An Improved Method for Isolating Cardiac Myocytes Useful for Patch-Clamp Studies

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Summary An improved method for isolating cardiac myocytes is described. This method consists of the initial dispersion of the myocytes with a new type of collagenase and the following treatment of the cells with a non-specific protease. This method gives more than 80% rod-shaped, viable, and quiescent cells, which are suitable for biochemical as well as electrophysiological experiments. Formation of a GΩ seal and subsequent whole-cell recording is easily performed using these cells.

Key words: dissociation of cells, isolated myocytes, patch clamp.

Since the pioneering work of Kono (1969) and Powell and Twist (1976), isolated cardiac cells have offered a useful preparation for cardiac electrophysiology. A number of isolation techniques have been developed to obtain single myocytes having intact electrical properties (for reviews see, Farmer et al., 1983; Bkaily et al., 1984; see also, Hume and Giles, 1981; Taniguchi et al., 1981; Bustamante et al., 1982; Bendukidze et al., 1985; Mitra and Morad, 1985). Most of these methods consist of perfusion of the heart with a low Ca²⁺ medium and subsequent dispersion of cells with crude collagenase and/or a proteolytic enzyme, such as trypsin or non-specific protease. However, it has become apparent that the viability of the myocytes thus isolated is variable and it is sometimes difficult to establish a high-resistance (GΩ) seal on these cells. We have been able to overcome these problems by using a new type of collagenase and subsequently treating the myocytes with a protease. The myocytes prepared by this method are useful not only for patch-clamp studies but also for biochemical and pharmacological studies, because of the high yield and maintained viability of the cells.

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Cell isolation procedure

The steps of the isolation procedure are as follows. 1) An adult white or wild-type female guinea-pig (weight, 300–600 g) is anesthetized with pentobarbital sodium (30 mg/kg i.p.), and the trachea is cannulated for artificial respiration. The chest is opened, the aorta is cannulated in situ to perfuse the coronary artery (on a Langendorff apparatus), and then the whole heart is dissected out. 2) The heart is perfused first with Tyrode solution at 37°C for about 3 min at a hydrostatic pressure of 60–70 cm (5–10 ml/min), second with nominally Ca-free Tyrode for 5–7 min, and finally with collagenase (0.12–0.20 mg/ml or 60–100 U/ml; Yakult, Japan) in nominally Ca-free Tyrode (50 ml), which is recirculated using a rotor pump for 10–15 min. Since the collagenase preparation obtained from Yakult company contained calcium of 6.4 mg/g, the concentration of Ca²⁺ in the enzyme solution was estimated to be 25–30 μM. Although increasing Ca²⁺ concentration in the collagenase solution (up to 70 μM) did not significantly affect the initial viability of myocytes, it tended to produce an uneven digestion of the heart. 3) The heart is washed with the storage solution (see below), and the ventricles (or atria) are cut into pieces (about 5 × 5 mm) and poured into a beaker. The myocytes are dispersed by gently shaking the beaker and filtration through a 105 μm stainless steel mesh. The myocytes are washed twice by centrifugation at 600–1,000 rpm (about 90 × g) for 5 min and kept at 4°C.

The myocytes thus isolated can be stored for up to 3 days and used for some biochemical and pharmacological studies. Although the cells may also be good for electrical measurements with a conventional microelectrode, the cells are often resistant to formation of GΩ seal with a patch-clamp pipette. In such cases, the cells are subsequently incubated with the storage solution containing both 0.05–0.20 mg/ml protease (Nagase, Alkaline protease NK-103; Yakult, AP-10; Sigma, type XIV; or Boehringer pronase), and 0.02 mg/ml deoxyribonuclease 1 (Sigma, type IV) for 10–15 min at 37°C. Then, cells are washed with the storage solution twice by centrifugation at 600–1,000 rpm for 5 min and stored at 4°C.

Tyrode solution (mM): NaCl 143; KCl 5.4; CaCl₂ 1.8; MgCl₂ 0.5; NaH₂PO₄ 0.25; HEPES 5; pH to 7.4 with NaOH. Storage solution: KOH 70; L-glutamic acid 50; KCl 40; taurine 20; KH₂PO₄ 20; MgCl₂ 3; glucose 10; HEPES 10; EGTA 0.5; pH to 7.4 with KOH.

Morphology

The yield of myocytes after the collagenase treatment was 0.5–1.4 × 10⁷ cells/g tissue (n = 3); this may correspond to 20–50% of tissue and is comparable with the values reported for previous methods (10–60%; reviewed by Farmer et al., 1983). Most of the myocytes dissociated with collagenase were rod-shaped and quiescent in the storage solution, and were Ca²⁺-tolerant in the Tyrode solution (Fig. 1A). These cells excluded trypan blue while other cells were rounded and stained clearly with the dye. The percentage of rod-shaped cells was 84.5 ± 1.7% (mean ± S.E., n = 6) in the Tyrode solution. Treatment of the isolated myocytes with protease did
not change the viability of cells; the rod-shaped cells were $85.8 \pm 2.0\%$ ($n = 6$). The percentage of rod-shaped cells was not significantly reduced when the cells were stored in the storage solution at $4^\circ C$ for 24 h.

The morphology of isolated myocytes was further examined under light and scanning electron microscopes (Fig. 1B, C). Figure 1B shows differential interference photomicrographs of myocytes treated with collagenase only (left column) and with collagenase + protease (right column). All these cells are regularly striated with a sarcomere length of 1.9–2.2 μm without protrusion (bleb) of the cell membrane. Myocytes without protease treatment show relatively sharp undulations of the membrane at both ends of the cells, and appear more "glossy" than the cells treated with protease. This might be explained by the fact that the surface coat (glycocalyx) of the myocytes is reduced by protease digestion (cf. Eisenberg and Klöckner, 1980; Bustamante et al., 1981, 1982). Using a scanning electron microscope, however, the cell surface was seen to be smooth both before and after protease treatment and no appreciable difference was observed (Fig. 1C).

To determine if the glycocalyx was modified with protease, myocytes were stained with fluorescein isothiocyanate-labelled concanavalin A (ConA–FITC), a lectin which binds to α-D-glucose and α-D-mannose. As illustrated in Fig. 1D, there is a clear tendency for the fluorescence of the cells to be reduced by protease treatment. Similar observations were obtained with alcian blue, which binds to acidic glycans (data not shown). These results support the idea that the glycocalyx is reduced by the protease treatment.

Electrophysiology

The electrophysiological properties of the isolated myocytes were investigated by the patch-clamp method (Hamill et al., 1981). In cells treated only with collagenase, to form a GΩ seal required a relatively large negative pressure (up to 50 cm H₂O) and the success rate was low (mostly <50%). In the protease-treated cells, however, a GΩ seal was obtained with a small suction (0–20 cm H₂O) and there was a high success rate (>90% with a 3–5 MΩ pipette containing 50 mM Ba²⁺). In the whole-cell current clamp mode, the resting membrane potential was about −85 mV and constant positive pulses elicited an action potential having a duration of 187 ms and an overshoot of 45 mV (Fig. 2A). Figure 2B and C show Ca²⁺ channel currents recorded in the whole-cell and cellattached patch modes, and the effects of isoprenaline (Iso) and acetylcholine (ACh) on these currents. In Fig. 2B, Ca²⁺ current ($I_{Ca}$), measured as a difference between the peak and steady-state currents during the depolarizing pulse, was increased by 50 nM Iso from 2.8 nA (sweep 1) to 6.0 nA (sweep 2), i.e., by 118%, and additional application of 1 μM ACh decreased $I_{Ca}$ to 4.2 nA (sweep 3), resulting in 56% inhibition. In the cell-attached mode shown in Fig. 2C, the same concentration of Iso increased the open-state probability of the Ca²⁺ channel, thereby increasing the peak of the mean current by 101%, an extent comparable to that in the whole-cell current. The comparable increase in the Ca²⁺ channel activity between the whole-cell and the
Fig. 1. Photomicrographs of isolated guinea-pig ventricular myocytes. A) Light microscopic appearance at low magnification. Protease-treated cells were perfused with the Tyrode solution. B) Differential interference micrographs of cells in the Tyrode solution. Cells were treated without (left column) and with protease (right column). C) Scanning electron micrographs. Cells were fixed with 3% (v/v) paraformaldehyde + 0.1% (v/v) glutaraldehyde and coated with gold. Left, without protease; right, with protease. D) Fluorescence photomicrographs with ConA-FITC. Cells were fixed in the same way as in C, treated with 0.2% (v/v) amylase to remove glycogen, and then incubated with 0.1 mg/ml ConA-FITC for 30 min. Left column, without protease; right column, with protease. Photographs were taken and processed under the same condition for these cells. Calibration: A, 200 μm; B and D, 50 μm; C, 2 μm.

cell-attached patch mode suggests that the channel regulation via the indirect pathway (phosphorylation) rather than the direct coupling of GTP-binding protein to the channel (YATANI and BROWN, 1989) is already dominant at this concentration of Iso. We have also been able to record other ionic currents, i.e., delayed outward \( K^+ \) current, inward rectifier \( K^+ \) current, ATP-dependent \( K^+ \) current, and \( Na^+ /Ca^{2+} \) exchange current in ventricular cells and ACh-activated \( K^+ \) current in atrial cells (data not shown). These results suggest that the electrical properties and
Fig. 2. Electrophysiology of the ventricular myocytes treated with protease. A) Action potential elicited by constant current pulses of 1 nA amplitude and 6 ms duration (bar) at a rate of 0.2 Hz. B) Effects of Iso and ACh on $I_{Ca}$. Current traces in response to voltage-clamp pulses from $-40$ to $0$ mV with a duration of 200 ms in control (1), with 50 nM Iso (2), and with 50 nM Iso + 1 μM ACh (3). Pipette solution for A and B (in mM): KOH, 110; KCl, 20; MgCl$_2$, 1; K$_2$ATP, 5; K$_2$ creatine phosphate, 5; aspartic acid, 90; EGTA, 10; and HEPES, 5. The pH was 7.4 and pCa was 8.2 ($10$ mM EGTA + $1.43$ mM Ca$^{2+}$). Bath solution was the Tyrode solution. C) Ca channel activity in the cell-attached mode and effects of Iso. Ca channels were activated by depolarizing pulses from $-60$ to $0$ mV for 200 ms at a rate of 0.5 Hz. The left column and the right one show 10 consecutive traces in control and after bath application of 50 nM Iso, respectively. The mean currents (averaged from 50 and 51 patch currents, respectively) are shown at the bottom. Pipette solution (mM): BaCl$_2$, 50; Choline Cl, 70; Bay K 8644, 0.003; EGTA, 0.5; and HEPES/CsOH buffer, 10; pH, 7.4. Bath solution (mM): K aspartate, 90; KCl, 30; KH$_2$PO$_4$, 10; EGTA, 1; and HEPES/KOH buffer, 10; pH, 7.4.

Their regulatory mechanisms are well preserved in the myocytes prepared with the present method.

In this study, we have reported an improved method for isolation of cardiac myocytes, which are suitable for patch clamp studies. The basic point of this method is to disperse the myocytes with a relatively pure collagenase and then to treat the cells with protease. Although most of the reported methods utilize crude collagenases and/or proteolytic enzymes to dissociate the myocytes suitable for patch-clamp studies (Hume and Giles, 1981; Bustamante et al., 1982; Bendukidze et al., 1985; Mitra and Morad, 1985), viability of the myocytes dissociated with these enzymes...
often varies noticeably from batch to batch. In our experience with selected batches of collagenase (Sigma, type I), the viability usually ranged from 10 to 70%. On the other hand, we have not experienced such a variability with the present method; Ca\textsuperscript{2+}-tolerant rod-shaped cells were 80–90%. This may be partly because the new collagenase (derived from Streptomyces genus) has a relatively low contamination of proteolytic enzymes, such as cascinase and trypic activity, and a low toxicity to various types of cells as compared to a collagenase derived from Clostridium genus (data from Yakult Co.). It should be noted that other factors, e.g., quality of water and age and sex of the animals, seem also to be important to obtain good cells, as pointed out by Lee (1987). Because of relatively high viability, the cells are also suitable for biochemical and pharmacological studies.

For the protease treatment of myocytes, we have used 5 types of protease and found that 4 preparations (Nagase, Alkaline protease NK-103; Yakult, AP-10; Sigma, type XIV; Boehringer pronase) are effective in improving the success rate of GΩ seal formation with patch-clamp pipettes. This supports the view that condition of the cell surface is important for the formation of a GΩ seal (cf. Corey and Stevens, 1983; Trube, 1983).

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