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Free calcium in sheep cardiac tissue and frog skeletal muscle measured with Ca^{2+}-selective microelectrodes

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Abstract. Microelectrodes filled with neutral carrier selective to Ca^{2+} were used to measure the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in sheep cardiac tissue and frog skeletal muscle. Calibration of the electrodes was performed in the presence of a solution resembling the cationic composition of the cytoplasm. $[Ca^{2+}]_i$ at rest in normal physiological saline (20–22° C) was 240 nM in Purkinje fibres, 270 nM in ventricular muscle, and 52 nM in skeletal muscle. In Purkinje fibres, elevation of $[Ca^{2+}]_o$ from 1.8 mM to 5.4 mM produced a 1.7-fold increase in $[Ca^{2+}]_i$. Elevation of $[Ca^{2+}]_o$ from 1.8 mM to 18 mM induced a 2.6-fold increase in $[Ca^{2+}]_i$. Exposure to Na^+-free solution (Li^+-substituted) gave rise to elevation of $[Ca^{2+}]_i$ by factors of 5.8 and 14 in ventricular muscle and Purkinje fibres, respectively. These latter changes in $[Ca^{2+}]_i$ were associated with the development of contractures which reached 34% and 172% of the corresponding twitch tension.

Key words: Calcium – Microelectrode – Cardiac Purkinje fibre – Cardiac ventricular muscle – Skeletal muscle

Introduction

A variety of cellular functions are modulated by the level of intracellular free calcium ($[Ca^{2+}]_i$). For example, regulation of muscle contraction, transmitter release, stimulus-secretion coupling, and intercellular coupling (for review, see Scarpa and Carafli 1978) all depend critically upon free $[Ca^{2+}]_i$. To serve in this regulatory capacity, the free $[Ca^{2+}]_i$ must fulfill certain requirements common to all rate-limiting substances. The concentration of free calcium ions must be low so that small changes can significantly influence cell functions. Secondly, there must be mechanism(s) that precisely control the free $[Ca^{2+}]_i$ so that cellular functions can adapt to a changing environment. The importance of calcium to muscle cells is emphasized by the number and redundancy of Ca-transport systems. Calcium is controlled at many levels: sarcolemmal, mitochondrial, and sarcoplasmic reticular. The level of free $[Ca^{2+}]_i$ appears to be exceedingly low and comparable to that of protons and free c-AMP. The accurate determination of free $[Ca^{2+}]_i$ would obviously aid our understanding of cellular control mechanisms but the scarcity of free Ca ions has necessitated sophisticated methods of detection.

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To date, several techniques have been employed to determine free $[Ca^{2+}]_i$ with each having intrinsic advantages and disadvantages. The photoprotein aequorin and other metallochromic and fluorescent indicators have had the widest application (for references, see Blinks et al. 1982; Tsien et al. 1982). These methods have had considerable success in measuring fast Ca^{2+}-transients but have not been as sensitive nor reliable for determining the absolute value of free $[Ca^{2+}]_i$. Recently, Ca^{2+}-selective ligands have been synthesized (Simon et al. 1978). The synthesis of these resins has enabled the construction of ion-selective microelectrodes suitable for the measurement of resting $[Ca^{2+}]_i$ (Tsien and Rink 190 and 1981). This paper presents our initial findings on the properties of these electrodes and their application to striated muscle preparations. Comparative studies have been performed on the resting $[Ca^{2+}]_i$ levels of sheep Purkinje fibres, sheep ventricular muscle, and frog skeletal muscle. In addition, a few experimental interventions demonstrate the applicability of Ca^{2+}-selective microelectrodes to slowly changing $[Ca^{2+}]_i$ levels. A brief account of our preliminary results has been published previously (Coray et al. 1980).

Methods

Preparations and set up

Sheep hearts (*Ovis merina*) were obtained from a local slaughterhouse and transported to the laboratory in chilled Tyrode solution (about 4° C). Free-running preparations of Purkinje fibres and trabecular muscle were dissected from both ventricles. Skeletal muscle fibres (m. semitendinosus) were dissected from frogs (*Rana temporaria*) which had been refrigerated at 4° C. The preparations were stored in Tyrode solution or Ringer solution at room temperature (20–22° C).

Experiments were performed in a Perspex chamber (volume 1.5 ml) superfused with either oxygenated Tyrode solution or Ringer solution (5 ml/min). The composition of the Tyrode solution was (mM): NaCl, 150; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.5; glucose, 5; Tris, 10 (adjusted to pH 7.4 with 1 N HCl). Ringer solution contained (mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; Na_2HPO_4, 2.15; NaH_2PO_4, 0.85 (pH 7.2). All experiments were performed at 20–22° C.

High impedance electrometers (F223A, 10^{15} Ω, and M 701, 10^{11} Ω, WP Instruments, New Haven, CT, USA) were used to monitor the potentials recorded by Ca^{2+}-selective microelectrodes (V_{CaE}) and conventional microelectrodes filled with 3 M KCl (V_m). The electrodes were

mounted in electrode holders containing electrode filling solution and a Ag/AgCl pellet. The bath was connected to ground (Ag/AgCl pellet) via a 3 M KCl-Agar bridge. Zero for both electrodes was defined as the bath potential when filled with Tyrode or Ringer. In some experiments, mechanical force was measured using a piezo-resistive transducer (AE 875, Aksjeselskapet Micro-Elektronikk, Horten, Norway). The preparation was supported by a Perspex pedestal, with one end attached to the transducer arm and the other end fixed to an anchoring hook.

Continuous recordings of membrane potential (V_m), the potential difference between V_{CaE} and V_m (V_{diff}), and in some cases mechanical force (T) were made on a chart recorder (Brush 2400, Gould Inc., Cleveland, OH, USA). In most experiments, V_{diff} and T were filtered with an RC circuit (corner frequency 10 Hz) to reduce high frequency noise. This was found to have no effect on the actual measurements.

Ca electrodes

Intracellular Ca^{2+} was measured using micro-electrodes filled with the neutral carrier selective for Ca^{2+}. Pipettes pulled from Pyrex capillary tubing (GC 120F, Clark Electromedical Instruments, Pangbourne, England) were bevelled on an air-driven rotating disk (FLM 80, A. W. Stähli, Pieterlen, Switzerland) and siliconized with N,N-dimethyl-trimethylsilylamine (TMSDMA; Fluka, Buchs, Switzerland) as previously described in detail (Hess et al. 1982). Initially, the electrodes were siliconized with dimethylchlorosilane (DMCS; Fluka) using the method developed by Coles and Tsacopoulos (1977). Pipettes with a tip diameter of approximately 1 µm were back-filled with calcium sensor resulting in a column a few millimeters in length. The sensor (kindly provided by Dr. D. Ammann) consisted of 10% (w/w) neutral ligand ETH 1001, 1% sodium tetraphenylborate dissolved in (o-nitrophenyl) octylether (Oehme et al. 1976). The shaft of the electrodes was filled with "internal solution" (for the composition, see below) whose *p*Ca was set to 6.4. After filling, the electrodes were conditioned by immersion in Tyrode solution for at least 1 h. After this treatment, some electrodes showed adequate responses and remained stable for several hours. The shank of the electrodes was painted to within 0.5 mm of the tip with silver-conductive paint (Dag 1415, Acheson Colloiden, Scheemda, Holland) such that it made contact with the bath solution. This minimized capacitative artifacts caused by small fluctuations of the fluid level in the experimental chamber. As a routine, new electrodes were prepared every day.

Subsequent to fabrication, the electrodes were screened for their response to changes in $[Ca^{2+}]$. The best were selected for experimentation. After each successful measurement, electrodes were re-calibrated carefully. Both manoeuvres were performed in a calibration chamber illustrated in Fig. 1. "Calibrating solutions" were transferred by pipette into a conical chamber in the Perspex block. In this manner, the old solution was forced out of the chamber into the waste container via a narrow U-shaped outlet. A 5 ml aliquot was found to be sufficient to replace the fluid sample. The fluid level, resulting from the balance between hydrostatic pressure in the chamber and surface tension in the outlet path, could be adjusted by tilting the Perspex block. The calibration chamber was grounded via a Ag/AgCl pellet. The potential difference between the Ca^{2+} electrode and a broken 3 M KCl electrode (tip resistance 2–5 MΩ) was recorded as a signal which was proportional to free $[Ca^{2+}]$.

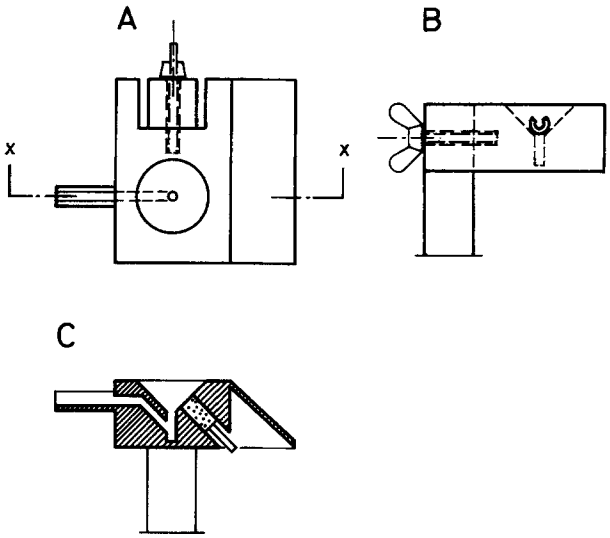

Fig. 1A–C. Calibration chamber. Top view (**A**), side view facing the outlet (**B**), and cross section (**C**) through the plane *x*-----*x* indicated in (**A**). A Perspex platform for the calibration fluid with a conically drilled cavity and a syphon-type outflow was connected to a vertical support with a magnetic stand. The platform was fixed to the support by means of an air screw. The fluid level in the chamber was set by the balance between hydrostatic pressure in the cavity and surface tension in the outlet

A constant interference method was used to calibrate the electrodes. This means that a series of calibrating solutions were employed which varied in $CaCl_2$ concentration but contained a constant ionic background chosen to mimic the cytoplasmic cation composition. The "internal solution" was based on previously reported values of intracellular ion concentrations (mM): NaCl, 7 (Lee and Fozzard 1975); KCl, 120 (Lee and Fozzard 1975); Tris or HEPES, 24 (adjusted to pH 7.2; Ellis and Thomas 1976); $MgCl_2$, 1; Ca^{2+} buffer substances, 4 (EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEDTA, N-(2-hydroxyethyl)-ethylene diamine-N,N′,N′-triacetic acid; NTA, nitrilotriacetic acid).

In order to know accurately the free Ca^{2+} in the calibrating solutions, the apparent Ca^{2+} stability constants (log K'_{Ca}) of the Ca^{2+} buffer substances were required. A pH-titrimetric method (Miller and Moisescu 1976) was adopted to determine experimentally log K'_{Ca} for EGTA under the appropriate conditions (4 mM EGTA; "internal solution" of ionic strength $\mu = 0.150$; pH 7.2; 20–22° C). The procedure involved determination of log K'_{Ca} at pH 5.2 and correction to pH 7.2. The value obtained for log K'_{Ca} was 6.796 ± 0.001 (mean ± SEM; $n = 10$). For comparison with tabulated values, Schwarzenbach's α-coefficient method (see Perrin and Dempsey 1974) was applied to calculate the absolute stability constant (log K_{Ca}) from log K'_{Ca} (concerning the use of "mixed" constants, see Martell and Smith 1974, pp xi–xii; Tsien and Rink 1980). The resulting log K_{Ca} was 10.96, and compared well with Schwarzenbach's constant of 10.97 (see Martell and Smith 1974). The values of log K'_{Ca} for HEDTA and NTA were computed from the tabulated absolute stability constants by using the reverse procedure

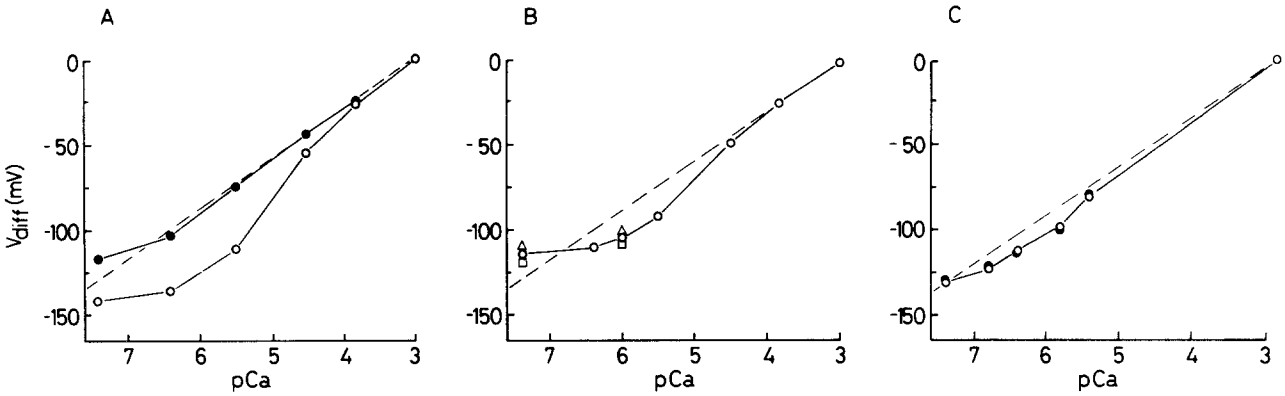

Fig. 2A–C. Calibration curves of different Ca^{2+}-selective microelectrodes. The potential difference between a Ca^{2+} electrode and a 3 M KCl reference electrode, V_{diff}, is plotted versus pCa of the calibrating fluids (see Methods section for composition). V_{diff} in the presence of 1 mM $CaCl_2$ was defined as zero mV. The dotted line represents the Nernstian slope for an ideal Ca^{2+} response (29 mV/pCa unit). **A** Influence of tip diameter. The electrode was calibrated before (○, tip diameter <1 μm) and after breaking the tip (●, tip diameter approximately 10 μm). **B** Influence of Na^+. Substantial effects were observed over the physiological range of pCa when the NaCl concentration was altered from 0 mM (□) to 7 mM (○) and 20 mM (△). **C** Influence of Mg^{2+}. No significant effects were seen when the $MgCl_2$ concentration was varied from 0 mM (○) to 5 mM (●)

(HEDTA: log $K_{Ca} = 8.3$, log $K'_{Ca} = 5.488$; NTA: log $K_{Ca} = 6.41$, log $K'_{Ca} = 3.778$; for log K_{Ca} values, see Martell and Smith 1974). $MgCl_2$ was omitted from the calibrating solutions containing HEDTA and NTA, because these compounds have a rather low selectivity for Ca^{2+} over Mg^{2+}.

Results

Properties of the Ca^{2+} electrodes

Considerable variation was observed in the performance of the Ca^{2+} electrodes. Figure 2 illustrates the calibration functions of three different electrodes having a suitable tip for intracellular measurements (tip diameter: 1 μm or slightly less). Several features were consistent findings: (1) Between pCa 3 and 4 the electrodes showed Nernstian responses (slope of the dotted line: 29 mV/pCa unit), (2) between pCa 4 and about 6, there was a consistent super-Nernstian slope. Figure 2A indicates that this behaviour was dependent upon the tip diameter of the electrode. The super-Nernstian response almost completely disappeared when the bevelled tip of approximately 1 μm diameter (unfilled circles) was broken to roughly 10 μm (filled circles). Above pCa 6 the response of the electrodes became progressively smaller. For our intracellular measurements we selected electrodes which yielded at least a 5 to 12 mV response between pCa 6 and 7.

The progressive flattening of the calibration curve with increasing pCa suggests interference from other cations. This effect is not surprising because the Ca^{2+} sensor is not perfectly selective for Ca^{2+} (for estimates of the selectivity coefficients, see Simon et al. 1978). The contribution to the interference from some of the background ions was assessed by individually varying the ionic species of the "internal solution". The most serious interference arose from Na^+. Fig. 2B shows that changing Na^+ from 7 mM (circles) to 0 mM (squares) increased the electrode potential by 4 mV at pCa 6 and by 5 mV at pCa 7.4. In contrast, increasing Na^+ from 7 mM (circles) to 20 mM (triangles) decreased the potential by 3.5 mV at pCa 6 and by 4.5 mV at pCa 7.4.

Figure 2C shows a similar test investigating the interference from Mg^{2+}. No changes in electrode response were detectable when Mg^{2+} was altered from 0 mM (unfilled circles) to 5 mM (filled circles). The interference from H^+ also was found to be negligible (curve not shown) over the range from pH 6.7 to 7.7.

Resting intracellular Ca^{2+} concentration

Figure 3 illustrates an experiment in which intracellular free [Ca^{2+}] was determined in a sheep Purkinje fibre. The original recordings are shown in Panel A. The upper trace represents the signal from the 3 M KCl electrode, V_m, and the lower trace the corresponding signal from the Ca^{2+} electrode, V_{CaE}. First the preparation was impaled with the reference electrode yielding a stable V_m of −66.5 mV. Then the Ca^{2+} electrode was impaled nearby (distance ~200 μm). A large V_{CaE} drop was observed which stabilized ~3 min later. The time required for the signal to reach a steady-state value of −216 mV presumably included both the chemical response time of the Ca^{2+} electrode and the "sealing process" of the intracellular electrode. There was no sign of any voltage perturbation on the V_m signal during insertion of the Ca^{2+} electrode suggesting that no serious damage was introduced by its entry. During the time period marked by the arrows (↓——↓), the potential difference between the two signals was recorded ($V_{CaE} - V_m$) which in turn was related to the free intracellular [Ca^{2+}]. The Ca^{2+} electrode was withdrawn subsequent to resumption of single ended recording. This manoeuvre again did not produce any detectable disturbances of V_m. The V_{CaE} trace soon reached its control level (± 1 mV) indicating the absence of a serious baseline shift. Withdrawal of the 3 M KCl electrode also demonstrated no significant zero shift. Figure 3B shows a plot of the calibration function of the Ca^{2+} electrode obtained immediately after the biological measurement. A pCa_i of 6.7 was determined from the calibration curve together with the measured V_{diff} of −149.5 mV.

A large number of similar experiments were performed on isolated Purkinje fibres. The collected results were subjected to rigorous selection criteria: (1) The reference

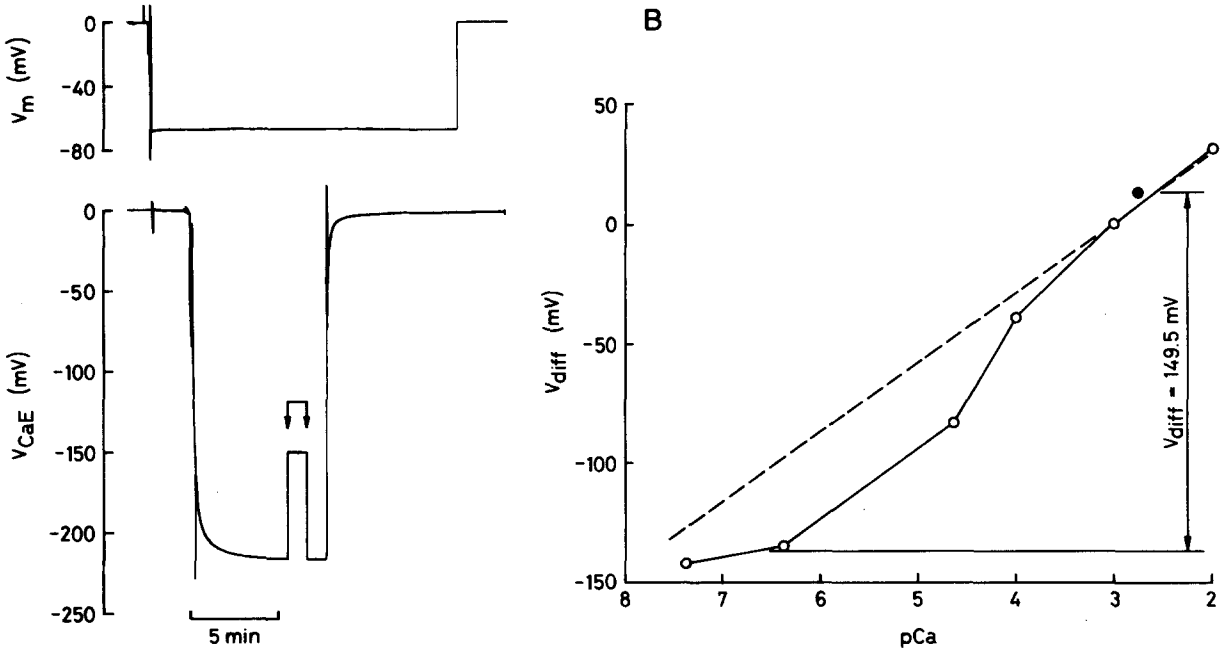

Fig. 3A, B. Measurement of the free intracellular Ca^{2+} in a sheep Purkinje fibre at rest. (**A** *top*) Membrane potential (V_m) recorded with a conventional microelectrode. (**A** *bottom*) Electromotive force (V_{CaE}) recorded with a Ca^{2+}-selective microelectrode. The difference signal ($\overleftrightarrow{\downarrow}$) between electrode recordings (V_{diff}) was −149.5 mV ($V_{CaE} = -216$ mV, $V_m = -66.5$ mV). **B** Calibration curve of the Ca^{2+} electrode obtained immediately after the intracellular measurement using "internal solutions" of variable *p*Ca (○). The potential difference in 1 mM $CaCl_2$ containing calibration solution was defined arbitrarily as zero mV. (●): V_{diff} in Tyrode solution. *Dotted line:* Nernstian slope of 29 mV/*p*Ca unit. The V_{diff} of −149.5 mV corresponds to a *p*Ca of 6.70 (free intracellular $[Ca^{2+}] = 200$ nM) P 15-1a

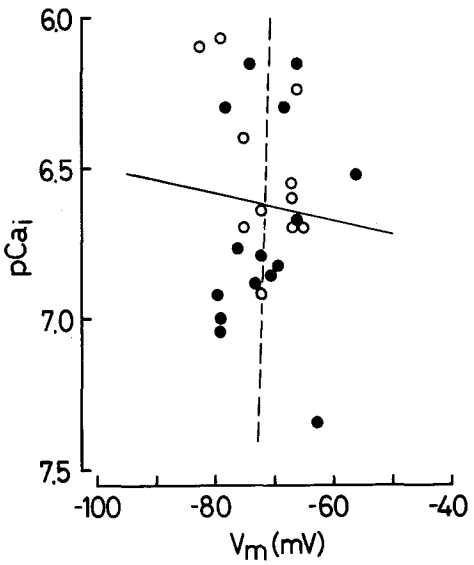

Fig. 4. Correlation between −log intracellular free $[Ca^{2+}]$ (pCa_i) and membrane potential (V_m). Each symbol represents an individual determination. *Filled circles:* Acceptable results from those experiments in which selection criteria (1), (2), and (3) were fulfilled (for further explanations, see p. 3). *Unfilled circles:* Data accepted from those experiments in which only criteria (1) and (2) were employed and fulfilled. *Solid line:* Linear regression pCa_i versus V_m. *Broken line:* Linear regression V_m versus pCa_i. The regression coefficient is not significantly different from zero ($r = 0.085$; $2\alpha > 0.01$)

potentials before and after impalement had to be identical to within 1 mV. This contraint was aimed at minimizing errors due to electrode drift. (2) The voltage perturbation on the V_m signal caused by the impalement with the Ca^{2+} electrode had to be transient or less than 1 mV. It is conceivable that the larger bevelled tip of the Ca^{2+} electrode might damage the impaled cell and thus local membrane depolarization was used as an indicator of such events. (3) The change in the V_{diff} signal had to be $\leqq 1.5$ mV upon depolarization of the preparation by Tyrode solution containing 15 mM KCl. This common-made rejection test allowed a determination of whether the transmembrane potential recorded by both electrodes was the same. Ideally, the V_{diff} signal should remain constant provided there are no direct effects on $[Ca^{2+}]_i$ associated with these potential changes.

Figure 4 shows a summary of the experimental results displaying the relationship between pCa_i and V_m. Each symbol represents a single determination satisfying the following criteria: for the filled circles, criteria (1), (2) and (3) were employed; for the unfilled circles, only criteria (1) and (2) were used. The reason only two criteria could be applied in the second group was because the common-made rejection test was not performed in all experiments. Since there was no significant difference between the two groups of results with regard to both pCa_i and V_m (Student's *t*-test: $P > 0.05$ and > 0.45, respectively), the data were pooled. The overall mean for pCa_i was 6.62 ± 0.06 (mean ± SEM, $n = 26$). This corresponds to an average free $[Ca^{2+}]_i$ of 240 nM. Individual pCa_i values ranged from 7.35 to 6.10. The mean membrane potential V_m was -71.4 ± 1.2 mV. There was no statistically significant correlation between pCa_i and V_m ($2\alpha > 0.1$; correlation coefficient $r = 0.085$). This suggests that any possible V_m dependency of pCa_i was below the resolution of the present method.

For comparison the resting free intracellular $[Ca^{2+}]$ also has been measured in two other types of muscular tissues, sheep ventricular muscle and frog skeletal muscle. In both instances, the properties of the Ca^{2+} electrodes and the selection criteria were identical to those described above.

For ventricular trabeculae the average pCa_i was 6.57 ± 0.17 (mean $\pm$ SEM; $n = 5$). This corresponded to a mean $[Ca^{2+}]_i$ of 270 nM. Individual results varied from pCa_i 6.96 to 6.05. The mean membrane potential was -63.0 ± 0.9 mV. In contrast, an average pCa_i of 7.29 ± 0.10 ($n = 8$) was determined in frog skeletal muscle. This was equivalent to a mean $[Ca^{2+}]_i$ of 52 nM. Individual pCa_i measurements varied from 7.70 to 6.85 whereas the mean membrane potential was -76.8 ± 1.5 mV.

Effect of $[Ca^{2+}]_o$

The question now arises as to how well the cytosolic free Ca^{2+} is maintained against extracellular perturbations. To investigate this point, Purkinje fibres were exposed to Tyrode solutions of varying $CaCl_2$ concentration. Figure 5B shows original pen recorder tracings of an experiment in which the $[Ca^{2+}]_o$ was elevated tenfold. An increase of $[Ca^{2+}]_o$ from 1.8 to 18 mM evoked small reversible increase of the free intracellular Ca^{2+} level from 80 to 180 nM (lower trace). The new steady state level of $[Ca^{2+}]_i$ was reached towards the end of a 7 min exposure period. This intervention also gave rise to a transient hyperpolarization of V_m by 5 mV (upper trace). Previous to altering the $[Ca^{2+}]_o$, the same preparation was subjected to a common-mode rejection test (illustrated in Fig. 5A). The extracellular K^+ was increased from 5.4 to 15 mM during a period of 4 min. This resulted in a reversible depolarization of V_m from -68 mV to -51.5 mV (upper trace). The absence of a measurable deflection on the V_{diff} signal (lower trace) agrees with the selection criteria defined on p. 3.

From this type of study the following mean values were obtained. A tenfold increas in $[Ca^{2+}]_o$ from 1.8 to 18 mM resulted in a 2.6-fold elevation of $[Ca^{2+}]_i$ ($n = 6$); a threefold increase in $[Ca^{2+}]_o$ from 1.8 to 5.4 yielded a 1.7-fold elevation of $[Ca^{2+}]_i$ ($n = 2$). In 5.4 mM $[Ca^{2+}]_o$, the average V_m was -71.5 mV; in 18 mM $[Ca^{2+}]_o$, V_m was -74.6 mV.

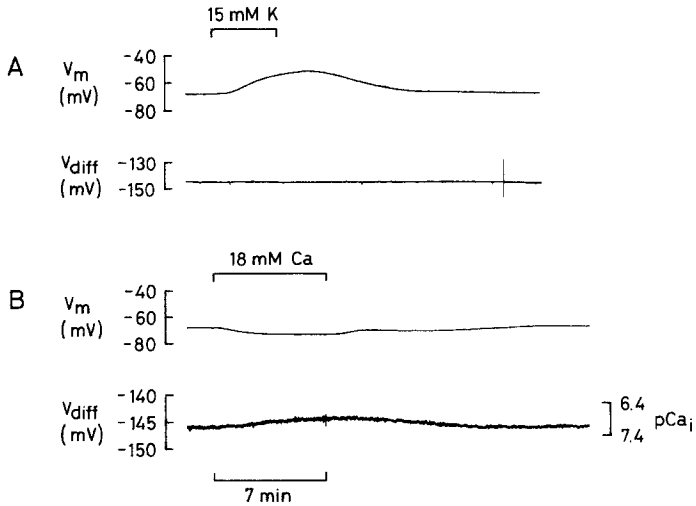

Fig. 5A,B. Effect of increasing $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ in sheep Purkinje fibre. **A** In the common-mode rejection test (elevation of $[K^+]_o$ from 5.4 mM to 15 mM), V_m depolarized from -68 mV to -51.5 mV (*upper trace*), but no detectable change of V_{diff} (*lower trace*) occurred. **B** Subsequent elevation of $[Ca^{2+}]_o$ from 1.8 mM to 18 mM produced a small hyperpolarization of V_m (*upper trace*) and a distinct and reversible increase in $[Ca^{2+}]_i$ from 80 to 180 nM (pCa_i 7.10 and 6.75, respectively). Note the different gain for V_{diff} in (**A**) and (**B**). P 15-1e

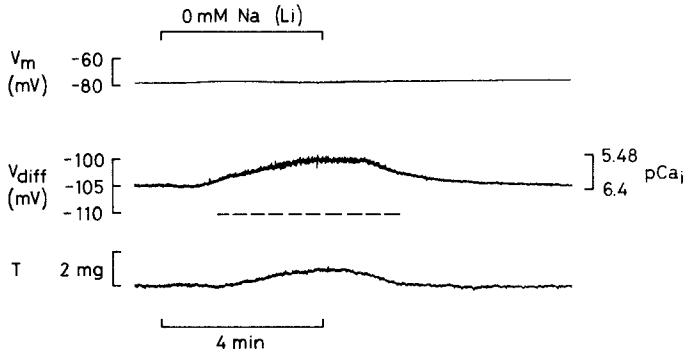

Fig. 6. Effects of 0 $[Na^+]_o$ on V_m, pCa_i and the resting tension (T) in a sheep ventricular trabecula. A mole for mole substitution of Na^+ for Li^+ led to a transient decrease in pCa_i from 6.30 to 5.60 (*middle trace*), corresponding to an increase in $[Ca^{2+}]_i$ from 500 to 2,500 nM. Associated with this Ca^{2+} was an increase in resting tension (*lower trace*). The broken line shows the level of the twitch tension in normal Tyrode solution measured previous to the Na^+-free intervention. P 32-5

Relationship between $[Ca^{2+}]_i$ and resting tension

In cardiac tissue, like other contractile structures, elevation of the free $[Ca^{2+}]_i$ beyond a critical threshold value leads to activation of the contractile proteins. A simple method for increasing $[Ca^{2+}]_i$ experimentally in such preparations consists of impairing the Na^+/Ca^{2+} exchange mechanism by lowering the $[Na^+]_o$ (see Mullins 1981). We used this approach to investigate the relationship between pCa_i and tension. Figure 6 illustrates an experiment in which a sheep ventricular trabecula was used to simultaneously measure tension and pCa_i. The muscle was exposed to Li^+-substituted Na^+-free Tyrode solution for a period of 4 min. As indicated in the middle panel, a slow increase in $[Ca^{2+}]_i$ developed which was fully reversible when normal Tyrode solution was re-admitted. The observed change in pCa_i from 6.30 to 5.60 corresponded to an alteration of free $[Ca^{2+}]_i$ from 500 to 2,500 nM. The lowest panel shows that the Ca^{2+} transient was associated with a mechanical response. A reversible contracture developed with a delay of about 40 s. When compared with the twitch previously measured in normal Tyrode solution at 0.3 Hz (indicated by the broken line in Fig. 6, lowest panel), the peak contracture tension reached 38% of the developed tension.

During exposure to Na^+-free solution small voltage fluctuations developed. They were clearly visible on the V_{diff} trace at high gain (Fig. 6, middle panel), barely dicernable on the V_m trace at lower gain (Fig. 6, upper panel), and absent on the V_{CaE} trace (not shown). Hence, the potential changes in the V_{diff} trace simply reflect fluctuations in V_m per se and were not measurable changes in free $[Ca^{2+}]_i$. Such fluctuations of V_m are thought to be caused by Ca^{2+}-induced release of Ca^{2+} from the sarcoplasmic reticulum under conditions of intracellular Ca^{2+} overload (Kass et al. 1978; Kass and Tsien 1982).

From this experiment and another of the same type, the following mean values were obtained: Control $pCa_i = 6.3$ ($[Ca^{2+}]_i = 500$ nM); Na^+-free solution, $pCa_i = 5.54$ ($[Ca^{2+}]_i = 2{,}900$ nM). Thus, the mean increase in $[Ca^{2+}]_i$ was 5.8-fold during $[Na^+]_o$ withdrawal. This elevation in $[Ca^{2+}]_i$ was accompanied by an average change of 34% for

the ratio $T_{Na\text{-}free}/T_{twitch}$ (contracture tension in Na^+-free solution versus twitch tension in normal Tyrode solution).

Similar experiments also have been performed on sheep Purkinje fibres. If anything, the observed changes in pCa_i and mechanical force developed more quickly in these preparations. The following mean values were obtained in four successful experiments: Control $pCa_i = 6.72$ ($[Ca^{2+}]_i = 190$ nM); Na^+-free solution, $pCa_i = 5.95$ ($[Ca^{2+}]_i = 2{,}600$ nM). The mean increase in $[Ca^{2+}]_i$ was 14-fold. The concurrent mechanical force measurements revealed a mean change of 172% for the ratio $T_{Na\text{-}free}/T_{twitch}$.

Discussion

Ca^{2+} electrodes

The individual performance of our Ca^{2+}-electrodes varied considerably even when electrodes fabricated the same day were compared. This prompted us to calibrate each electrode individually and to introduce strict criteria for their utilization. The calibration procedure attempted to address the problems of interference from other intracellular cations and uncertainties in the Ca-buffer association constant. The Ca^{2+}-electrodes were calibrated under conditions resembling those encountered during the intracellular Ca^{2+} determinations (i.e. with respect to temperature and ionic background). In this way, numerical corrections were unnecessary. In addition, we tried to determine experimentally the apparent stability constant of EGTA in a medium resembling the intracellular fluid. The underlying problems will be discussed within this framework.

Temperature. Preliminary tests revealed that the electrodes respond to temperature changes in an inconsistent manner. Therefore, both the calibrations and the intracellular measurements were performed at the same temperature. For convenience, we chose room temperature (20–22°C). Among the investigators who carried out Ca^{2+} determinations in muscle preparations, several have used a different temperature for their calibration and actual measurements (Lee et al. 1980; Marban et al. 1980; Bers and Ellis 1982; Lee and Dagostino 1982). However, Marban et al. (1980) and Lee and Dagostino (1982) corrected their results for the temperature difference.

Background ions. Based on determinations of selectivity coefficients for the various Ca^{2+} resins (Amman et al. 1979), Ca^{2+} electrodes cannot be expected to respond exclusively to Ca^{2+}. Thus, possible interference from K^+ and Na^+ (mainly) must be resolved when performing intracellular measurements. The influence of these ions rises with decreasing $[Ca^{2+}]$ and can be substantial under intracellular conditions (see Fig. 2B). This suggests that considerable errors might occur when calibration and measurements are performed in different ionic backgrounds. This problem may be overcome by using a constant interference calibration method. This involves use of an "internal solution" whose cationic composition resembles the cytoplasm. Of course, the reliability of this approach depends on the assumptions made for the concentrations of the individual ions. We based our "internal solution" on data available from other ion-selective microelectrode measurements (see Methods section). A similar approach was taken by Coray and McGuigan (1981), Sheu and Fozzard (1982) and Lee and Dagostino (1982). Originally, Lee's group (Lee et al. 1980) ignored NaCl effects. All other investigators used only KCl as a background (Marban et al. 1980; Tsien and Rink 1980; Bers and Ellis 1982; Lopez et al. 1983). Among these groups, Marban et al. (1980) introduced a numerical correction for the absence of NaCl.

Ca^{2+} buffers. One consequence of using the constant interference method is that the appropriate stability constants for the Ca^{2+} buffers are not readily available. The tabulated stability constants originally were determined in a single salt medium (KCl, ionic strength 0.1 M) and thus may not be applicable to our mixed salt medium (ionic strength 0.15 M). Therefore, we tried to determine the apparent stability constant of EGTA in the presence of "internal solution" under our experimental conditions. EGTA was chosen because it buffers the putative physiological range of pCa. Our measurements revealed a log K'_{Ca} of 6.796. From this an absolute stability constant (log K_{Ca}) was calculated by means of the α-coefficient method. The value obtained was close to the one reported by Schwarzenbach (10.96 versus 10.97). Therefore, we felt confident in computing log K'_{Ca} for the other Ca^{2+} ligands used in our solutions (HEDTA and NTA) from the tabulated log K_{Ca} values.

As pointed out by Tsien and Rink (1980; see also Blinks et al. 1982), there has been a great deal of controversy in the biological literature concerning the application of tabulated stability constants for Ca^{2+} chelators. A major misunderstanding apparently arose from the existence of two kinds of proton association constants, namely stoichiometric and mixed constants (see also Martell and Smith 1974; pp xi–xii).

Ca^{2+} chelators effectively buffer the free Ca^{2+} over a pCa_i range equivalent to $pK_d \pm 1$ (equilibrium dissociation constant). Beyond this range the buffering is poor and the measurements uncertain. This point has been neglected by several investigators in that EGTA was used as sole chelator to buffer from pCa_i 8 to 5 (Lee et al. 1980; Bers and Ellis 1982; Lee and Dagostino 1982; Sheu and Fozzard 1982).

Calibration functions. Using the above calibration procedure, we have observed a super-Nernstian response for Ca^{2+} microelectrodes between pCa 4 and 6 (see Figs. 2 and 3B). Similar observations also have been reported by Tsien and Rink (1980). Interestingly, this has not been a reported finding among the other groups who have used exactly the same Ca^{2+} cocktail. The reasons for this discrepancy remain unclear. Dagostino and Lee (1982) claim to have seen this behaviour only shortly after fabrication of the electrodes but not a few hours later. In contrast, our observations on the electrodes suggest that the *entire* calibration curve flattens with increasing time, and the non-linear behaviour persists. Tsien and Rink (1980) originally proposed that the exchangeable inorganic cation in the cocktail (Na^+) might be responsible for this effect. However, the observation that the super-Nernstian response disappears with larger tip sizes (see Fig. 2A) would not support this hypothesis. Tsien and Rink (1981) have subsequently suggested that the reduction of shunt resistance through the wall of the glass might be responsible for the super-Nernstian behaviour. They report that embedding the Ca^{2+} sensor in a PVC matrix eliminates the extra slope. Moreover, this modification seemed to improve the stability of the electrodes and increased the response to low Ca^{2+} levels (see also Lanter et al. 1981).

Table 1. Resting $[Ca^{2+}]_i$ in muscular tissues measured with Ca^{2+}-selective microelectrodes

Tissue	Species	$[Ca^{2+}]_i$ (nM)	Temp. (°C)	$[Ca^{2+}]_o$ (mM)	$[K^+]_o$ (mM)	References
Purkinje fibre	sheep	75–590	34	2.0	6.0	Bers and Ellis (1982)
	sheep	290	34	1.8	5.4	Lado et al. (1982)
	dog	350	36	1.8	5.4	Lee and Dagostino (1982)
	sheep	288	34	1.8	4.0	Sheu and Fozzard (1982)
	sheep	240	22	1.8	5.4	this paper
Ventricular muscle	rabbit	119	35	1.8	4.0	Lee et al. (1980)
	ferret	260	36	1.8	4.0	Marban et al. (1980)
	guinea pig	270	22	1.8	4.0	Coray and McGuigan (1981)
	sheep	272	34	1.8	4.0	Sheu and Fozzard (1982)
	sheep	270	22	1.8	5.4	this paper
Skeletal muscle	frog	160	20	1.5	2.5	Tsien and Rink (1980)
	frog	120	21	1.8	2.5	López et al. (1983)
	frog	52	22	1.8	2.5	this paper

Some authors expressed their intracellular Ca^{2+} values as activities. For convenience, their data has been converted into concentrations using an activity coefficient of 0.32. No corrections were applied for the use of different apparent stability constants for the Ca^{2+} buffers

The slopes of our calibration curves were rather flat over the physiological range of $[Ca^{2+}]_i$ but our calibration solutions also contained Na^+ (see Figs. 2 and 3B). Steeper slopes by other investigators may be linked to the absence of Na^+ in the calibration solutions (see above).

Basal $[Ca^{2+}]$

A major goal of this study was to perform comparative measurements of intracellular $[Ca^{2+}]$ in a variety of muscular preparations. The same tool (Ca^{2+}-selective microelectrodes) was used to investigate the resting Ca^{2+} level in tissues which differ widely with respect to cell size and topology (single cells versus multicellular tissues). This approach may generate more coherence in the data and allow more meaningful interpretations of possible differences. Table 1 lists our mean values and those obtained by other investigators who used similar Ca^{2+} electrodes.

The table allows several comparisons. Starting with our own data, statistical analysis showed no significant difference in $[Ca^{2+}]_i$ between sheep Purkinje fibres and ventricular muscle. If anything, membrane damage due to imperfect impalement and subsequent overestimates of $[Ca^{2+}]_i$ might be more pronounced in ventricular muscle. This is because ventricular muscle has a larger input resistance than Purkinje fibres (see e.g. Tanaka and Sasaki 1966; Pressler et al. 1982). The lack of a significant difference in $[Ca^{2+}]_i$ suggests that our selection criteria for acceptance of data (see p. 3) may have minimized the presence of artifactually high results.

In addition, our measurements suggest that the skeletal muscle from frog has a significantly lower $[Ca^{2+}]_i$ than either cardiac preparation. The question arises if this difference is genuine or not. In this comparison, the direction of the difference is such that it could be explained in terms of localized membrane damage caused in small cells versus large cells. Even though we cannot exclude completely the contribution of this possibility, we believe that if this were a serious problem, there should also have been a difference in $[Ca^{2+}]_i$ between ventricular muscle and Purkinje fibres. Another factor could be an erroneous "internal solution". Indeed, more recent studies indicate that $[Na^+]_i$ might be larger than originally assumed for the construction of our "internal solution", i.e. 7 mM. For example, $[Na^+]_i$ in guinea pig ventricular muscle was reported to be 11 mM (Cohen et al. 1982), and in frog skeletal muscle as much as 16 mM (Schümperli et al. 1982). According to Fig. 2B, such an underestimate in $[Na^+]_i$ would be equivalent to a slight overestimate in $[Ca^{2+}]_i$ in skeletal muscle because the $[Na^+]_i$ in skeletal muscle seems to be larger than in cardiac tissue. The changes incurred by an error for Na^+ in the calibration solution are in the wrong direction to explain the difference in free $[Ca^{2+}]_i$.

It is also conceivable that cardiac tissue has a larger $[Ca^{2+}]_i$ per se. This would seem plausible considering two possible differences between the physiology of the two tissues. One possibility is that the Ca^{2+}-sensitivity of the contractile proteins is different in the two types of muscle. However, this conflicts with data from comparative studies using frog skeletal muscle and rat ventricular muscle. No quantitative differences were observed in the relationship between tonic tension and *p*Ca (Kerrick and Donaldson 1975; Fabiato and Fabiato 1978). Nevertheless, more recent data have re-opened the question since species and developmental differences in this relationship have been noted in cardiac tissue (see Fabiato 1982). Another potential reason for a larger $[Ca^{2+}]_i$ is that cardiac preparations may not operate at the bottom of the tension/*p*Ca curve under physiological conditions. There are a number of observations in the literature supporting this view. For example, it as been reported that alterations in $[Ca^{2+}]_o$ (Chapman et al. 1983), $[Na^+]_o$ (Horackova and Vassort 1979) or $[K^+]_o$ (Eisner and Lederer 1979) may lead to a decrease in tonic tension. These interventions have been shown to act via modification of $[Na^+]_i$ and $[Ca^{2+}]_i$ through the Na^{2+}/Ca^{2+} exchange mechanism. Such a mechanism seems to be very important, in cardiac tissue (for references, see Mullins 1981), but much less so in skeletal muscle (Schümperli et al. 1982; but see also Blaustein 1974) and thus might account for the difference in free $[Ca^{2+}]_i$.

Table 1 shows that our Ca^{2+} values for both Purkinje fibres and ventricular muscle do not deviate considerably from those reported by other investigators. This is rather surprising in view of the extensive differences in conditions used to calibrate the electrodes and to perform the actual

measurements. In case of skeletal muscle, our $[Ca^{2+}]_i$ is lower than any other reported value. This discrepancy could easily be explained in terms of differences in the calibration procedure (see above).

Optical methods have also been used to estimate intracellular Ca^{2+} (for review see Blinks et al 1982). While these techniques have proven to be most successful for the detection of relatively fast Ca^{2+} transients, they are not easy to apply to the measurement of absolute Ca^{2+} levels. So far only aequorin data is available for the resting $[Ca^{2+}]_i$ in the muscle preparations discussed in this paper. Wier and Hess (1984) reported a value of 290 nM for canine Purkinje fibres and Snowdowne (1979) found 90 nM in frog skeletal muscle.

Regulation of the free $[Ca^{2+}]_i$

In the three tissues investigated, the free $[Ca^{2+}]_i$ was too low to be compatible with a passive distribution across the sarcolemma. For example, in Purkinje fibres, the predicted $[Ca^{2+}]_i$ would have been 6.5 μM whereas the observed value was 240 nM. This discrepancy suggests the operation of powerful transport mechanisms for removing large quantities of Ca^{2+} from the cytoplasm into either the extracellular space and/or subcellular compartments (for review, see Sulakhe and St. Louis 1980; Borle 1981). This view is in accordance with the changes in $[Ca^{2+}]_i$ observed in Purkinje fibres exposed to high $[Ca^{2+}]_o$ solutions. Based on a passive distribution one would expect a 3-fold (8-fold) increase in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ is elevated from 1.8 to 5.4 mM (18 mM). Instead, increases in $[Ca^{2+}]_i$ by factors of 1.7 and 2.6 were observed experimentally.

In both types of cardiac tissue, Na^+ withdrawal led to an elevation in $[Ca^{2+}]_i$ which was associated with a contracture. Obviously, the $[Ca^{2+}]_i$ reached a level which was well beyond the threshold for contractile activation. Applying identical techniques to ventricular muscle, Lee et al. (1980), Marban et al. (1980), and Sheu and Fozzard (1982) obtained qualitatively similar results for partial or complete replacement of extracellular Na^+.

Using aequorin as an indicator for intracelluar Ca^{2+}, Allen et al. (1983) recently reported similar findings for ferret ventricular muscle. It is generally accepted that the increase in $[Ca^{2+}]_i$ under this condition is due to impairment of the Na^+/Ca^{2+} exchange mechanism (see e.g. Mullins 1981).

Our comparative study suggested that specialized tissues from the same heart may respond in a quantitatively different way to the Na^+-free intervention. Both the increase in $[Ca^{2+}]_i$ and the ratio $T_{Na\text{-}free}/T_{twitch}$ were much larger in Purkinje fibres than in ventricular muscle. Concerning the elevation of $[Ca^{2+}]_i$, it is conceivable that the two types of tissue differ in their apparatus to regulate $[Ca^{2+}]_i$ (Borle 1981; Page 1978). With respect to the mechanical response, our observation was somewhat surprising considering the lower Ca^{2+} sensitivity of the myofibrils in Purkinje fibres (see Fabiato 1982).

Recently, asynchronous fluctuations of intracellular Ca^{2+} were observed in Ca^{2+} overloaded cardiac preparations (Orchard et al. 1983; Wier et al. 1983). These fluctuations were accompanied by mechanical force oscillations of the same characteristic frequency (Orchard et al. 1983). Wier et al. (1983) demonstrated that the frequency and the amplitude of the Ca^{2+} oscillations grew larger with increasing $[Ca^{2+}]_i$. Since this corresponds to a rightward shift of the Ca^{2+}-tension relationship, less developed tension would be expected at a given mean $[Ca^{2+}]_i$. However, this finding seems to conflict with our observations on Purkinje fibres during Na^+ withdrawal, i.e. a larger elevation in $[Ca^{2+}]_i$ and a larger $T_{Na\text{-}free}/T_{twitch}$ ratio than in ventricular muscle. Future investigations will be needed to further resolve this matter.

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