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Short communication

An estimate of the calcium content of the sarcoplasmic reticulum in rat ventricular myocytes

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Summary. The aim of this paper was to estimate the Ca content of the sarcoplasmic reticulum (s.r.) and to compare this with the amount of Ca which enters the cell via the calcium current in systole. The s.r. Ca content was measured electrophysiologically in voltage-clamped rat ventricular myocytes. Rapid application of caffeine produced a transient increase of $[Ca^{2+}]_i$ which was accompanied by a transient inward Na-Ca exchange current. The integral of this current gives a measure of the Ca^{2+} pumped out of the cell by Na-Ca exchange. Ni^{2+} (5 mM) inhibited the current and decreased the rate of fall of $[Ca^{2+}]_i$ to 32% of the control suggesting that Na-Ca exchange is responsible for 68% of Ca removal from the cytoplasm following the addition of caffeine. Correcting for the Na-Ca independent Ca removal suggests that the s.r. Ca content is equivalent to about 120 μmol per litre cell. Furthermore we estimate that, during systole, Ca entry into the cell via the sarcolemmal calcium current is equal to about 6% of the Ca content of the s.r.

Introduction

In cardiac muscle, the rise of intracellular Ca concentration ($[Ca^{2+}]_i$) which activates contraction comes from two sources. (i) The extracellular fluid; (ii) the sarcoplasmic reticulum. The former is thought to result largely from Ca entry through the L type Ca channels. The magnitude of the Ca entry via the Ca current (I_{Ca}) can be readily estimated from voltage clamp records. It is, however, more difficult to assess the Ca content of the s.r. Previous measurements have used a variety of techniques including radioactive Ca efflux and electron microprobe analysis (see [2] for review). There are problems associated with these methods. For example with the electron microprobe technique it may be difficult to resolve the smallest parts of the s.r. In the present paper we use an electrophysiological technique based on the fact that caffeine releases calcium from the s.r. and that this calcium is pumped out of the cell on an electrogenic Na-Ca exchange [3]. The charge carried by this exchange current can therefore be used to give an estimate of the calcium content of the s.r. We also compare this estimate of the total amount of Ca in the s.r. with that of the calcium which enters the cell through the sarcolemmal Ca current.

A preliminary version of some of the data in this paper has been presented to the Physiological Society [8].

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Materials and methods

The experiments were performed on rat ventricular myocytes isolated as described previously using a collagenase and protease dissociation [5]. Rats were killed by stunning and cervical dislocation.

Isolated cells were loaded with the acetoxymethyl (AM) ester of indo-1 and fluorescence measured using apparatus described previously [5]. Briefly, cells were placed in a bath on the stage of an inverted microscope. Fluorescence was excited at 340 nm and collected at 400 and 500nm. The ratio of emission at 400 and 500 nm gives a measure of $[Ca^{2+}]_i$. Voltage clamp control was imposed with the whole cell patch clamp technique. In order to ensure that the s.r. was consistently loaded with calcium, caffeine-dependent currents were measured after a 1 min rest at a holding potential of -80 mV.

Solutions. The experimental solution contained (mM): NaCl, 134; KCl, 4; $MgCl_2$, 1; HEPES, 10; glucose, 11; $CaCl_2$, 1; titrated to pH 7.4 with NaOH. 5 mM 4-aminopyridine (to inhibit the transient outward current) and 0.1 mM $BaCl_2$ were added to this solution to block K currents. All experiments were carried out at 27 °C. The patch pipettes were filled with a solution containing (mM): CsCl, 120; tetraethylammonium chloride, 20; NaCl, 12; HEPES, 10; $MgCl_2$, 5; EGTA, 0.1; K_2ATP, 5; titrated to pH 7.2.

Results

In preliminary experiments in which caffeine (10-20 mM) was applied to release Ca^{2+} ions from the s.r., we observed a transient increase of inward current which

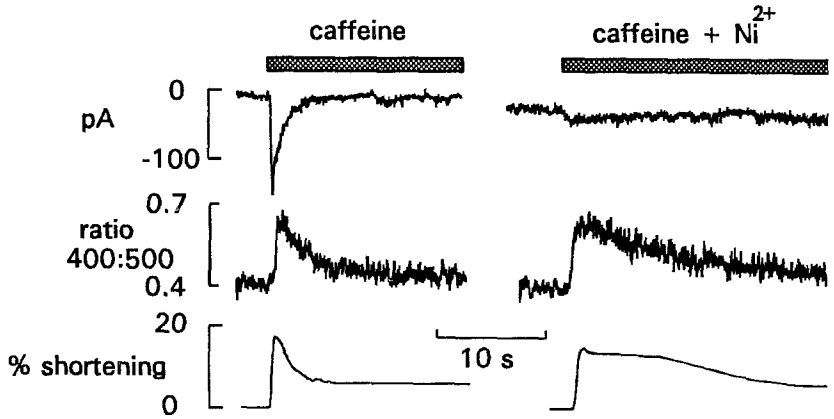

Fig. 1. The effects of caffeine on membrane current. The traces show (from top to bottom): current; indo-1 ratio of emission at 400:500 nm; cell shortening. Caffeine (20 mM) was applied for the period shown. The membrane potential was held at -80 mV throughout. The left hand panel shows a control caffeine application. Caffeine was then removed. The right hand panel shows the effects of adding caffeine after the cell had been exposed to Ni^{2+} (5 mM) for 3 min.

decayed within 1-2 sec at -80 mV. This was, however, often followed by a maintained outward current (not shown). The addition of Ba^{2+} (0.1 mM) to the extracellular solution abolished this maintained component of current. It is likely that the maintained current represents inhibition of the inward rectifier. In all experiments presented in this paper 0.1 mM Ba^{2+} and 5 mM 4 aminopyridine were included in the external solution to avoid this problem. Fig. 1 shows the effects on membrane current, $[Ca^{2+}]_i$ and contraction of adding caffeine (20 mM). As shown previously [3], this produces a transient increase of $[Ca^{2+}]_i$ and contraction which is accompanied by an inward current. This caffeine-evoked current has been ascribed to electrogenic Na-Ca exchange [3]. In agreement with previous work, it is inhibited by Ni^{2+} ions. The inhibition of the current is accompanied by a decrease in the rate of relaxation of $[Ca^{2+}]_i$ and contraction. It should be noted, however, that, although the current is almost completely inhibited, the rate of fall of $[Ca^{2+}]_i$ is decreased by a smaller factor. On average Ni^{2+} decreased the rate constant of fall of $[Ca^{2+}]_i$ to $31.5 \pm 1.4\%$ (n=11) of the control.

The caffeine-evoked current is compared with I_{Ca} in Fig. 2. I_{Ca} is considerably larger and briefer than is the caffeine-induced current. The cumulative integral traces show that the charge transferred by the caffeine-evoked current is about 3 times greater than is that carried by I_{Ca}. On average the charge carried by I_{Ca} was 21.5 $\pm$ 2.3 pC (n=13) and that by the caffeine-evoked current 94 $\pm$ 14 pC. The average ratio of the charge carried by I_{Ca} to that by the caffeine-evoked current was 0.29 $\pm$ 0.05 (n=13).

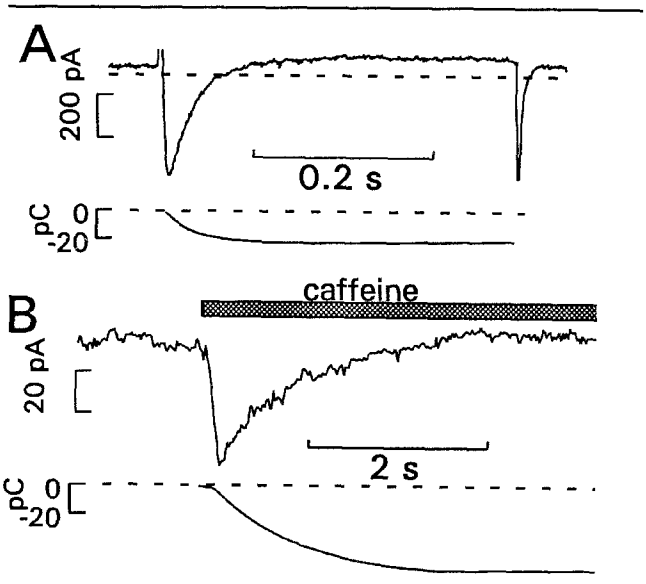

Fig. 2. Comparison of the calcium current (I_{Ca}) with that evoked by caffeine. In both panels traces show: top, current; bottom, cumulative integral of current. **A.** I_{Ca}. The membrane was depolarized from -40 to 0 mV at 0.33 Hz. **B.** Caffeine-evoked current. The membrane potential was held at -80 mV throughout and caffeine (20 mM) applied as shown above.

Discussion

The results presented in this paper provide an electrophysiological estimate of the Ca content of the s.r. This is based on the amount of Ca which can be released by caffeine. There are two possible sources of error: (i) currents other than Na-Ca exchange may be activated by the application of caffeine; (ii) mechanisms other than Na-Ca exchange may remove Ca^{2+} ions from the cell. We will consider these in turn. There are two sorts of confounding current changes which could be produced by caffeine. Caffeine will activate $[Ca^{2+}]_i$-dependent currents producing a transient current change with a similar timecourse to that of $[Ca^{2+}]_i$. In addition caffeine may, independent of any changes of $[Ca^{2+}]_i$, affect membrane currents. Evidence for a $[Ca^{2+}]_i$-independent action was provided by the maintained shift of outward current observed in initial experiments. However, this was abolished by Ba^{2+} and is not a problem. We are therefore left with the question of how much of the transient inward current which is produced by caffeine is due to Na-Ca exchange as opposed to other $[Ca^{2+}]_i$-dependent current mechanisms. Our experiments show that any contaminating current must also be Ni^{2+} sensitive. Furthermore, in other experiments (not shown) we have

found that the integral of the charge transported at 0 mV is about 82% of that at -80 mV. This is consistent with a Na-Ca exchange mechanism pumping out roughly the same amount of Ca^{2+} from the cell in both cases but is inconsistent with activation of a Ca-dependent conductance. Specifically one would expect the current activated by a K conductance to be much greater at 0 than at -80 mV whereas that for a Na or non-specific cation conductance would show a reverse potential dependence.

It is clear from results such as that of Fig. 1 that, although Ni almost completely inhibits the caffeine-evoked current, it only decreases the rate of fall of $[Ca^{2+}]_i$ by 68%. This is in agreement with previous work also on rat ventricular myocytes where it was shown that inhibition of Na-Ca exchange either by complete removal of external Na or with Ni^{2+} only decreased the rate of fall of $[Ca^{2+}]_i$ by about 50% [6]. This contrasts with studies on rabbit cardiac muscle where it was estimated that the Na-Ca exchange has a rate which contributes about 95% of Ca removal [1]. The present result suggests that, in the rat ventricular myocyte, factors other than Na-Ca exchange make a significant contribution to removal of Ca from the cytoplasm. These other processes will include the surface membrane Ca-ATPase and mitochondrial Ca sequestration. It is important to be able to correct for the amount of Ca which is removed by them. The effects of Ni^{2+} suggest that the exchange is responsible for removing 68% of the calcium load. Therefore the total amount of Ca removed from the cytoplasm is 1.47 times that estimated from the calcium carried by the Na-Ca exchange. It should be noted that the rate constants used in this calculation are based on indo-1 ratio measurements rather than on the rate of fall of $[Ca^{2+}]_i$ itself. Although the systolic level of $[Ca^{2+}]_i$ is below the saturating level of the indicator [5], any non linearity in the relationship between indo-1 ratio and $[Ca^{2+}]_i$ will mean that the time constants for the indo-1 signal will be different from those of $[Ca^{2+}]_i$ itself. Nevertheless, since the decay phase of the indo-1 ratio during the caffeine response can be well fitted by a single exponential (not shown) and the control and Ni responses occur over the same range of $[Ca^{2+}]_i$, this means that the time taken for $[Ca^{2+}]_i$ to fall between any two levels will be slowed in proportion to the slowing of the indo-1 ratio response.

We can now estimate the total Ca content of the s.r. From the mean surface capacitance (60 pF) and assuming a surface to volume ratio of 0.5 μm^{-1} [7] and a specific capacitance of 1 $\mu F.cm^{-2}$, we calculate that the charge transported by Na-Ca exchange flux corresponds to a Ca flux of 81 $\mu mol.l^{-1}$. With the correction for non Na-Ca exchange processes removing Ca, this suggests that caffeine application results in the release of 120 $\mu mol.l^{-1}$ Ca from the s.r. Under the conditions used in the present experiments (in particular the low stimulation rate) it is likely that the s.r. is maximally loaded with calcium and this figure may approximate to the maximum Ca content of the s.r. It should be noted that, although our estimate of the s.r. Ca content is lower than some others [2], it is comparable to the amount of Ca estimated to be required for a normal contraction [4].

Correcting the ratio of the charge carried by I_{Ca} to that carried by I_{ex} for the fact that (i) I_{Ca} carries two and I_{ex} one charge per Ca^{2+} and (ii) the Na-Ca independent Ca removal, we calculate that the ratio amount of Ca^{2+} entering on I_{Ca}: amount stored in the s.r. is 0.10. In the present experiments I_{Ca} was elicited by depolarization to 0 mV for a time which is long enough for it to completely inactivate. Under physiological conditions the rat action potential is comparatively brief and it is likely that I_{Ca} will be decreased by deactivation during repolarization. We have estimated this effect by applying a command potential of a similar shape to the rat action potential (not shown). The calcium current flowing during the action potential was estimated as the difference current produced by nifedipine (10 μM) or cadmium (0.25 mM). On average we found that the calcium current calculated in this manner was 0.58 ± 0.06 (n=6) of the current evoked by a step depolarization to 0 mV. This suggests that the calcium current flowing during the action potential will produce an entry of calcium equal to 6% of the calcium content of the s.r. This small contribution of the calcium current is consistent with the large reduction of the systolic Ca transient produced in this species by drugs which interfere with s.r. function.

In conclusion, the technique presented in this paper provide a quantitative, on-line and non-destructive way to monitor s.r. Ca content.

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