A Microfluorometric Method for Simultaneous Measurement of Changes in Cytosolic Free Calcium Concentration and pH in Single Cardiac Myocytes

Izumi SHIBUYA, Kiyoji MATSUYAMA, Kazuhiro TANAKA,
and Katsuhiko DOI

Department of Physiology, Yamagata University School of Medicine,
Yamagata, 900-23 Japan

Summary We describe a new system to measure changes in cytosolic free Ca concentration ($[Ca^{2+}]_i$) and pH (pH_i) simultaneously using the dyes fura-2 and BCECF, and a random access camera with computer-operated synchronously-changing excitation and emission filters. Fluorescence intensities for fura-2 and BCECF were recorded from rat cardiac myocytes and calibrated *in vivo* using multiple-point determinations. $[Ca^{2+}]_i$ and pH_i were 88.6 ± 32.8 nM and 7.16 ± 0.12 , respectively.

Key words: cytosolic free calcium concentration, cytosolic pH, microfluorometry.

Changes in $[Ca^{2+}]_i$ and pH_i are known to crucially alter cellular functions in various cells. In cardiac myocytes, $[Ca^{2+}]_i$, pH_i and contractile force are reported to be closely correlated: low pH has inhibitory effects on (a) Ca^{2+} activated force development (BOUNTRA and VAUGHAN-JONES, 1989); (b) the slow inward Ca^{2+} current (IRISAWA and SATO, 1986); (c) Ca^{2+} binding to troponin (BLANCHARD and SOLARO, 1984); and (d) Ca^{2+} release and uptake by sarcoplasmic reticulum (ORCHARD, 1987). In ischemic cardiac myocytes, it has been suggested that the loss of force development during ischemia is caused by intracellular acidification (VANHELL *et al.*, 1989). On the other hand, an increase in $[Ca^{2+}]_i$ produces intracellular acidification (VAUGHAN-JONES *et al.*, 1983). Thus, the interplay between $[Ca^{2+}]_i$ and pH_i is important in controlling contractile function under normal as well as pathological conditions.

In order to understand the complex mechanisms regulating contractile function in cardiac myocytes, it is important to follow the changes in both $[Ca^{2+}]_i$ and pH_i simultaneously and quantitatively under various cellular conditions. To date, the simultaneous measurement of $[Ca^{2+}]_i$ and pH_i has been reported by FUJIMOTO

et al. (1989) by the combined use of Ca^{2+} - and pH-sensitive microelectrodes. Recent development of fluorescent dyes such as fura-2 (GRYNKIEWICZ *et al.*, 1985) and BCECF (RINK *et al.*, 1982) has enabled measurements of $[\text{Ca}^{2+}]_i$ and pH_i with greater time and spatial resolution in intact cell preparations, and MIYATA *et al.* (1989) have successfully measured $[\text{Ca}^{2+}]_i$ and pH_i from cardiac myocytes simultaneously loaded with these two dyes. To measure changes in $[\text{Ca}^{2+}]_i$ and pH_i simultaneously and continuously over a time scale of seconds using these two dyes, precise control of excitation and emission filters becomes crucial. In this paper, we describe the development of a system for simultaneous and continuous measurement of $[\text{Ca}^{2+}]_i$ and pH_i from myocytes loaded with fura-2 and BCECF by controlling the movements of the optical system in a synchronized manner. To record fluorescence, we used a random access camera system, with which up to 100 data points can be obtained independently in the visual field. To confirm the quantitative accuracy of $[\text{Ca}^{2+}]_i$ and pH_i values obtained by this method, we used "multiple-point" *in vivo* calibrations for both dyes, and examined possible errors due to interference between $[\text{Ca}^{2+}]_i$ and pH_i measurements.

1. Isolation of single cardiac myocytes

Single cardiac myocytes were isolated from the ventricles of male Sprague-Dawley rats weighing 250–350 g by a modified method of STEMMER *et al.* (1989). The medium used for the isolation procedure was a modified Krebs-Henseleit buffer (KHB: 120 NaCl, 24 NaHCO_3 , 3.8 KCl, 1 KH_2PO_4 , 12 MgCl_2 , 2.5 Na-pyruvate, and 10 glucose, in mM, and saturated with 5% CO_2 in O_2 at 37°C, pH 7.4). Under pentobarbital anesthesia (5 mg/100 g, i.p.), the heart was removed and perfused retrogradely via the aorta with KHB containing 1.8 mM CaCl_2 at a rate of 10 ml/min for 10 min. The perfusion was then switched to KHB containing 10 μM CaCl_2 (low Ca^{2+} solution) for 10 min, and this low Ca^{2+} solution (60 ml) containing collagenase (Yakult; 0.1 mg/ml) was recirculated through the heart for an additional 30 min. Then, the ventricles were minced in 20 ml of this collagenase solution containing 1.5% bovine serum albumin (BSA) and shaken vigorously for 30 min at 35°C. The resultant cell and tissue suspension were filtered through a nylon mesh (pore size, 250 μm) and allowed to settle. Light debris was then removed from the supernatant fraction. The myocytes were resuspended in KHB containing 10 μM CaCl_2 , 10 mM HEPES, and 0.1% BSA (saturated with 100% O_2 at room temperature, pH 7.4; storage solution) and stored at room temperature until use.

2. Loading of fluorescent dyes

To obtain optimal and constant loading of fura-2 and BCECF, the density of the myocyte suspension was adjusted to about 10^5 cells/ml before loading with acetoxymethyl esters of fura-2 (fura-2/AM; 5 μM) and BCECF (BCECF/AM; 1 μM) at 30°C for 1 h. After loading, myocytes were washed twice with dye-free storage solution.

3. Recording apparatus

Schematic illustration of the recording apparatus is shown in Fig. 1A. Light emitted from a 150 W Xenon lamp was passed through excitation filters (380 and 340 nm for fura-2; and 500 and 440 nm for BCECF) held in a filter wheel, and introduced into an inverted microscope (Diaphoto-TMD, Nikon). The beam was

Fig. 1. A: Schematic illustration of the apparatus used for the simultaneous measurement of $[Ca^{2+}]_i$ and pH_i . Abbreviations are as follows: CH, camera head; CCU, camera control unit; PC1 and PC2, personal computers; M1 and M2, stepping motors; XL, Xe lamp; L1-3, quartz lenses; FW, filter wheel; EF, excitation filter; NDF, neutral density filter; DM, dichroic mirror; EMF, emission filter; FC, filter cassette; and RC, reaction chamber. B: Schematic illustration of the optical system. Abbreviations are as follows: FC1, filter cassette containing NDF, DM, and EMF for fura-2; FC2, filter cassette containing NDF, DM, and EMF for BCECF; and EC, electrical circuit to control stepping motors. The other abbreviations are the same as in A.

passed through neutral density filters (transmittance 63% for fura-2; 10% for BCECF) held in two separate filter cassettes (FC1 and FC2), and reflected by dichroic mirrors (reflecting wavelengths $<400\text{ nm}$ for fura-2; $<510\text{ nm}$ for BCECF) also held in the two filter cassettes, onto myocytes in a reaction chamber via an objective lens (CF Fluor 20x, Nikon). The temperature of the solution in the chamber was precisely regulated at $37 \pm 0.2^\circ\text{C}$. The fluorescence from the sample was introduced into a random access camera after being passed through the dichroic mirrors and emission filters (passing wavelengths $>420\text{ nm}$ for fura-2; $>520\text{ nm}$ for BCECF) which were also held in the two filter cassettes.

4. *Synchronized control of optics*

In order to record time courses of changes in fluorescence from both fura-2 and BCECF simultaneously, dichroic mirrors and emission filters were changed in accordance with the change in excitation filters. For this purpose, we developed original computer programs to select filters in both excitation and emission sides in a synchronized manner. Figure 1B shows a schematic illustration of the optical system. The computer, PC2, was connected to dual stepping motors: M1 to select one of the excitation filters (F1 and F4) set in the filter wheel, and M2 to select either of the two filter cassettes, FC1 and FC2. M1 directly drove the filter wheel, and M2 controlled the movement of an aluminum plate on which the filter cassettes were fixed via a cam. The plate could be moved along a rail guide which was fixed to the microscope so that the filter cassettes could be changed smoothly. When F1 and F2 were selected, FC1 was positioned in the light path. Immediately after the measurement at F2, M2 moved the plate to place FC2 in the position, and F3 and F4 were selected. Finally, M2 moved the plate back to select FC1 and the cycle was repeated. Each time an excitation filter stopped at the light path (for about 100 ms), PC2 sent a trigger pulse to initiate data sampling. The whole cycle can be completed within 2 s.

5. *Photon counting system*

The fluorescence was recorded with a random access camera, C3329 system (Hamamatsu Photonics; Fig. 1A), which consists of a camera head, a camera control unit and a personal computer. The camera head consists of a photoemissive cathode, deflection coils to control the direction of the photoelectron, a fixed aperture which allows electron only from selected positions in the visual field to pass, and dynodes and an anode to amplify the photon signal. The x - and y -coordinates of the position (up to 100 points) and the gate time (time during which the camera remains at a selected position) can be set from the computer, PC 1. We usually selected 5 to 20 points and a gate time of 5 ms. The current reaching the anode is digitized with a 16-bit photonocounter and the digitized data are sent to PC1. This system has several distinct advantages over conventional SIT or CCD cameras as follows: (a) random access to selected positions in the visual field which greatly shortens the time required for measurement and analysis; (b) high signal/

noise ratio because of the use of photon counting; (c) broad dynamic range of about 10^5 ; (d) variable sensitivity, obtained by varying gate time (gate time of 100 μ s gives an equivalent sensitivity to SIT); (e) no afterimage as occurs in SIT cameras.

6. Calibration of $[Ca^{2+}]_i$ and pH_i

Since both fura-2/AM and BCECF/AM deesterified in the cytosol have been reported to show different spectral properties from these dyes in a protein-free aqueous solution (SCANLON *et al.*, 1987, for fura-2; ALPERN, 1985, for BCECF) leading to erroneous quantitation, multiple-point *in vivo* calibrations were used for both dyes. Unlike MIYATA *et al.* (1989), we found that calibration curves obtained with single- and dual-dye loading were significantly different. We did not subtract fluorescence intensities obtained from single-dye loading as a background, because final intracellular concentrations of the dyes loaded simultaneously were smaller than those loaded separately, and were difficult to estimate. Therefore, both dyes were loaded for both $[Ca^{2+}]_i$ and pH_i calibration procedures.

1) *Calibration of $[Ca^{2+}]_i$.* In cardiac myocytes, *in vivo* calibration with the calcium ionophore, ionomycin, is extremely difficult (see for example, EISNER *et al.*, 1989) because of potent calcium extrusion and sequestering reactions (LI *et al.*, 1987). We used a modified method of LI *et al.* (1987), who obtained consistent changes of fura-2 signals with ionomycin by suppressing energy metabolism of cardiac myocytes. Myocytes were deenergized by incubation in a high K^+ HEPES-buffered solution (130 mM KCl, 20 mM NaCl, 10 mM HEPES, pH 7.05) containing 2 mM NaCN, 0.5 mM monoiodoacetic acid, and 2 μ M carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) for at least 30 min. The $[Ca^{2+}]_i$ calibration solutions with free extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) up to 10 μ M were prepared in the high K^+ solution, using EGTA (5 mM) as a buffer and assuming the apparent dissociation constant for the Ca^{2+} /EGTA complex to be 214 nM at pH 7.05 and 37°C (GRYNKIEWICZ *et al.*, 1985). After the incubation, ionomycin (2 μ M final concentration) was added to the myocyte suspension in the chamber to equilibrate $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$.

Figure 2A shows time courses of the change in the ratio (R_F) between fluorescence intensities of fura-2 at 340 and 380 nm excitation (F340 and F380, respectively) due to ionomycin addition. To confirm that the equilibrium between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ had been reached, the detergent digitonin (1 μ g/ml final concentration) was added to the suspension. Both F340 and F380 decreased rapidly after digitonin addition due to dye leakage; however, R_F remained unchanged, indicating that $[Ca^{2+}]_i$ was very close to $[Ca^{2+}]_o$. Averaged R_F values in the plateau phase were plotted against the negative logarithm of $[Ca^{2+}]_i$ (pCa_i) in Fig. 2B. pCa_i can be calculated from R_F over a wide range of $[Ca^{2+}]_i$, using the following equation:

$$pCa_i = pK_F - \log \{ (R_F - R_{Fmin}) / (R_{Fmax} - R_F) \} , \quad (1)$$

Fig. 2. A: Typical examples of time courses of the change in R_F (F340/F380) in $[Ca^{2+}]_i$ calibration. Myocytes were loaded with BCECF and fura-2, deenergized, and transferred into the $[Ca^{2+}]_i$ calibration solution in the reaction chamber. Ionomycin ($2\mu M$ final concentration) was added to the chamber at the arrow. The numbers in the figure represent $[Ca^{2+}]_o$ in each experiment. B: The relation between R_F and pCa_i obtained from myocytes ($n=131$) loaded with dual-dyes. Data are shown as mean $\pm$ S.E.M. C: A typical example of time courses of changes in $F500$, $F440$, and R_B (F500/F440) in pH_i calibration. Myocytes were loaded with BCECF and fura-2, treated with a high K^+ , HCO_3^- -buffered solution containing nigericin ($10\mu g/ml$), and transferred into the reaction chamber. CO_2/O_2 gas mixtures were subsequently injected into the reaction chamber at the times indicated by the arrows. The numbers at the arrows represent fractions of CO_2 (in %). The fluorescence intensity is shown as original values of digital output from the C3329 system. D: The relation between R_B and pH_i obtained from myocytes ($n=192$) loaded with dual-dyes. Data at pH 5.76 and 8.87 were obtained separately from myocytes suspended in solutions buffered with MES and CHES. Data are shown as mean $\pm$ S.E.M. The calibration curves obtained with single-dye loading are shown with broken lines in both B and D, and the calibration equations were obtained by non-linear curve fitting using PC-SAS programs.

where pK_F is the negative logarithm of the apparent dissociation constant (K_F) for fura-2, R_{Fmax} and R_{Fmin} are R_F s for unbound and bound forms of the fura-2/ Ca^{2+} complex, respectively. K_F corresponds to $K_d \times \beta$ in the equation of GRYNKIEWICZ

et al. (1985), where K_d is the dissociation constant for the fura-2/ Ca^{2+} complex, and β is the ratio between maximum and minimum fluorescence intensities of fura-2 at 380 nm excitation. pK_F , R_{Fmax} , and R_{Fmin} were estimated to be 5.69, 4.26, and 0.45, respectively, by non-linear curve fitting between pCa_i and R_F . The calibration curve obtained from single-dye loading is also shown with a broken line in Fig. 2B.

Our calibration method is distinct from the method of LI *et al.* (1987) in that they measured ratios at two points, R_{Fmax} and R_{Fmin} , with ionomycin, and used K_d from an *in vitro* calibration. However, we found that K_d (187 nM) calculated by dividing K_F by β obtained from single-dye loading was significantly smaller than K_d of fura-2 in an aqueous solution (224 nM by GRYNKIEWICZ *et al.*, 1985; 257 nM in our *in vitro* calibration), indicating that the use of an *in vitro* K_d would lead to an overestimation of $[Ca^{2+}]_i$ by 20 to 40%. Moreover, since $[Ca^{2+}]_i$ values reported to date (for example, LI *et al.*, 1987; MIYATA *et al.*, 1989) fall in the range well below K_F (where the slope is very shallow), quantitation of $[Ca^{2+}]_i$ using R_{Fmax} , R_{Fmin} , and β , together with K_d from an *in vitro* calibration would cause considerable error. Therefore, we measured R_F for several $[Ca^{2+}]_i$ (multiple-points covering the most relevant $[Ca^{2+}]_i$ range) and estimated K_F , R_{Fmax} , and R_{Fmin} by non-linear curve fitting.

2) *Calibration of pH_i .* For pH_i calibration, the H^+/K^+ exchanger, nigericin (THOMAS *et al.*, 1979) was used. After loading with the two dyes, myocytes were suspended in a high K^+ , HCO_3^- -buffered solution (130 mM KCl, 24 mM $NaHCO_3$, 1 mM, EGTA) containing 10 μ g/ml nigericin for 10 min. Then, 10 μ l of the suspension were transferred into the reaction chamber and spread to a thin liquid layer (about 200 μ m). To change the extracellular pH (pH_o), warmed (37°C), humidified gas mixtures containing various CO_2 concentrations in O_2 were sequentially injected into the chamber with syringes.

Figure 2C shows time courses of changes in fluorescence intensities of BCECF at 500 and 440 nm (F500 and F440, respectively), and the ratio between F500 and F440 (R_B), due to the gas injections. This pH calibration method was more convenient and was less prone to mechanical noise than a method using perfusion of solutions with different pHs. Figure 2D shows the relation between pH_i and R_B thus obtained. pH_i can be calculated from R_B over a wide range of pH_i , using the following equation similar to Eq. (1):

$$pH_i = pK_B + \log \{ (R_B - R_{Bmin}) / (R_{Bmax} - R_B) \}, \quad (2)$$

where pK_B is the apparent pK for BCECF, R_{Bmax} and R_{Bmin} are maximum and minimum R_B s, respectively. pK_B , R_{Bmax} , and R_{Bmin} were estimated to be 7.13, 5.60, and 0.68, respectively, by non-linear curve fitting between pH_i and R_B .

7. Evaluation of the method

To evaluate the accuracy of this method, it is necessary to confirm that (a) the signals of fura-2 and BCECF are not affected directly by pH_i and $[Ca^{2+}]_i$, respectively; and (b) the interference between fura-2 and BCECF signals does not

affect quantitations of $[Ca^{2+}]_i$ and pH_i . To test these, $[Ca^{2+}]_i$ -independent effects of pH_i on the fura-2 signals and pH_i -independent effects of $[Ca^{2+}]_i$ on the BCECF signals were examined in myocytes with single-dye loading. Secondly, pH_i -dependencies of BCECF signals at the fura-2 excitation wavelengths due to pH_i change (F_{B340} and F_{B380}) and $[Ca^{2+}]_i$ -dependencies of fura-2 signals at the BCECF excitation wavelengths due to $[Ca^{2+}]_i$ change (F_{F500} and F_{F440}) were measured in myocytes with single-dye loading. Using these tests, we concluded that measurements of $[Ca^{2+}]_i$ and pH_i are virtually independent of each other over useful ranges of $[Ca^{2+}]_i$ and pH_i .

1) *Effects of pH_i on fura-2 signals and effects of $[Ca^{2+}]_i$ on BCECF signals.* Figure 3 shows $[Ca^{2+}]_i$ -independent effects of pH_i on F340, F380, and R_F measured in fura-2 loaded myocytes ($[Ca^{2+}]_i$ equilibrated with $[Ca^{2+}]_o$; $10\ \mu M$). Both F340 and F380 did not change when pH_i was between 6.6 and 8.3. R_F decreased only when pH_i became 6.1. This pH effect was negligible at a low $[Ca^{2+}]_i$ (less than 10 nM; EGTA added). In BCECF-loaded myocytes, changes in $[Ca^{2+}]_i$ from 11.3 nM to 1 mM had no effect on the BCECF signals over a wide range of pH_i . It was concluded that fura-2 signals were independent of pH_i when pH_i was between 6.6 and 8.3 and $[Ca^{2+}]_i$ was below $10\ \mu M$, and that BCECF signals were completely independent of $[Ca^{2+}]_i$.

Fig. 3. $[Ca^{2+}]_i$ -independent effects of pH_i on fura-2 signals. Typical examples of time courses of changes in F340, F380, and R_F (F_{340}/F_{380}) are shown. Myocytes were loaded with fura-2 alone, treated with a high K^+ , HCO_3^- -buffered solution containing nigericin ($10\ \mu g/ml$) and ionomycin ($2\ \mu M$), and transferred into the reaction chamber. $CaCl_2$ ($10\ \mu M$ final concentration) was added to a nominally Ca^{2+} -free solution without EGTA. The numbers at the arrows represent final pH_i achieved by CO_2/O_2 gas injections. The fluorescence intensity is shown as original values of digital output from the C3329 system.

2) *Interference between BCECF and fura-2 signals.* F_{B340} increased and F_{B380} decreased with an increase in pH_i ; however, when pH_i was between 6.1 and 8.3, the changes in F_{B340} and F_{B380} was smaller than 2% of F_{B340} and F_{B380} of fura-2 itself. There was no clear $[Ca^{2+}]_i$ -dependency in F_{F500} ; however, F_{F440} decreased significantly when $[Ca^{2+}]_i$ was higher than $10 \mu M$. When $[Ca^{2+}]_i$ was lower than $10 \mu M$, the changes in F_{F500} and F_{F440} were smaller than 1% of F_{F500} and F_{F440} of BCECF itself. These data indicate that the interference between fura-2 and BCECF signals is negligible when pH_i is between 6.1 and 8.3 and $[Ca^{2+}]_i$ is below $10 \mu M$; maximal errors in R_F and R_B due to the interference were estimated to be smaller than 3%.

8. $[Ca^{2+}]_i$ and pH_i of quiescent myocytes

The mean and S.D. of $[Ca^{2+}]_i$ and pH_i simultaneously measured from quiescent ventricular myocytes ($[Ca^{2+}]_o$ 1 mM; pH_o 7.4) were 88.6 ± 32.8 nM and 7.16 ± 0.12 , respectively ($n=320$). Both values showed Gaussian distributions. This pH_i value coincides well with pH_i (7.15 ± 0.04) obtained by MIYATA *et al.* (1989); however, $[Ca^{2+}]_i$ is about twofold higher than their value (49 ± 8 nM). Other groups reported $[Ca^{2+}]_i$ of 112 ± 14 nM (LI *et al.*, 1987) and 131 ± 47 nM (BORZAK *et al.*, 1990) by the use of an *in vivo* calibration. The discrepancy between our $[Ca^{2+}]_i$ and theirs could be explained by their use of K_d from an *in vitro* calibration. We found no significant correlation between our resting $[Ca^{2+}]_i$ and pH_i values.

In conclusion, this system has enabled us to make on-line measurements of changes in both $[Ca^{2+}]_i$ and pH_i simultaneously and quantitatively from multiple individual cells in the visual field over wide ranges of $[Ca^{2+}]_i$ and pH_i , suggesting that it will be a useful tool to study the interplay between $[Ca^{2+}]_i$ and pH_i , and to analyze complex mechanisms controlling cellular functions in cardiac myocytes (and other cells) not only under physiological but also under pathophysiological conditions.

The authors are grateful to Dr. Sathapana Kongsamut, Department of Pharmacology, Yale University, for his critical reviewing of this manuscript and to Drs. Hiroshi Kato and Ken-ichi Ito, Department of Physiology, Yamagata University, for kindly providing us information on the C3329 system. This work was supported by Grants-in-Aid for Scientific Research to K. Doi (63480114) and to I. Shibuya (01770050) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

- ALPERN, R. J. (1985) Mechanism of basolateral membrane $H^+/OH^-/HCO_3^-$ transport in the rat proximal convoluted tubule. *J. Gen. Physiol.*, **86**: 613-636.
- BLANCHARD, E. M. and SOLARO, R. J. (1984) Inhibition of the activation and troponin calcium binding of dog cardiac myofibrils by acidic pH. *Circ. Res.*, **55**: 382-391.
- BORZAK, S., KELLY, R. A., KRAMER, B. K., MATOBA, Y., MARSH, J. D., and REERS, M. (1990) *In situ* calibration of fura-2 and BCECF fluorescence in adult rat ventricular

- myocytes. *Am. J. Physiol.*, **259**: H973-H981.
- BOUNTRA, C. and VAUGHAN-JONES, R. D. (1989) Effect of intracellular and extracellular pH on contraction in isolated mammalian cardiac muscle. *J. Physiol. (Lond.)*, **418**: 163-187.
- EISNER, D. A., NICHOLS, C. G., O'NEILL, S. C., SMITH, G. L., and VALDEOLMILLOS, M. (1989) The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *J. Physiol. (Lond.)*, **411**: 393-418.
- FUJIMOTO, M., KUBOTA, T., HAGIWARA, N., KUBOKAWA, M., OHNO-SHOSAKU, T., and KOTERA, K. (1989) Relationship between cytosolic activities of calcium and pH in frog proximal tubules. *Jpn. J. Physiol.*, **39**: 273-296.
- GRYNKIEWICZ, G., POENIE, M., and TSIEN, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**: 3440-3450.
- IRISAWA, H. and SATO, R. (1986) Intra- and extracellular actions of proton on the calcium current of isolated guinea pig ventricular cells. *Circ. Res.*, **59**: 348-355.
- LI, Q., ALTSCHULD, R. A., and STOKES, B. T. (1987) Quantitation of intracellular free calcium in single adult cardiomyocytes by fura-2 fluorescence microscopy: Calibration of fura-2 ratios. *Biochem. Biophys. Res. Commun.*, **147**: 120-126.
- MIYATA, H., HAYASHI, H., SUZUKI, S., NODA, N., KOBAYASHI, A., FUJIWAKE, H., HIRANO, M., and YAMAZAKI, N. (1989) Dual loading of the fluorescent indicator fura-2 and 2,6-biscarboxyethyl-5(6)-carboxyfluorescein (BCEFC) in isolated myocytes. *Biochem. Biophys. Res. Commun.*, **163**: 500-505.
- ORCHARD, C. H. (1987) The role of the sarcoplasmic reticulum in the response of ferret and rat heart muscle to acidosis. *J. Physiol. (Lond.)*, **384**: 431-449.
- RINK, T. J., TSIEN, R. Y., and POZZAN, T. (1982) Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell Biol.*, **95**: 189-196.
- SCANLON, M., WILLIAMS, D. A., and FAY, F. S. (1987) A Ca^{2+} -insensitive form of fura-2 associated with polymorphonuclear leukocytes. *J. Biol. Chem.*, **262**: 6308-6312.
- STEMMER, P., AKERA, T., BRODY, T. M., RANDON, D., and WATANABE, A. G. (1989) Isolation and enrichment of Ca^{2+} -tolerant myocytes for biochemical experiments from guinea-pig heart. *Life Sci.*, **44**: 1231-1237.
- THOMAS, J. A., BUCHSBAUM, R. N., ZIMNIAK, A., and RACKER, E. (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. *Biochemistry*, **18**: 2210-2218.
- VANHEEL, B., LEYBAERT, L., HEMPTINNE, A. D., and LEUSEN, I. (1989) Simulated ischemia and intracellular pH in isolated ventricular muscle. *Am. J. Physiol.*, **257**: C365-C376.
- VAUGHAN-JONES, R. D., LEDERER, W. J., and EISNER, D. A. (1983) Ca^{2+} ions can affect intracellular pH in mammalian cardiac muscle. *Nature*, **301**: 522-524.