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DUAL LOADING OF THE FLUORESCENT INDICATOR FURA-2 AND 2,7-BISCARBOXYETHYL-5(6)-CARBOXYFLUORESCEIN (BCECF) IN ISOLATED MYOCYTES

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Isolated rat heart myocytes were loaded with both the Ca^{2+} sensitive fluorescent probe fura-2/AM and the fluorescent pH indicator 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF/AM). Changes in $[Ca^{2+}]i$ and pHi were measured simultaneously using digitized video fluorescence microscopy. In measurement of $[Ca^{2+}]i$ and pHi, the ratios of dual-loaded cells were not different from single-loaded cells. Using this method, $[Ca^{2+}]i$ and pHi in myocytes were 48±7 nM and 7.17±0.05. It is concluded that $[Ca^{2+}]i$ and pHi could be measured simultaneously in isolated myocyte using dual-loading of fura-2 and BCECF.

Intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in cardiac myocytes is influenced by many ions such as Na^+, Mg^{2+} and H^+ (1,2). An increase in $[Ca^{2+}]i$ has been reported to be associated with a decrease in pHi and vice versa. On the other side, intracellular alkalinization has been shown to reduce $[Ca^{2+}]i$, whereas acidification has been shown to increase $[Ca^{2+}]i$. This is an important problem since intracellular pH (pHi) and $[Ca^{2+}]i$, for example, interact to affect contraction (3,4). The fluorescent Ca^{2+} indicator fura-2 has been used to monitor $[Ca^{2+}]i$ in a wide variety of cells (5). The analogus fluorescent pH indicator BCECF has been also used to measure changes in pHi (6). When these probes are loaded, the lipid-soluble acetoxymethyl ester is taken up by the cells and hydrolyzed by cellular esterases to produce the relative non-permeant free acid. These fluorescent indicators have been, however, used independently (7-10). In the present study we investigated whether isolated myocytes could be loaded with both fura-2 and BCECF, and that the fluorescence of these probes coupled with digitized video microscopy could provide a continuous recording of $[Ca^{2+}]i$ and pHi simultaneously. Using the

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ratio imaging technique, fura-2 emission at >500 nm was measured after excitation at 340 nm and 380 nm, while BCECF fluorescence was measured at 505-560 nm when excited at 490 nm and 450 nm. Subsequently, the ratios of 340/380 nm and 490/450 nm in single-loaded and dual-loaded cells were compared.

MATERIALS AND METHODS

Calcium-tolerant adult rat heart myocytes were isolated as previously described (11). The cells were loaded with 5μM fura-2/AM and/or 0.5μM BCECF/AM for 40 min at 37 ℃. The cells were washed three times with modified Krebs solution before study, and was placed in an experimental chamber, which was mounted on the stage of a Nikon TMD inverted microscope and a Nikon Fluor 20x, (numerical aperture:0.75) objective. The myocytes were superfused with modified Krebs solution(mM); NaCl 113.1, KCl 4.6, $CaCl_2$ 2.45, $MgCl_2$ 1.2, $NaHPO_4$ 3.5, $NaHCO_3$ 21.9, and glucose 5, equilibrated with 95% O_2-5% CO_2(pH 7.4). Exitation light was provided by 300W Xenon lamp and passed through an interference and neutral density filter to select wavelength and intensity. A low-light, intensified silicon target (SIT) camera (Hamamatsu Photonic K.K.) collected fluorescent images that were fed to a computer (ARGUS;Hamamatsu Photonic K.K.). All filters had half-bandwidths of 10 nm. After passing the filters the exciting light was reflected by a dichroic mirror. This had a half-pass wavelength of 400 nm for fura-2 and 510 nm for BCECF, which was changed manually. In measurement of $[Ca^{2+}]i$, fura-2 fluorescence elicited the cells at 340 nm and 380 nm was measured at 500nm (emission; bandpass 20 nm). Fluorescence ratios were obtained by dividing pixel by pixel, the 340 nm image after background subtraction by the 380 nm image after background subtraction. In measurement of pHi, the fluorescent signal was obtained with excitation wavelengths at 490 nm and 450 nm, and emission wavelength at 505-560 nm. Fluorescent ratios were also obtained by dividing pixel by pixel, the 490 nm image after background subtraction by the 450 nm image after background subtraction.

Calibration of fura-2 fluorescence. We used *in vivo* calibration according to the methods described by Li et al (12). Briefly, the cells were loaded with fluorescent probes, and were then superfused with a glucose-free buffer containing the metabolic poisons carbonyl cyanide m-chlorophenylhydrazone(CCCP;5.0μM; an inhibitor of oxidative phosphorylation) and amytal(3.3mM; an inhibitor of NADH dehydrogenase) for 15-20min to deplete stores of intracellular ATP. After this the cells had a "brick-like" appearance and did not contract further upon the exposure to Ca^{2+}. R_{max} was determined by adding the Ca^{2+} ionomycin(10μM) and $CaCl_2$(5mM). R_{min} was determined by adding the Ca^{2+} ionomycin(10μM) and 10 mM EGTA to the external solution. $[Ca^{2+}]i$ is related to the ratio of measured fluorescence signals elicited at two excitation wavelengths according to the following equation (5). $[Ca^{2+}]i=Kd\beta x(R-R_{min})/(R_{max}-R)$. The measured ratios of the cell could be converted directly to $[Ca^{2+}]i$ with this equation.

RESULTS AND DISCUSSION

In this study, isolated rat heart myocytes were loaded with 5μM fura-2/AM and 0.5μM BCECF/AM. This was because BCECF was much brighter probe due to its high extinction coefficient and

emission quantum efficiency. In addition, BCECF fluorescence was obtained with exitation wavelengths at 340 nm and 380 nm. Cell viability was not different between dual-loaded and single-loaded cells.

(1)Effects of BCECF loading on $[Ca^{2+}]i$ measurement

Table 1 shows the relative fluorescence and fluorescence ratio in single-loaded and dual-loaded cells. The absolute values of the fluorescence in single cells were measured from an area of 55 μm^2. The average absolute value of fura-2 fluorescence intensity excited at 340 nm was almost equal to that of BCECF fluorescence intensity excited at 490 nm. The intrinsic fluorescence of the non-loaded cells (autofluorescence) was low (5% at 340 nm and 3% at 380 nm, of the total signal from fura-2-loaded cells), and BCECF-loaded cells showed similar fluorescence as non-loaded cells in $[Ca^{2+}]i$ measurement unit (340 nm:5%, 380 nm:3%). When Ca^{2+}tolerant rat heart myocytes were loaded with fura-2, there was no difference in ratios (340nm/380nm) between fura-2-loaded cells and dual-loaded cells (0.38±0.02 and 0.38±0.02, respectively).

(2)Effects of fura-2 loading on pHi measurement

Using BCECF, fluorescence has generally been excited at 490-500nm at which an alkalosis increases fluorescence, and at 450 nm at which changes in pH have little effect (6). Table 1 also shows that the intrinsic fluorescence intensity from

Table 1. The relative fluorescence intensity (R.F.I.) and fluorescence ratio in single-loaded and dual-loaded cells. In $[Ca^{2+}]i$ measurement, the percentage of the relative fluorescence was calculated by diviving the values of fura-2 signal by that of fura-2-loaded cells. In pHi measurement, the percentage of the relative fluorescence was calculated by dividing the values of BCECF signal by that of only BCECF-loaded cells. Values are mean(SE).

		Ca^{2+} Measurement Unit		pH Measurement Unit	
	N	R.F.I (340/380)	Ratio (340/380)	R.F.I (490/450)	Ratio (490/450
Non-loaded	18	5(0)/3(0)		4(0)/4(0)	
Fura-2	10	100(8)/100(7)	0.38(0.02)	10(1)/10(1)	
BCECF	10	5(1)/3(0)		100(9)/100(10)	1.09(0.02)
Dual-loaded	16	99(9)/98(7)	0.38(0.02)	102(13)/97(11)	1.11(0.03)

non-loaded cells (autofluorescence) in pHi measurement unit was less than 4% (490 nm:4%, 450 nm:4%) of the total signal from BCECF-loaded cells, but that from fura-2-loaded cells showed a little increase in intrinsic fluorescence (490nm:10%, 450nm:10%). The increase was likely to represent background signal of opticus. But the total signal of BCECF was high enough to exceed the level of intrinsic fluorescence by seven-fold (12). There was no statistical difference in ratios (490nm/450nm) between BCECF-loaded cells and dual-loaded cells (1.09±0.02 and 1.11±0.03, respectively).

(3)Calibration of fura-2 fluorescence

It is, however, difficult to calibrate the fluorescence changes produced by fura-2 added as the AM ester in cardiac muscle. This is because: [1] compartmentation of fura-2. [2] incomplete de-esterification of fura-2/AM. Therefore we used *in vivo* calibration as described in the method. Table 2 summarizes the results of an experiment where the R_{max} and R_{min} values were compared between fura-2-loaded and dual-loaded cells. Agreement between fura-2 and dual loading was quite acceptable. By applying the fura-2 fluorescence ratios in Table 1 to this calibration, $[Ca^{2+}]i$ in fura-2 and dual-loaded cells were 49±8 nM and 48±7 nM, respectively.

(4)Calibration of BCECF fluorescence

The fluorescent pH indicator BCECF has been used to monitor changes in cytoplasmic pHi, and spontaneous leakage rate of BCECF has been shown to be slow (14). The fluorescence intensity ratio signal from BCECF has been reported to be unaltered by changes in

Table 2. R_{max} and R_{min} in $[Ca^{2+}]i$ measurement. ATP was depleted by 15-20 min perfusion with amytal/CCCP. R_{max} and R_{min} were determined after ionomycin/saturating calcium or ionomycin/EGTA, respectively. Data are expressed as mean±SE.

	Fura-2	Fura-2+BCECF
R_{max}	4.54±0.43 (n=19)	4.63±0.41 (n=24)
R_{min}	0.24±0.01 (n=9)	0.24±0.02 (n=15)
$[Ca^{2+}]i$	49±8 nM (n=10)	48±7 nM (n=16)

extracellular Ca^{2+} concentration from 50nM to 10μM (15). Figure 1 shows *in vivo* calibration curves generated in BCECF-loaded, and dual-loaded myocytes by the addition of 10 μg/ml nigericin, a K^+/H^+ ionophore, in 130mM KCl, 1mM $MgCl_2$, 15mM MES (2-[N-Morpholino] ethnesulfonic acid), 15mM Hepes buffer at 37° C. The pH was adjusted appropriately with KOH. The BCECF or dual-loaded cells were incubated with the solution for at least 5-10 min to allow complete equilibration before recording the data. Fluorescence ratios were linearly related to pH from 6.5 to 7.5, and there was no statistical difference between BCECF-loaded and dual-loaded cells. The BCECF fluorescence ratios in cells perfused with the control Krebs solution correspond to 7.15±0.04 in BCECF-loading and 7.17±0.05 in dual-loading, by applying this calibration. Our estimates of pHi were compatible to those obtained using BCECF in rat myocytes (7) and in cultured chick ventricular cells (8).

In summary, we have developed a new method for measuring the Ca^{2+}sensitive fura-2 fluorescence signal and pH-sensitive BCECF fluorescence signal in rat myocytes simultaneously. Using this method, $[Ca^{2+}]i$ and pHi in rat heart myocytes were 48±7 nM and 7.17±0.05. On the other side, $[Ca^{2+}]i$ and pHi in single-loaded cells were 49±8 nM and 7.15±0.04. This technique should be applicable to other cell types, and $[Ca^{2+}]i$ and pHi could be measured under comparable condition. This represents a substantial advantage to investigate the interaction of $[Ca^{2+}]i$ and pHi in a cell.

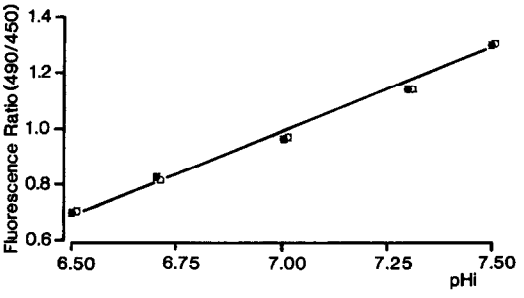

Figure 1. Relationship between apparent intracellular pH of myocytes and intracellular BCECF fluorescence intensity ratio in BCECF-loaded (O) and dual-loaded cells (●). Difference between BCECF-loaded and dual-loaded groups was not significant. Each value represents mean±SE of 6 cells.

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