DETERMINATION OF IONIC CALCIUM IN FROG SKELETAL MUSCLE FIBERS

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ABSTRACT Ionic calcium concentrations were measured in frog skeletal muscle fibers using Ca-selective microelectrodes. In fibers with resting membrane potentials more negative than −85 mV, the mean pCa value was 6.94 (0.12 μM). In fibers depolarized to −73 mV with 10-mM K the mean pCa was 6.43 (0.37 μM). This increase in the intracellular [Ca^{2+}] could be related to the higher oxygen consumption and heat production (Solandt effect) reported to occur under these conditions. Caffeine, 3 mM, also produced an increase in the free ionic calcium to a pCa of 6.52 (0.31 μM) without changes in the membrane potential. Lower caffeine concentrations, 1 and 2 mM, did not change the fiber pCa. Lower Ca concentrations in the external medium effectively reduced the internal ionic calcium to an estimated pCa of 7.43 (0.03 μM).

INTRODUCTION

The recent availability of calcium-specific neutral ligand resins (Simon et al., 1978) has permitted the development of highly selective Ca^{2+}-sensitive microelectrodes suitable for the measurement of intracellular free calcium concentration in a variety of preparations (Tsien and Rink, 1980; Alvarez-Leefmans, et al., 1981). In the case of muscle fibers these techniques are particularly interesting because of the paramount importance of Ca^{2+} ions in contractile activation. In this paper, we report measurements of the free myoplasmic calcium concentration in quiescent muscle fiber using microelectrodes with tip diameters of <0.4 μm and a Ca resin cocktail kindly donated by Professor W. Simon. Besides obtaining information about the intracellular resting calcium concentration, we have also measured the changes in myoplasmic calcium that occur when muscle fibers are moderately depolarized with potassium, which could be associated with the Solandt effect (Hegnauer et al., 1934; Solandt, 1936), and when they are exposed to doses of caffeine that are sufficient to potentiate contractions but do not induce contractures (Sandow and Brust, 1966).

MATERIAL AND METHODS

Whole satorius muscles dissected from the frog *Rana pipiens* were mounted in a Lucite chamber with the ventral surface exposed for microelectrode penetration. Individual muscle fibers were impaled first with a 10-MΩ microelectrode filled with 3-M KCl to measure the membrane potential (V_m) and again after several seconds with the Ca^{2+}-sensitive microelectrode. This procedure allowed us to estimate the possible damage caused by the insertion of the second microelectrode into the fiber. The Ca^{2+} microelectrode registered a potential (V_{CaE}) given by the sum of the fiber membrane potential and the Ca^{2+}-specific potential V_{Ca}. V_m was subtracted electronically from V_{CaE} via an amplifier (F-D223; W-P Instruments, Inc., New Haven, CT) to obtain E_{Ca}. Both V_m and V_{CaE} or E_{Ca} were registered on a pen recorder (7132A; Hewlett-Packard Co., Palo Alto, CA). All the experiments were carried out at room temperature (20–21°C).

Solutions

The Ringer's solution used for these studies contained the following in millimolar concentration: NaCl 115, KCl 2.5, $CaCl_2$ 1.8, and Tris 5. In the experiments with high K^+, the solution was prepared keeping the [K] × [Cl] product constant (Hodgkin and Horowicz, 1959). The pH was 7.4. The low Ca^{2+} solution was similar to the standard Ringer's solution except that $CaCl_2$ was substituted by 3-mM $MgCl_2$, and 3-mM EGTA were added. In some experiments, caffeine or tetracaine was added to the standard Ringer's solution at concentrations indicated in Table I and in the figure legends. The calibrating solutions of different pCa were similar to those used by Tsien and Rink (1980) and Alvarez-Leefmans et al. (1981).

Ca^{2+}-selective Electrodes

Glass capillaries with filaments (GCI20F-6; Clark Instrument, Inc., Dearborn, MI) were washed with HCl and carefully rinsed with distilled water before being pulled with a vertical puller (M30; Narishige, Tokyo, Japan). The microelectrodes were heated to 250°C and then treated with tri-*n*-butylchlorosilane vapors for 20 min (Tsien and Rink, 1980). The tips of the microelectrodes were filled by application of a small drop of the sensor resin cocktail (10% of the neutral ligand *N,N'*-di[11-ethoxycarbonyl]undecyl-*N,N'*,4,5 -tetramethyl-3,6-dioxaoctane-1,8-dioamide, and 1% sodium tetraphenylborate in [*o*-nitrophenyl]octyl ether kindly sup-

TABLE I
MEMBRANE POTENTIAL AND $[Ca^{2+}]_i$ UNDER DIFFERENT EXPERIMENTAL CONDITIONS

	V_m	pCa	Ca^{2+}
	$-mV$		μM
Normal Ringer's solution	−91 ± 3.5 (n = 60)	6.92 ± 0.04	0.12 ± 0.01
Caffeine (1 mM)	−91 ± 3.2 (n = 5)	6.96 ± 0.06	0.11 ± 0.01
Caffeine (2 mM)	−91 ± 4.0 (n = 5)	6.94 ± 0.09	0.11 ± 0.02
Caffeine (3 mM)	−91 ± 3.5 (n = 6)	6.52 ± 0.13	0.30 ± 0.09
K^+ (10 mM)	−72 ± 2.8 (n = 3)	6.42 ± 0.08	0.38 ± 0.06
Low Ca (EGTA)	−84 ± 2.8 (n = 3)	7.45 ± 0.07	0.04 ± 0.006*

All values represent the mean ± SD.
*The differences between the Ca^{2+} values obtained in 3-mM caffeine, high K^+, and low Ca^{2+} and those obtained in normal Ringer's solution are significant with a $P < 0.001, 0.0001, 0.0001$, respectively.

plied by Professor W. Simon) at the distal end of the electrodes. The drop reached the tip by capillar action. Then the microelectrode was backfilled with a solution of pCa 7. The sensor columns obtained by this procedure were ~6-mm long. Measurement of the microelectrode tip diameters was carried out with a scanning electron microscope (500; Philips Electronic Instruments, Inc., Mahwah, NJ) at 20 kV with a resolution of 100 Å. A population of twenty microelectrode tips pulled on different days with the same puller adjustment showed an outside diameter of 0.382 ± 0.003 μm.

Electrode Calibration

The Ca^{2+}-sensitive microelectrodes were calibrated before and after intracellular measurement using solutions of Ca^{2+} ranging from pCa 3 to ∞ made up with the Ca^{2+} buffer, NTA (Nitrilotriacetic acid), pCa 4–5, HE EDTA (*N*-[2-hydroxyethyl]-ethylenediamine-*N*,*N*',*N*'-triacetic acid) pCa 6, and EGTA (ethyleneglycol-bis[B-aminoethylether] -*N*,*N*' -tetra-acetic acid) pCa 7 (Tsien and Rink, 1980; Alvarez-Leefmans et al., 1981; Bers, 1982). Each was individually tested and calibrated with the Ca^{2+}-buffered solutions. The potential measured in the calibration solutions was plotted against the pCa, and only those microelectrodes that showed a Nernstian behavior (29.3 mV per decade change in $[Ca^{2+}]$) between pCa 3 and 7 were used experimentally. The microelectrodes retained their responsiveness over periods of 24–48 hr. At very low Ca^{2+} concentration, pCa 8–∞, Na^+ interfered slightly with the measured value of V_{CaE}. Thus, for instance at pCa ∞, where the Na^+ interference was maximal, 30-mM Na^+ in the calibration solution induced an increase in the electrode potential of 5 mV; caffeine and tetracaine, at the doses used in the present study, were found to have no interference with the function of the microelectrodes.

RESULTS

Fig. 1 shows simultaneous measurements of membrane potential and and myoplasmic calcium concentration in a

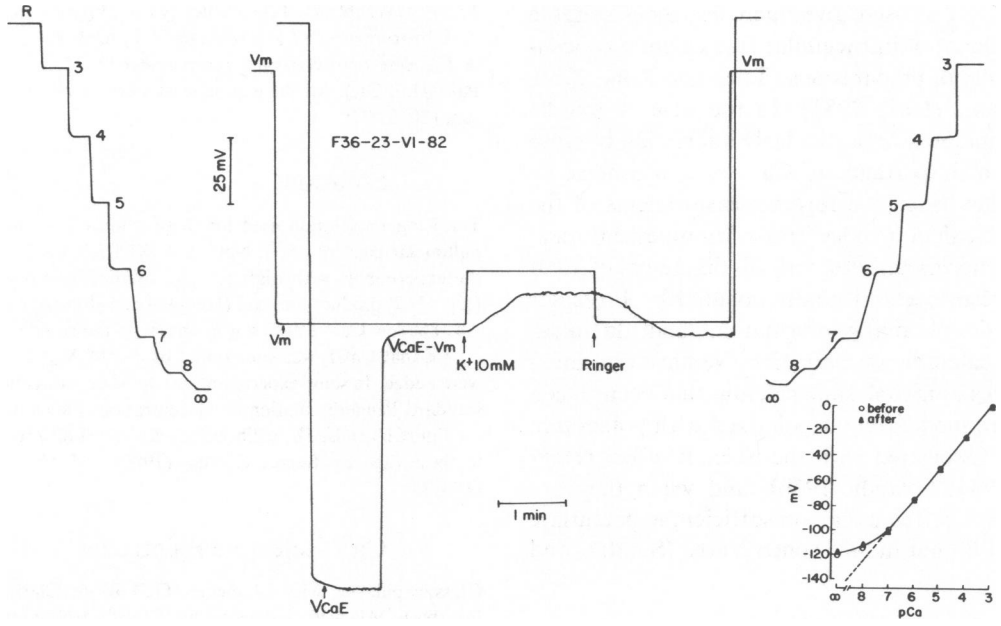

FIGURE 1 Simultaneous measurements of membrane potential (V_m) and cytoplasmic calcium ion activity ($V_{CaE} - V_m$) in a skeletal muscle fiber. On the left-hand side is the preimpalement calibration for the electrode. V_m is the membrane potential recorded with a conventional 10-M microelectrode filled with 3-M KCl. V_{CaE} is the potential recorded with the calcium-selective electrode. After reaching a stable potential, the signal was subtracted electronically ($V_{CaE} - V_m$); V_{Ca} represents the cytoplasmic free Ca^{2+}. The application of 10-mM K^+ induced a moderate depolarization that was followed by an increase in the cytoplasmic Ca^{2+} activity. On the right-hand side is the postimpalement calibration. The inset shows the microelectrode potentials measured at different calcium concentrations before and after impalement. --- represents the ideal Nerstian response to Ca^{2+}.

muscle fiber. The fiber was first impaled with the voltage microelectrode, and after the potential recorded with this microelectrode (V_m) had reached a stable value (−95 mV in this case), the muscle fiber was impaled with the calcium microelectrode (V_{CaE}). The second penetration did not cause a change in the resting membrane potential level. After the second electrode had reached a steady level, the potential from both electrodes was electronically substracted to obtain V_{Ca}. The potential thus obtained represents the cytosolic calcium ion activity, which in this fiber gave a pCa of 6.92 (0.12 μM). In sixty fibers of different muscles with a mean membrane potential value of −91 ± 0.45 mV the average resting pCa was 6.92 (0.12 ± 0.02 μM). Fig. 1 shows that exposure of the muscle fiber to a solution containing 10-mM K^+ caused an immediate depolarization to a new level, −75 mV. This membrane depolarization was followed by a slower change of the Ca^{2+}-selective microelectrode potential that corresponds to a new pCa value of 6.42 (0.38 μM). This increase was reversed after repolarizing the membrane as shown in Fig. 1. At the left and right ends of Fig. 1 the precalibration and postcalibration recordings of the microelectrode are shown; the figure insert shows the corresponding calibration curves.

Caffeine is able to affect muscle contractility without changing the membrane potential (Sandow and Brust, 1966). This drug induces contractures when used at relatively high concentrations (>3 mM) and twitch potentiation at lower concentrations (Sandow and Brust, 1966; Lüttgau and Oetliker, 1968). The first effect is thought to be caused by a caffeine-induced release of Ca^{2+} from the sarcoplasmic reticulum (SR); the latter could be due either to a subthreshold release of Ca^{2+} from the SR or to an increased release of calcium during contractile activation (Caputo et al., 1981). To test these possibilities experiments at different caffeine concentrations were performed. Fig. 2 shows a typical experiment. After reaching steady values for the membrane potential (V_m) of −95mV and measuring a value pCa of 6.93 (0.12 μM), we exposed the muscle fiber to 2-mM caffeine, which caused no change in the pCa level. Subsequent exposure to 3-mM caffeine caused a significant increase in the intracellular free calcium level from 0.12 to 0.31 μM. Interestingly the effect induced by 3-mM caffeine on the intracellular calcium ion activity was blocked by 1-mM tetracaine.

In principle a reduction of the free intracellular Ca^{2+} concentration should be obtained by drastically reducing the external Ca^{2+} using a solution prepared with 3-mM EGTA and no added Ca. Such an effect was indeed observed in several experiments. After the normal Ringer's solution was replaced by a low-Ca solution, the membrane was rapidly depolarized by 7 mV. This was followed by a slow decrease in the cytoplasmic calcium concentration, which reached a steady level of 0.04 ± 0.01 μM. Table I summarizes the results obtained in these experiments in fibers bathed with normal Ringer's solution and those treated with caffeine (1–3 mM), potassium (10 mM), and low Ca^{2+} solutions.

DISCUSSION

Our experiments show that Ca^{2+}-sensitive microelectrodes represent a convenient method for the determination of intracellular calcium in skeletal muscle. Our mean resting level of free Ca^{2+} in the myoplasm was 0.12 μM (pCa

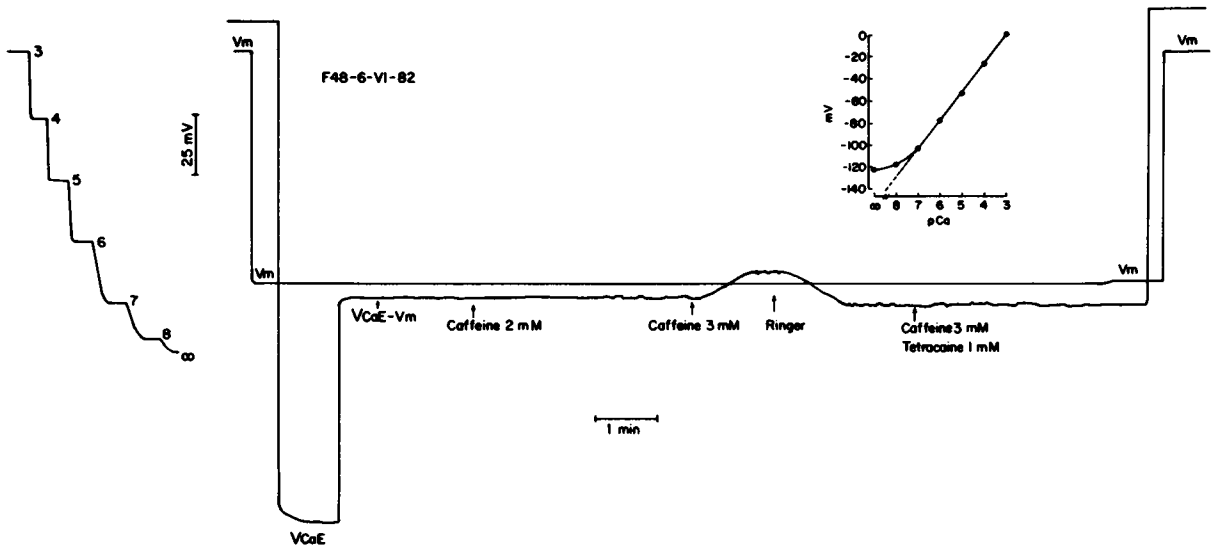

FIGURE 2 A typical response obtained with a conventional microelectrode (V_m) and a Ca^{2+}-selective microelectrode (V_{CaE}) from a skeletal muscle fiber. On the left-hand side is shown the calibration curve for the Ca^{2+} microelectrode before impalement. After the determination of the resting calcium activity, we treated the muscle fiber with 2-mM caffeine, which does not induce a detectable increase on the intracellular free calcium activity. However, the application of 3-mM caffeine produced a marked enhancement of the resting Ca^{2+} activity that is blocked when 1-mM tetracaine is applied simultaneously with 3-mM caffeine.

6.92), a value somewhat higher than 0.055 μM reported by Coray et al. (1980), but slightly lower than that reported by Tsien et al., (1980) (0.15 μM). Both values were determined with the aid of Ca^{2+}-sensitive microelectrodes. Our value is also higher than the 0.08 μM, obtained with the photoprotein aequorin (Blinks et al., 1980). Note that in our experiments great care was taken in collecting data from experiments in which (*a*) the Ca microelectrodes showed a Nernstian behavior in the pCa range between 3 and 7; (*b*) the electrodes tip diameters were <0.4 μm; and (*c*) the fibers had membrane potentials higher than −85 mV. The $[Ca^{2+}]_i$ value reported here is similar to those obtained in a variety of cells (Coray et al., 1980; Alvarez-Leefmans et al., 1981) including the squid giant axon (DiPolo et al., 1976; DiPolo et al., 1983).

The metabolic rate in a skeletal muscle fiber can be increased by exposure to Ringer's solution containing a moderately high concentration of K^+ (Solandt effect). Under such experimental conditions there is a marked increase in muscle oxygen consumption (Hegnauer et al., 1934) and in heat production (Solandt, 1936). The experiments reported in this paper offer a direct demonstration that these effects are probably linked to an increase in the level of free intracellular calcium as previously postulated by other authors (Novotný and Vyskocil, 1966).

An interesting result is the effect of caffeine on the intracellular free $[Ca^{2+}]$. 1 or 2-mM caffeine did not change the basal level of intracellular Ca^{2+}, although this alkaloid at such low concentration acts as an effective potentiator of muscle contraction (Sandow and Brust, 1966). This demonstrates that the potentiating effect of caffeine is exerted at the level of the excitation-contraction coupling mechanism and not mediated by a subthreshold liberation of Ca^{2+}, which would add to the calcium liberated during contractile activation. On the other hand, 3-mM caffeine caused a marked increase in the resting ionic calcium, which indicates that at such a concentration caffeine might induce a dual effect: (*a*) increasing the cytosolic calcium level, and (*b*) enhancing the release of Ca^{2+} during the process of muscle activation. In agreement with the results of other authors, tetracaine was found to be an effective antagonist of caffeine. (Lüttgau and Oetliker, 1968).

Finally treatment of muscle fiber with Ca^{2+}-free EGTA solution caused a reduction in the level of the intracellular free calcium (Table I). This indicates the importance of the transport mechanisms that, at the level of the fiber membrane, maintain a balance of fluxes to keep a low intracellular calcium concentration under normal conditions. The alternate possibility of a leak of EGTA into the fibers, which could cause a reduction of the intracellular free Ca^{2+}, although less likely, should also be considered.

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