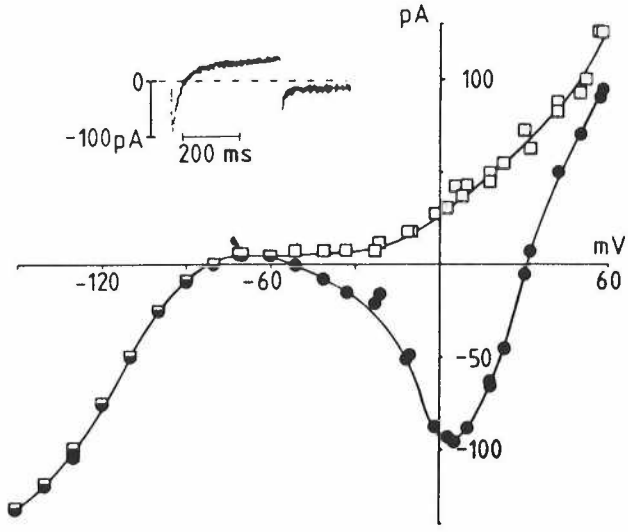

Figure 3. Current components in single cells from toad atrium. Inset: current record obtained in a cell in experimental solution # 1 with 6×10^{-7} TTX, depolarized to + 3 mV by a 400 ms pulse. Holding potential − 80 mV. (B) Current voltaje realtions obtained from peak currents (•) and currents at the end of the pulse (□) in records similar to the one shown in (A).

K currents were measured at the end of 400 ms pulses. For negative potentials, this measurement gives essentially the background K current (see squares in figure 3) while,

for more positive potentials, both background and time dependent K currents are measured. Figure 3 shows a strong inward rectification of K currents between −150 and −50 mV. Input resistance of the cell was measured at −80 mV from the slope of the background current at this potential and ranged from 500 to 1,200 Mohms.

A more complete study of Ca current was undertaken in conditions where contamination by other ionic currents was totally eliminated. While TTX was used to block fast Na inward current, Cs ions were used in place of K ions to block all K currents.[13-5-6] For this purpose, the cells were bathed in ES#2, containing 6 x 10^{-7} M TTX and the patch electrode was filled with MIS#2 containing Cs instead of K (see Methods). Figure 4 (inset) shows an individual current trace obtained with a depolarization of 78 mV and 400 ms duration from −80 mV holding potential. Ca current is the only time and voltage dependent current left functional under such conditions (see fill circles in figure 4). The inset of figure 4 also shows that after perfusing the cell with 10^{-4} M $CdCl_2$, this current completely disappeared and left a small time and voltage independent background current. The current-voltage relationship for peak current and current at 200 ms in the absence and presence of 10^{-4} M $CdCl_2$, is shown in this figure. The background current (square in figure 4), is a linear function of membrane potential and all the rectification that was observed in ES#1 (square in figure 3) is eliminated by Cs. Besides, the amplitude of the background current is strongly diminished in Cs. This leads to a larger cell input resistance which ranged from 1 to 3 Gohms at −80 mV. Zero-current potential (i.e. resting potential) was −60 ± 5 mV (mean ± s.d., N = 6). The peak current-voltage relationship in the presence of Cd in the external solution (open circles in figure 4) was identical to that for the current measured at 200 ms. The net amplitude of the Ca currents were obtained by two differents procedures: In one case the Ca current is given by the difference between the peak value of total currents obtained in the absence and in the presence of Cd (fill circles in figure 5). In the other case, when Cs has been added inside and outside of the cell, and Cd is not present, the net amplitude of the Ca current becomes identical to the difference between the value of the peak current during the first miliseconds and the value of the current at 200 ms after the application of the pulse (squares in figure 5). It can be seen that the use of a Ca blocker is not required to accurately measure net Ca current in our conditions. The peak current-voltage relationship of figure 5 shows that no Ca current was activated below −40 mV depolarization and that reversal potential for that current was near + 55 mV. This is less than expected from the calculated equilibrium potential for Ca ions under our conditions and could result either from a participation of other ions to the total inward current measured[14] (but see ref. 11) or to an accumulation of Ca ions at the inner side of the membrane.

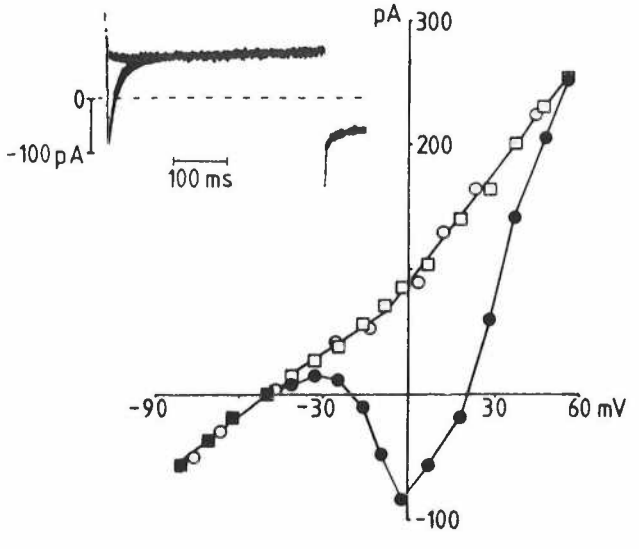

Figure 4. Measurement of I_{Ca} in single cells isolated from toad atrium. Inset: current records obtained in the presence (upper) and the absence (lower) of 0.1 mM $CdCl_2$, using a depolarizing pulse of 400 ms duration which changed the membrane potential to −2 mV. B. Current voltage relationships of currents in the presence and absence of 0.1 mM $CdCl_2$. Currents were elicited by 400 ms voltage steps from a holding potential of -80 mV. (●) Peak current in absence of $CdCl_2$. (□) Quasi-steady state current at 200 ms (I_{200}) in the absence of $CdCl_2$. (○) Peak currents and I_{200} in the presence of 0.5 mM $CdCl_2$.

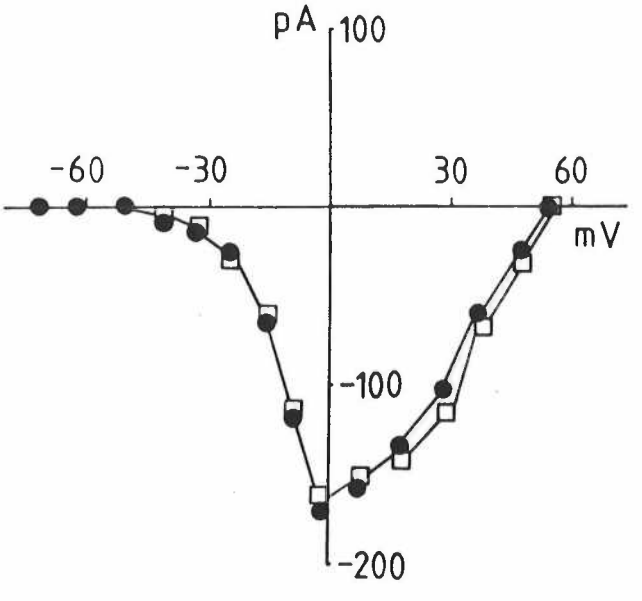

Figure 5. Net I_{Ca} of toad atrial cells. (○) Net I_{Ca} determined by substracting I_{200} (squares in figure 4) from peak currents (filled circles in figure 4). (●) Cd-sensitive current obtained by substracting peak current in the presence of Cd (filled circles in figure 4) from the peak currents in the absence of Cd (empty circles in figure 4).

Inactivation of Ca current was studied using a double pulse protocol.[13-6] Every 4 sec, a 200 ms prepulse to various potentials ranging from −70 to +110 mV was followed by a 200 ms test pulse to 0 mV (figure 6A). Prepulse and test pulse were separated by a 5 ms return to −80 mV holding potential. The degree of inactivation achieved at the potential of the prepulse is determined by the diminution of the Ca current measured during the 0 mV test pulse as compared to the current at 0 mV in the absence of prepulse. Inactivation was absent below −60 mV and was almost complete at −20 mV with half-inactivation occuring between −30 and −40 mV. With prepulses above 0 mV, inactivation was reduced due to a reduction in the influx of Ca current as the membrane potential is approaching the reversal potential for the Ca current. This behavior of the Ca current, originally described in the molluscan neuron by Tillotson[17] was attributed to a Ca-mediated inactivation process.[13]

Reactivation (recovery from inactivation) of Ca current was studied at −80 mV using an other double pulse protocol. The cell was depolarized every 4 sec to 0 mV for a duration of 200 ms (prepulse), return to −80 mV holding potential for various intervals ranging from 20 to 500 ms, and then depolarized by a second 200-ms pulse to 0 mV (test pulse). The ratio between the amplitude of Ca current elicited by the test pulse and Ca current elicited by the prepulse is plotted as a function of the interval of time between the two pulses (figure 6B). The reactivation followed approximately an exponential function of time, half reactivation time being about 80 ms at −80 mV and 23 °C.

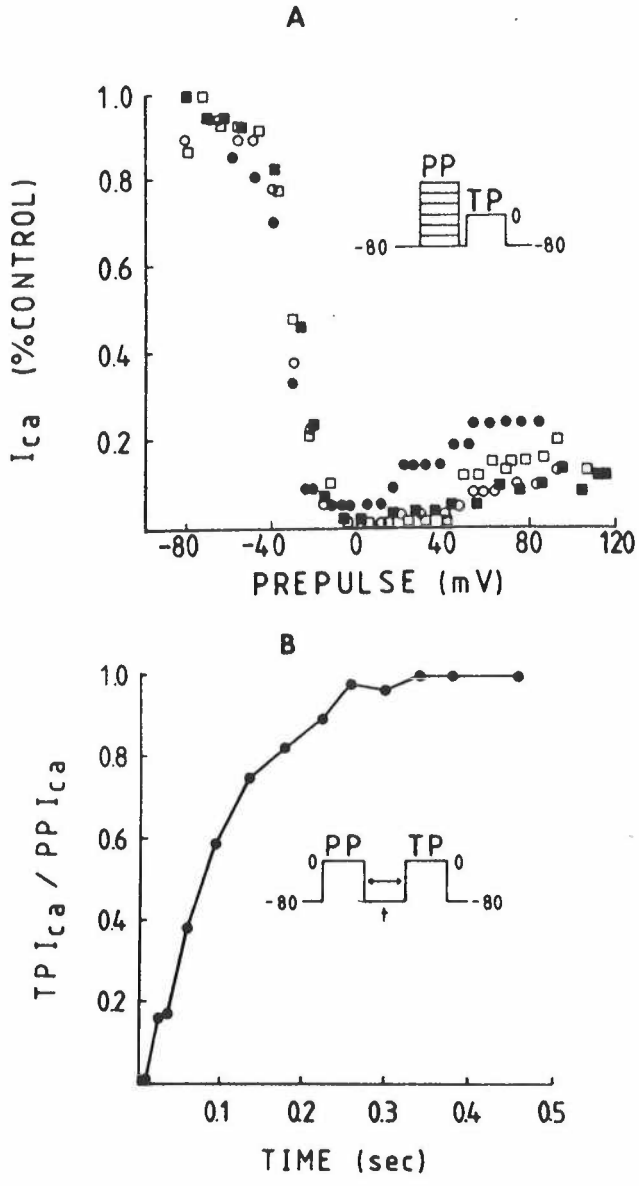

Figure 6. Inactivation and reactivation of I_{Ca}. (A) Inactivation as measured by a double pulse protocol. The amplitude of I_{Ca} elicited by a test pulse (TP) is plotted as a percentage of the I_{Ca} evoked by a step from −80 to 0 mV in the absence of a prepulse. The symbols correspond to different cells. (B) Reactivation. The ratio between the amplitude of I_{Ca} elicited by a test pulse (TP) and I_{Ca} elicited by a prepulse (PP) is plotted as a function of the interval between the pulses. The results are from a single experiment.

DISCUSSION

Cell dissociation techniques provide useful tools for correlating cellular properties to the function of the whole organ. Besides, they allow a more direct access to the fundamental mechanisms regulating the physiological properties of the cells. However, before starting a complete investigation on one of those mechanisms, it is necessary to verify that the properties of the single cells are being unaffected by the dissociation as well as by the recording methods.

Here we present a simple enzymatic dissociation technique for obtaining single atrial cells from the adult tropical toad *Bufo marinus*. The method gives a large number of rod-shaped, Ca-tolerant cells that anatomically resemble cells dissociated from other amphibian species.[1-10-16] The electrical properties of the cells were recorded using the whole-cell patch clamp technique.[7] Action potential characteristics and ionic currents were recorded with low resistance patch electrodes filled with a solution designed to mimic the intracellular medium.[5-6]

Action potential characteristics of toad atrial cells are similar to those recorded with the double sucrose gap technique on isolated bundles of atrial cells from *Rana pipiens* or *Rana esculenta* (e.g. refs. 15, 8 y 12). They also resemble those recorded on single bullfrog *(Rana castebiana)* atrial cells using a smaller patch electrode filled with 3 M KCl.[9] However, our mean action potential duration (481 ms) was significantly shorter than that measured by Hume and Giles (722 ms)[9] using a higher extracellular Ca concentration.

Na and K currents recorded here do not differ from those obtained in single bullfrog atrial cells.[10] Also, similar Ca currents to those presented here were recorded in single frog atrial cells bathed in normal Ringer solution.[2-10] When Cs ions are substituted for K ions inside and outside a toad atrial cell, the Ca currents resemble those recorded under the same conditions in single ventricular cells from *Rana esculenta*.[5-6] Besides, inactivation and reactivation properties of Ca current were found similar to those recorded with the double sucrose gap technique on multicellular preparations from frog atrium.[13]

In conclusion, toad atrial tissue could be easily dissociated into single cells. These cells possess electrical properties that resembled those recorded on other amphibian species, both from single cells and from multicellular preparations. Thus, it seems that our dissociation and recording techniques are suitable for more advanced studies of the mechanisms underlying the cardiac electrical activity.

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