Regulatory Volume Decrease of Cardiac Myocytes Induced by β-Adrenergic Activation of the Cl^- Channel in Guinea Pig

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ABSTRACT A new method was developed to automatically measure the thickness of a single ventricular myocyte of guinea-pig heart. A fine marker was attached on the cell's upper surface and changes in its vertical position were measured by focusing it under the microscope. When the osmolarity of the bath solution was varied, the cell thickness reached a new steady level without any obvious regulatory volume change within the period of observation up to 15 min. The cell thickness was 7.8 ± 0.2 μm (n = 94) in the control Tyrode solution and was varied to 130.4 ± 3.1% (n = 10), 119.1 ± 1.1% (n = 50), 87.2 ± 1.9% (n = 9), and 75.6 ± 3.2% (n = 5) of control at 50, 70, 130, and 200% osmolarity, respectively. The application of a Cl^- channel blocker, 500 μM anthracene-9-carboxylic acid (9AC) did not modify these osmotic volume changes. We discovered that the application of isoprenaline induced a regulatory volume decrease (RVD) in cells inflated by hypotonic solutions. This isoprenaline-induced RVD was inhibited by antagonizing β-adrenergic stimulation with acetylcholine. The isoprenaline-induced RVD was mimicked by the external application of 8-bromoadenosine 3′:5′-cyclic monophosphate. The RVD was inhibited by blocking the cAMP-dependent Cl^- channel ($I_{Cl,\ cAMP}$) with 9AC but was insensitive to 4, 4′-diisothiocyanostilbene-2, 2′-dissulphonate (DIDS). Taken together these data suggest an involvement of $I_{Cl,\ cAMP}$ activation in the RVD. Whole cell voltage clamp experiments revealed activation of $I_{Cl,\ cAMP}$ by isoprenaline under the comparable conditions. The cardiac cell volume may be regulated by the autonomic nervous activity.

KEY WORDS: cell volume regulation • cAMP-dependent Cl^- current • β-adrenergic stimulation • ventricular myocyte • guinea-pig heart

INTRODUCTION

During ischemia, cardiac cell swelling occurs before the onset of irreversible injury (Leaf, 1973; Macknight and Leaf, 1977; Tranum-Jensen et al., 1981; Jennings et al., 1985). Depletion of energy stores, consequent failure of active membrane transport, such as Na^+-K^+ pump, and accumulation of anaerobic metabolites, such as lactate, H^+, inorganic phosphate, glycolytic intermediates, etc., induce myocyte swelling. This is a vicious cycle since cell swelling compresses the vascular bed, increasing ischemia, and results in a decrease of compliance of the chamber (Vogel et al., 1986). Although a local increase of catecholamines in ischemic tissues is known to occur (Videbaek et al., 1972; Karwatowska-Krynska and Beresewicz, 1983), its physiological relevance in relation to cell swelling is unclear.

The Cl^- current ($I_{Cl,\ cAMP}$)[1] activated by the stimulation of β-adrenergic receptors (Bahinski et al., 1989; Harvey and Hume, 1989; Matsuoka et al., 1990) acts through a membrane protein of the ATP-binding cassette (ABC) superfamily (Hart et al., 1996) and is very unique in that it works only under the activation of the β-adrenergic receptors. As a result of the $I_{Cl,\ cAMP}$ activation, a significant change in resting membrane potential was demonstrated by Egan et al. (1988). Furthermore, the action potential prolongation due to sympathetic increase of Ca^{2+} current is minimized by the activation of $I_{Cl,\ cAMP}$ (Harvey et al., 1990; Takano and Noma, 1992). In addition to these electrophysiological consequences, we hypothesize that the $I_{Cl,\ cAMP}$ activation by β-adrenergic agonists may be responsible for a regulatory volume decrease (RVD), since activation of I_{Cl} usually results in osmotically obliged water efflux in various tissues (Hoffmann and Simonsen, 1989; Sarkadi and Parker, 1991; Zhang et al., 1993).

To test this hypothesis, it is prerequisite to be able to continuously measure the cell volume. It has been reported that when dissociated cardiac myocytes swell in hypotonic solution, changes in their width and thickness are significantly greater than changes in cell length (Roos, 1986; Drewnowska and Baumgarten, 1991). We have developed a new method designed to continuously measure variations in the thickness of a single ventricular cell by focusing on a fine marker attached to the cell's upper surface. Using this new method, we have revealed that the application of iso-

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[1]*Abbreviations used in this paper:* 8-Br-cAMP, 8-bromoadenosine 3′:5′-cyclic monophosphate; 9AC, anthracene-9-carboxylic acid; ACh, acetylcholine; DIDS, 4, 4′-diisothiocyanostilbene-2, 2′-dissulphonate; I-V, current-voltage; $I_{Ca,\ L}$, L-type Ca^{2+} current; $I_{Cl,\ cAMP}$, the cAMP-dependent Cl^- current, I_{Cl}, Cl^- current; $I_{Cl,\ swell}$, swelling-induced Cl^- current; I_K, delayed rectifier K^+ current; $I_{K,1}$, inward rectifier K^+ current; RVD, regulatory volume decrease; RVI, regulatory volume increase.

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prenaline induced RVD in osmotically swollen cells. These findings strongly suggest an autonomic neural regulation of cardiac cell volume.

MATERIALS AND METHODS

Preparation

Single ventricular myocytes were isolated with essentially the same enzymatic dissociation technique as described previously (Powell et al., 1980, Matsuoka et al., 1990). Briefly, adult guinea pigs weighing 200–300 g were anesthetized with sodium pentobarbitone (40 mg/kg). Under artificial respiration the chest cavity was opened and the aorta was cannulated in situ to start perfusion of the coronary vessels with the control Tyrode solution. The heart was then excised, fixed on a Langendorff-type apparatus, and perfused with ~50 ml nominally Ca^{2+}-free Tyrode solution and then with the same solution containing 0.04% collagenase (type I; Sigma Chemical Co., St. Louis, MO) for 20 min. After enzymatic perfusion, single myocytes were dispersed from the left ventricle in a high-K^+, low-Cl^- solution (a modified KB solution, Isenberg and Klöckner, 1982) and stored in a cell culture medium (Eagle's medium; Flow Laboratories, McLean, VA) at room temperature (24–26°C) for later use. The high-K^+, low-Cl^- solution contained in mM: KCl, 25; K-glutamate, 70; KH_2PO_4, 10; $MgCl_2$, 1; taurine, 10; glucose, 11; EGTA, 0.5; and HEPES, 10; (pH = 7.3 with KOH).

Solutions

The composition of the control Tyrode solution was (in mM): NaCl, 140.0; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_2, 0.3; glucose, 5.5; and HEPES, 5.0, pH adjusted to 7.4 with NaOH. The hypotonic and the hypertonic solutions were prepared as described before (Sasaki et al., 1994; Wang et al., 1996). In brief, the hypotonic solutions of 70 and 50% osmolarity were made by decreasing the concentration of NaCl to 100 and 70 mM in the control Tyrode solution, respectively. In these experiments, control solution was either the control Tyrode solution in preliminary experiments or an isotonic solution prepared by adding mannitol to the hypotonic solutions to adjust the osmolarity to 100%. When the bath solution was switched from the control Tyrode solution to the isotonic solution adjusted with mannitol, no obvious change was noticed in the cell thickness. The hypertonic solutions of 130 and 200% osmolarity were prepared by adding 90 and 280 mM mannitol to the control Tyrode solution, respectively. In all experiments the temperature of the bath solution was kept at 35 ± 0.5°C by passing the solutions through a water jacket prior to the inlet of the recording chamber.

For the whole cell voltage clamp experiments, the pipette solution contained in mM: cesium aspartate, 100.0; CsCl, 10; tetraethylammonium chloride (TEA-Cl), 20.0; $MgCl_2$, 0.5; Mg^{2+}-ATP, 5.0; Na_2-phosphocreatine, 5; EGTA, 10; HEPES, 10; pH = 7.3 with CsOH. CsCl 5 mM and nicardipine 2 μM were added to the external solutions to block the inward rectifier K^+ channel and L-type Ca^{2+} channel, respectively.

Isoprenaline ((±)-isoproterenol; Sigma Chemical Co.), acetylcholine chloride (ACh; Sigma), 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP; Sigma), anthracene-9-carboxylic acid (9AC; Tokyokasei, Tokyo, Japan), and 4, 4′-diisothiocyanostilbene-2, 2′-dissulphonate (DIDS; Dojindo, Kumamoto, Japan) were added to the external solutions when necessary. Isoprenaline was dissolved in distilled water and prepared as a 1 mM stock solution containing an equal weight of ascorbic acid. ACh was prepared as a 10 mM stock solution in distilled water. 9AC stock solution was prepared in dimethyl sulfoxide (DMSO) solution. The final concentration of DMSO in external solutions was <0.1%. All chemicals were prepared freshly before experimentation. The experiments with 8-Br-cAMP were performed in relative darkness to avoid photo-destruction.

Measurements of Cell Thickness

The optical system for measuring the variation in cell thickness included an inverted microscope (TMD300; Nikon, Tokyo, Japan) and a photomultiplier tube (P100; Nikon). The variation in cell thickness was measured from the vertical position of the objective glass, which focused on a fine marker attached to the upper surface of a myocyte. The lower surface of the myocyte was fixed to the glass bottom of the recording chamber mounted on an inverted microscope. To facilitate cell attachment, the glass surface was cleaned beforehand with chromic acid and coated with 100 μg/liter poly-lysine solution (poly-L-lysine; Sigma). A drop of cell suspension was added to a bath of the control Tyrode solution and after the myocytes settled on the glass bottom, a suspension of small carbon particles (3–7 μm in diameter) was perfused within the chamber.

A single myocyte marked with a carbon particle near the middle of the cell is shown in Fig. 1 *A*. If no myocyte with an appropriate marker was found, we lifted a suitable carbon particle using the tip of a conventional glass pipette and positioned it on a myocyte as a marker. The image of the marker was directed through the side-port of the microscope to an adjustable rectangular aperture and then to the photomultiplier tube. The size of the aperture was adjusted so as to be slightly smaller than the image of marker. Thus, when the marker image was in focus, the output of the photomultiplier (light intensity) was at a minimum in the relationship between light intensity and focus position of the objective glass as shown in Fig. 1 *B*. An almost exponential increase of the light intensity was obtained by moving the objective glass (×100) up and down (5–10 μm) across the marker focus level. An on-line computer (PC98; NEC) calculated the first derivative of the relationship between the focus position and light intensity, and the minimum light position was determined from the intersection of the first derivative curve with the abscissa (Fig. 1 *B*). During the experiment, the objective glass was moved up and down automatically by connecting the handle for fine adjustment of focus to an electric motor, which was driven by a sinusoidal electric signal every 6 s.

The basal thickness of each ventricular myocyte was measured by eye at a magnification power of 1,000 (100 × 10) at the beginning of the experiment. We measured the cell thickness as the difference of plane of focus of two particles; one is on the top of the cell and the other on the surface of the bottom of recording chamber. When the accuracy of the eye measurements was examined by repeating measurements 6 times for a given marker, the standard deviation of the marker focus level was within 0.5 μm.

Data are given as mean ± SEM. Statistical significance was determined by Student's unpaired *t* test.

It should be noted that the present study measures changes in the cell thickness as an estimator of the cell volume change. However, precise determination of the cell volume is beyond the scope of the present study, since the present study measured neither the width nor length of myocytes. The aim of the new method is to reveal cell inflation or shrinkage based on an assumption that the increase or decrease of the cell thickness reflects parallel changes of the cell volume.

Source of Artifact

Drift in the light intensity passing through the aperture was attributable to various mechanisms, such as changes in the depth of the bathing solution and movement of the marker attached on

the cell. These problems were largely solved by searching for the intensity minimum of the light signal. Cell movement was inhibited by sticking cells to the poly-lysine-coated glass bottom of the recording chamber (the glass was first thoroughly cleaned with chromic acid). Those cells which showed marked changes in the cell shape or obvious bleb formation during the perfusion of the hypotonic solution were discarded. Any change in the temperature of the recording bath caused a vertical movement of the bottom of the recording chamber. Also, changes in the ambient air temperature caused variations of the distance between the stage and the objective glass. Thus, control of the temperature of both the perfusion solution and the room air was essential with the present technique. After excluding these source of artifact, the stability of the optical system and the consistency of determining the light minimum by computer was tested by recording the vertical position of a fine marker simply placed on the bottom of the recording chamber perfused with the control saline as shown in Fig. 1 *C*. Small scatter of measurements is evident from the standard deviation of 0.07 μm in the experiment shown in Fig. 1 *C*.

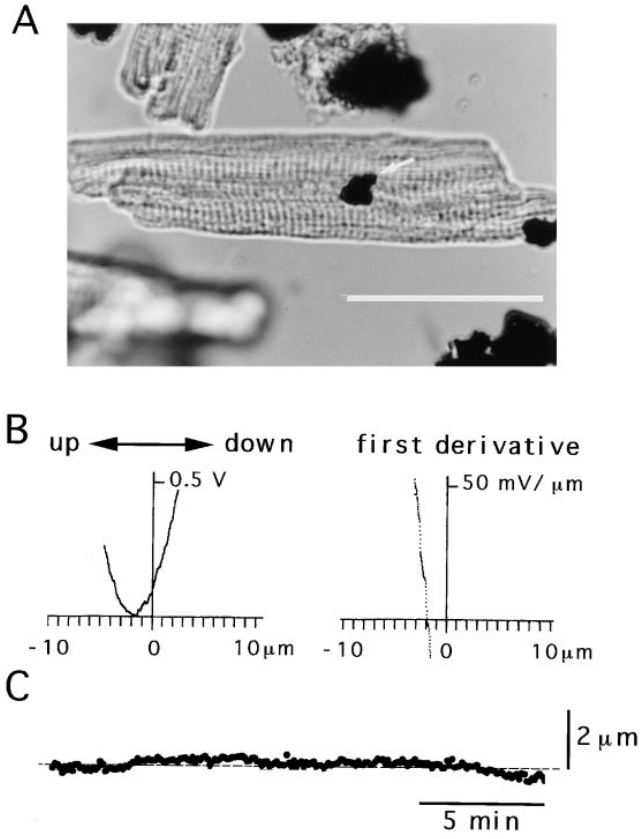

FIGURE 1. A representative measurement of the vertical position of the marker. (*A*) A ventricular myocyte with a small carbon particle (*arrow*) on its upper surface. The horizontal bar indicates 50 μm. (*B*) An example of the relationship between light intensity (*ordinate*) and focus position (*abscissa*). In this relationship, the focus position is determined with reference to a given zero (arbitrary) position of the objective glass, and positive sign is assigned to the downward movement of the objective glass. The first derivative of the relationship is shown in the right panel. Home-written computer software determined the focus level of the marker and plotted the time course of change on the computer graphic display every time one cycle of objective glass movement was completed. (*C*) Changes of the focus position of a marker attached to the bottom of the recording chamber were recorded. The dotted line indicates the basal level. See text for explanation.

Whole Cell Voltage Clamp

Activation of $I_{Cl,\ cAMP}$ was measured by the whole-cell patch clamp technique (Hamill et al., 1981) using a patch-clamp amplifier (Axopatch-ID; Foster, CA). The electrode resistance was 2–4 MΩ when filled with the internal solution. The current-voltage (I-V) curve was measured by applying a ramp pulse, which consisted of a positive slope from −80 to +60 mV followed by a negative slope to −140 mV with a dV/dt of 200 mV/s. During the rising phase of the ramp pulse, the Na^+ channel was inactivated, and the I-V relation was obtained from the falling phase. The membrane current and potential were sampled by an online computer and also by a digital tape recorder. The liquid junction potential of −10 mV between the pipette solution and the bath solution was corrected for all membrane potential recordings.

RESULTS

Cell Swelling and Shrinkage in Hypotonic and Hypertonic Solutions

To examine both the accuracy of the new method and spontaneous RVD in the cardiac myocyte, we measured the cell thickness in solutions of various osmolarities. In Fig. 2 *A*, cell thickness was 10 μm in the control Tyrode solution and started to increase immediately after applying 70% hypotonic solution. A new steady level was reached within 130 s. The increase in cell thickness (~3 μm) was reversed after switching back to the control Tyrode solution. In the present study, we failed to observe any spontaneous RVD in 70 or 50% hypotonic solution within the observation period of 1–15 min ($n >$ 50). Also, no regulatory volume increase (RVI) was observed after cell shrinkage induced by exposure to 130% hypertonic solution ($n = 3$, 10 min observation). These findings are consistent with the previous study in adult cardiac myocytes (Drewnowska and Baumgarten, 1991).

The change in cell thickness in response to varying the osmolarity was graded and reversible as shown in the typical recording in Fig. 2 *B*. When changes were normalized to the basal cell thickness of 9 μm for the myocyte used in Fig. 2 *B*, values were 108, 123, and 88%, for solutions of 70, 50, and 130% osmolarity, respectively. Fig. 2 *C* (○) summarizes the relationship between relative cell thickness and osmolarity. The average of control thickness of guinea-pig ventricular myocyte used was 7.8 ± 0.2 μm ($n = 94$), and the cell thickness was increased to 130.4 ± 3.1% ($n = 10$) and 119.1 ± 1.1% ($n = 50$) of control in 50 and 70% hypotonic solutions, respectively. In 130 and 200% hypertonic solutions, the cell thickness decreased to 87.2 ± 1.9% ($n = 9$) and 75.6 ± 3.2% ($n = 5$), respectively. These findings are consistent with other reports (Roos, 1986; Drewnowska and Baumgarten, 1991; Tseng,

1992). The mean increase in cell thickness to 119.1 ± 1.1% (n = 50) in 70% hypotonic solution is similar to the change in the cell area (118%) we previously measured in the same preparation (Wang et al., 1996).

In many other tissues, spontaneous RVD is explained by a delayed activation of a swelling-induced Cl^- current ($I_{Cl,\ swell}$) (Sarkadi and Parker, 1991), a current which is also present in cardiac myocytes (Tseng, 1992; Vandenberg et al., 1994). Our electrophysiological study (Sasaki et al., 1994) confirmed the activation of $I_{Cl,\ swell}$, but it occurred later than the activation of both the delayed K^+ current and the Na^+/K^+ pump in 70% hypotonic solution. Therefore, it is unlikely that activation of $I_{Cl,\ swell}$ was so rapid that volume regulation occurred during the swelling phase, and no delayed RVD was observed. To confirm a lack of involvement of $I_{Cl,\ swell}$ in volume regulation, the cell thickness-osmolarity relationship was also measured in the presence of 9AC, a potent blocker of $I_{Cl,\ swell}$ (Tseng, 1992; Vandenberg et al., 1994) as shown in Fig. 2 *C* (●). The relative thickness (*closed circles*) were almost superimposable with the control experiments in the absence of 9AC. Also no significant difference was found in the time course of cell thickness change between the control and 9AC experiments. It is concluded that volume regulation through activation of $I_{Cl,\ swell}$ did not occur within the experimental time period of hypotonic or hypertonic perfusion in the present study.

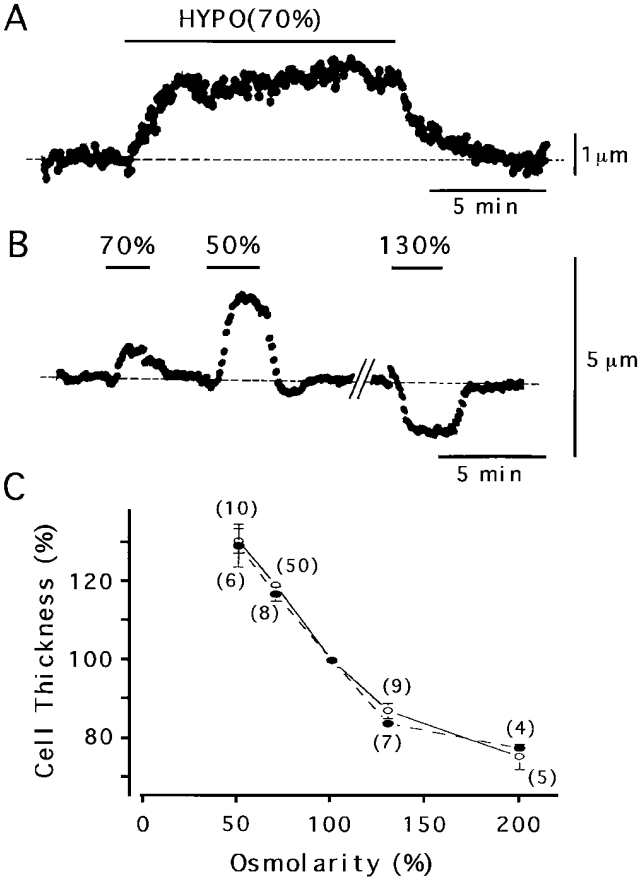

FIGURE 2. Changes in cell thickness at various external solution osmolarities. (*A*) Superfusion of a cell with hypotonic (70%) solution for about 12 min is indicated by the horizontal bar. HYPO(70%), 70% hypotonic solution. Note the absence of any spontaneous RVD. The intermittent line indicates the control cell thickness (10 μm in this experiment), which was measured at the beginning of the experiment in all of following figures. (*B*) Superfusion with solutions of varying osmotic strength as indicated above the trace. The control cell thickness was 9 μm. (*C*) An osmolarity-response curve. The cell thickness obtained at various external osmolarities was normalized to control cell thickness in each experiment. (*open circles*) In the absence of 9AC; (*closed circles*) in the presence of 500 μM 9AC. Data points represent mean ± SEM (numbers are indicated alongside each data point).

Regulatory Volume Decrease Induced by Isoprenaline

We next tested our hypothesis that activation of $I_{Cl,\ cAMP}$ by β-adrenergic agonists may induce RVD in osmotically inflated cells. Since isoprenaline >50 nM fully activates $I_{Cl,\ cAMP}$ (Tareen et al., 1991), isoprenaline 50 nM was applied after the cell thickness had reached a new steady level in 70% hypotonic solution (Fig. 3 *A*). It can be seen that on application of isoprenaline cell thickness gradually decreased back toward the control level. This isoprenaline-induced RVD was observed in 11 out of 18 cells (61%, with 50 nM isoprenaline). The observation rate was not elevated by increasing the isoprenaline concentration to 0.1 μM (9 of 16 cells, 56%) or 1 μM (5 of 11, 45%). In 14 out of 25 cells, complete recovery (~100%) to the control level was observed during the RVD response, and it took 320 ± 62 s (n = 14) after the application of isoprenaline. In the rest of cells, the increase in the cell thickness was reversible only by 56.3 ± 3.5% (n = 11).

The application of isoprenaline to cells bathed in the control Tyrode solution did not cause a consistent change in the cell thickness. The experiment shown in Fig. 3 *B* represents the most frequent observation that no obvious change occurred in the cell depth (12 out of 16 experiments). In 4 other experiments, increases by 9, 15, and 28%, (Fig. 3 *C*) or a decrease (13%) were observed in the cell thickness.

Involvement of the cAMP-dependent Pathway in RVD

RVD induced by isoprenaline might be mediated by the cAMP-dependent pathway after activation of β-adrenergic receptor. Therefore, we tested whether RVD could be induced by the application of the membrane-permeable derivative of cAMP, 8-Br-cAMP. Fig. 4 demonstrates a representative experiment. The basal cell thickness was 6 μm in the isoosmotic solution, and increased by 1.2 μm in 70% hypotonic solution. During swelling, the bath application of 0.5 mM 8-Br-cAMP induced an evident RVD. It should be noted that after

RVD was induced in hypotonic solutions, returning to 100% osmotic solution caused an undershoot of cell thickness below the control level as expected from a loss of solute from the intracellular medium during RVD. The RVD response induced by application of 8-Br-cAMP was observed in all 5 cells tested.

It is well established that the production of cAMP through activation of adenylate cyclase by β-adrenergic receptor activation is blocked by stimulating the muscarinic receptor. As shown in Fig. 5 *A*, the RVD induced by isoprenaline was, as expected, abolished by applying 5.5 μM ACh simultaneously with isoprenaline ($n = 5$). To confirm the reproducibility of the hypotonic response, the increase in the cell thickness was compared between the first and second applications of 70% hypotonic solution in 11 cells. In the average, the increase in the cell thickness was 117.8 ± 1.9% with the first application and 118.5 ± 1.3% with the second application.

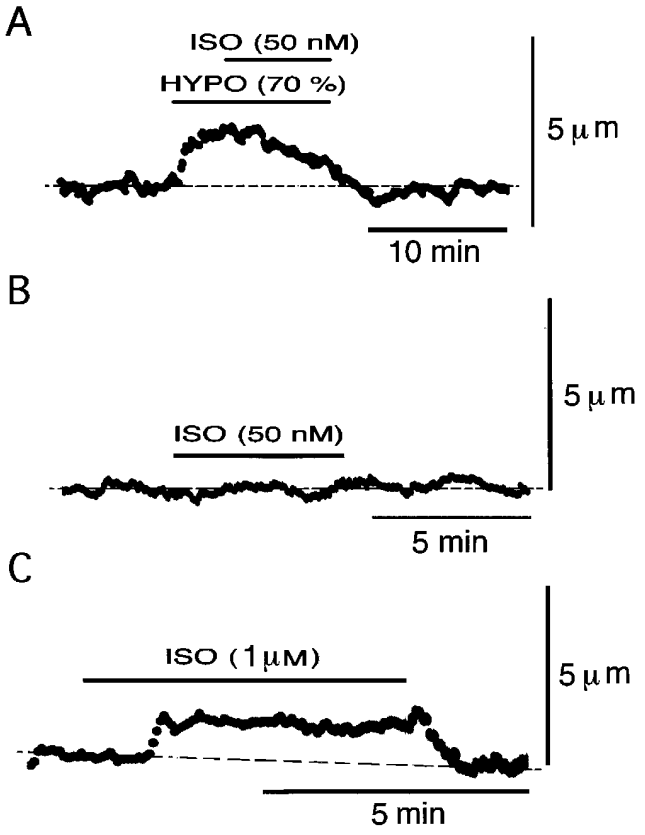

FIGURE 3. Effects of isoprenaline on cell thickness in the hypotonic (*A*) and in the control solution (*B* and *C*). (*A*) RVD induced by isoprenaline in osmotically inflated cells. Application of solutions is indicated by the horizontal bars. (*ISO*) Isoprenaline and (*HYPO(70%)*) 70% hypotonic solution. Control cell thickness was 6 μm. (*B*) No obvious change or an increase in cell thickness was induced by isoprenaline in the control Tyrode solution in most experiments. (*C*) An exceptional recording, which indicated an increase of cell thickness by isoprenaline in the control Tyrode solution. The dotted lines indicate the basal cell thickness levels.

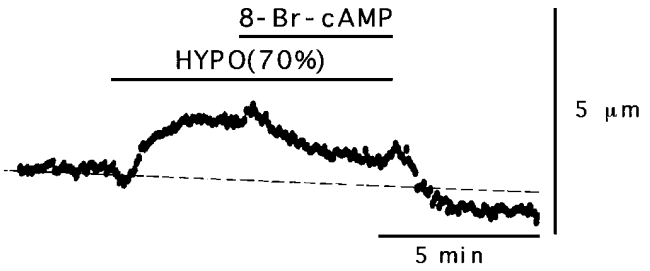

FIGURE 4. RVD induced by 8-Br-cAMP in osmotically inflated cells. Application of solutions is indicated by the horizontal bars. The cell thickness in the control Tyrode solution was 6 μm. The dotted line indicates the basal cell thickness.

The application of ACh alone did not cause any obvious changes in cell thickness ($n = 3$, Fig. 5 *B*).

Involvement of $I_{Cl,cAMP}$ in Isoprenaline-induced RVD

β-adrenergic stimulation increases the amplitude of various time- and voltage-dependent current systems, such as L-type Ca^{2+} current ($I_{Ca,L}$) and delayed rectifier K^+ current (I_K) (Kameyama et al., 1985) in addition to the time-independent $I_{Cl,cAMP}$ in guinea pig ventricular cells. It is unlikely that $I_{Ca,L}$ or I_K was involved in isoprenaline-induced RVD, since these currents would not be expected to be activated at the resting membrane potential. To confirm the involvement of $I_{Cl,cAMP}$, effects of isoprenaline were compared in the absence and presence of 9AC, a blocker of $I_{Cl,cAMP}$ (Harvey et al., 1990; Levesque et al., 1993). In the experiment shown in Fig. 6, the isoprenaline-induced RVD was confirmed first in 70% hypotonic solution. In the presence of 500

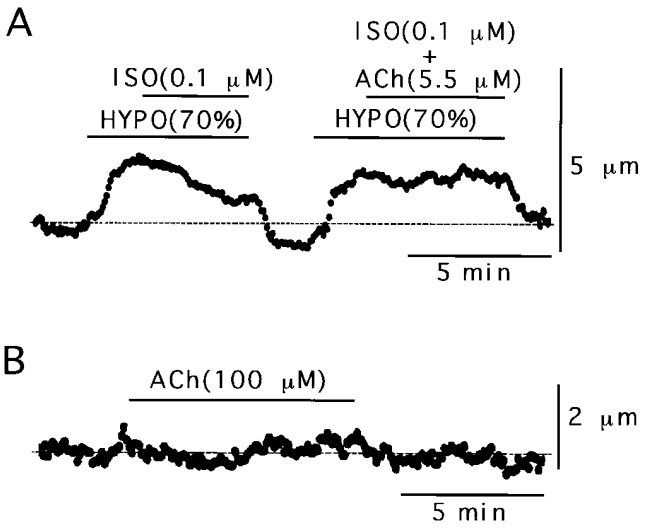

FIGURE 5. Effects of ACh on cell thickness. (*A*) Application of 5.5 μM ACh prevented isoprenaline-induced RVD in a cell in which isoprenaline had previously provoked such a response. The cell thickness in the control Tyrode solution was 11 μm. (*B*) ACh alone failed to induce a marked change in the cell thickness. The dotted lines indicate the basal cell thickness.

μM 9AC, the cell swelling was induced to the same extent by superfusing the same cell with 70% hypotonic solution. However, the application of isoprenaline failed to induce RVD ($n = 8$). In 5 out of 8 cells RVD induced by application of isoprenaline was completely abolished by 9AC; and in remaining 3 cells the RVD was partly blocked by 9AC.

Although we could not observe spontaneous RVD attributable to an activation of $I_{Cl,\ swell}$ in the control experiment (Fig. 2), the β-adrenergic stimulation might have enhanced the activation of $I_{Cl,\ swell}$ to induce RVD as demonstrated in the human ventricular cell (Oz and Sorota, 1995). This possibility was examined by using DIDS (1 mM), which causes a rapid and complete inhibition of $I_{Cl,\ swell}$ without inhibiting $I_{Cl,\ cAMP}$ (Vandenberg et al., 1994). We first confirmed the ineffectiveness of DIDS on $I_{Cl,\ cAMP}$ with the whole-cell patch clamp method. The I-V relations in Fig. 7 *A* were recorded by a ramp pulse before and during the application of isoprenaline (1 μM). The marked increase in the membrane conductance (●) is due to the activation of $I_{Cl,\ cAMP}$ and was not influenced by the additional application of DIDS (1 mM) (■). Essentially the same observations were obtained in 4 cells, confirming that $I_{Cl,\ cAMP}$ is not inhibited by DIDS. We then examined if isoprenaline could induce RVD in the presence of the same concentration of DIDS. In the experiment shown in Fig. 7 *B*, after the cell thickness increased to a steady level in the 70% hypotonic solution, application of 1 μM isoprenaline induced RVD. The isoprenaline-induced RVD was confirmed in 6 out of 7 cells in the presence of DIDS. These findings support the involvement of $I_{Cl,\ cAMP}$ in the isoprenaline-induced RVD.

Activation of $I_{Cl,cAMP}$ in Hypotonic Solution

Because of the technical complexity involved, it was difficult to conduct concurrent recordings of cell thickness and whole cell membrane current. Thus, to prove the activation of $I_{Cl,\ cAMP}$ in the hypotonic solutions, whole cell voltage clamp was conducted without automatic measurement of the cell thickness. In the experiment shown in Fig. 8, $I_{Ca,\ L,}$ $I_{K,1}$, and I_K were blocked (see MATERIALS AND METHODS), and the I-V relation was measured by a ramp command pulse from a holding potential of −80 mV. Exchange of the bath solution from the isotonic solution (70% $[Na^+]_o$ supplemented with mannitol) to 70% $[Na^+]_o$ hypotonic solution hardly changed the I-V relationship. Subsequent application of isoprenaline, however, induced an obvious increase in the membrane conductance. The isoprenaline-induced current showed a slight outward rectification, and the reversal potential was near the Cl^- equilibrium potential (E_{Cl} = −32 mV) expected from the Cl^- compositions of the internal and external solutions. It should also be noted that the I-V relationship before the application of isoprenaline is superimpos-

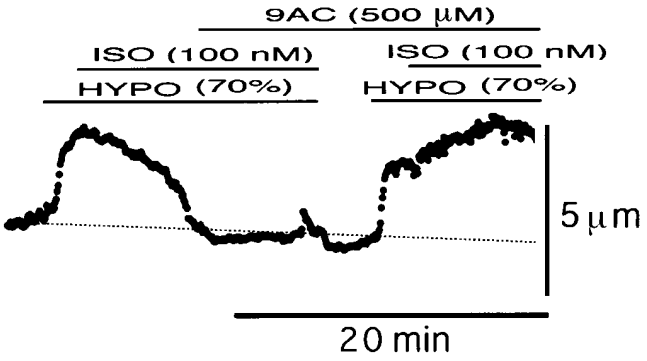

FIGURE 6. Prevention of isoprenaline-induced RVD by 9AC. Application of solutions is indicated by the horizontal bars. Application of 500 μM 9AC prevents isoprenaline-induced RVD in a cell in which isoprenaline had previously been shown to induce RVD. The dotted line indicates the basal cell thickness.

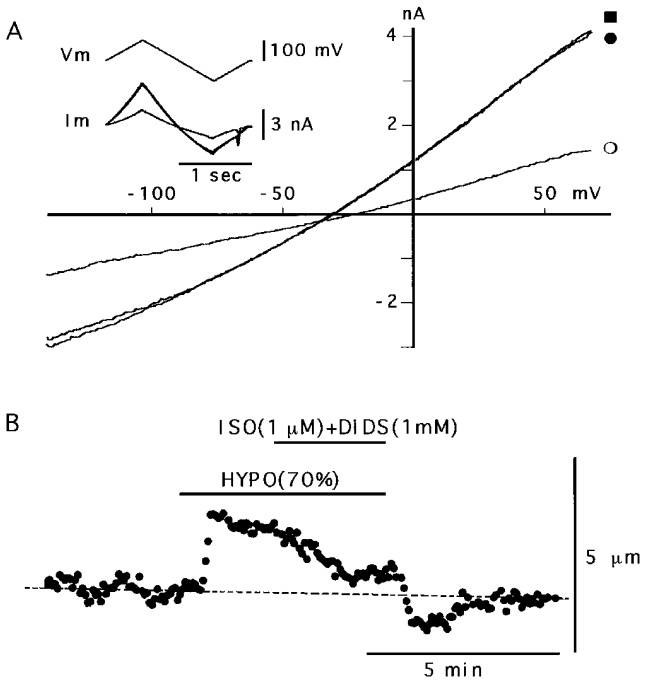

FIGURE 7. $I_{Cl,cAMP}$ and isoprenaline-induced RVD observed in the presence of DIDS. (*A*) Effects of DIDS on $I_{Cl,\ cAMP}$. The inset shows the original current recordings (I_m) with the ramp command pulse (V_m) obtained after blocking $I_{Ca,L}$, $I_{K,1}$, and I_K. The ramp pulse started from −40 mV holding potential to +60 mV and then to −140 mV. The I-V curves in the main panel were determined from the descending limb of the ramp pulse. The current recordings superimposed were control (○), in the presence of isoprenaline (1 μM) (●) and in the presence of both isoprenaline (1 μM) and DIDS (1 mM) (■). (*B*) Isoprenaline-induced RVD in the presence of DIDS. Application of solutions is indicated by the horizontal bars. The cell thickness in the control Tyrode solution was 9.5 μm and the increase of the cell thickness is 1.8 μm. The dotted line indicates the basal cell thickness.

able with that in the isotonic external solution, indicating that $I_{Cl,\ swell}$ was not activated at least before the application of isoprenaline.

Changes in the cell thickness was measured only by eye during the experiment. On average, cell thickness increased to 133.1 ± 4.2% (n = 7) in the hypotonic solution (70%) from the basal thickness of 7.7 ± 0.4 μm. In 3 experiments the RVD induced by isoprenaline nearly completely returned cell thickness to control levels, in 2 experiments the RVD was incomplete (~73 and 60%) and in the remaining 2 experiments the RVD could not be detected. These findings confirmed the involvement of $I_{Cl,\ cAMP}$ in RVD response induced by isoprenaline.

DISCUSSION

Summary and Conclusion

The present study has demonstrated that spontaneous RVD does not occur in osmotically inflated guinea pig ventricular myocytes within 15 min of swelling. However, RVD can be induced by the β-adrenergic stimulation. The involvement of $I_{Cl,\ cAMP}$ activation in RVD is strongly suggested by the results of the present study: i.e., the application of isoprenaline or 8-Br-cAMP, both activators of $I_{Cl,\ cAMP}$, was able to induce RVD and, further, the isoprenaline-induced RVD was blocked by 9AC and by ACh (Bahinski et al., 1989; Harvey and Hume, 1989; Matsuoka et al., 1990; Harvey et al., 1990; Tareen et al., 1991; Levesque et al., 1993). The former blocks the $I_{Cl,\ cAMP}$ channel directly, and the latter antagonizes the activation of adenylate cyclase mediated by the GTP-binding protein (Gi). The activation of $I_{Cl,\ cAMP}$, which is different from $I_{Cl,\ swell}$, by the β-adrenergic stimulation is well established in the guinea-pig ventricular myocytes (Vandenberg et al., 1994). Furthermore, the whole cell voltage clamp experiments confirmed the activation of $I_{Cl,\ cAMP}$ during RVD even in the osmotically inflated myocytes.

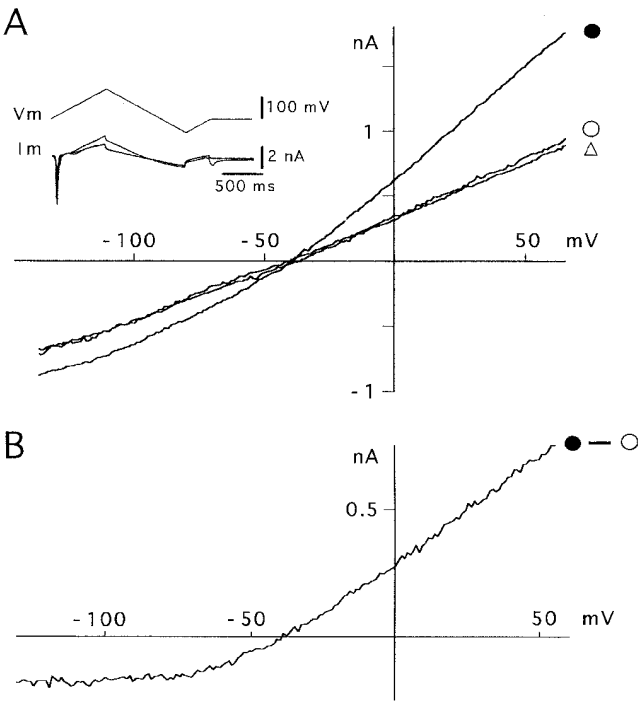

FIGURE 8. Activation of $I_{Cl,\ cAMP}$ by isoprenaline in hypotonic (70%) solution. (*A*) The inset shows the original current recordings (I_m) with the ramp pulse (V_m). The ramp started from a holding potential of −80 mV to +60 mV and then to −140 mV. Three current recordings were superimposed, control (○), in the hypotonic solution (△), and during the application of 50 nM isoprenaline (●). The I-V curves in the main panel were determined from the descending limb of the ramp pulse. (*B*) The isoprenaline-induced current was obtained by subtracting the control current from that in the presence of isoprenaline.

In the human ventricular myocytes, forskolin induced no extra Cl^- current in control, but increased $I_{Cl,\ swell}$ when $I_{Cl,\ swell}$ was induced beforehand by cell swelling (Oz and Sorota, 1995). However, in the present study the Cl^- current was activated by isoprenaline when no $I_{Cl,\ swell}$ was observed in the 70% osmotic solution (Fig. 8) and the Cl^- current was not blocked by DIDS (Fig. 7 *A*), which causes a rapid and complete inhibition of $I_{Cl,\ swell}$ (Vandenberg et al., 1994). Thus, the Cl^- current in Fig. 7 *A* and Fig. 8 is considered to be $I_{Cl,\ cAMP}$. The isoprenaline-induced RVD in the presence of DIDS also confirmed the involvement of $I_{Cl,\ cAMP}$. Isoprenaline also activates Na^+-K^+ pump (Desilets and Baumgarten, 1986) and Na^+-K^+-$2Cl^-$ co-transporter (Sarkadi and Parker, 1991), but it is unlikely that 9AC blocked these ion transporters.

The isoprenaline-induced RVD can be explained as follows. The value of E_{Cl} in the cardiac myocyte ranges from −45 to −65 mV (Vaughan-Jones, 1982), therefore, at a resting membrane potential near −90 mV, intracellular Cl^- is driven in the outward direction through the membrane. Thus, intracellular Cl^- should be extruded via activation of $I_{Cl,\ cAMP}$ channel and depolarize the membrane. Then, the membrane depolarization induces an extra efflux of cellular K^+, which is driven by the increased electromotive force largely through inward rectifier K^+ channels. The outward movement of K^+ and Cl^- results in an outflow of cellular water and thus in RVD. This view is consistent with the experimental finding of the undershoot of cell volume on returning to the isoosmotic solution after RVD was established.

The isoprenaline-induced RVD during the whole cell voltage clamp in present study is apparently inconsistent with the above hypothesis, since $I_{K,1}$ was blocked by replacing the intracellular K^+ with Cs^+ and also by the application of TEA. Furthermore, the membrane potential was maintained constantly during activation of

$I_{Cl,\ cAMP}$, so that we cannot expect the passive, depolarization-driven extra-movement of Cs^+ across the cell membrane, for example, through the nonselective cation background current system described in the same preparation (Kiyosue et al., 1993). One possible explanation is that the inward current applied by the clamp amplifier through the whole cell clamp electrode would be carried largely by a movement of Cs^+ from the cytoplasm to the pipette interior, since the mobility of the major counter ion (aspartate) would be relatively smaller than Cs^+ in the pipette solution used in the present study. Thus, the efflux of Cl^- through the cell membrane driven by the holding potential of −80 mV would be accompanied with an efflux of Cs^+ into the pipette solution, resulting in the loss of cellular solute together with cellular water. This hypothesis is evidently a subject for future experimentation.

Dissociation between the activation of Cl^- channel and RVD has been described. Hall et al. (1995) found that in chick embryonic heart cells RVD was not observed even when $I_{Cl,\ swell}$ was activated in the osmotically inflated cell and that RVD occurred under the perforated whole cell condition without activation of $I_{Cl,\ swell}$. Furthermore, in the present study 30–40% cells did not show RVD on application of isoprenaline, even though activation of $I_{Cl,\ cAMP}$ occurred consistently. A possible reason for the dissociation of RVD from the activation of I_{Cl} might be due to multiplex mechanisms of the cell volume regulation, such as the compromised amino acid loss and taurine transport (Zhang et al., 1993; Rasmusson et al., 1993), activation of Na^+-K^+ pump, Na^+-K^+-$2Cl^-$, Na^+-Cl^-, and Na^+-Ca^{2+} cotransport (Drewnowska and Baumgarten, 1991; Whalley et al., 1993; Tosteson and Hoffman, 1960; Smith et al., 1993) and the Na-H exchange (Whalley et al., 1994). When the cell begins to swell, the swelling automatically activates the Na^+-K^+ pump, moving more ions to the exterior and carrying water with them. Therefore, the Na^+-K^+ pump performs a continual surveillance role in maintaining normal cell volume. Our previous study (Sasaki et al., 1994) indeed demonstrated an increase in Na^+-K^+ pump current by 66% in swollen guinea-pig ventricular cell. Interestingly, this increase in pump current was observed in less than half of myocytes examined, depending on the Na^+ concentration within the internal solution. Some of the above mechanisms might be activated simultaneously with the development of cell swelling to bring the cell volume toward the equilibrium throughout the time course of cell swelling, so that a full-blown RVD could not be observed on the application of isoprenaline even if $I_{Cl,\ cAMP}$ was activated. An alternative explanation for the incomplete RVD might be a change in the cell shape during the superfusion of the hypotonic solution, which was not completely reversible on washing off the hypotonic solution. In the experiments observing RVD by the β-stimulation, we occasionally failed to observe the undershoot of the cell thickness on returning to the isoosmotic solution. Although we have no clear explanation, unknown drift of the recorded basal level might be suggested.

Physiological Relevance of RVD in Cardiac Myocytes

In many other tissues, spontaneous RVD is explained by the activation of $I_{Cl,\ swell}$ (Hoffmann and Simonsen, 1989; Sarkadi and Parker, 1991; Zhang et al., 1993). Cell swelling activates a Cl^- conductance along with K^+ and/or nonselective cation conductance, so that the outward movement of KCl, driven by the K^+ and Cl^- gradients, results in osmotically obliged water efflux and thus RVD. In cardiac myocytes it has been consistently shown that spontaneous RVD does not occur during hypotonic cell swelling (Drewnowska and Baumgarten, 1991), except in the cultured myocytes (Zhang et al., 1993). However, since $I_{Cl,\ swell}$ also exists in cardiac myocytes (Tseng, 1992; Sasaki et al., 1994; Vandenberg et al., 1994), the spontaneous RVD might potentially be induced through activation of $I_{Cl,\ swell}$ if the hypotonic stimulation was prolonged beyond the experimental time period in the present study. It should be noted that in the rabbit ventricular myocytes the application of 9-AC increased cell swelling by 44% induced by the 60% hypotonic solution, suggesting the involvement of $I_{Cl,\ swell}$ in the volume regulation (Suleymanian et al., 1994). This finding is different than the result in the present study (Fig. 2), and suggests species difference in the contribution of $I_{Cl,\ swell}$. Under physiological conditions, the heart is continuously under the influence of both adrenergic and muscarinic stimulation. This tonic activity of the sympathetic nerve causes a continuous, but moderate activation of $I_{Cl,\ cAMP}$. Since ACh antagonized the β-adrenergic stimulation, it seems that autonomic nerves may regulate cardiac cell volume in addition to their role in regulation of membrane excitability.

According to the above hypothesis, activation of $I_{Cl,cAMP}$ should have resulted in osmotically obliged water efflux and thus in a volume decrease even in the control Tyrode solution. However, we observed a slight cell shrinkage only in one cell (out of 16 cells) in the present study, and the more frequent observation was no change in the cell thickness. The failure of cAMP and forskolin to affect isosmotic cell volume has been documented previously in rabbit ventricular (Clemo et al., 1992) and atrial myocytes (Clemo and Baumgarten, 1995). At present we have no clear explanation for the volume effect of isoprenaline in the control Tyrode solution. It is interesting that the blockage of the Na^+ -K^+ pump did not always result in the cell swelling (Smith et al., 1993).

Egan et al. (1988) demonstrated that pressure ejection of a high concentration (1 μM) of isoprenaline from a drug-loaded micropipette produced depolarizations which were often large enough to initiate repetitive action potential discharge. These depolarizations were explained by activation of an inward current, which was later attributed to the activation of $I_{Cl,\ cAMP}$ (Matsuoka et al., 1990). However, the physiological function of $I_{Cl,\ cAMP}$ has, to date, remained unclear, since membrane excitability is not dramatically changed by a moderate activation of $I_{Cl,\ cAMP}$. Namely, basal activity of $I_{Cl,\ cAMP}$ of dissociated myocytes is negligibly small in the absence of the β-adrenergic agonists, and the increase of membrane Cl^- conductance by a moderate stimulation of the β-adrenergic receptor is minimal in contrast to the large K^+ conductance through the inward rectifier K^+ channels. The plateau of the ventricular action potential is only slightly shortened by the activation of $I_{Cl,\ cAMP}$ (Takano and Noma, 1992). The increase of the delayed rectifier K^+ current may also cancel the prolonging effect of the increased $I_{Ca,\ L}$. Thus, we propose that a major physiological function of the $I_{Cl,\ cAMP}$ channel is to mediate the autonomic neural regulation of the cell volume.

The importance of myocardial cell volume regulation may better be understood under pathological conditions. During ischemia cell swelling reduces chamber compliance (Vogel et al., 1986), thus reducing both systolic and diastolic function; it may also occlude coronary capillaries further augmenting ischemia. A plethora of changes occur during myocardial ischemia, but one well known consequence is the release of catecholamines (Videbaek et al., 1972; Karwatowska-Krynska and Beresewicz, 1983). RVD induced through activation of $I_{Cl,\ cAMP}$ by an increase in catecholamine level may operate to relieve the myocytes from the vicious cycle of ischemia resulting in cell swelling, in turn, leading to a worsening of ischemia.

Methodology of Measuring the Cell Volume

Based on the osmotically inactive fraction of 34% in the cardiac myocytes (Drewnowska and Baumgarten, 1991), a 28% swelling is expected on switching from 100 to 70% for a perfect osmometer. According to our previous study (Wang et al., 1996), the cell area increased to 118%. If the shape of cross section of a myocyte remains the same, the mean increase in cell thickness to 119.1 ± 1.1% in 70% hypotonic solution gives a volume increase by 43%, which is obviously overestimation. It may be speculated that the average increase in the cell thickness along the cell edge might be smaller than at the center (118%).

The new method of measuring the cell thickness was performed using conventional apparatus for measuring Ca^{2+} signals in the present study, and can be combined potentially with the whole cell voltage clamp using the patch clamp technique. Automatic measurement of cell height was also achieved by focusing on fluorescent microbeads placed on both the apical and basal surfaces of cultured epithelial cells (Van Driessche et al., 1993). Similar but manual measurement of the thickness of ventricular myocytes was performed previously using small (2–3 μm) particles placed on the chamber surface and top of the cell (Drewnowska and Baumgarten, 1991). The cell height was measured as the distance between these two markers. This method may be advantageous to our method, where the depth of the maker only on the top of the cell was measured, and therefore the measurement was affected by a temperature-dependent change in the distance between the stage of the microscope and the objective glass (in the range of several mm). The time resolution of 6 s and the accuracy higher than 0.1 μm may be similar to our method. The value of the standard error of mean was within 0.1 μm if the measurement of the marker position was done without using myocytes in the present study (see MATERIALS AND METHODS).

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