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Small Changes of Cytosolic Sodium in Rat Ventricular Myocytes Measured with SBFI in Emission Ratio Mode

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A. Baartscheer, C. A. Schumacher and J. W. T. Fiolet. Small Changes of Cytosolic Sodium in Rat Ventricular Myocytes Measured with SBFI in Emission Ratio Mode. *Journal of Molecular and Cellular Cardiology* (1997) **29**, 3375–3383. The spectral properties of SBFI (sodium-binding benzofurzan isophthalate) were re-examined to arrive at a more specific and sensitive method to measure small changes of intracellular sodium ($[Na^+]_i$) particularly at low concentration. Relative to spectra of SBFI in protein- and cell-free solution, binding of SBFI to intracellular proteins caused a shift of excitation and emission spectra, and increased quantum efficiency. Excitation of SBFI at 340 nm caused an exclusively sodium-dependent fluorescence from 400–420 nm, and hardly any change of fluorescence above 530 nm upon replacing sodium by potassium. Due to these spectral and quantum efficiency changes, SBFI excitated at 340 nm can be used in a dual emission ratio mode to measure $[Na^+]_i$. In dual emission ratio mode (410 and 590 nm, respectively), the fluorescence ratio increased by a factor of 13 upon replacing sodium for potassium. The apparent equilibrium constant measured in single isolated rat ventricular myocytes was 22.5 ± 0.3 mmol/l. Control $[Na^+]_i$ was 9.6 ± 0.4 mmol/l. After abrupt reduction of extracellular sodium from 156 to 29 or 11 mmol/l, $[Na^+]_i$ decreased mono-exponentially to 2.5 ± 0.3 and 1.9 ± 0.3 mmol/l, respectively, with a rate constant of about 0.02/s. We conclude that SBFI used in dual emission mode provides a more sensitive and more specific method to measure small changes of $[Na^+]_i$ in single myocytes down to cytosolic sodium concentration as low as about 1 mmol/l.

Key Words: Myocytes; Intracellular sodium; SBFI; Fluorescence; Spectra.

Introduction

The trans-sarcolemmal electrochemical potential difference of sodium ions, being a determinant of the driving force of ion transport systems such as the Na^+/K^+-ATPase, the Na^+/Ca^{2+}-exchanger, and the fast Na^{2+}-channel (Albitz *et al.*, 1991), modulates the currents generated by these electrogenic transport mechanisms (Gadsby *et al.*, 1985; Mechmann and Pott, 1986; Miura and Kimura, 1989; Janvier and Boyett, 1996). Also, $[Na^+]_i$ directly activates a K^+-current (Kameyama *et al.*, 1984; Luk and Carmeliet, 1990). Through Na^+/Ca^{2+}- and Na^+/H^+-exchanger activity, cytosolic calcium and pH are very sensitive to relatively small changes of $[Na^+]_i$ (Deitmer and Ellis, 1980; Borzak *et al.*, 1992; Fiolet *et al.*, 1995; Baartscheer *et al.*, 1996). Consequently, the inotropic state of the heart, which is regulated by both Ca^{2+} and H^+ ions (Lee and Levi, 1991), is also very sensitive to relatively small changes of $[Na^+]_i$.

Presently available methods used to measure $[Na^+]_i$ have a number of disadvantages. Because potentials measured with Na^+-sensitive micro-electrodes, which disrupt cell membranes, consist of membrane potential (V_m), and sodium potential (V_{Na}), separate measurement of V_m is required. Moreover, changes of V_{Na} usually are small compared to V_m (Desilets and Baumgarten, 1986). With NMR, relatively large quantities of tissue are required,

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and time resolution is limited (Springer *et al.*, 1987; Wittenberg and Gupta, 1987). The sodium-sensitive fluorescent indicator SBFI is used to measure $[Na^+]_i$ in dual wavelength excitation ratio mode (340/380 nm) and emission, measured around 500 nm (Harootunian *et al.*, 1989). However, with SBFI specificity, problems exist (Donoso *et al.*, 1992; Harrison, 1992), and sensitivity to small changes of $[Na^+]_i$ is rather low. The sensitivity problems are even more pronounced intracellularly, because spectral properties of intracellular SBFI differ from those in solution (Harootunian *et al.*, 1989; Borzak *et al.*, 1992). However, the use of SBFI offers the attractive opportunity that it allows measurement of $[Na^+]_i$ on the single-cell level with relatively high time-resolution and without sarcolemmal disruption (Borzak *et al.*, 1992; Levi *et al.*, 1994).

The aim of the present study is to develop a more sensitive and more specific method to measure $[Na^+]_i$ using SBFI in dual wavelength emission mode.

Materials and Methods

Isolation of ventricular myocytes

This investigation conformed with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Ventricular myocytes were isolated from hearts of male Wistar rats (weight about 250 g), as described previously (ter Welle *et al.*, 1988). After isolation, myocytes were kept at 37°C in separate vials, each containing about 1.5 mg of cell protein and 5 ml of solution I containing (mmol/l): $[Na^+]$ 156, $[K^+]$ 4.7, $[Ca^{2+}]$ 1.3, $[Mg^{2+}]$ 2.0, $[Cl^-]$ 150.6, $[HCO_3^-]$ 4.3, $[HPO_4^-]$ 1.4, [HEPES] 17, [Glucose] 11 and 1% (w/v) albumin, pH 7.3. The average fraction of rod shaped myocytes was 82% (76–86%); 40% of the rounded cells excluded trypan blue. The yield of viable myocytes was about 3×10^6 myocytes per g wet weight.

Crude myocyte protein was obtained by hypoosmotic shock in a small volume of distilled water containing 20 μg/ml of digitonin. After lysis and centrifugation, the supernatant was freeze-dried. The freeze-dried pellet was dissolved (final concentration about 4 mg protein per ml) and dialysed for 24 h against a solution II of the following composition (mmol/l): $[Cl^-]$ 144, $[HCO_3^-]$ 4.3, $[HPO_4^-]$ 1.4, [EGTA] 2, [HEPES] 17, [Glucose] 11 and $[Na^+]+[K^+]$ 150, pH 7.3.

Myocytes were incubated for 120 min at 37°C under regular gentle shaking in solution I with 10 μmol/l of the acetoxymethylester of SBFI (SBFI/AM, Molecular Probes Inc., S-1263) and 0.01% (w/v) pluronic F-127. After sedimentation, myocytes were washed twice and resuspended in 5 ml solution I without albumin and kept for 15 min to ensure complete de-esterification of all residual intracellular SBFI/AM.

Excitation and emission spectra of SBFI in suspensions of myocytes

SBFI loaded myocytes were resuspended in 5 ml of solution II and allowed to sediment for 1 min again. 25 μl of the loosely packed myocyte suspension (containing about 1 mg cell protein) was added to a 37°C temperature-controlled chamber (Fig. 1 in Fiolet *et al.*, 1995) containing 1.3 ml of solution II of either zero K^+ or zero Na^+ (sum concentration 150 mmol/l). After 1 min, intra- and extracellular sodium and potassium were equilibrated by addition of 2 μmol/l gramicidin, 30 μmol/l monensin and 1 mmol/l ouabain (Levi *et al.*, 1994). Extracellular spectra of SBFI were obtained in cell-free solution II in the presence and absence of crude protein isolated from myocytes (4 mg/ml) containing 500 nmol/l SBFI.

Excitation and emission spectra were obtained with a step-motor driven Zeiss monochromator (M4QIII) at a rate of 1 nm/s and digitized with a sample frequency of 8 Hz. Spectra were appropriately corrected for background spectra of non-loaded myocyte suspensions under the same conditions. No corrections were applied for non-linearity of exciting light intensity (Osram XBO 100 W) or spectral characteristics of PMTs (Hamamatsu R2949).

SBFI fluorescence in single myocytes in dual wavelength emission mode

SBFI-loaded myocytes were attached to a poly-D-lysine (0.1 mg/ml)-treated cover slip. The cover slip was placed on the stage of an inverted fluorescence microscope (Nikon Diaphot). The stage was temperature controlled (37°C). A temperature-controlled (37°C) perfusion chamber (height 0.4 mm, diameter 10 mm, volume 30 μl) was positioned on the cover slip; the contents of the chamber could be completely replaced within 200 ms. SBFI fluorescence was excited (Osram XBO 100 W) at 340 nm through the 40 × objective at a rate of 1 Hz, with flashes of 100 ms duration. Emitted light passed a

barrier filter of 400 nm, a 450 nm dichroic mirror and two narrow band interference filters of 410 nm (F_{410}) and 590 nm (F_{590}) placed in front of two PMTs (Hamamatsu R-2949). During each flash, signals were sampled at a rate of 10^4 Hz and averaged. Averaged signals were corrected for background signals and of non-loaded myocytes. Background was determined after removal of the myocyte at the end of each experiment. Auto-fluorescence was determined separately in each myocyte preparation from the average fluorescence of three non-loaded myocytes. Relative to signals of loaded control myocytes, autofluorescence was $15\% \pm 1.5$ at 410 nm and $0.5 \pm 0.1\%$ at 590 nm.

Fluorescence signals of each individual SBFI loaded myocyte were calibrated at the end of an experiment by superfusion with solution II containing gramicidin, monensin and ouabain (see above) at two concentrations of extracellular sodium ($[Na^+]_o$, 0 and 150 mmol/l, respectively). $[Na^+]_i$ was calculated according to:

$$[Na^+]_i = K_d \times \beta \frac{R - R_{min}}{R_{max} - R} \quad [1]$$

in which K_d is the apparent intracellular dissociation constant, β is the ratio of fluorescence signals in sodium free and sodium saturated cells measured at 590 nm, R the ratio of corrected F_{410} and F_{590}, R_{min} and R_{max} are minimal and maximal ratios in sodium free and sodium saturated cells, respectively.

$K_d \times \beta$ and the ratio R_{max}/R_{min} were determined in 73 myocytes from six different preparations treated with solution II containing different $[Na^+]_o$ and in addition gramicidin, monensin and ouabain to equilibrate $[Na^+]_i$ and $[Na^+]_o$ (see above). Each myocyte was superfused with up to six different concentrations. After about 2 min of ion equilibration, the fluorescence ratio was determined. Calculated ratio values were plotted as a function of $[Na^+]_o$. Using equation [1], a hyperbolic fit was applied to the data (Tablecurve Jandell Scientific) and $K_d \times \beta$ and the ratio R_{max}/R_{min} were calculated.

Results

Figure 1 shows intra- (a) and extracellular (b) and (c) excitation spectra of SFI recorded at 516 nm. In (c), the solution contained 4 mg/ml of crude myocyte protein. Solid lines represent "sodium spectra" (150 mmol/l Na^+, 0 mmol/l K^+) and dotted lines "zero sodium spectra" (150 mmol/l K^+, 0 mmol/l Na^+). The "zero sodium spectra" were normalized to the peaks of the respective sodium spectra. Relative to the extracellular sodium spectrum, there was a shift of the peak of the intracellular excitation spectrum of about 10 nm to longer wavelengths. Although no such shift was found for the zero sodium spectra, the quantum efficiency of the intracellular zero sodium spectrum was about twice as high. When proteins from lysed myocytes were added to the cell free solution, the characteristics of the extracellular excitation spectra became similar to those of the intracellular spectra. Addition of 0.35% albumin had similar effects, although the increase of the quantum efficiency of the zero sodium spectrum was more pronounced (not shown).

The excitation wavelengths of SBFI commonly used in dual excitation ratio mode are 340 and 380 nm, respectively (Satoh *et al.*, 1991; Jung *et al.*, 1992). Figure 2 shows intracellular emission spectra of SBFI excited at these wavelengths. Sodium spectra and zero sodium spectra are represented by solid and dotted lines, respectively. With excitation at 340 nm (a) the emission of the sodium spectrum at wavelengths shorter than 500 nm is higher than that of the zero sodium spectrum; in the range between 400–430 nm, there is hardly any fluorescence in the zero sodium spectrum. Above 550 nm, emission spectra were hardly sodium sensitive. With excitation at 380 nm (b) there is no spectral shift and the sodium spectrum has a uniformly lower intensity than the zero sodium spectrum over almost the entire wavelength range. At wavelengths shorter than about 440 nm, fluorescence intensities were low and almost similar.

These spectral properties indicate that when excited at 340 nm, fluorescence of intracellular SBFI measured around 420 nm is mainly sodium—rather than potassium-dependent. This is further demonstrated in Figure 3(a), which shows intracellular excitation spectra of SBFI recorded at 420 nm. The intracellular sodium spectrum had a peak around 350 nm. The corresponding zero sodium spectrum had a very low quantum efficiency, with a 13 times lower peak intensity than the sodium spectrum. Figure 3(b) shows that the excitation spectra of SBFI in cell free solution recorded at 420 were identical to the intracellular spectra [compare (a)]. Figure 3(b) also shows that when potassium was completely replaced by choline in cell free solution, the zero sodium spectrum remained unchanged. Similarly, reduction of potassium to 100 mmol/l without compensation by choline had no effect on the zero sodium spectrum (not shown). In the physiological range, the presence of neither magnesium nor calcium (2.5 mmol/l and

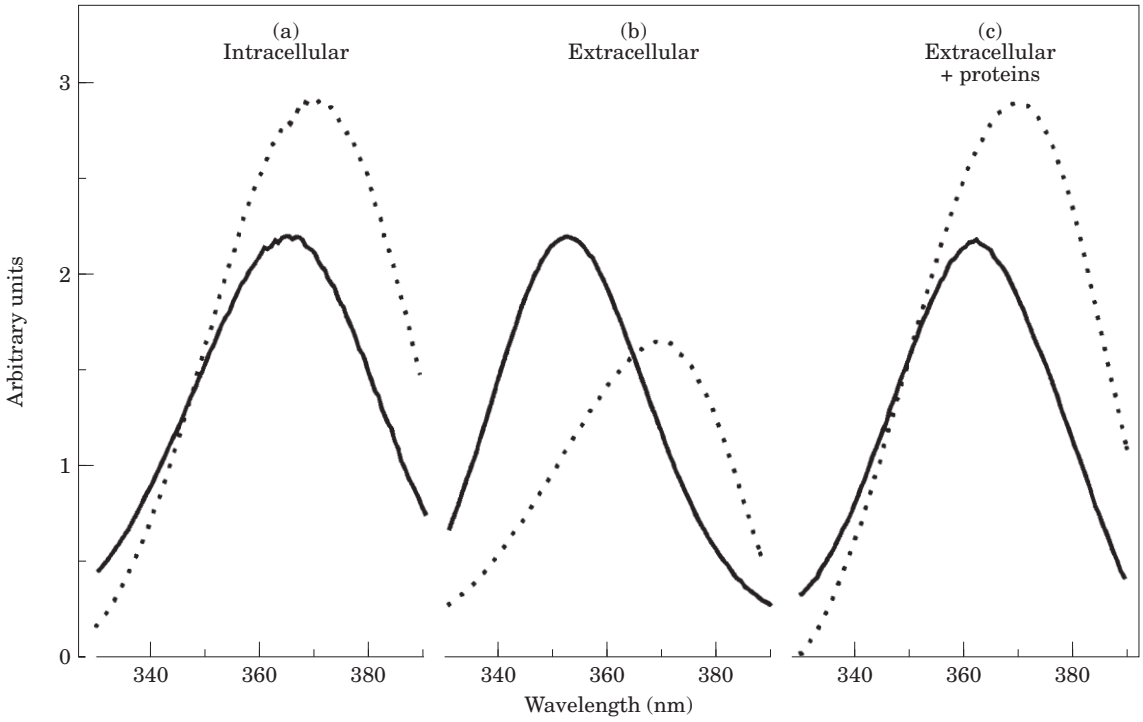

Figure 1 Excitation spectra of SBFI recorded at an emission wavelength of 516 nm corrected for background and autofluorescence. Solid lines $[Na^+]=150$, $[K^+]=0$ mmol/l, dotted lines $[K^+]=150$, $[Na^+]=0$ mmol/l. (a) intracellular spectra with peak values at 364 and 372 nm for "sodium and potassium spectra, respectively"; (b) extracellular spectra with peak values at 353 and 370 nm; (c) extracellular spectra in the presence of crude myocyte proteins (4 mg/ml) with peak values at 363 and 370 nm. The "zero sodium spectra" were normalized to the peaks of the respective "sodium spectra".

10 μmol/l, respectively) measurably affected any of the spectra. However, concentrations of calcium in the millimolar range (2.5 mmol/l) increased the peak fluorescence of the zero sodium spectrum up to about 30% of the peak intensity of the sodium spectrum (not shown). Therefore, intracellular SBFI fluorescence excited at 340 nm and measured around 420 nm seems to be entirely sodium-specific.

It therefore appears that intracellular SBFI used in dual wavelength emission ratio mode is suitable and possibly advantageous to measure cytosolic sodium in single cells. Fluorescence was excited at 340 nm, and recorded at 410 and 590 nm, respectively (at the latter wavelength, fluorescence was hardly sensitive to changes in either sodium or potassium, see Fig. 2). In order to be able to calculate $[Na^+]_i$, values for K_d, β, R_{max}/R_{min} were determined in single myocytes, which were superfused with solution II containing different concentrations of sodium and a mixture of gramicidin, monensin and ouabain to equilibrate extra- and intracellular sodium (Levi *et al.*, 1994). Figure 4 shows the fluorescence ratio (F_{410}/F_{590}) as a function of $[Na^+]_o$. The inset shows the part of the physiologically relevant range of sodium concentration. The solid line represents a hyperbolic fit to the data using equation [1]. The correlation coefficient was 0.98 and the standard error was 0.05. Variable values were: $K_d \times \beta = 25.3 \pm 0.3$, $R_{max} = 1.31 \pm 0.06$ (asymptote given by the dotted line in Fig. 4) and $R_{min} = 0.14 \pm 0.03$ (all means $\pm$ S.D.). The average value of β was 1.12. Therefore, the apparent intracellular K_d of SBFI was 22.5 mmol/l. The average ratio of R_{max}/R_{min} was 9.6.

Figure 5 shows steady-state $[Na^+]_i$ in control non-stimulated myocytes (curve 1) and its change after abrupt reduction of extracellular $[Na^+]_o$ from 156 to 29 (curve 2) and 11 mmol/l (curve 3); SBFI was used in dual emission ratio mode as above. Steady-state $[Na^+]_i$ was 9.6 ± 0.3 mmol/l (mean $\pm$ S.D., $n=6$). $[Na^+]_i$ decreased monoexponentially to 2.5 ± 0.3 and 1.9 ± 0.3 mmol/l, respectively (means $\pm$ S.D., $n=10$) upon reduction of $[Na^+]_o$, with a rate constant of about 0.02/s. Calibration was performed in each individual myocyte by superfusion with zero and 150 mmol/l sodium solutions (see Materials and Methods) giving

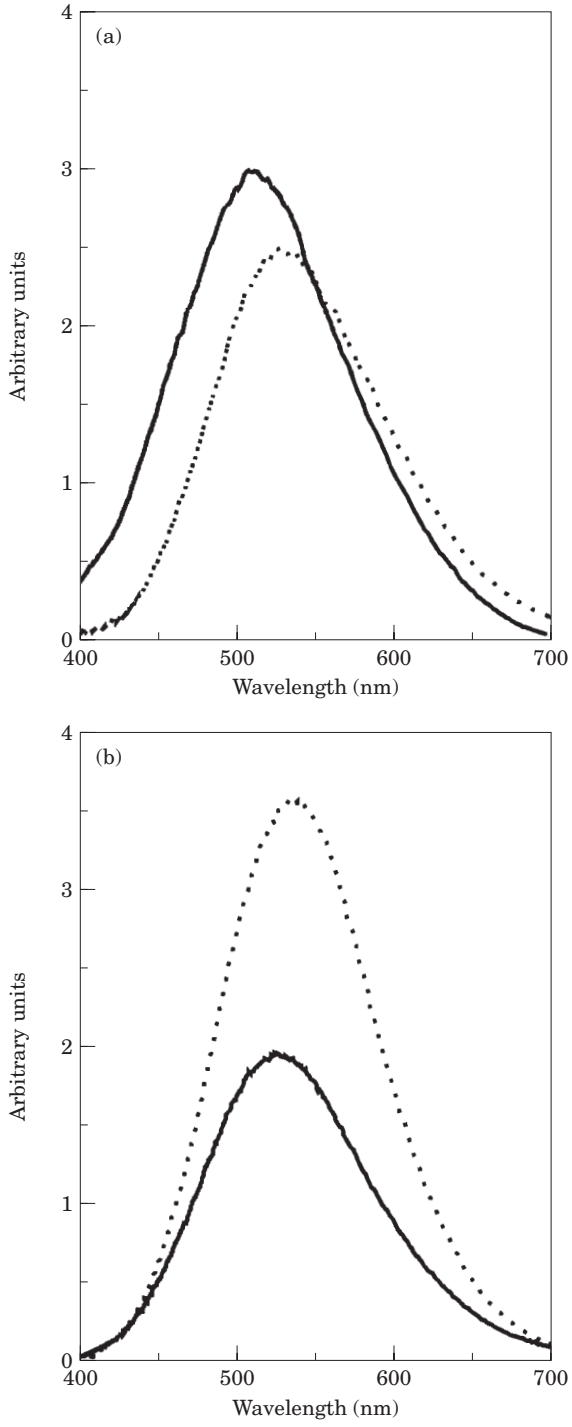

Figure 2 Intracellular emission spectra of SBFI corrected for background and autofluorescence. Solid lines $[Na^+]=150$, $[K^+]=0$ mmol/l, dotted lines $[K^+]=150$, $[Na^+]=0$ mmol/l. (a) excitation wavelength is 340 nm, (b) excitation wavelength is 380 nm. The peak values of the "sodium spectra" were 509 and 525 nm when the excitation wavelength is 340 and 380 nm, respectively, and the peak value of the "zero sodium spectra" is 530 nm.

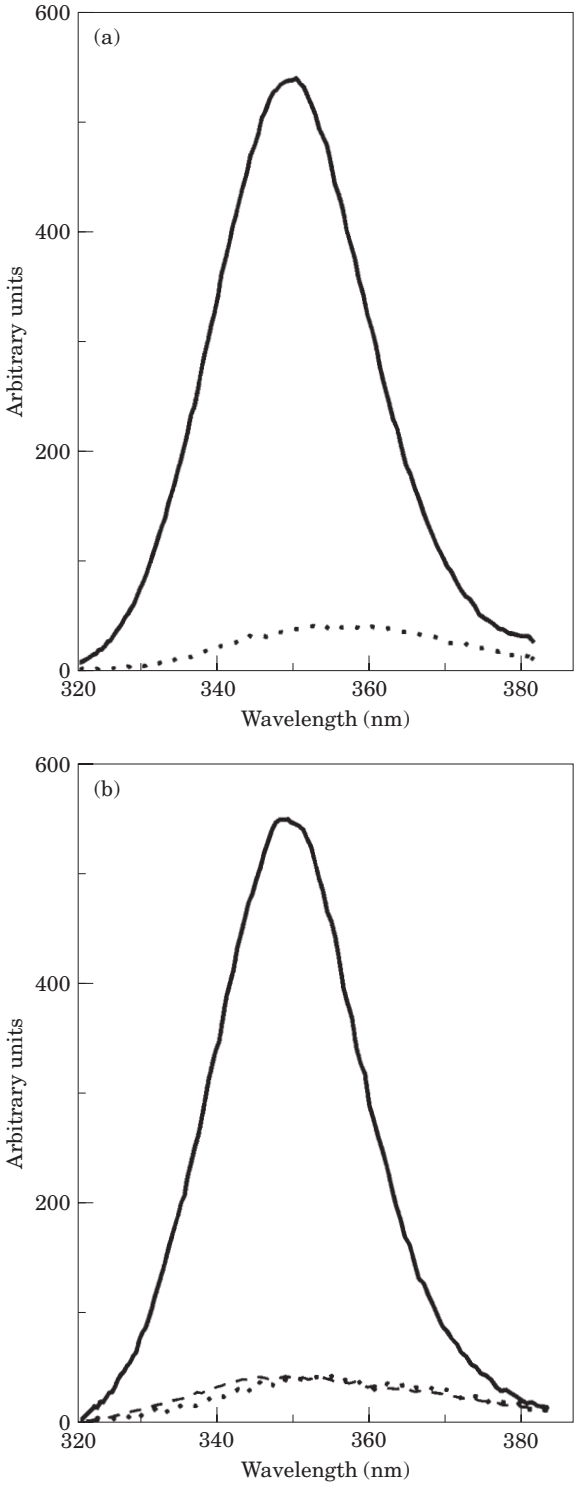

Figure 3 Intracellular (a) and extracellular (b) excitation spectra of SBFI recorded at an emission wavelength of 420 nm corrected for background and autofluorescence. Solid lines $[Na^+]=150$, $[K^+]=0$ mmol/l, dotted lines $[K^+]=150$, $[Na^+]=0$ mmol/l, dashed line $[Na^+]=0$, $[K^+]=0$, $[Choline^+]=150$ mmol/l. The peak values are 349 and 357 nm for the "sodium spectra" and "zero sodium spectra" respectively.

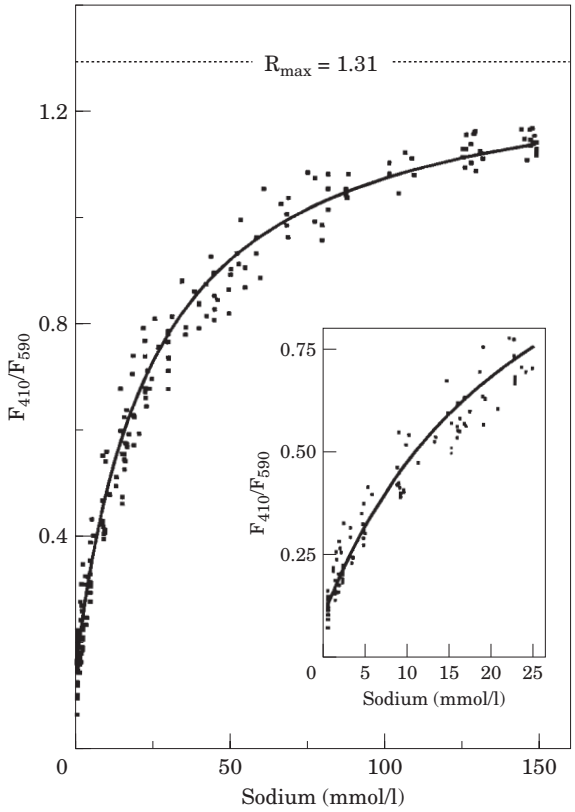

Figure 4 The ratios of the fluorescence of 410 and 590 nm of single myocytes after equilibration of the intracellular and extracellular $[Na^+]$ and $[K^+]$ (corrected for background and autofluorescence) plotted against the extracellular $[Na^+]$. $[Na^+]$ plus $[K^+]$ is constant (150 mmol/l). The solid line is the result of a fit procedure using equation [1] with as variables (R_{min}, R_{max} and $\beta \times K_d$). The value of these values $\pm$ S.D. were R_{max}/R_{min} is 9.6 ($1.34 \pm 0.06/0.14 \pm 0.03$) and $\beta \times K_d$ is 25.3 ± 0.3. The fit had a correlation coefficient of 0.98. The Fit standard error was 0.05. The inset shows the physiological range of sodium. The dotted line represents the maximal value of the fitted curve.

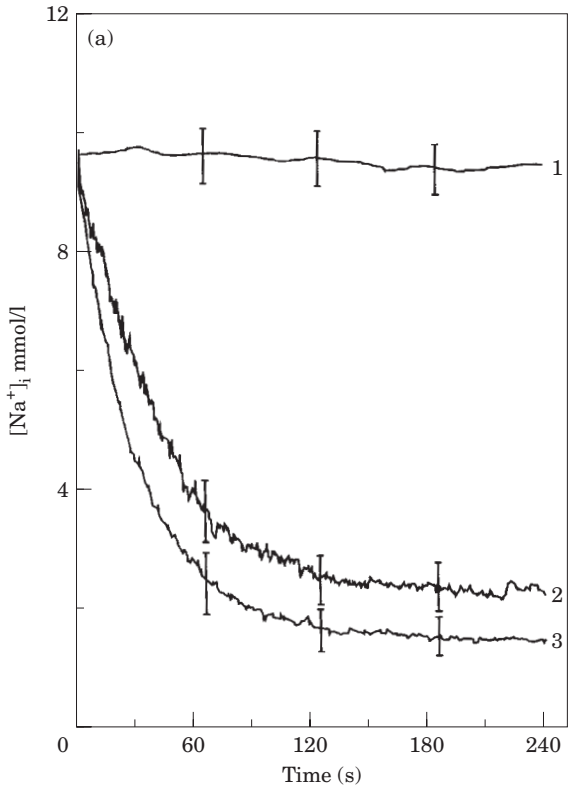

Figure 5 $[Na^+]_i$ ($\pm$ S.D.) in single myocytes in steady state with $[Na^+]_o$ of 156 mmol/l (curve 1 $n = 6$) and the time course of change of $[Na^+]_i$ after a reduction of $[Na^+]_o$ from 156 to 29 (curve 2 $n = 10$) and 11 mmol/l (curve 3 $n = 8$). The solutions were osmolatically compensated by the addition of cholinechloride. The $[Na^+]_i$ is calculated using an emission ratio method.

values for R_{min} and R_{150}. The highest concentration of sodium that can completely equilibrate intra- and extracellularly is 150 mmol/l (see Discussion), which is insufficiently high to give R_{max}. Therefore, R_{max} was calculated from equation [1] with $K_d \times \beta$ as determined above (Fig. 4), substituting 150 mmol/l for $[Na^+]_i$, R_{min} and measured R_{150}.

Discussion

A relatively small change of intracellular sodium activity may have large consequences for intracellular ionic conditions and for Ca^{2+} homeostasis in particular, mediated by sodium dependent exchangers and the sarcolemmal sodium pump. This is particularly relevant in pathophysiological conditions such as, for instance, in heart failure, where it has been suggested that small changes in $[Na^+]_i$ underlie abnormalities in Ca^{2+} metabolism (Vermeulen, 1996). The introduction of the fluorescent indicator SBFI made it possible to study the role of $[Na^+]_i$ in isolated cells (Harootunian *et al.*, 1989; Minta and Tsien, 1989). This fluorescent probe is commonly used in dual excitation ratio mode; excitation wavelengths 340 and 380 nm, emission wavelength around 500 nm. However, the excitation ratio method is rather insensitive to small changes of cytosolic sodium especially at low levels of $[Na^+]_i$ and is not entirely sodium-specific (Borzak *et al.*, 1992; Levi *et al.*, 1994). We therefore re-examined the intracellular spectral characteristics of SBFI fluorescence in order to develop a more sensitive and more specific method to measure $[Na^+]_i$. Excitation spectra in free solution indicate that with excitation at 340 nm, an increase of $[Na^+]_o$ causes an increase of 500 nm fluorescence [Donoso *et al.*, 1992; see also Fig. 1(b)]. However,

intracellularly, an increase of $[Na^+]_i$ causes hardly any change [Harootunian *et al.*, 1989; Satoh *et al.*, 1991; see also Fig. 1(a)]. This different response is caused by a shift of the intracellular sodium spectrum [Harootunian *et al.*, 1989; Borzak *et al.*, 1992; Donoso *et al.*, 1992; see also Fig. 1(a)] combined with the relative increase in quantum efficiency of the zero sodium spectra. This is presumably caused by binding SBFI to intracellular proteins. Indeed, in free solution containing cytosolic proteins, the same spectral shifts and change of quantum efficiency were observed [Fig. 1(c)]. It cannot be excluded that in addition, high molecular weight compounds other than protein contribute to the spectral changes. The observation, however, that albumin had similar effects, points to an important role for proteins. Previously, we also demonstrated protein-dependent spectral changes of intracellular indo-1 (Fiolet *et al.*, 1995). In this respect, it may be speculated that binding of SBFI to intracellular non-soluble proteins or membranes (partly) accounts for the residual fluorescence in digitonin treated myocytes, which usually is ascribed to compartmentalization of the dye (Harootunian *et al.*, 1989; Satoh *et al.*, 1991; Donoso *et al.*, 1992).

There are little data on emission spectra of SBFI in literature; only those in cell-free solution emission spectra of SBFI were reported (Minta and Tsien, 1989). To our knowledge, Figure 2 shows for the first time intracellular emission spectra of SBFI, which clearly demonstrate the rationale for the original dual excitation mode with emission measured at 500 nm. However, the spectra obtained with excitation at 340 nm [Fig. 2(a)] indicate the applicability of a dual emission mode with potential advantages over the dual excitation mode; with emission wavelengths below 430 and around 590 nm, respectively. The spectra suggest that fluorescence recorded around 410 nm is highly sodium-specific and that changes of sodium concentration may be more sensitively measured, although at the expense of lower absolute fluorescence intensities. This is rather convincingly demonstrated in the intracellular excitation spectra recorded at 410 nm [Fig. 3(a)], which shows that the peak of the sodium spectrum is about 13 times higher than that of the zero sodium spectrum. In dual excitation mode, an increase of sodium in the physiological range from 0–20 mmol/l causes a change of fluorescence ratio of about 1.27 (Borzak *et al.*, 1992; Levi *et al.*, 1994). In dual emission mode, the change of ratio was 4.51 with a corresponding change of sodium (see also Fig. 4). With regard to specificity, it could be argued that the sodium spectrum is in fact entirely K^+-specific rather than Na^+-specific; all SBFI fluorescence could be potassium-dependently quenched. However, this is very unlikely for the following reasons. First, the extracellular zero sodium spectrum remained unchanged upon replacement of potassium by choline [Fig. 3(b)]. Of course, an intracellular choline spectrum would be preferable, but is not feasible, because extracellular choline could never completely replace potassium. However, because the intra- and extracellular sodium spectra and the zero sodium spectra are identical, it seems unlikely that a difference between intra- and extracellular choline spectrum would exist. Secondly, in free solution with potassium held constant sodium spectra with intermediate peak values were obtained with intermediate sodium concentrations (not shown). Thirdly, reduction of potassium from 150 to 100 mmol/l at constant sodium concentration no change of zero sodium spectrum occurred. Regarding specificity, we also found that neither magnesium nor calcium in the physiologically relevant range of concentrations, had any measurable influence. K_d values for SBFI of 60 and 38 mmol/l of magnesium and calcium, respectively, are in accordance with this observation (Minta and Tsien, 1989). Because, at the second emission wavelength, an approximate 590-nm sodium-dependent change of fluorescence is rather small, problems related to ion specificity would hardly play a role [Fig. 2(a)].

The value of the apparent K_d might depend on the method. In this method, we determined $\beta \times K_d$ (see equation [1]) in single myocytes equilibrated with different sodium concentrations up to 150 mmol/l sodium. To obtain equilibration, we used the approach of Levi *et al.* (1994). A concentration of 150 mmol/l is the maximum sodium concentration that can equilibrate due to requirement of electroneutral exchange with intracellular potassium (about 140 mmol/l). We calculated a value for K_d of 22.5 mmol/l, which is substantially less than that previously found in rat ventricular myocytes (30 mmol/l) using SBFI in excitation ratio mode (Donoso *et al.*, 1992). It may be speculated that the potassium sensitivity of fluorescence signals in dual excitation mode, contributes to the observed difference. Such an explanation would be supported by the observation that the addition of potassium to only sodium-containing cell-free solutions increased the apparent K_d of SBFI for sodium (Minta and Tsien, 1989).

With SBFI in dual emission mode, we found steady state $[Na^+]_i$ of 9.5 mmol/l in electrically non-stimulated myocytes. This is a little lower than values reported by others (10–20 mmol/l) using

SBFI in dual excitation modes (Borzak *et al.*, 1992; Donoso *et al.*, 1992; Levi *et al.*, 1994). However, it is well within the range found with other methods such as micro-electrodes and NMR (Fozzard, 1986; Shattock and Bers, 1989). A value as high as 20 mmol/l though seems unlikely, because it would implicate reversed mode operation of the Na/Ca-exchanger. This would not be in accordance with results we previously obtained showing that inhibition of the Na/Ca exchanger in non-stimulated myocytes caused an increase of $[Ca]_i$ (Baartscheer *et al.*, 1996). On the other hand, Bers (1989) put forward the hypothesis that at least in electrically stimulated rat myocytes the Na/Ca-exchanger would operate in reversed mode during diastole. More recently, Bassani and Bers (1994) concluded that the Na/Ca-exchanger must be near equilibrium because during 3 min of rest following stimulation $[Ca]_i$, SR-calcium content and post-rest potentiation remained unchanged with inhibited exchanger.

In dynamic conditions, we measured a mono-exponential decrease of $[Na^+]_i$ down to about 2 mmol/l after abrupt reduction of $[Na^+]_o$. This concentration as well as the kinetics compare well with data obtained under similar experimental conditions using ion-sensitive micro-electrodes (Allen *et al.*, 1983; Chapman *et al.*, 1983). The overall methodological variability of the dual emission ratio method with SBFI in the physiological range of sodium concentration may be estimated from the standard deviations calculated for the single cell measurements (Fig. 5). At higher concentrations, the impact of an error introduced by uncertainty in the extrapolated value of R_{max} depends on the actual $[Na^+]_i$. At low $[Na^+]_i$, methodological variation is proportional to errors in R_{max}. At $[Na^+]_i$ higher than K_d, errors become progressively more severe with erroneous estimates of R_{max}. It is not certain whether a pathophysiological condition could affect the intracellular spectral properties of SBFI, in which case it would contribute to systematic errors. Because it is well known, for instance, that low pH in free solution affects SBFI-fluorescence, intracellular acidosis during ischemia might be expected to change spectral properties of SBFI, either directly or indirectly, by affecting protein binding. Therefore, in such conditions, calibrations should be performed with solutions approaching the intracellular (patho)physiological conditions as much as possible.

Another possible important source of systematic errors may result from compartmentalization of SBFI. The fraction of SBFI compartmentalized is still uncertain, estimates ranging from 10 (Sato *et al.*, 1991) to as high as 50% (Donoso *et al.*, 1992). Regardless of the method used (dual excitation or dual emission mode), when calibrating with known extracellular concentrations of sodium (c.f. Fig. 4), compartmentalization is immaterial for correct calculation of cytosolic sodium, provided two criteria are met (see also Donoso *et al.*, 1991): (1) extracellular and cytosolic sodium concentrations must completely equilibrate under the calibration conditions (gramicidin, monensin etc.); and (2) the relationship between cytosolic and mitochondrial sodium must not be affected by the calibration conditions. Sufficient evidence has been presented to conclude that the first criterion is met indeed (Levi *et al.*, 1994). To our knowledge, no evidence relating to the second criterion has ever been presented. Donoso *et al.* (1992) concluded that SBFI was 50% compartmentalized and calculated that under physiological conditions, mitochondrial sodium was twice as low as the cytosolic concentration. In isolated mitochondria, Jung *et al.* (1992) also reported that matrix sodium was lower than cytosolic sodium both in non-respiring and respiring mitochondria, which maintained gradients of 2 and 8 respectively. More importantly, they also found that mitochondrial sodium always increased linearly with extramitochondrial sodium. It seems not unreasonable to assume that this linearity also holds in our calibration conditions. Should the second criterion not apply, the data reported by Donoso *et al.* (1991) allow calculation of worst case errors due to compartmentalization; calculated cytosolic sodium concentrations of 3 and 20 mmol/l should be corrected to 4.0 and 25.3 mmol/l, respectively.

In summary: intracellular spectral characteristics of SBFI change due to protein binding in such a way that this fluorescent probe for measurement of $[Na^+]_i$ can be used in dual emission mode instead of the commonly used dual excitation mode. The dual emission mode provides the following advantages: cross-sensitivity of fluorescence signals to potassium is much reduced, the relative sensitivity to small changes of $[Na^+]_i$ is enhanced and K_d for sodium is closer to the physiological range of $[Na^+]_i$. Therefore, this is the preferred method to measure small changes of $[Na^+]_i$ in single myocytes, especially at low absolute concentrations.

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