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# Cell swelling impairs dye coupling in adult rat ventricular myocytes. Cell volume as a regulator of cell communication

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## Abstract

The influence of cell swelling on cell communication was investigated in cardiomyocytes isolated from the ventricle of adult rats. Measurements of dye coupling were performed in cell pairs using intracellular dialysis of Lucifer Yellow CH. The pipette was attached to one cell of the pair and after a gig ohm seal was achieved, the membrane was ruptured by a brief suction allowing the dye to diffuse from the pipette into the cell. Fluorescence of the dye in the injected as well as in non-dialyzed cell of the pair was continuously monitored. The results indicate that in cell pairs exposed to hypotonic solution the cell volume was increased by about 60% within 35 min and the dye coupling was significantly reduced by cell swelling. Calculation of gap junction permeability (P(j)) assuming an the intracellular volume accessible to intracellular diffusion of the dye as 12% of total cell volume, showed an average P(j) value of $0.16 \pm 0.04 \times 10^{-4}$ cm/s (n = 35) in the control and $0.89 \pm 1.1 \times 10^{-5}$ cm (n = 40) for cells exposed to hypotonic solution ($P < 0.05$). Similar results were found assuming intracellular volumes accessible to the dye of 20 and 30% of total cell volume, respectively. Cell swelling did not change the rate of intracellular diffusion of the dye. The results, which indicate that cell volume is an important regulator of gap junction permeability, have important implications to myocardial ischemia and heart failure as well as to heart pharmacology because changes in cell volume caused by drugs and transmitters can impair cell communication with consequent generation of slow conduction and cardiac arrhythmias.

## Introduction

It is well known that a mechanical stimulus applied to the precordium can cause ventricular premature beats, ventricular tachycardia or even ventricular fibrillation [1].

On the other hand, acute stretch of cardiac muscle may play a role in cardiac arrhythmias generated during myocardial ischemia through the activation of membrane ionic channels like the IClswell and the IK-ATP channels [2, 3]. No information is available on the influence of cell swelling on intercellular communication in cardiac muscle.

The spread of impulses through the gap junctions is essential for impulse propagation and electrical synchronization of the heart beat [4]. The gap junction channel is commonly described as a weakly selective ion channel permeable to hydrophilic molecules of about 1 KD [5].

Several studies indicated that Lucifer Yellow CH−, a non-toxic-substituted naphthalimide with two sulfonated groups (mol weight 457 Da), diffuses through the cytoplasm and gap junctions but does not cross the surface cell membrane [4, 6]. This means Lucifer Yellow CH is an important fluorescent probe useful in studies of intercellular communication when introduced into the cell.

The junctional permeability in cardiac muscle is modulated by different factors like intracellular Ca concentration [7] and cAMP [4, 6, 8]. Our knowledge of the influence of cell swelling on gap junctional permeability is, however, scanty. This is of fundamental importance to heart cell biology because it is well known that during myocardial ischemia, which is characterized by cell swelling, cell coupling is abolished [9] providing the substrate for the generation of slow conduction and cardiac arrhythmias.

Recent studies indicate that cell swelling induces early after depolarization and finally blocks impulse propagation in the failing heart [10]. These findings raise the possibility that cell volume is involved in the regulation of intercellular communication in cardiac muscle. It is then important to investigate if the gap junctional permeability is impaired by cell swelling. In the present work this problem was investigated in ventricular myocytes isolated from adult rat heart.

## Methods

Normal male adults Sprague–Dawley rats (250 g body weights) were used. The animals were kept in the Animal House at constant temperature (24°C) and humidity following the recommendations of NIH. Animals were kept on a normal laboratory animal diet and given tap water ad libitum. The animals were anesthetized with sodium pentobarbital (50 mg/kg, ip), and the heart was removed with the animals under deep anesthesia.

### Cell isolation procedure

Cells were obtained from the ventricle of normal adult rats following the method of Powell and Twist [11] and Tani- gushi et al. [12]. The heart was removed and immediately perfused with normal Krebs solution containing: (mM): NaCl 136.5; KCl 5.4; CaCl2 1.8; MgCl2 0.53; NaH2 PO4 0.3; NaHCO3 11.9; glucose 5.5; HEPES 5, pH adjusted to 7.3. After 20 min a Ca-free solution containing 0.4% collagenase (Worthington Biochemical Corp) was recirculated through the heart for 1 h. The collagenase solution was washed out with 100 ml of recovery solution containing (mM): taurine 10; oxalic acid 10; glutamic acid 70; KCl 25; KH2 PO4 10; glucose 10; EGTA 0.5; pH 7.4. All solutions were oxygenated with 100% O2. Ventricles were minced (1–2-mm thick slices) and the resulting solution was agitated gently and the suspension was filtered through nylon gauze and the filtrate centrifuged for 4 min at 22 g.

The cell pellets were then resuspended in normal Krebs solution. Suction pipettes were pulled from microhematocrit tubing by means of a controlled puller (Narashige). The pipettes were prepared immediately before the experiments and filled with the following solution (mM): cesium aspartate 120; NaCl 10; MgCl2 3; EGTA 10; tetraethylammonium chloride 20; Na2 ATP 5; HEPES 5; pH 7.3. Large patch electrodes were used to facilitate the diffusion of Lucifer Yellow CH between the electrode lumen and the cell interior.

### Measurements of cell width, length, and volume

Measurements of cell length and width were made in quiescent ventricular myocytes immersed in Krebs solution using an inverted phase contrast microscope (Nikon) and a high-resolution camera (Paxcam). Images were obtained with a Pax-it imaging management system and stored into a computer. The volume of the cardiomyocytes was calculated by the following equation [13]: V. pwdl = 4 where w is the width, d is the depth, and l the length of the myocyte. Moreover, it was assumed that the cells were elliptical cylinders and that the depth (d) was 1/3 of the value of width [14]. Validation of these measurements was performed using a high-resolution video camera to measure the cell area and width.

### Experimental procedures

All experiments were performed in a small chamber mounted on the stage of an inverted phase-contrast microscope (Diaphot, Nikon). Ventricular cells were placed in a modified cultured dish (volume 0.75 ml) in an open-perfusion microincubator (Model PDMI-2, Medical Systems). Cells were allowed to adhere to the bottom of the chamber for 15 min and were superfused with normal Krebs solution (3 ml/min) that permits a complete change of the bath in less than 500 ms. A video system (Diaphot) made possible to inspect the cells and the pipettes throughout the experiments. The electrical measurements were carried out using the patch clamp technique in a whole cell configuration with an Axon (model 200B) patch-clamp amplifier. The leak currents were digitally subtracted by the P/N method (n = 5–6). Experiments performed without leak subtraction indicated low and stable leak currents. Series resistance originated from the tips of the micropipettes was compensated for electronically at the beginning of the experiment.

**Measurements of dye coupling—**Cell pairs of ventricular myocytes were used. Patch pipettes with a diameter of 2–5 lm were filled with a solution (mM): cesium aspartate 120; NaCl 10; MgCl2 3; EGTA 10; tetraethylammonium chloride 20; Na2 ATP 5; HEPES 5 pH 7.3 containing 4% of Lucifer Yellow CH. The pipette was attached to one cell of the pair and after a gig ohm seal was achieved the membrane was ruptured by a brief suction allowing the dye to diffuse from the pipette into the cell. Subsequently, the dye was allowed to diffuse out of the pipette into the dialyzed cell as well as in adjacent cells. Fluorescence of the dye in the injected and non-dialyzed cell of the pair was continuously monitored using a Nikon inverted microscope (Tokyo) equipped with a NIS-Elements Documentation System, Camera and a mercury-100 W lamp. Cells were observed using an objective with a magnitude of × 63. Images were captured in both bright field and fluorescence modes. The concentration of the dye in each cell was measured with NIS-Elements Documentation System and computer system. Because bleaching has been found with many fluorescent dyes [4] a low light intensity was used. Under these conditions bleaching was found to be very small and did not interfere with the measurements of cell communication. In some experiments, the influence of hypertonic solution on cell volume and dye coupling was investigated. For this, the pipette solution was made hypertonic and Lucifer Yellow CH (4%) was added to it.

### Drugs

Lucifer Yellow CH was from Sigma Chemical Co, St. Louis, MO.

### Statistical analysis

Data are expressed as mean ± SEM. Student’s t test was used. Comparison between groups was done by analysis of variance (ANOVA). Differences were considered significant when $P < 0.05$.

## Results

As shown in Fig. 1, Lucifer Yellow CH dialyzed into the cell spreads easily to nearby cells reaching full equilibration between two adjacent cells within 10–12 min. Since Lucifer Yellow CH (mol wt 457 Da) does not diffuse through the surface cell membrane [6], the intercellular diffusion of the dye indicates the presence of permeable gap junctions.

To investigate the influence of cell swelling on intercellular communication cells were exposed to hypotonic solution (prepared by diluting the normal solution to one-third) and measurements of cell volume were performed every 2 min. As shown in Fig. 2 the cell volume was significantly increased (60%) reaching a maximal value around 35 min. At the

end of this time, Lucifer Yellow CH was dialyzed into one cell of the pair and measurements of fluorescence were performed in the dialyzed cell and in a nearby cell as a function of time. As shown in Fig. 3, hypotonic solution caused a drastic reduction in dye coupling. A quantitative estimation of the gap junction permeability was made using the following equation [15]:

$$P_j = V_{cell}/A_j \times K_j$$

where $V_{cell}$ is the cell volume that is accessible to Lucifer Yellow CH, $A_j$ is area of the gap junctional membrane, and $K_j$ is the rate constant of the transjunctional diffusion [15]. Morphometric studies performed on ventricular tissues [16] indicate that $A_j$ contributes to 17% of the cross-sectional area of the rat cardiomyocyte (177 l m2) [17], and that myofibrils, mitochondria, sarcoplasmic reticulum, and nucleus occupy about 88% of the total cell volume leaving 12% of total cell volume free for the dye diffusion through the cytoplasm. The junctional permeability ($P_j$) was then calculated for controls and after 35 min of exposure to hypotonic solution. As shown in Table 1, $P_j$ calculated taking $V_{cell}$ as 12% of total cell volume, was appreciably reduced from $0.16 \pm 0.04 \times 10^{-4}$ cm/s (n = 35) in the control, to $0.89 \pm 1.1 \times 10^{-5}$ cm/s (n = 40) after 35 min of exposure to hypotonic solution. Table 1 also includes values of $P_j$ assuming $V_{cell}$ as 20 and 30% of total cell volume, respectively. As it can be seen, the values of $P_j$ were significantly reduced by cell swelling.

In other experiments, cell swelling produced by intracellular dialysis of a hypertonic solution (prepared by increasing the NaCl concentration of intracellular solution 233 to 200 mM) into just one cell of a single pair, also caused, as expected, an increase in cell volume of the dialyzed cell as well as cell uncoupling as shown in Fig. 4. Interestingly, parallel measurements of cell volume performed in the normal adjacent cell not dialyzed with hypertonic solution, showed a decrease in cell volume (not shown). The possibility that the diffusion of Lucifer Yellow CH throughout the cytoplasm is reduced in swollen cells and consequently enhancing the time required by the dye to reach the non-dialyzed cell was investigated by measuring the time course of the fluorescence in the injected cell under isosmotic and hypotonic conditions. As shown in Fig. 5, there is no significant change in the time course in swollen cell when compared with controls. Results from 18 cells (3 animals) showed no significant results ($P > 0.05$).

## Discussion

The present results indicate, by the first time, that cell swelling severely impairs cell communication in rat ventricular cardiomyocytes, a conclusion supported by the drastic decline in the spread of Lucifer Yellow CH from cell-to-cell. Previous studies demonstrated that Lucifer Yellow CH is an appropriate fluorescent probe for studies of intercellular communication because the dye does not cross the surface cell membrane but flows easily through the gap junctions [4,6]. Moreover, the possibility that the fall in dye coupling be related to a decrease in sarcoplasmic diffusion of Lucifer yellow CH was discarded. These findings lead us to conclude that cell swelling reduces the gap junction permeability. Recent findings showed that cell swelling impairs and finally blocks the impulse propagation in the whole ventricle of cardiomyopathic hamsters [10] as well as in the rat ventricle (De Mello, unpublished). The decrease of impulse propagation might be related to: (a) a decline in gap junctional conductance; (b) a fall in surface membrane resistance. The activation of ionic channels including the swelling-activated chloride channel (IClswell) caused by cell swelling [10,18] seems to indicate that a decline in surface cellmembrane resistance is, at least in part, related to the fall in impulse propagation. Indeed, the coupling coefficient (Cc)

is equal to gj/gj ? gjn, where gj is the junctional conductance and gjn is the conductance of the surface cell membrane. Moreover, the possibility that an increase in membrane capacitance be involved in the decline of impulse propagation in swollen cells cannot be discarded. It is known that acute stretch causes an unfolding of slack membrane and integration of caveolae with possible consequences for membrane excitability. Mills et al. [19] showed that passive ventricular volume loading to 30 mmHg causes unfolding of the slack membrane and an integration of caveolae into the sarcolemma. Consequently, membrane proteins in the caveolae are now in contact with the extracellular fluid with possible changes in cardiac electrophysiology [1,19]. These stretch-associated effects would result in increased capacitive electrical load with consequent decline of conduction.

It is important to emphasize, however, that acute membrane stretch and cell swelling are not the same and can produce different changes in cellular functions [20]. Cell swelling, for instance, changes the intracellular ionic concentrations and activates signaling pathways different from those elicited by mechanic stress [21,20].

The mechanism by which cell swelling impairs the gap junction permeability is not known but it is probably multifactorial. Cell swelling, stimulates protein kinase C [21] and enhances tyrosine phosphorylation of several proteins, a finding particularly relevant because it is known that Ang II changes the gap junction conductance as well as the inward calcium current in the heart through the activation of PKC and tyrosine kinases [22,23]. It is well known that ion channel proteins may be phosphorylated and that this phosphorylation can change the functional properties of ion channels [24]. Connexin43 which is the most important connexin in mammalian heart ventricle is a phosphoprotein [25]. Previous studies indicated that cAMP and consequent PKA activation increases the gap junction conductance and permeability in the heart [6,8]. It is known that an increase in the intracellular free calcium reduces the junctional conductance in cardiac muscle [7] and that cell swelling increments the intracellular calcium [26]. The role of the increase in free $Ca^{+2}$ in the decline in dye coupling, however, seems unlikely due to the use of EGTA (10 mM) in the pipette solution. Further studies will be needed to clarify the mechanisms involved in the decline of junctional permeability in cardiac muscle induced by cell swelling.

## Conclusions

The present results demonstrate that cell volume is an important regulator of gap junction permeability in cardiac muscle. These findings indicate that under pathological conditions like myocardial ischemia and heart failure which are characterized by cell swelling, the impairment of cell coupling and impulse propagation is, in part, related to the increase of cell volume. Implications are extended to cardiac pharmacology because drugs and peptides like angiotensin II that increase heart cell volume, also reduces cell communication. These findings open a new window on studies of intercellular communication particularly during pathological conditions. It is known that oxygenated myocytes possess the ability to decrease the cell volume enhanced by hypo-osmotic challenge by activation of regulatory volume decrease (RVD) mechanisms that are largely dependent on opening of chloride channels [22,27]. Therefore, inhibition of RVD leads to impairment of chemical and electrical communication between cardiac cells with serious consequences for electrical and mechanical function of the heart.

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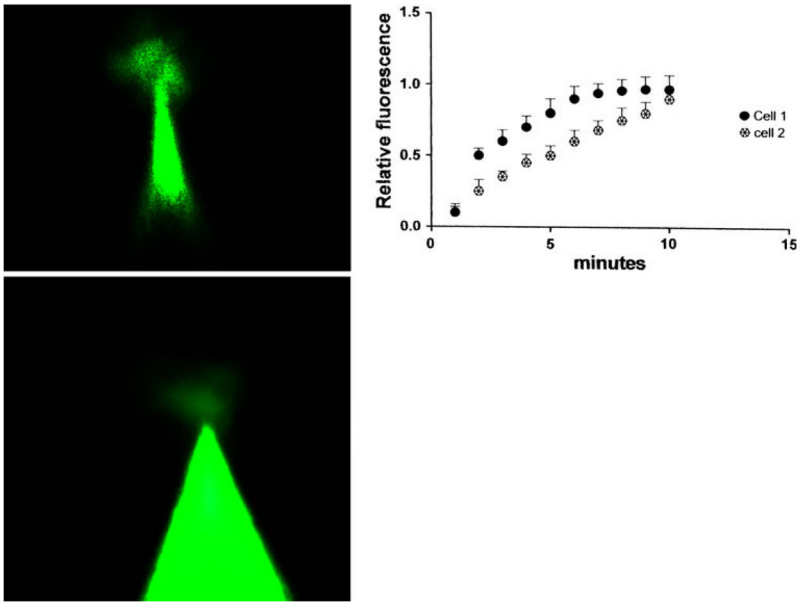


**Fig. 1.**

Left-Top shows Lucifer Yellow CH spreading in a cell pair immersed in normal Krebs solution. Bottom beginning of intercellular diffusion of the dye recorded in the first minutes of the experiment. Right plot of fluorescence intensity versus time. Lucifer Yellow CH was dialyzed into cell 1 and measurements of fluorescence intensity were performed in cell 1 and nearby cell 2, respectively, as a function of time. Each point represents the average from 40 cells (4 animals). Vertical line at each point SEM ($P<0.05$)

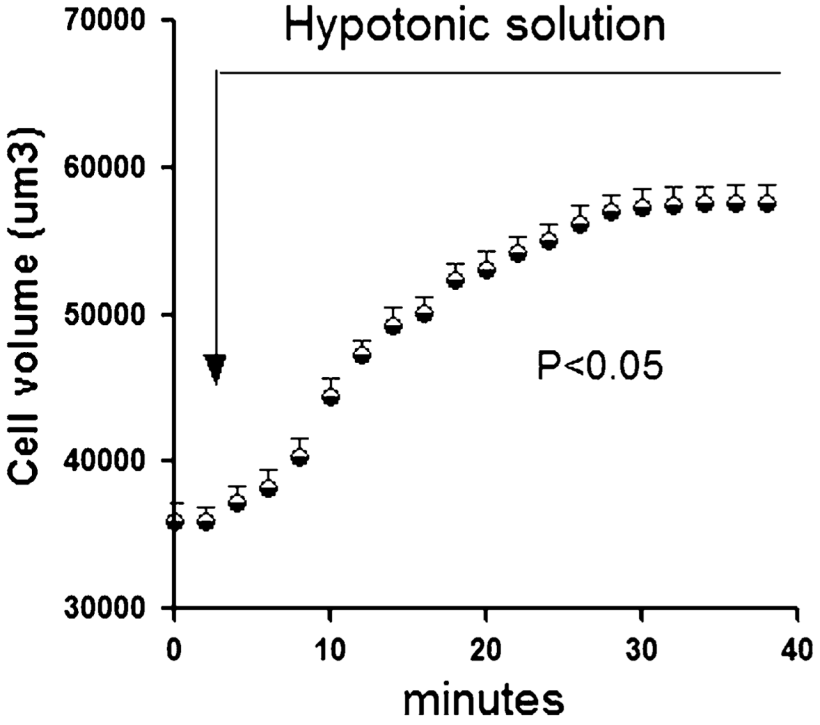


**Fig. 2.**
Influence of hypotonic solution on cell volume of rat ventricular myocytes. Each point is average from 38 cells (6 animals). Vertical line at each point SEM (P<0.05)

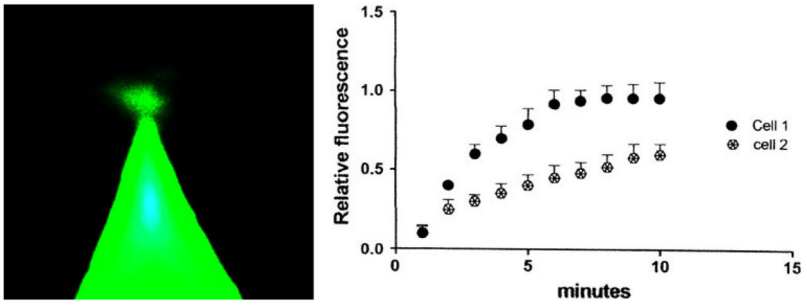


**Fig. 3.**

Left shows lack of intercellular diffusion of Lucifer Yellow CH recorded from a single cell pair previously superfused with hypotonic solution for 35 min. Right plot of fluorescence intensity as a function of time recorded for cells 1 and 2, respectively, and previously exposed to hypotonic solution for 35 min. Each point is the average from 43 cells(4 animals). *Vertical line* at each point SEM ($P<0.05$)

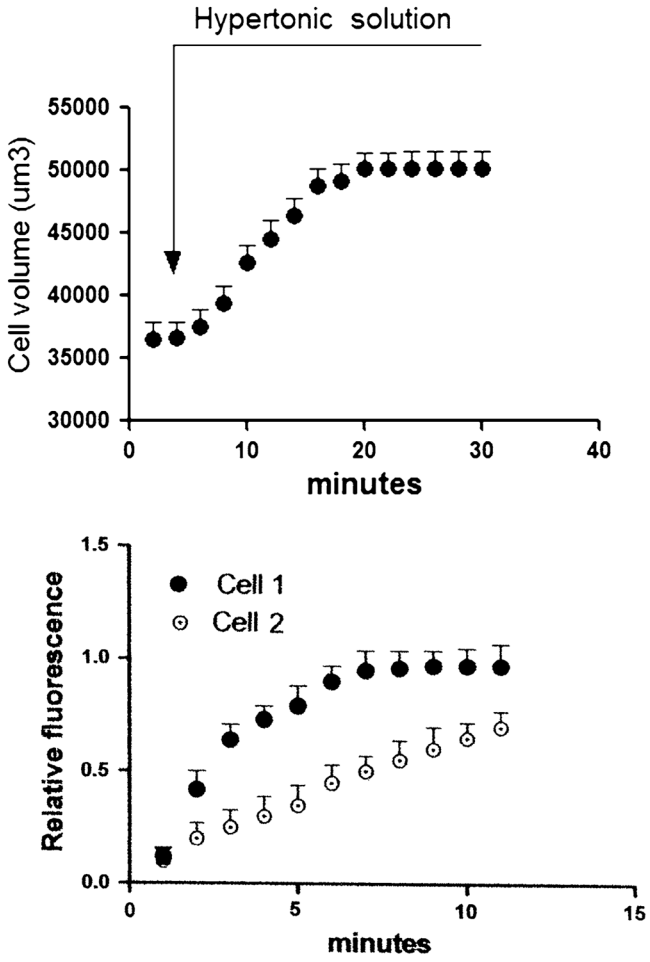


**Fig. 4.**

Top shows the increase in cell volume elicited by dialysis of hypertonic solution into a rat cardiomyocyte from a cell pair immersed in normal Krebs solution Each point is the average from 33 cell pairs (3 animals). *Vertical line* at each point SEM ($P<0.05$). Bottom plot of fluorescence intensity as a function of time for cell 1 dialyzed with hypertonic solution containing the dye. As it can be seen a drastic decline in intercellular coupling was produced. Each point is the average from 37 cells (4 animals). *Vertical line* at each point SEM ($P<0.05$)

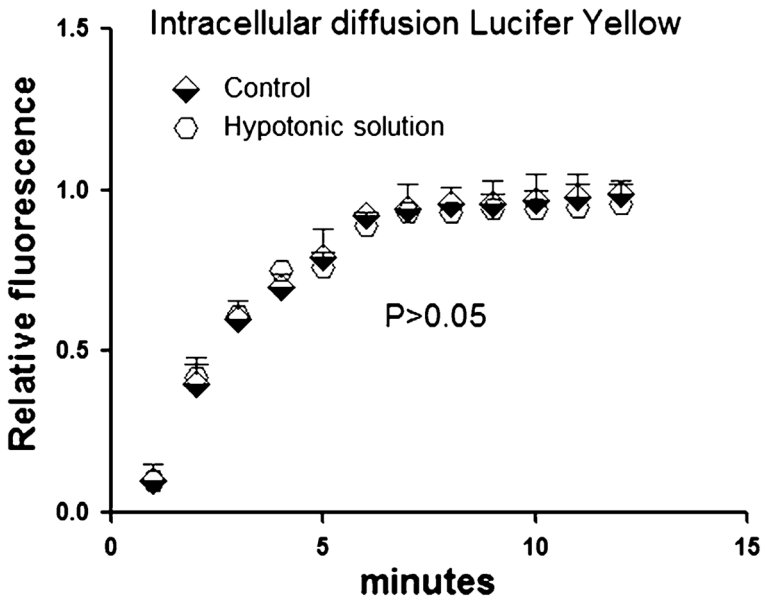


**Fig. 5.**

Lack of influence of hypotonic solution of intracellular diffusion of Lucifer Yellow CH in cells of normal rat ventricle. Each point is the average from 18 cells (3 animals). *Vertical line* at each point SEM (P>0.05)

## Table 1

Influence of hypotonic solution on cell volume and gap junction permeability ($P_j$) cm/s

| | Control | P | Hypotonic solution |
|---|---|---|---|
| **Total cell volume** | 36000 ± 1100 | <0.05 | 57600 ± 980 |
| | ($\mu m^3$) | | ($\mu m^3$) |
| | (n=30) | | (n=34) |
| | (5 animals) | | (5 animals) |
| | **$P_j$** | **P** | **$P_j$** |
| $V_{cell}$ 12% | $0.16 \pm 0.04 \times 10^{-4}$ | <0.05 | $0.89 \pm 1.1 \times 10^{-5}$ |
| | (n = 35) | | (n = 40) |
| | (6 animals) | | (6 animals) |
| $V_{cell}$ 20% | $0.27 \pm 0.05 \times 10^{-4}$ | <0.05 | $1.4 \pm 1.3 \times 10^{-5}$ |
| $V_{cell}$ 30% | $0.37 \pm 0.04 \times 10^{-4}$ | <0.05 | $2.2 \pm 1.6 \times 10^{-5}$ |

Vcell is the fraction of total cell volume occupied by Lucifer Yellow CH inside the cell and taken as 12, 20, and 30%, respectively of the total cell volume [15]. Aj was taken as 17% of the cross-sectional area of the rat cardiomyocyte (177 lm2) [17]