

Experimental Aspects of Isolation of Myocytes from Adult Guinea-pig Hearts

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Abstract. Ionic channels can now be effectively studied on enzymatically isolated cardiac myocytes by means of the patch clamp technique. Three procedures reported to give consistently high yields of Ca-tolerant myocytes were tested for applicability to calcium channel studies under our laboratory conditions. None of them was found to be suitable for direct use. Therefore, a modified method for isolation of myocytes from adult guinea-pig hearts was developed. Calcium channel currents measured in Ca-tolerant myocytes isolated by this procedure have been presented and problems of myocytes isolation and of patch-clamp measurement discussed.

Key words: Guinea-pig heart — Ca-tolerant myocytes — Patch clamp — Calcium currents — Enzymatic digestion

Introduction

Isolated heart muscle cell is currently a very popular preparation to study ionic channels (Noble and Powell 1987). This popularity arose as a consequence of two methodological breakthroughs: first, isolation of cells from cardiac tissue using enzymatic digestion (Dow et al. 1981; Watanabe et al. 1986), and, second, invention of the patch-clamp technique (Hamill et al. 1981; Sakmann and Neher 1983). Single cell studies of this type opened a new era in cardiac electrophysiology (Noble 1984; Reuter 1984; Tsien et al. 1987).

For those entering this field, patch clamp is the technique of unanimous vote. On the other hand, to choose the most suitable method of enzymatic digestion can be a problem. In different laboratories, myocytes tolerant to (at least) physiological concentrations of Ca^{2+} ions are prepared using different procedures or their modifications and little is reported regarding comparative aspects (Farmer et al. 1983).

The aim of this paper is to report our experience with three substantially differing methods used to isolate myocytes from adult guinea-pig hearts and to

describe the technique which we found to be most convenient under our conditions.

Part of this work has been presented in preliminary form (Zahradniková et al. 1988).

Materials and Methods

Isolation of cells

Guinea pigs of either sex, weighing 200–400 g were used. Collagenase type I (Sigma, USA) or Collagenasa cruda (USOL, Czechoslovakia); hyaluronidase (Serva, FRG or USOL, Czechoslovakia); pronase E (Koch-Light, Great Britain); bovine serum albumin (Armour, USA or Imuna, Czechoslovakia) and aprotinin (Serva, FRG or Léčiva, Czechoslovakia) were from the sources given in parentheses. Eagle's MEM (USOL, Czechoslovakia) was supplemented with 10% horse or fetal calf serum (Serva, FRG or Institute for veterinary studies, Czechoslovakia); when 100% O₂ was used to saturate solutions, NaHCO₃ in the medium was replaced by 5 mmol/l HEPES (pH adjusted to 7.35 with NaOH). Other chemicals used were of the highest purity available.

Isotonic solutions prepared from analytical grade NaCl were found to contain up to 75 $\mu\text{mol/l}$ Ca²⁺. Therefore, for further use NaCl was recrystallized twice from bidistilled water. The first crystallization was in the presence of 3 mmol/l EDTA (pH adjusted to 7.0 with NaOH).

The tonicity of solutions was measured using a semimicroosmometer (Knauer, FRG), and the concentration of free Ca²⁺ was measured with a Ca-selective electrode (Radiometer, Denmark). Calibrating solutions for the Ca-selective electrode contained 150 mmol/l NaCl and 10 mmol/l HEPES (pH adjusted to 7.4 with NaOH) as the background electrolyte.

The enzyme solutions were filtered through a Millipore filter (pore size 0.22 μm); other solutions used for perfusion were filtered through a sintered glass filter (pore size 4–16 μm). Solutions for the isolation procedures according to Kao et al. (1980), Isenberg and Klöckner (1982), and Mitra and Morad (1985) were prepared as described in the respective original reports (cf. Table 1). The composition of solutions used for the resulting method is given in Table 2, and the flow chart of the procedure is shown in Table 3.

Animals were fasted overnight. If used, heparin (500 U/100 g) and sodium pentobarbital (10 mg/100 g) were administered intraperitoneally. The temperature of the heart perfusion and incubation media was 37° C with the solutions being saturated either with 95% O₂ — 5% CO₂ or with 100% O₂. The hearts were perfused in a retrograde manner on a Langendorff-type apparatus (column height ~ 60 cm) with a four-way valve and flow regulation. The cells were harvested by centrifugation (65 g/3 min).

Viability of the cells was assessed by determining the fraction of rod-shaped, non-beating cells at magnification 64 \times , and by inspection of their morphology at magnification 640 \times (striation, presence of blebs, granulation, and rounded edges) in "enzyme" solution (immediately after dispersion) and in Eagle's MEM (after isolation and 30 min later). The ability of cells to form tight seals and the presence of functional Ca channels either in cell-attached patches or in the whole-cell configuration were definite criteria.

Experimental setup

Cells were placed in a perfusion chamber (volume 300 μl) which allowed rapid switching between several (up to 6) solutions. The exchange of the solutions surrounding the measured cell was

Table 1. A brief overview of the compared isolation procedures.

METHOD	FIRST PERFUSION O ₂ , 37°C	LOW Ca PERFUSION O ₂ , 37°C	ENZYMATIC PERFUSION O ₂ , 37°C	STABILISATION
Isenberg and Klöckner (1982)		5 min 1 μ M Ca ²⁺ pH 6.9 20 mM taurine 5mM pyruvate	20 min 30 μ M Ca ²⁺ pH 6.9 "low Ca" + 0.1% collagenase 0.1% hyaluronidase	perfus. 37°C, 5 min storage 4°C 0.03 μ M Ca ²⁺ pH 7.2 "Kraftbrühe" with isotonic K ⁺
combined Kao et al. (1980) and Farmer et al. (1983)	5 min 1mM Ca ²⁺ pH 7.4	5 min 10 μ M Ca ²⁺ pH 7.4	perfus. 5 min incub. 2 \times 10 min 50 μ M Ca ²⁺ pH 7.4 60 mM taurine 0.1% collagenase 0.04% hyaluronidase	transfer in 3 steps into MEM storage room temp.
Mitra and Morad (1985)		5 min 10 μ M Ca ²⁺ pH 7.3	8 min 200 μ M Ca ²⁺ pH 7.3 0.2% collagenase 0.03% pronase	perfus. 37°C, 5 min storage 37°C 200 μ M Ca ²⁺ pH 7.3

complete within 5 s. Experiments were carried out at laboratory temperature (25°C).

Ionic currents were measured using the technique of patch clamp (Hamill et al. 1981). Pipettes from borosilicate glass, pulled in two steps and fire-polished to o.d. 1.5–2.0 μm were used. Their resistance was 1.5–2.5 M Ω . For cell-attached recordings, the pipettes were coated with Sylgard prior to fire-polishing. Single-channel and whole-cell currents were measured using a List Electronic EPC-7 apparatus (FRG), compensated for linear current components, and filtered at 3 kHz (integral Ca currents) or at 1 kHz (single channel currents). Data acquisition and analysis were carried out using a laboratory work station connected to a mainframe computer, equipped with a 12-bit laboratory interface unit (Karhánek et al. 1987; Zahradniková 1987).

Results

Comparative experiments

The efficiency of three different procedures for yield of Ca-tolerant myocytes from the heart of adult guinea-pig was tested; the methods included those described by Isenberg and Klöckner (1982), Kao et al. (1980), and Mitra and Morad (1985). The common features of all these methods include retrograde perfusion of the excised heart and the use of collagenase as the main proteolytic enzyme. Substantial differences concern concentrations of free Ca^{2+} in individual solutions, supplements used to fortify the perfusing solutions, and the cell stabilising step (see Table 1).

Although effort was spent to follow the original procedures as closely as possible, some deviations of minor importance were introduced. First, the animal was always killed by cervical dislocation, then the heart was quickly dissected and cleaned in oxygenised Krebs-Henseleit (K-H) solution (with heparin added) at 37°C. Next, perfusion always started with the K-H solution without supplements for 5 minutes, which was sufficient to wash out the blood. The "low Ca" solution in Kao's procedure was also without supplements (Farmer et al. 1983; B. Nilius, personal communication). The solutions contained 25 mmol/l sodium bicarbonate and were saturated with 95% O_2 — 5% CO_2 . From the procedure of Isenberg and Klöckner (1982), β -hydroxybutyrate was omitted from the KB medium. The same batch of collagenase (Sigma, type I) was used in all three procedures.

The best yields of Ca-tolerant myocytes were obtained with the procedure of Isenberg and Klöckner (1982): about 80% rod-shaped cells (16 experiments). Only slightly worse results were obtained with the simplified procedure of Kao et al. (1982), but at a much lower price and within a shorter time (~60%, 19 experiments). The method of Mitra and Morad (1985) did not give satisfactory results in our hands (~20%; 5 experiments). In contrast to their experience, strong contractions of the perfused heart were always observed in our experiments after switching from "low Ca" to "enzyme" solution (0.2 mmol/l free

Ca²⁺ as recommended; pH was carefully set to 7.3). It is very probable that the batch of collagenase and/or pronase used in our experiments was not suitable for this procedure.

The procedures according to both Isenberg and Klöckner (1982) and Kao et al. (1980) gave acceptable results as concerns the amounts and the morphological appearance of dissociated myocytes. Tight seal contacts ($\sim 10\text{ G}\Omega$) were obtained with a sufficiently high frequency ($> 60\%$ of attempts), the method of Isenberg and Klöckner (1982) being slightly better in this respect. However, independent of the isolation procedure, Ca currents in the whole-cell configuration were often small (about 0.5 nA), they disappeared within 5–10 minutes, and sometimes were not even measurable. At the same time, integral Na and K currents were well preserved, even in rounded cells. In the cell-attached mode, with 110 mmol/l BaCl₂ in the pipette, single Ca channel currents were seen only occasionally. Most often only large currents, resembling membrane or seal instabilities, eventually similar to activity of several high-conductance channels were observed. Their polarity reversed at zero membrane potential.

Tuning of the method

General

Based on the above experience we decided to adapt the procedure of Kao et al. (1980), simplified according to Farmer et al. (1983) for our laboratory conditions. Three problems remained open for solution: First, about 30% of isolations were not successful (not included in the above evaluation). Second, for the sake of convenience, the isolation procedure had to be adapted so as to employ enzymes and other biochemicals of home production (USOL, Imuna, and Léčiva, Czechoslovakia). Third, the procedure had to be further developed to supply cells consistently suitable for Ca-channel studies.

To solve these problems, careful checkups of all experimental steps (Watanabe et al. 1986) was included as described in Methods. With respect to the multifactorial nature of this task we did not attempt evaluating the effects of individual modifications quantitatively. Rather, individual steps were evaluated subjectively.

The first problem, a large percentage of unsuccessful isolations (yields $< 40\%$) was found to be partly due to faults in the perfusion procedure (incomplete blood washout from the ventricular cavity, prolonged exposure to "low Ca" solution, obstruction of the coronary arteries by air bubbles) and to the composition of solutions (pCa, osmolality, missing constituents). Neverthe-

Table 2. The composition of the solutions used for cell isolation.

	"K-H"	"low Ca"	"enzyme 1"	"enzyme 2"
NaCl	145	145	145	145
KCl	2.7	2.7	2.7	2.7
KH ₂ PO ₄	1.4	1.4	1.4	1.4
MgSO ₄	1.4	1.4	1.4	1.4
CaCl ₂	1	0	0	0
HEPES	10	10	10	10
EGTA	0	0	0.1	0.1
glucose	20	20	20	20
taurine	0	0	60	60
collagenase	0	0	0.1%	0.1%
hyaluronidase	0	0	0.04%	0.04%
albumin	0	0	1%	3%
aprotinin	0	0	500 TIU/ml	500 TIU/ml
pH	7.35	7.3	7.3	7.3
free Ca ²⁺	1	0.005	0.06	0.06
tonicity	310	310	370	370

The concentrations are in mmol/l, if not indicated otherwise.

less, other factors were also disclosed as important: Hearts of animals with body weight exceeding 300 g gave lower yields of viable cells, and the ability of the cells to form tight seals was reduced. Killing of animals under deep pentobarbital anesthesia turned to be much more suitable than cervical dislocation, as the latter often resulted in the appearance of ischemic regions. Heparinisation of the animal and filtration of the perfusing solutions were also found to be beneficial. Surprisingly, but consistently, yields above 10% were not obtained if solutions were prepared with high quality deionized water (0.055 μ S/cm; Barnstead — NANOpure) instead of bidistilled water. Quite opposite experience was reported by Lee (1987). The perfusion solutions and Eagle's MEM were prepared without NaHCO₃, with 5 mmol/l HEPES, and were saturated with pure O₂.

Enzymatic digestion

If collagenase type I in the "enzyme" solution was simply replaced with collagenasa cruda, contractions of the perfused heart were elicited and whitening of the muscle was observed. As a result, all dissociated cells were damaged. Two major causes were found to account for the above observations:

1) SDS — polyacrylamide gel electrophoresis of both enzyme types before and after 30 min incubation at 37° C revealed that collagenasa cruda is relatively rich in middle molecular weight proteins. These proteins had proteolytic activity as was seen from increased number of bands in the low molecular weight region

after the incubation. The adverse effect of the contaminating proteases was suppressed by dissolving collagenasa cruda in cold "low Ca" solution (cf. Table 2) containing 500 TIU/ml of aprotinin, and by placing the solution in the perfusion column 2 min prior to its application to the heart.

2) Determination of free Ca^{2+} concentration in collagenasa cruda showed about 5 times higher contents of Ca^{2+} than in type I collagenase; in a 0.1% solution, free Ca^{2+} ranged from 150 to 200 $\mu\text{mol/l}$. The concentration of free Ca^{2+} in the "enzyme" solution was lowered by additions, under pCa monitoring, of a stock EGTA solution (10 mmol/l EGTA and 1 mmol/l CaCl_2 , pH adjusted to 7.3 with NaOH). Setting free Ca^{2+} below 30 $\mu\text{mol/l}$ led to a decrease of collagenase activity and decreased cell yields. The optimal concentration of free Ca^{2+} was found to be 50–70 $\mu\text{mol/l}$. Under these conditions, heart contractions were short-lasting and very weak (just visible), and the activity of collagenase was sufficient to disperse the heart tissue.

Hyaluronidase, aprotinin and albumin of either source were found to be equally suitable for use in the "enzyme" solution.

Low Ca treatment

The formulation of the "enzyme" solution was optimised using "low Ca" solution containing 10 $\mu\text{mol/l}$ free Ca^{2+} , applied for 5 min during the second perfusing step.

The treatment of the heart with "low Ca" solution weakens intercellular contacts. If this process is thought of as a chemical equilibrium, then it must be faster upon a greater shift from the equilibrium. With a fast loosening of the contacts the time during which the cell can be damaged (e.g. by Na overload) is shortened. This reasoning led us to decrease both the free Ca^{2+} concentration in "low Ca" solution and the time of its perfusion. With free Ca^{2+} set to about 5 $\mu\text{mol/l}$, 4 minute perfusion was found sufficiently long. Of this time, 2–3 minutes at a perfusion rate of 2 drops/s are needed for a complete wash-out of Ca^{2+} from the heart, as was measured with a Ca-selective electrode. Heart swelling was observed at the end of the fourth minute, thus indicating loosening of the cell-to-cell contacts.

If the "low Ca" solution containing 5 $\mu\text{mol/l}$ free Ca^{2+} was applied more than five minutes, the yield of rod-shaped cells was relatively high but the tolerance to millimolar Ca concentrations was strongly decreased, probably because of Na overload. On the other hand, if Ca concentration in "low Ca" solution was increased to 20 $\mu\text{mol/l}$, intercellular contacts were not made completely free within 5 minutes. As a result, cells dissipated after enzyme treatment were all hypercontracted. It may be supposed that the cells were mechanically damaged by contractions at the beginning of perfusion with the enzyme.

Table 3. Description of the isolation procedure.

Step No.	Description
1	Heparin 50 U/100 g i.p., wait 30–60 min; sodium pentobarbital 10 mg/100 g i.p., wait 4–10 min
2	Dissect the heart; transfer to “K-H” solution with heparin; clean from fat, veins and pericardium; cannulate aorta to perfusing apparatus; 37°C; O ₂
3	Perfuse with “K-H” solution at 60 cm pressure until blood washout (2–5 min); 37°C; O ₂
4	Perfuse with “low Ca” solution at 2 drops/s for 4 min; 37°C; O ₂
5	Perfuse with “enzyme 1” solution at 1 drop/s for 5 min; 37°C; O ₂
6	Dissect ventricles and cut them open along the septum; shake gently in “enzyme 1” solution (30 s; 37°C; O ₂); evaluate dispersed cells microscopically. If yield < 20%, stop isolation.
7	Incubate the remaining tissue in 10 ml of “enzyme 2” solution (37°C; O ₂ ; shake gently). Every 5 min transfer undigested tissue to fresh enzyme and go to next step with the dispersed cells.
8	Filter the suspension through a nylon mesh; dilute with 10 ml of MEM (37°C; O ₂) and sediment at 65 × g (3 min). Disperse the pellet in 10 ml of MEM (37°C; O ₂) and sediment as before. Finally, disperse the pellet in MEM and place in monolayers on Petri dishes. Store at laboratory temperature. 3–5 fractions of cells are usually obtained.

In steps 1 to 3, the heart was beating continuously.

The composition of the “K-H”, “low Ca”, “enzyme 1”, and “enzyme 2” solutions is given in Table 2.

Stabilization of cells

Freshly dissociated myocytes are not equally tolerant to calcium. This is not essential for electrophysiological experiments, where healthy looking cells can be selectively chosen for measurements. Thus, we found sufficient to follow the combined procedure of Kao et al. (1980) and Farmer et al. (1983). The tolerance to calcium had not to be increased by incubating the myocytes in enriched KB medium (Isenberg and Klöckner 1982).

Myocytes suspended in the “enzyme” solution were transferred in three steps to Eagle’s MEM supplemented with horse serum (Table 3). By this technique isolated myocytes were washed off of enzymes, cellular debris, and badly damaged cells. Suspensions of purified myocytes from individual digests were finally placed on Petri dishes and kept at laboratory temperature (25°C) at least for 30 min before the experiments. Sometimes healthy cells failed to give tight contacts with patch pipettes at these early times. Instead, after applying suction to the pipette, resistance increased to 10–20 MΩ only and then the membrane ruptured. After standing for ~2 hours, the cells stabilised and readily formed gigaohm contacts.

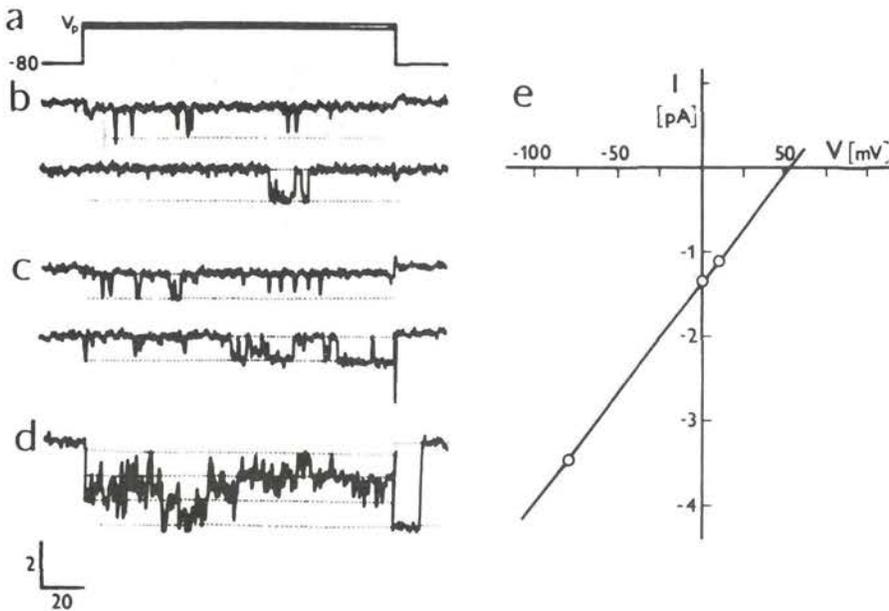


Fig. 1. Unitary Ba currents through single L-type calcium channels. Ca channels were activated by depolarizing voltage pulses (a) from a holding potential of -80 mV to 0 mV (b) and $+10$ mV (c). (d) Simultaneous activity of 4 channels in another patch, $V_p = +10$ mV. (e) Current-voltage relation from experiments shown in (b) — (d). Conductance 25.5 pS. Solutions (in mmol/l); pipette: 110 BaCl₂, 10 HEPES, pH 7.4 ; bath: 140 K methanesulfonate, 10 MgCl₂, 10 HEPES, 10 EGTA, pH 7.35 . Calibration bars are in pA and ms for current and time, respectively.

Calcium channels measurements

Myocytes isolated in the described manner (Table 3) survived for more than six hours.

In the single-channel experiments (Fig. 1), cells gave gigaohm seals in 70% of the attempts (total of 135 cells). Immediately after forming a giga-seal, large current fluctuations were occasionally present but they ceased in several seconds. 68% of the isolated membrane patches displayed no signs of channel activity in spite of stable tight seals. In 16% of the patches single L-type calcium channels were stable for at least 20 min (up to above 120 min). In the remaining 16% of the patches, activity of Ca channels lasted for several minutes only, then ceased and did not reappear in the next 5 min. Typically, there were 1 to 2 channels in a patch (maximum 4). The unitary Ba currents presented were chosen to illustrate short- and long-lived openings and the voltage dependence

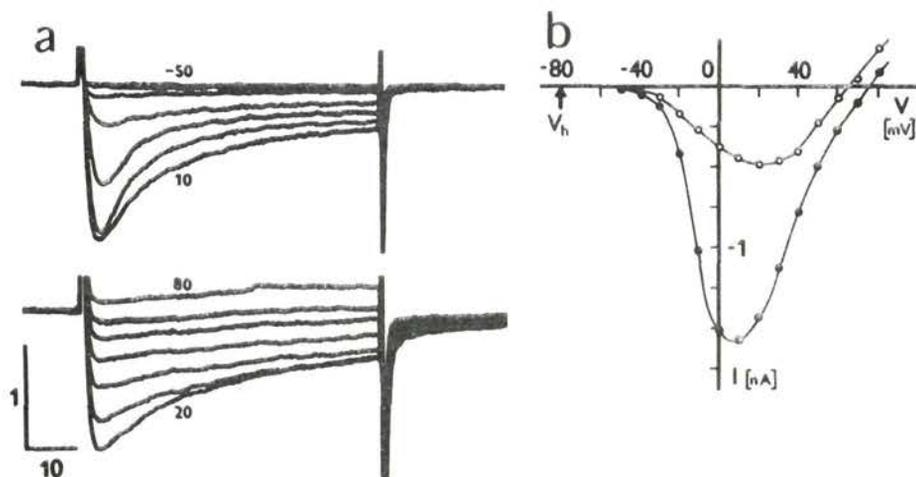


Fig. 2. L-type Ca channel currents. (a) A family of Ca currents in response to 60 ms depolarizing pulses from a holding potential of -80 mV to $-50 \div +10$ (above) and $+20 \div 80$ mV (below) in 10 mV steps. (b) Current-voltage relationships for peak (full circles) and end currents (open circles). Solutions (in mmol/l); pipette: 65 Cs methanesulfonate, 10 Cs glutamate, 5 Cs₂HPO₄, 20 CsCl, 20 TEACl, 1 MgSO₄, 10 taurine, 10 HEPES, 5 EGTA, 3 ATP, 0.05 cAMP, pH 7.2; bath: 3 CaCl₂, 1 MgCl₂, 40 CsCl, 120 Cs methanesulfonate, 5 HEPES, 5 4-aminopyridine, pH 7.3. The fast capacitive transients were blanked to improve presentation. Calibration bars are in nA and ms for current and time, respectively.

of current amplitude. The record shown in Fig. 1*d* was taken from another patch with 4 active channels to show single-channel "tail" current. The single-channel conductance determined from these two patches was 25.5 pS (Fig. 1*e*). When pipettes were filled with isotonic SrCl₂ solution, the single channel conductance amounted to about 14 pS.

In the whole-cell experiments (total of 507 cells) a gigaseal was achieved in 49% of cases. Of these, whole cell configuration was formed in 48% of cases. Pure calcium currents were observed in 84%, and 53% of them were stable for more than 15 min. The stability of integral Ca currents depended on the composition of the pipette solution, but measurements stable for 20–30 min were quite usual. The ionic compositions of the internal and external solution were formulated for direct measurements of pure Ca currents. Two types of Ca currents could be observed at sufficiently negative holding potentials (Figs. 2 and 3). Maximal amplitude of the L-type Ca channel current in 3 mol/l external Ca²⁺ was typically 1–2 nA in cells with a capacitance of about 100 pF. Calcium currents peaked at about +10 mV and reversed their polarity around +60 mV (Fig. 2). Activation of T-type calcium channels could be easily observed in cells

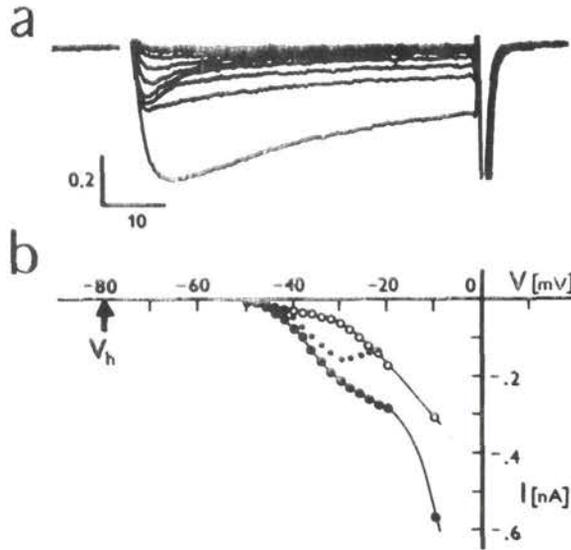


Fig. 3. T-type Ca channel currents. (a) Currents were elicited by 60 ms depolarizations from a holding potential of -80 mV to $-50 \div -20$ mV in 2 mV steps (for clarity, only every second record is shown), and finally to -10 mV.

To improve presentation the first 2 ms in the pulse are not plotted. Calibration bars are in nA and ms for current and time, respectively. (b) Current-voltage relationships for peak currents (full circles), currents at the end of the pulse (open circles), and their difference (points). In the voltage range of -50 to -22 mV this difference current may be considered the pure T-channel current. For solutions see legend to Fig. 3.

with smaller L-type currents at depolarizations above -45 mV (Fig. 3). Their amplitude reached about 0.2 nA at -20 mV. At increasing depolarizations (above -10 mV) L-type channel currents became dominating. The T-channel current can be identified under closer inspection also in Fig. 2a. It is seen at the -30 mV trace as a rapidly activating inward transient (cf. times to peak in Fig. 3a). At this threshold potential, the L-type Ca current is not expected to inactivate.

Discussion

Isolation of Ca-tolerant myocytes is a multifaceted problem (Parrat 1985; Chapmann and Tunstall 1987) and, as might be expected, different laboratories have gained different experience (for a review see Dow et al. 1981; Farmer et al. 1983). To our experience, the following general conditions are important:

- homogenous perfusion of the heart (Farmer et al. 1983)
- fast and complete loosening of gap junctions (Alto and Dhalla 1979)
- correctly balanced enzyme activity at low free Ca^{2+}
- graded recovery of free Ca^{2+} from micromolar to the millimolar range (Farmer et al. 1983)
- stabilization of the cells in supplemented media (Kao et al. 1980; Isenberg and Klöckner 1982)
- use of young animals with low collagen crosslinking (Alberts et al. 1983; but see Lee 1987).

If one or more of the above requirements were not met, a decreased tolerance to calcium ensued which manifested itself by:

- rounded and disintegrated cells in the "enzyme" solution (mechanical damage at regions of intercellular communication)
- hypercontractures immediately elicited by millimolar Ca^{2+} concentrations, and rounded cells with blebs (Na overload)
- development of granulation, rounded edges, stiffness, membrane fragility or low Ca currents after staying in high Ca solution (metabolic deprivation).

There are apparently several ways how to meet the above conditions; the appropriate method depends upon the laboratory conditions and the aim of the study. To our knowledge, only one report (Trube 1983) has dealt so far with a comparison of different approaches to myocyte preparation using the patch-clamp technique. Trube pointed to two puzzles: Tight seal contacts could be obtained in spite of both, the presence of glycocalyx covering the surface of the myocytes, and the high surface density of T-tubule openings. The glycocalyx has been shown to be entirely removed by the procedure of Isenberg and Klöckner (1982); on the other hand, approx. 50 nm thick glycocalyx remains after the isolation by the procedure of Kao et al. (1980) and Farmer et al. (1983). According to current understanding of tight seal formation (Corey and Stevens 1983), the presence of a hydrophilic layer between the glass surface of the pipette and the cell lipidic membrane is expected to decrease the shunt resistance of such a seal below gigaohm values. Therefore, the lipid membrane of the cell may be supposed to bud through the glycocalyx loosened by enzyme treatment. This corresponds to the usually observed course of gigaohm contact formation (Hamill et al. 1981) when, after applying suction to the pipette, the resistance increases slowly to values of 30 to 100 $\text{M}\Omega$ (sometimes keeping this intermediate value for many seconds), and then abruptly and spontaneously reaches values of about 10 $\text{G}\Omega$. At the same time, the T-tubule lumen is expected to be stretched, pulled out and healed over by a mechanism observed during excision of isolated membrane patches (Hamill et al. 1981). This hypothesis is supported by the measured capacitance of the isolated membrane patch corresponding to the capacitance of a smooth membrane plane. Current fluctuations just after

gigaseal formation could be ascribed to the healing process. In these events elasticity of the membrane plays an important role. If decreased (e.g. in consequence to an improper metabolic state), the membrane breaks up without forming a tight seal.

The records of Ca channel currents presented herein demonstrate that the isolation technique tailored to our conditions gives reliable results. Both, single channel and integral currents display characteristics comparable to those reported for guinea-pig myocytes isolated by different procedures (Mitra and Morad 1986; Nilius et al. 1986; Hamilton et al. 1987; Hume and Uehara 1985; Trautwein and Pelzer 1985; Isenberg et al. 1987; Lee 1987).

After establishing the whole-cell configuration, the lifetime of Ca currents is thought to be determined mainly by the composition of the internal (pipette) solution, and by pipette tip diameter (Belles et al. 1988; Kameyama et al. 1985). To our experience, the preexperimental metabolic state of the isolated cell is very important (Lee 1987), as under otherwise equal conditions, the period of stable recording ranged from 5 min to 2 hours.

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