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FLUORESCENCE MEASUREMENTS OF CYTOPLASMIC AND MITOCHONDRIAL SODIUM CONCENTRATION IN RAT VENTRICULAR MYOCYTES

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SUMMARY

1. The fluorescent Na^+ indicator SBFI was incorporated into isolated ventricular myocytes using the acetoxymethyl (AM) ester.

2. The excitation spectrum was found to be shifted about 20 nm in the cell compared to *in vitro*. In the cell, an increase of $[Na^+]$ decreased fluorescence at 380 nm (F_{380}) and had no effect at 340 nm (F_{340}). The ratio ($R = F_{340}/F_{380}$) was used as a measure of $[Na^+]_i$.

3. *In vivo* calibration of SBFI for $[Na^+]_i$ was obtained by equilibrating $[Na^+]$ across the plasma membrane with a divalent-free solution in the presence of gramicidin D.

4. Selective removal of the surface membrane with saponin or digitonin released only about 50% of the indicator. Following saponin treatment, cyanide or carbonylcyanide *m*-chlorphenylhydrazone (CCCP) increased the apparent $[Na^+]$ measured by the remaining (presumably mitochondrial) SBFI. It is suggested that mitochondrial $[Na^+]$ is normally less than cytoplasmic.

5. Attempts to examine the effects of metabolic inhibition on $[Na^+]_i$ were hampered by changes of autofluorescence due to changes of [NADH]. It is shown that this effect can be corrected for using the isosbestic signal (excited at 340 nm).

6. Inhibition of both aerobic metabolism (with CN^-) and glycolysis (glucose removal or iodoacetate) produced a gradual increase of $[Na^+]_i$. This began before the resting contracture developed and may (via Na^+–Ca^{2+} exchange) account for some of the rise of diastolic $[Ca^{2+}]_i$ seen in previous work. The rise of $[Na^+]_i$ began at about the same time as the decrease of systolic contraction and therefore at a time when $[ATP]_i$ had begun to fall.

INTRODUCTION

The importance of sodium ions in the regulation of normal cardiac function has long been recognized. Through Na^+–Ca^{2+} and Na^+–H^+ exchanges, $[Na^+]_i$ is involved in the regulation of both intracellular calcium concentration and intracellular pH

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(pH_i; Reuter & Seitz, 1968; Deitmer & Ellis, 1980). The activity of both exchangers depends on the maintenance of the sodium electrochemical gradient by the Na^+–K^+-ATPase. In the beating heart adequate contractility depends on the interrelation of these three ions. Ion-sensitive fluorescent probes able to measure $[Ca^{2+}]_i$ and pH_i in single isolated cardiac myocytes have greatly increased our understanding of the intracellular regulation of these ions in heart muscle. The lack of a similar indicator for sodium has restricted measurements of intracellular sodium concentration, $[Na^+]_i$, to the use of Na^+-selective microelectrodes with the limitations and difficulties inherent to this technique. The recent introduction of the fluorescent probe SBFI (Minta & Tsien, 1989), a sodium indicator which can be easily loaded into the cell as the acetoxymethyl (AM) ester, should allow a similar increase in our knowledge of $[Na^+]_i$ regulation. However work with other fluorescent indicators has shown that there are problems due to incomplete hydrolysis of AM esters (Highsmith, Bloebaum & Snowdowne, 1986) and non-cytoplasmic localization (Almers & Neher, 1985; Spurgeon *et al.* 1990). Therefore, the initial aim of the present work was to examine the utility of SBFI as an intracellular Na^+ indicator in isolated cardiac myocytes.

If the heart is made ischaemic profound changes in intracellular ion concentrations occur, impairing the heart function and leading to the development of an irreversible contracture (Allen & Orchard, 1987). These changes have been studied in isolated ventricular myocytes in which the production of ATP has been blocked by inhibiting glycolysis and oxidative phosphorylation. Experiments using photoproteins or fluorescent probes in metabolically inhibited myocytes have measured increases in both $[Ca^{2+}]_i$ and $[H^+]_i$ (Allshire, Piper, Cuthbertson & Cobbold, 1987; Smith & Allen, 1988; Stern *et al.* 1988; Eisner, Nichols, O'Neill, Smith & Valdeolmillos, 1989) that precede the development of the contracture. It is possible that these changes could result from an increase in $[Na^+]_i$ due to inhibition of the Na^+–K^+-ATPase. So far direct measurements of $[Na^+]_i$ (using ion-sensitive microelectrodes) have produced contradictory results (Kleber, 1983; MacLeod, 1989).

In this study we have measured the changes in $[Na^+]_i$ following metabolic inhibition in single, isolated myocytes from rat ventricle using SBFI. We also report some of the properties of the dye and discuss the suitability of this dye for use in cardiac myocytes. Preliminary accounts of some of the data in this paper have been presented to the Physiological Society (Donoso, Eisner & O'Neill, 1990*a*; Donoso, Mill, O'Neill & Eisner, 1990*b*).

METHODS

Rat ventricular myocytes were isolated as previously described (Eisner *et al.* 1989). Briefly, rats were killed by cervical dislocation and the hearts were retroperfused through the aorta with a nominally Ca-free solution (for composition see below) to which collagenase and protease were added after all blood had been washed out of the coronary vessels. Perfusion with the enzyme solution was continued for 6 min. At the end of the enzymatic digestion the hearts were perfused with a nominally Ca-free solution in which 50 mM-taurine replaced 25 mM-NaCl. Perfusion with taurine improved the calcium tolerance and the survival of the isolated cells (Isenberg & Klockner, 1982).

Cells were loaded with the acetoxymethyl (AM) ester of SBFI (Molecular Probes) at a final concentration of 10 μM for 1 h at room temperature. The SBFI-AM was added from a stock solution made in 25% pluronic acid in dry dimethyl sulphoxide (DMSO). The uptake of the indicator was stopped by dilution and the loaded cells were kept at room temperature (21 °C).

Fluorescence measurements

Loaded cells were placed on a bath with a cover-slip base on an inverted microscope modified for epifluorescence. Fluorescence was excited alternately at 380 and 340 nm. Rather than using a spinning filter wheel (Eisner *et al.* 1989) we used a simple motor to switch between 340 and 380 nm filters. Each filter was present for 1 s and the light recorded over this period was integrated. The final value of the integral was displayed continuously. Emitted fluorescence was measured at 530 nm.

Solutions

The experimental solution contained (mM): NaCl, 134; KCl, 4; $MgCl_2$, 1; HEPES, 10; $CaCl_2$, 1; glucose, 10; titrated to pH 7·4 with NaOH. Two calibration solutions were prepared to contain (mM): NaCl or KCl, 140; HEPES, 10; EGTA, 1; glucose, 10; gramicidin D, 0·2 μg/ml; titrated to pH 7·4 with either NaOH or KOH. The final calibration solution of the required $[Na^+]$ was prepared by mixing these two stocks in the appropriate proportions. The solutions were equilibrated with air and all experiments were carried out at 27 °C. In some experiments either glucose was omitted or 0·5 mM-iodoacetate added to inhibit glycolysis and cyanide (2 mM) added to inhibit respiration.

Permeabilized cells

In some experiments cells were 'permeabilized' in a solution that contained (mM); NaCl, 10; KCl, 80; $MgCl_2$, 5; KH_2PO_4, 1; EGTA, 1; succinate, 10; ATP, 1·8; mannitol, 100; HEPES, 25; adjusted to pH 7·1 with KOH. Assuming a contaminating Ca^{2+} of 10 μM the pCa of this solution would be approximately 8·5. Digitonin (0·25 mg/ml) or saponin (25 μg/ml) were applied until the fluorescence had declined to a steady level representing the full release of cytosolic dye. Where needed the mitochondrial uncoupler CCCP (carbonylcyanide *m*-chlorophenylhydrazone) was added to a final concentration of 10 μM.

With the exception of Fig. 8 the cells were not electrically stimulated. All statistics are given as means ± S.E.M.

RESULTS

The *in vitro* behaviour of the free acid of SBFI is shown in Fig. 1*B*. This excitation spectrum shows that increasing $[Na^+]$ (at constant $[Na^+]+[K^+]$) increases fluorescence excited by wavelengths less than about 360 nm and decreases it at longer wavelengths (similar results were achieved when measurements of the excitation spectrum of the free acid were made on the microscope stage). These spectral changes are similar to those shown in earlier work (Harootunian, Kao, Eckert & Tsien, 1989). Therefore *in vitro*, the fluorescence excited at 340 nm (F_{340}) increases with $[Na^+]_i$ and that excited at 380 nm (F_{380}) decreases. Consequently the ratio ($R = F_{340}/F_{380}$) increases with increasing $[Na^+]_i$. This point was examined in a single rat ventricular myocyte in the experiment illustrated in Fig. 1*A*. Here SBFI was loaded into the cell as the acetoxymethyl (AM) ester. In this experiment $[Na^+]_i$ was elevated by the application of ouabain (1 mM) to inhibit the Na^+–K^+-ATPase and Ni^{2+} (5 mM) to inhibit Na^{2+}–Ca^{2+} exchange. The resulting increase in $[Na^+]_i$ is signalled by an increase of the ratio of 340:380 nm excited fluorescence. This change in the ratio is brought about by a decrease in F_{380}. However there is no effect on F_{340}. These changes in fluorescence are in marked contrast to the behaviour of the dye *in vitro*.

Calibration of SBFI

The above results show that the spectral properties of SBFI are different *in vivo* from those found *in vitro*. Calibration of the indicator must therefore be done in the cell. This was done using the method illustrated in Fig. 1*A* in which the cell is exposed

to a solution which contains no divalent cations. This allows Na^+ ions to enter through Ca^{2+} channels and elevates $[Na^+]_i$ (Chapman, Fozzard, Friedlander & January, 1986). In addition gramicidin D (0·2 μg/ml) was added to further increase the Na^+ permeability. Control experiments showed that the addition of ouabain

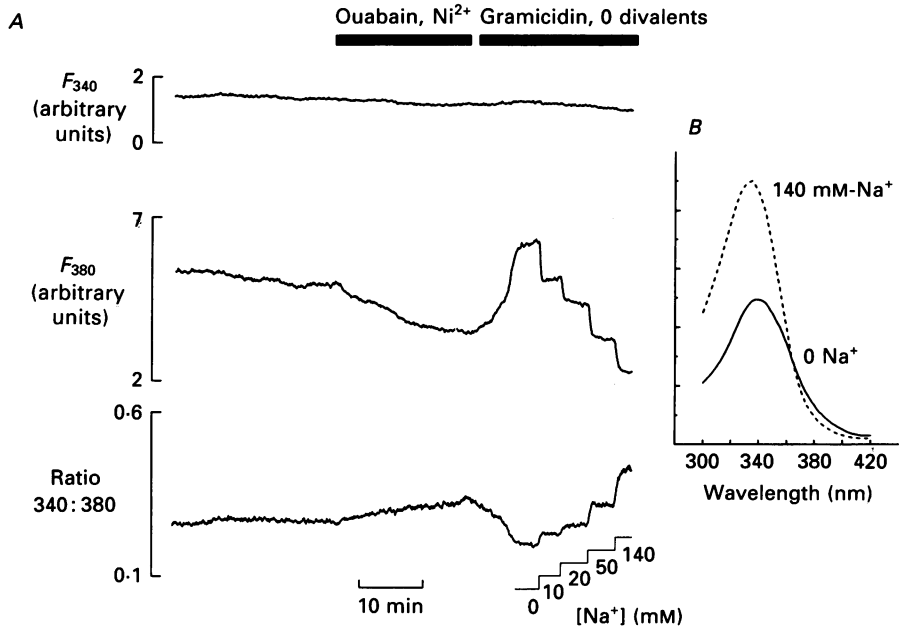

Fig. 1. The effects of changes of $[Na^+]$ on fluorescence of SBFI measured *in vivo* and *in vitro*. *A*, *in vivo*. Traces show measurements of SBFI fluorescence from a single rat ventricular myocyte which had been loaded with the AM ester of SBFI. Traces show (from top to bottom): F_{340}; F_{380}; ratio (F_{340}/F_{380}). Solution changes are denoted above the record. The first horizontal bar shows the effects of adding ouabain (1 mM) and $NiCl_2$ (5 mM). The second bar shows the calibration. Here the cell was exposed to a divalent cation-free solution (1 mM-EGTA) containing gramicidin (0·2 μg/ml). $[Na^+]_o$ was changed (at constant $[Na]+[K^+]$). *B*, *in vitro*. The traces were obtained from a 0·5 μM solution of the free acid of SBFI. Traces show excitation spectra obtained in either 0 mM (—) or 140 mM-Na^+ (----). In the Na-free solution, Na^+ was replaced by K^+. The spectra have not been corrected for any variation in the properties of the spectrometer with wavelength.

(1 mM) had no effect on $[Na^+]_i$ in this calibrating solution showing that $[Na^+]_i$ was at equilibrium with the extracellular sodium concentration, $[Na^+]_o$. The figure shows that changing $[Na^+]_o$ produces rapid changes of $[Na^+]_i$. These were used to calibrate the previous part of the experiment.

We have attempted to measure the apparent affinity of the indicator for intracellular Na^+. This is complicated by the fact that the indicator is sensitive to K^+ as well as Na^+ ions and the calibration involves changing $[Na^+]_i$ at constant $[Na^+]_i+[K^+]_i$. We make the following assumptions: (i) SBFI can bind either Na^+ or K^+; (ii) the fluorescence of the Na-bound form is identical to that of the K-bound; (iii) a given concentration of K^+ is equivalent to a concentration of α times that of Na^+. In other words 1 mM-K^+ produces the same fluorescence as α mM-Na^+, (iv) $[Na^+]_i+[K^+]_i = 140$ mM.

Finally, we assume that the fluorescence (F) of SBFI at any wavelength can be expressed as:

$$F = A + BX/(D+X), \tag{1}$$

where A and B are functions of wavelength, D is a constant and $X = [Na^+]_i + \alpha[K^+]_i$.

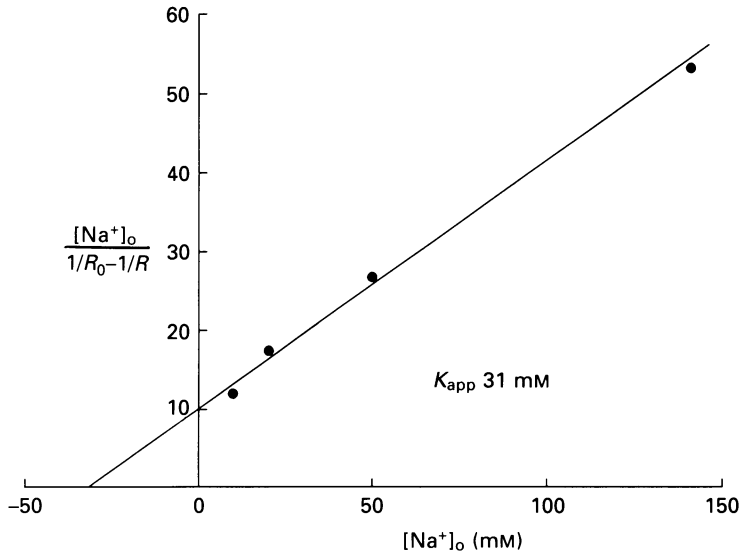

Fig. 2. Measurement of the apparent K_d (K_{app}) of SBFI for Na^+ The record shows data obtained from the calibration illustrated in Fig. 1*A*. The graph is a Hanes plot and the K_{app} is given by the x-axis intercept.

It can be shown that:

$$F = A + B\left(1 - \frac{D}{D+[Na^+](1-\alpha)+140\alpha}\right). \tag{2}$$

In the special case when $[Na^+]_i = 0$ (and $[K^+]_i = 140$ mM) then:

$$F_{0Na} = A + B[1 - D/(D+140\alpha)]. \tag{3}$$

It follows that:

$$F - F_{0Na} = F_{max}[Na^+]_i/([Na^+]_i + K_{app}), \tag{4}$$

where $F_{max} = BD/(D+140\alpha)$ and $K_{app} = (D+140\alpha)/(1-\alpha)$, K_{app} being the apparent dissociation constant of SBFI for sodium. This therefore shows that, so long as the fluorescence in the absence of Na^+ is subtracted from the measured levels, then simple Michaelis behaviour will be observed even though both Na^+ and K^+ are changing. The above equation can therefore be used to interpolate between measured calibration points. One problem with the use of this method is that often there is a loss of signal during the calibration procedure. We have circumvented this by using $1/R$ (which is equal to F_{380}/F_{340}) rather than F_{380}. Since F_{340} is independent of $[Na^+]_i$ $1/R$ gives a measure of F_{380} corrected for any loss of signal. Figure 2 shows a Hanes plot of the data from the calibration of a single cell. The value of K_{app} found here is

31 mM. In twelve cells a value for K_{app} of 29·3 ± 2·7 mM was obtained. This value is slightly higher than that of 17 mM *in vitro* (Minta & Tsien, 1989). This difference may reflect the influence of some, unidentified cytoplasmic constituent.

Using the method of calibration described above, we have found a $[Na^+]_i$ of 10·7 ± 1·2 mM (n = 13). Isolation of single cardiac myocytes into a solution rich in

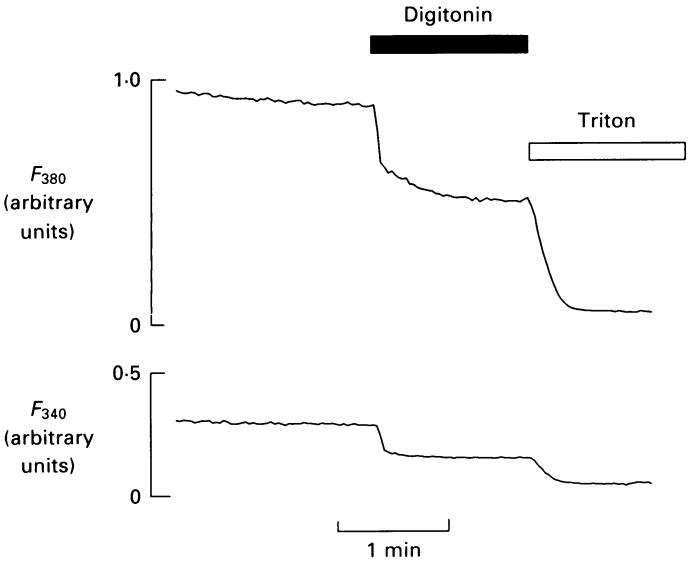

Fig. 3. The effects of removing the surface membrane on the fluorescence from SBFI. Traces show: top, F_{380}; bottom, F_{340}. Digitonin (0·25 mg/ml) and Triton X-100 (1% v/v) were added as shown above the record.

taurine, has been shown to produce a lower $[Na^+]_i$ (Chapman & Rodrigo, 1990). In this study, when the taurine perfusion step was omitted, $[Na^+]_i$ was 21·0 ± 2·0 mM (n = 12).

It has been shown for other fluorescent indicators e.g. Fura-2 (Highsmith *et al.* 1986) and Indo-1 (Spurgeon *et al.* 1990) that, when the indicator is added as the AM ester, some of it is de-esterified in intracellular organelles rather than in the cytoplasm. We have investigated whether this occurs with SBFI. Figure 3 shows the effect of selectively removing with digitonin the surface membrane of a cell loaded with the ester form of SBFI. On application of digitonin both fluorescence signals fell by about 50% within 1 min, remaining constant thereafter. The remaining fluorescence was lost when the intracellular membranes were solubilized by application of the non-ionic detergent Triton X-100. In other experiments we have used saponin (25 μg/ml) and found that saponin released 43·2 ± 2% (n = 12) of the fluorescence (measured at 340 nm to avoid confusion from changes of $[Na^+]$). This result indicates that up to half of the SBFI introduced into a cell as the membrane permeant AM ester is located inside intracellular organelles.

One likely location for the non-cytoplasmic dye is within mitochondria. In order to determine whether the dye was located within mitochondria we removed the surface membrane of SBFI-AM loaded cells with saponin (25 μg/ml) and measured

the response of the remaining fluorescence to inhibitors of mitochondrial function (Fig. 4). As soon as the fluorescence signals started to decrease, saponin was removed from the perfusion solution and a new stable fluorescence ratio was obtained. The subsequent addition of CN^- to inhibit the mitochondrial respiratory chain produced

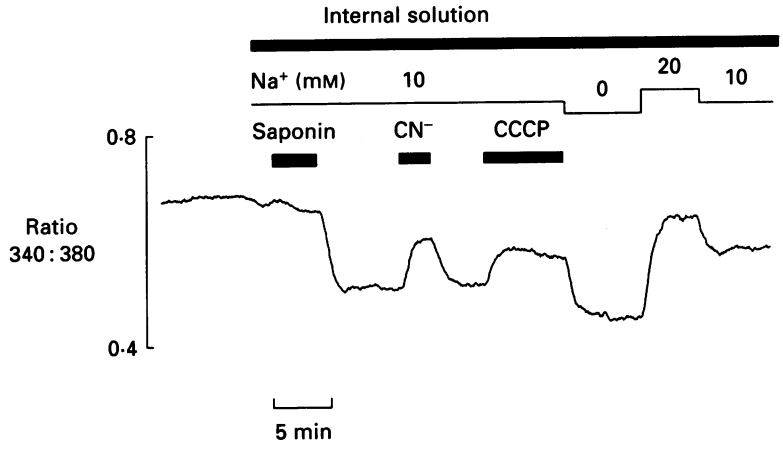

Fig. 4. The effects of various interventions on the fluorescence of SBFI-loaded skinned cells. The records shows the F_{340}/F_{380} ratio. The cell was initially superfused with a standard extracellular solution. At the point indicated by the horizontal bar this was then replaced with a mock intracellular solution containing 10 mM-Na^+ (see Methods for details). Saponin (0·25 μg/ml) was added until there was a large decrease of fluorescence (not shown) which was accompanied by a fall in the ratio. The CN^- (2 mM) or CCCP (10 μM) were added for the periods indicated above. Changes in superfusate [Na^+] are indicated above the record.

a reversible increase in the fluorescence ratio. There are three possible explanations for this increase in ratio: (i) It may result from changes of autofluorescence due to changes of [NADH] since this molecule absorbs and emits light over the wavelengths used in the present work. (ii) SBFI is somewhat pH sensitive (Minta & Tsien, 1989) and the change of ratio could result from the large changes of mitochondrial pH expected under these conditions. (iii) It could represent a real change of intramitochondrial [Na^+]. Subsequent experiments were designed to distinguish between these possibilities.

One result which argues against explanation (i) is that the mitochondrial uncoupler CCCP has qualitatively similar effects to those of CN^- on the ratio (Fig. 4). However CCCP will *decrease* [NADH] in contrast to the *increase* produced by CN^- (see Fig. 6*B*). The fact that both CN^- and CCCP produce the same effect on the ratio means that it cannot therefore result from changes of [NADH]. The experiment illustrated in Fig. 5 was designed to investigate possible effects of changes of intramitochondrial pH. Under control conditions, the intramitochondrial pH in rat myocytes will be about one pH unit more alkaline than that of the cytoplasm and will equilibrate with that of the cytoplasm in the presence of CCCP (Reers, Kelly & Smith, 1989). Figure 5 shows that the addition of CCCP decreased F_{340} and F_{380}. The decrease at 340 nm is due to changes of [NADH] (see later). However the important part of this figure shows the effects of subsequently changing the pH of the solution

(and therefore the intramitochondrial pH) to 8·0. If the effect of CCCP to increase the ratio is due to the intramitochondrial acidification then the pH 8·0 solution should decrease the ratio. The figure shows, however, that there is a small increase in the ratio. Therefore the effects of CCCP and CN^- on the ratio are not due to changes of

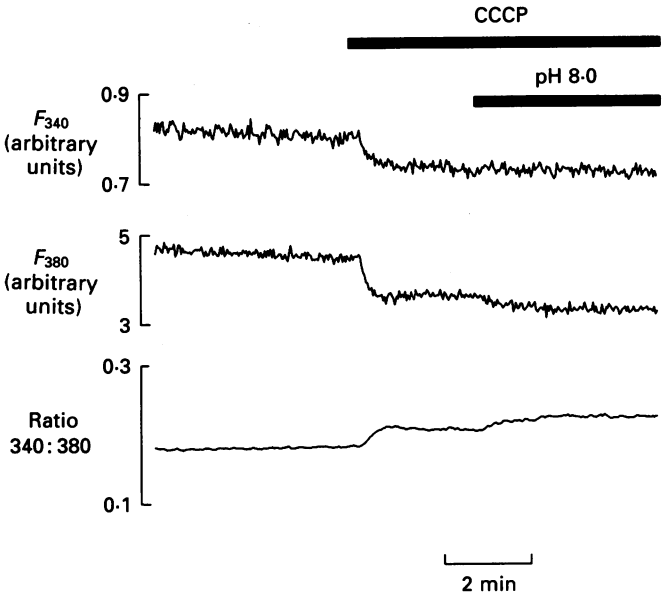

Fig. 5. Comparison of the effects of CCCP with those of altered pH on SBFI-loaded mitochondria. Traces show from top to bottom: F_{340}, F_{380}, ratio. The cell had been skinned with saponin (0·25 μg/ml) 1 min before the record began. CCCP (10 μM) was added and the external pH changed from 7·0 to 8·0 for the periods shown.

pH. These considerations favour the hypothesis that the increase of ratio produced by CCCP (or indeed CN^-) is a consequence of a rise of intramitochondrial $[Na^+]$.

The effects of metabolic inhibition on $[Na^+]_i$

Our main interest was to use SBFI as a probe for changes in $[Na^+]_i$ during metabolic inhibition under conditions identical to those previously used to measure changes in both $[Ca^{2+}]_i$ and pH_i which take place before irreversible contracture. Early experiments showed a serious problem in the interpretation of such experiments.

The experiment illustrated in Fig. 6*A* shows that some of the changes of fluorescence cannot be attributed to the effects of changes of $[Na^+]_i$ (whether cytoplasmic or mitochondrial) on SBFI. The application of CN^- increases the F_{340}/F_{380} ratio suggesting an increase of $[Na^+]_i$; however this is accompanied by increases in both the raw fluorescence signals, an effect which (cf. Fig. 1) cannot be due to an increase of $[Na^+]_i$ alone. This is then followed by a decrease of F_{380} with no change of F_{340}. This slower phase may be due to an increase of $[Na^+]_i$. The explanation of the unexpected early increase of fluorescence is suggested by the records of Fig. 6*B* which shows measurements taken from a cell which was not loaded

with SBFI. Here the application of CN^- increases both F_{340} and F_{380}. This increase of fluorescence has been reported previously and is due to accumulation of NADH (from NAD) as a result of inhibition of mitochondrial electron transport (Eng, Lynch & Balaban 1989). The application of the mitochondrial uncoupler CCCP decreases fluorescence as expected from the decrease of [NADH].

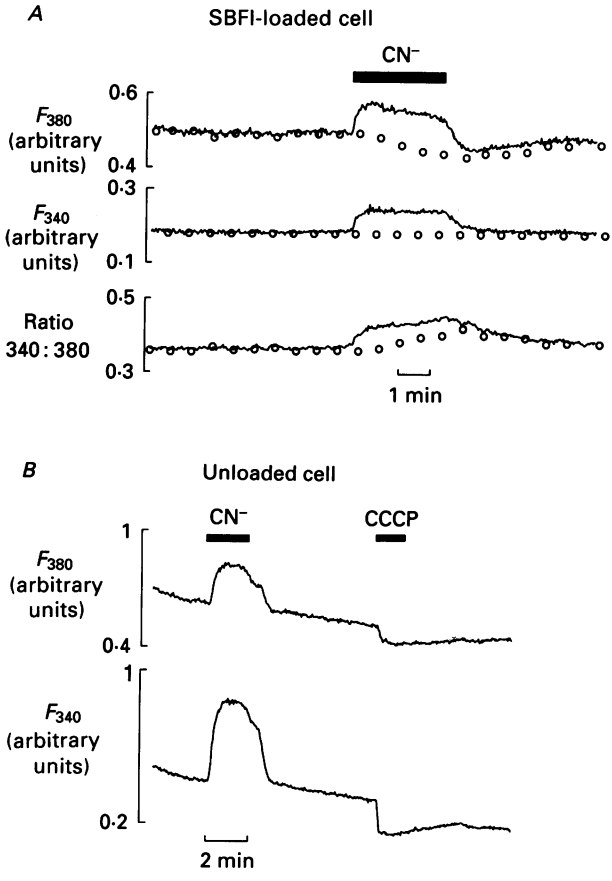

Fig. 6. The effects of CN^- on loaded and unloaded cells. *A*, SBFI-loaded cell. The traces show from top to bottom: F_{380}, F_{340} and the ratio. Cyanide (2 mM) was applied for the period indicated above the record. The open circles on the records show the corrections made for changes of [NADH] as described in the text. *B*, unloaded cell. The traces show F_{380} (top) and F_{340} (bottom). Cyanide (2 mM) and CCCP (10 μM) were applied as shown above.

Since changes of $[Na^+]_i$ have no effect on F_{340}, the changes of F_{340} produced by CN^- in a SBFI-loaded cell must be due to changes of autofluorescence whereas the changes at 380 nm will be due to both autofluorescence and Na^+-dependent changes of SBFI fluorescence ΔF_{380} (SBFI). The former will depend on the change of [NADH] and a scaling, wavelength-dependent factor (a_λ).

Specifically:

$$\Delta F_{340} = a_{340} \times \Delta[\text{NADH}];$$

$$\Delta F_{380} = a_{380} \times \Delta[\text{NADH}] + \Delta F_{380}(\text{SBFI}).$$

Rearranging: ΔF_{380} (SBFI) $= \Delta F_{380} - \Delta F_{340} a_{380}/a_{340}$.

The value of a_{380}/a_{340} can be obtained from experiments on unloaded cells. The exact value depends, of course, on the precise filters used and was measured from

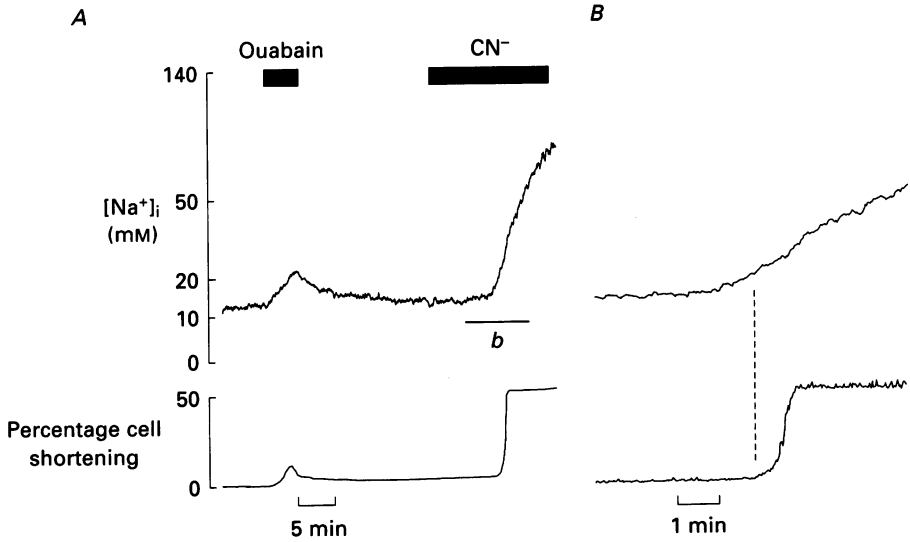

Fig. 7. The effects of metabolic inhibition on $[Na^+]_i$ and cell length. *A*, comparison of the effects of ouabain with those of metabolic inhibition. Traces show $[Na^+]_i$ calculated from the SBFI ratio (top), diastolic cell length (bottom). Ouabain (1 mM) and CN^- (2 mM) were added as shown above. All solutions were glucose free. The ratio record has been corrected (as described in Fig. 6) for the changes of [NADH]. *B*, this shows the region of *A* indicated *b* on an expanded time scale. The vertical dashed line shows when the diastolic contracture began.

experiments such as that of Fig. 6*B*. The open circles in Fig. 6*A* show the corrected fluorescence and ratio signals. It should be noted that the correct ratio rises more gradually than was suggested by the raw signals.

The relationship between $[Na^+]_i$ and contraction during metabolic inhibition

In this series of experiments we investigated the temporal relationship between the rise of $[Na^+]_i$ produced by metabolic inhibition and the changes of contraction. We have compared this with the effects of a manoeuvre which simply elevates $[Na^+]_i$ with no primary effect on metabolism: inhibition of the Na^+–K^+ pump. As shown in Fig. 7 the application of ouabain causes a reversible increase of $[Na^+]_i$ which is associated with a reduction in resting cell length which is largely reversible. When the cell is exposed to CN^- to inhibit oxidative phosphorylation (in the absence of glucose) there is a gradual increase of $[Na^+]_i$. This rise of $[Na^+]_i$ begins before the diastolic contracture develops and then continues even after the contracture is complete. Experiments in eight cells showed that the $[Na^+]_i$ had risen by $7{\cdot}1 \pm 1{\cdot}2$ mM before the irreversible contracture began in metabolic blockade. That this increase of

$[Na^+]_i$ may contribute to the onset of the irreversible contracture is indicated by the cell shortening produced by a similar increase of $[Na^+]_i$ induced by ouabain. Similar results were obtained when glycolysis was inhibited by the addition of iodoacetate.

The experiment illustrated in Fig. 8 shows the relationship between the twitch and $[Na^+]_i$ during metabolic inhibition. The decline of the twitch coincides with the rise

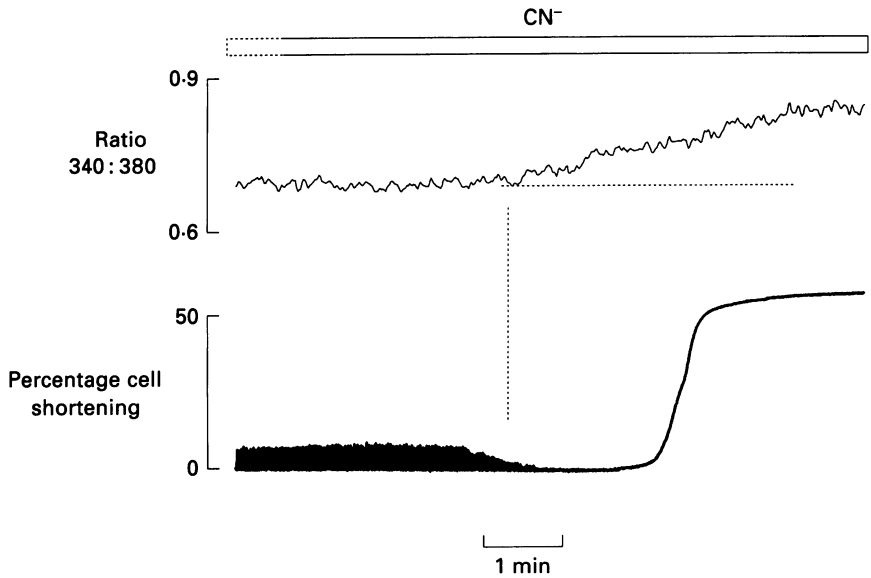

Fig. 8. The effects of metabolic inhibition on twitch and $[Na^+]_i$. Traces show: top, $[Na^+]_i$; bottom, cell length. The cell was stimulated throughout at 1 Hz. Ten minutes before the start of the figure CN^- (2 mM) was added and glucose removed. The vertical dashed line indicates the point at which $[Na^+]_i$ had begun to increase.

of $[Na^+]_i$. As was the case in Fig. 7, the increase of $[Na^+]_i$ precedes the development of the diastolic contracture.

DISCUSSION

Measurement of $[Na^+]_i$ with SBFI

In the present work we have investigated the suitability of the fluorescent dye SBFI for measuring $[Na^+]_i$ in single, isolated rat cardiac myocytes. We found that it is possible to record changes in $[Na^+]_i$ in cardiac cells loaded with the membrane permeant acetoxymethyl (AM) ester form of SBFI. In agreement with previous reports we have found that there is a shift in the excitation spectrum of SBFI inside the cell (Harootunian *et al.* 1989). Figure 1 shows, however, that in cardiac cells, the shift is larger than previously seen in other cell types such that the 340 nm excited fluorescence, which shows marked changes with sodium *in vitro* (Fig. 1*B*), is close to the isosbestic wavelength when the dye is located inside the cell. This spectral shift prevents any comparison with fluorescence signals obtained from *in vitro* measurements in standard solutions and makes it necessary to carry out the calibration of fluorescence signals *in situ* at the end of each experiment. We have done this by

equilibrating Na^+ ions across the membrane by a combination of a divalent-free solution (which allows Na^+ ions to move through L-type Ca^{2+} channels) and the ionophore gramicidin D. In this way we have calculated a K_{app} of the dye for sodium of about 30 mM, which is appropriate for measuring $[Na^+]_i$ in cardiac cells.

The resting $[Na^+]_i$ of 21 mM that we found in hearts perfused in the absence of taurine confirms previous findings of a high $[Na^+]_i$ in the rat. This value lies within the range previously reported in studies using Na^+-selective electrodes of between 17 and 40 mM (units of concentration) in the rat ventricle (Wasserstrom, 1983; Szabo & Armstrong, 1984; Shattock & Bers, 1989). The inclusion of taurine in the perfusion medium at the end of the enzymatic digestion reduced $[Na^+]_i$ approximately by half: a similar effect has been found in guinea-pig ventricular myocytes (Chapman & Rodrigo, 1990).

As discussed below, much of the SBFI is located in the mitochondria. As mitochondrial $[Na^+]$ is less than cytoplasmic (see below), it might be thought that this will give an erroneously low value for $[Na^+]_i$. Whether or not this occurs depends on whether the calibration procedure affects the relationship between cytoplasmic and mitochondrial $[Na^+]$. It is likely that the low concentration of gramicidin and divalent-free solution will have no effect on the mitochondria in which case the calibration will give an accurate measurement of cytoplasmic $[Na^+]$.

Incorporation of SBFI into mitochondria

In the experiments shown in this study, the cells were loaded with the acetoxymethyl (AM) ester form of SBFI at, typically, 10 μM for about 1 h. In these conditions we were able to obtain a reasonably high signal with a minimum of contamination from autofluorescence. SBFI-loaded cells were usually 7–15 times brighter than unloaded cells. It is, however, known that loading of fluorescent probes in this way can lead to compartmentalization of the dye inside intracellular organelles (Highsmith *et al.* 1986; Spurgeon *et al.* 1990). Indeed, we have found that about half of the SBFI in our cells is contained within intracellular organelles, as disruption of the surface membrane of the cell with either digitonin or saponin leads to the loss of only half of the fluorescence signal (Fig. 3), the remaining fluorescence is lost on exposure to Triton X-100. The signal from the cell, therefore, is composed of two separate pools of roughly equal size; in the cytoplasm and in intracellular organelles.

The experiments on permeabilized cells showed that both CN^- and CCCP increased the apparent $[Na^+]$. The application of either CN^- or CCCP will affect only the mitochondria since the inclusion of ATP in the internal solution should ensure the normal function of all other intracellular organelles. This suggests that the SBFI was located in the mitochondria. This appears reasonable as the mitochondria account for 35% of the cell volume in cardiac muscle (McCallister & Page, 1973) and also contain the esterases necessary to hydrolyse and hence trap similar indicators (Brierley, Davis, Cragoe & Jung, 1989; Jung, Davis & Brierley, 1989; McCormack, Brown & Dawes, 1989). It might be argued that changes of mitochondrial NADH contribute to the changes of fluorescence in these experiments. However the following arguments suggest that this is not a significant problem. (i) Since these experiments were performed in the presence of succinate, [NADH] should be near

maximum thus limiting the scope for increase in the presence of CN (Chance, 1976). (ii) Similar changes take place in the presence of rotenone (not shown) which will prevent changes in the redox state of NADH by inhibiting electron transfer at complex I of the respiratory chain. (iii) CCCP should decrease [NADH] and give the opposite effect on autofluorescence to CN^-. The experiments showed, however, that both had the same effect on the ratio in SBFI-loaded cells.

The fact that mitochondrial [Na^+] was increased by metabolic inhibitors suggests that normal respiring mitochondria have a [Na^+] less than that of the cytoplasm. It seems likely that this is maintained by the active Na^+–H^+ exchange which is established by the mitochondrial pH gradient. Some estimate of the value of mitochondrial [Na^+] can be obtained from experiments such as that illustrated in Fig. 4. We assume that, in the presence of CN^- or CCCP, mitochondrial [Na^+] equals that in the external solution (10 mM). We assume also that in Na-free solution mitochondrial [Na^+] is zero. The value of mitochondrial [Na^+] can then be calculated. With this approach we estimate that (with 10 mM-Na^+ outside) the mitochondrial [Na^+] is $5{\cdot}1 \pm 0{\cdot}2$ mM ($n = 6$). Ten millimolar Na^+ is close to the value found for $[Na^+]_i$ in taurine-isolated cells and this therefore suggests that mitochondrial [Na^+] may be about half of that in the cytoplasm. These results can be compared with those using electron microprobe analysis to measure [Na^+]. That technique gives concentrations with respect to dry weight. After conversion to millimolar units it was found that in cardiac muscle mitochondrial [Na^+] is either 0·5 (Wendt-Gallitelli & Isenberg, 1989) or 0·7 (Wendt-Gallitelli & Isenberg, 1991) of that in the cytoplasm. On the other hand, work on skeletal and smooth muscle found mitochondrial and cytoplasmic [Na^+] to be identical (Somlyo, Gonzalez Serratos, Shuman, McClellan & Somlyo. 1981; Somlyo, Somlyo & Shuman, 1979).

It should be pointed out that in our permeabilized cell experiments the cytoplasmic calcium is very low (about pCa 8·5). As the mitochondrial membrane contains a Na^+–Ca^{2+} exchange mechanism it is inevitable that the absence of calcium from the cytoplasm will change the way in which the mitochondria regulate Na^+. One would expect that Ca^{2+} ions will enter the mitochondrion through Ca^{2+} channels and then be pumped out by the Na^+–Ca^{2+} exchange resulting in an increase of intramitochondrial Na^+ concentration. The inclusion of calcium in our permeabilized cell solutions is difficult as even slight activation of the contractile filaments in these mechanically unloaded cells leads to irreversible contraction and the loss of the mitochondria from the permeabilized cell.

This study shows that SBFI can be used to follow changes of sodium in the mitochondria. The fluorescent indicator BCECF has been used to measure the activity of Na^+–H^+ exchange in isolated mitochondria (Brierley *et al.* 1989) but BCECF can be used only after dissipating the pH gradient of normally respiring mitochondria in order to provide an internal source of H^+ to support the inward exchange of Na^+ and place the mitochondrial pH in a region appropriate to measure with BCECF. So with BCECF, Na^+–H^+ exchange can only be measured in uncoupled mitochondria and in the reverse mode. The present study shows a technique which allows measurement of mitochondrial [Na^+] in respiring mitochondria, under conditions where the bathing solution can be rapidly exchanged. This will allow further study of the mitochondrial regulation of [Na^+].

The effects of metabolic inhibition on $[Na^+]_i$

We have previously reported the effect of metabolic inhibition on $[Ca^{2+}]_i$ and pH_i in single cardiac cells using Fura-2 and BCECF (Eisner *et al.* 1989). We were interested to know how $[Na^+]_i$ was affected under the same conditions to see if changes in $[Na^+]_i$ could explain the rise in $[Ca^{2+}]_i$ and $[H^+]_i$ and the contracture previously reported. The experiments were initially hampered by large changes of autofluorescence due to changes of [NADH] which produced an abrupt increase in the SBFI ratio. It was possible to correct for this by assuming that all the change of F_{340} is due to autofluorescence.

The experiments on permeabilized cells show that metabolic inhibition will increase mitochondrial $[Na^+]$. This appears to be a rapid process compared to the time course of sodium equilibration across the surface membrane. One would therefore expect that, in the intact cell, there will be a corresponding decrease of cytoplasmic $[Na^+]$. This may not have been observed because: (i) there are similar amounts of SBFI in the mitochondria and cytoplasm and (ii) the $[Na^+]$ in both compartments lies on the linear region of the $[Na^+]$–response curve. This effect may, however, account for observations that ischaemia can *decrease* $[Na^+]_i$ as measured with a Na^+-sensitive microelectrode and therefore only from the cytoplasm (Kleber, 1983). After the initial rapid change we would expect mitochondrial sodium to follow changes of $[Na^+]_i$.

From Fig. 8 it is apparent that the twitch decreases as $[Na^+]_i$ rises in metabolic blockade. This decrease of the twitch is due to a fall of $[ATP]_i$ which increases the membrane potassium conductance leading to a shortening and eventual failure of the action potential and consequent reduction and abolition of the twitch (Lederer, Nichols & Smith, 1989). Therefore $[ATP]_i$ has begun to fall at the time $[Na^+]_i$ rises. The magnitude of the fall of $[ATP]_i$ is, however, harder to estimate. Although single channel studies suggested that $[ATP]_i$ had to fall to below 1–2 mM to activate the K^+ conductance (Noma & Shibasaki, 1985), more recent nuclear magnetic resonance studies have shown that the action potential shortens before there is any great fall of $[ATP]_i$ (Elliott, Smith & Allen 1989). The $K_{0.5}$ for ATP of the Na^+–K^+ pump is about 100 μM (Glynn & Karlish, 1976). If we assume that the sodium influx is constant and that pump rate is proportional to $[Na^+]_i$, then for the Na^+–K^+ pump to be responsible for the rise of $[Na^+]_i$ observed when the cell goes into contracture (from 12 to 19 mM) would require the pump to be inhibited to 63% of the control rate. This would require $[ATP]_i$ to fall to 170 μM. Since the increase of inorganic phosphate (P_i) further inhibits the Na^+–K^+ pump (Eisner & Richards, 1982) a smaller reduction of [ATP] will be required to inhibit the Na^+–K^+ pump. This estimate can be compared with the fall of [ATP] required to produce rigor. In permeabilized rat ventricular muscle at pCa 7·0, a reduction of [ATP] to 300 μM produced a rigor equivalent to 10% of maximum force (Fabiato & Fabiato, 1975). It is therefore plausible to suggest that the increase of $[Na^+]_i$ is due to the effects of a decrease of $[ATP]_i$ acting on the Na^+–K^+ pump.

The data show that the gradual increase of $[Na^+]_i$ begins before the cell goes into a contracture. These results are similar to those obtained in the Purkinje fibre (MacLeod, 1989). It is likely that this increase of $[Na^+]_i$ is responsible (via Na^+–Ca^{2+} exchange) for the early increase of $[Ca^{2+}]_i$ which is observed before the contracture develops and along with low ATP-induced rigor may contribute to its onset (Eisner *et al.* 1989). It is also possible that (via Na^+–H^+ exchange) it contributes to the

observed acidosis (Eisner *et al.* 1989). It should, however, be noted that, metabolic inhibition has been reported to elevate $[Na^+]_i$ with no effect on $[Ca^{2+}]_i$ (Guarnieri, 1987). The lack of effect on $[Ca^{2+}]_i$ in this last study was attributed to either a decrease of Ca^{2+} entry or, alternatively, to an inhibition of Na^+–Ca^{2+} exchange. We would only point out that, if Na^+–Ca^{2+} exchange is inhibited this, by itself, should cause an increase of $[Ca^{2+}]_i$.

The present work reports the use of the fluorescent dye SBFI to measure $[Na^+]_i$ in cardiac muscle. The introduction of the dye into cells using the membrane permeant form and the problems of compartmentalization and interpretation of the intracellular records which arise from this technique are reported and discussed. We also report the use of SBFI to measure the intramitochondrial $[Na^+]$ and present data showing that the mitochondria maintain a $[Na^+]$ lower than that in the cytoplasm. Experiments investigating the effect of metabolic inhibition show that there is a small increase in $[Na^+]_i$ which occurs as the twitch disappears before the development of the irreversible contracture. Although we have found certain problems in its use we conclude that SBFI is a useful tool for the measurement of $[Na^+]_i$ in cardiac cells if care is taken in the interpretation of results.

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