Ca Entry and Contraction as Studied in Isolated Bovine Ventricular Myocytes

Gerrit Isenberg

II. Physiologisches Institut, Universität des Saarlandes 6650 Homburg/Saar, Bundesrepublik Deutschland

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Single bovine ventricular myocytes were superfused with Tyrode solution containing 1.8 mM $CaCl_2$. The cells did not bear external load and contracted isotonically. Contraction and relaxation were characterized by the shortening and relengthening of the sarcomeres which resembled in their time course the isometric twitches of bovine papillary muscles. Resemblance was also found in regard to positive inotropic interventions as increase in the stimulation frequency, exposure to elevated $[Ca]_0$ or to adrenaline.

A two-microelectrode voltage-clamp technique was applied to the single myocyte. The transmembrane Ca inward current I_{Ca} was defined as difference current sensitive to 5 mM Ni or to 2 μM D600. During a voltage step from −45 to +5 mV, I_{Ca} peaked within 3 ms to −6 nA, afterwards it decayed to 15% of peak amplitude (incomplete inactivation with a 2 exponential time course). Experiments in Na-free media suggested that Na entry does not significantly contaminate I_{Ca}. Therefore, Ca entry could be calculated from I_{Ca}. The increment in total intracellular Ca concentration ($\Delta[Ca]_i^T$) was estimated by referring Ca entry to the cell volume (50 pl).

Within 100 ms $\Delta[Ca]_i^T$ came to 25 μM at control conditions, to 55 μM at $[Ca]_0 = 3.6$ mM and to 88 μM when 0.1 μM adrenaline were present. The $\Delta[Ca]_i^T$ values were sufficient to activate contraction without the necessity of Ca-release from SR.

Despite the new data, the relationship between Ca entry and activation of contraction was complex: during the "positive Herztreppe" I_{Ca} slightly attenuated but contractility doubled. Therefore, the old EC-model (M. Morad and Y. Goldman, Progr. Biophys. Mol. Biol. **27**, 257 (1973)) was adapted. The Ca-entry's capability to load and to overload the intracellular Ca store (SR) is discussed.

Introduction

In heart muscle, activator Ca is derived both from extracellular space and from the internal store of the sarcoplasmic reticulum. During excitation Ca^{2+} enters through the sarcolemmal membrane, but the amount of this Ca-entry is insufficient by itself to activate the myofilaments; thus, contraction can be activated only if additional Ca is released from the SR (for rev. see [1–4]). The conclusion is mainly based on voltage-clamp studies analysing Ca entry via the transmembraneous Ca inward current [5–10]. Unfortunately, the multicellular nature of ventricular trabeculae or papillary muscles prevents a truly uniform voltage control, and with this limitation some controversy exists over the validity of the conclusions [9, 11–13]. Therefore, the problem of Ca entry via I_{Ca} will be analysed here by applying the voltage clamp to isolated ventricular cells.

Reprint requests to Gerrit Isenberg.

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Materials and Methods

The procedure of cell isolation has been published elsewhere in detail [14, 15]. Briefly, chunks of adult bovine left ventricular tissue were incubated in a medium containing 30 μM [Ca] as well as hyaluronidase (0.1%) and collagenase (0.1%). The isolated cells were stored in an ATP-containing KCl-medium to become Ca-tolerant. For the experiment, some of these cells were transferred to a small chamber (volume 1 ml), and were continuously superfused with a Tyrode solution composed of 150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES (adjusted with NaOH to pH 7.4, pre-warmed to 35 °C). A single myocyte was impaled with 1 or 2 microelectrodes and stimulated at 0.5 Hz. The myocyte contracted in response to the stimuli. The contractions were investigated with a TV-camera-tape system adapted to an inverted microscope. A ZEISS LD 40 objective delivered an image with final enlargement of 2600. A photodiode array was put over the monitor image to measure time course of contraction (see Fig. 1).

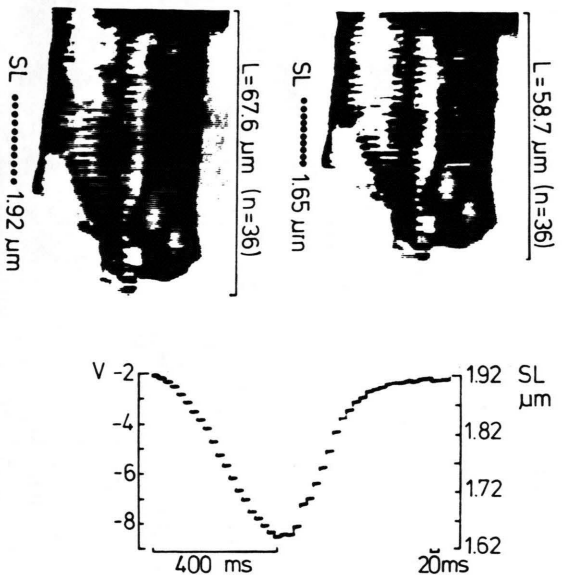

Fig. 1. Contraction as evaluated with the TV-system. Top: single images from the TV tape. Half of a myocyte is seen, and an impaled microelectrode (black shadow). During contraction, the cell ends move towards the microelectrode (fix point). The distance L between cell end and tip shortens. L devided by the numbers of sarcomeres ($n = 36$) gives the sarcomere length (SL) which is 1.92 µm at diastole and 1.65 µm at systole. Below: the output signal V of an array of photodiodes is proportional to the shortening of L. Off line, it can be calibrated with the S information from the TV-tape. The time resolution is limited by the image frequency of 50 Hz.

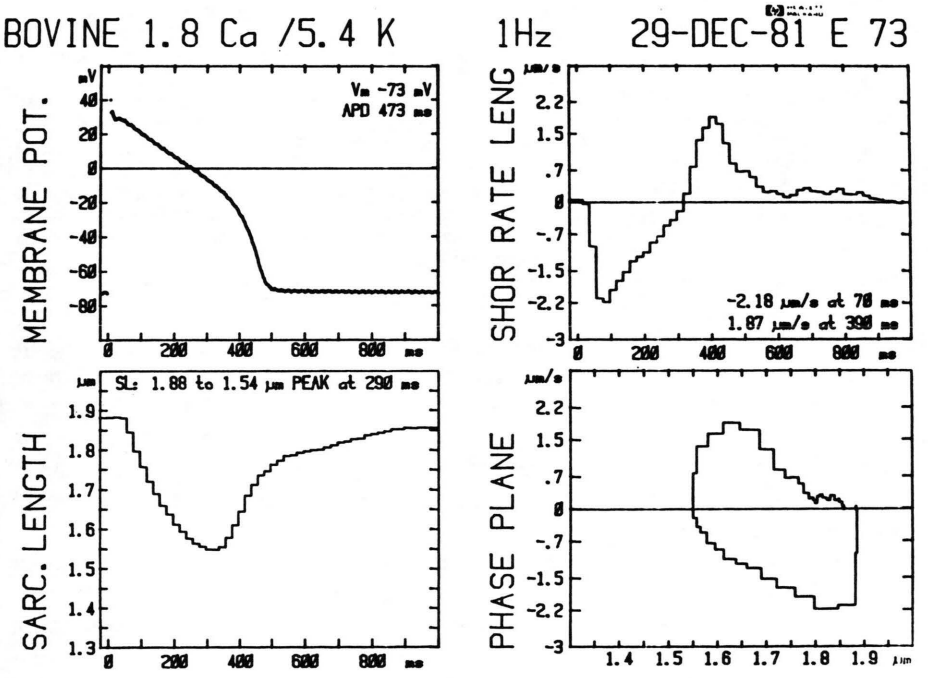

Fig. 2. Excitation and contraction of an isolated bovine ventricular myocyte. The part labelled with "membrane pot" shows an AP which starts $t = 0$ from a resting potential (V_m) of −73 mV, comes to an overshoot of + 33 mV and repolarizes with a well pronounced plateau. Action potential duration (APD) of 473 ms is measured at repolarization to 90% of V_m. The part sarc. length indicates isotonic shortening (downward deflection) and re-lengthening of the average SL. – Rate of shortening and re-lengthening (part in the upper right) is the difference quotient ΔSL over 20 ms. In the phase plane it is plotted as a function of actual SL.

The signals were stored on digital tape. Off line, they were calibrated (see Fig. 1), evaluated and plotted by a mini-computer. The non-linearity of the records was controlled with object micrometers to be less than 8%.

Results

Excitation. In comparing the resting and action potentials (AP's) recorded from single bovine ventricular myocytes with the data published for multicellular bovine ventricular tissue [16, 17], no peculiar abnormalities were found. (For a detailed comparison see [18].) Usually, the resting potentials were between −70 and −80 mV. The AP's lasted for 300–500 ms, their shape was trapezoidal (Fig. 2) due to a "plateau". (The plateau results mainly from the Ca inward current I_{Ca} [19, 20]. As long as the plateau does not repolarize to potentials negative to −30 mV, I_{Ca} will transport Ca ions into the cell.)

Normally, the plateau is preceded by a "sodium spike" which is characterized by a fast depolarization (200 V/s) to the overshoot (+35 mV in Fig. 2). The sodium spike component of the AP is based on the sodium inward current, I_{Na} [20]. It can be eliminated either by superfusing the cells with a Na-free medium (Fig. 6) or by depolarizing them to −45 mV, either by voltage clamp ("holding potential" in the experiment of Fig. 6) or by media containing 20 mM KCl (Fig. 3). In the latter case the AP is called "slow" AP because the upstroke rises with a rate of 10 V/s or less. Slow AP's starting from −45 mV without sodium spike components can trigger normal contractions (Fig. 3, compare [21]). The same is true for clamp steps depolarizing from −45 mV to *e.g.* +5 mV [7, 10, 22].

Contraction. In diastole, the unloaded bovine ventricular myocyte has in average a sarcomere length (SL) of 1.88 ± 0.05 µm (mean ± S.D. from 65 cells). During systole, the SL shortens to a minimum of about 1.7 µm if the contraction is weak and to less than 1.5 µm when the contraction is strong. Sometimes contractility will be indicated by the "extent of shortening" which is defined as (diastolic SL-minimum systolic SL)/diastolic SL. With the SL numbers given above, the extent of shortening is 0.095 when the contraction is weak, it is 0.2 when the contraction is strong.

Time course. Contraction follows excitation with a latency of 10–20 ms. (The exact evaluation of the latency is beside the resolution of the TV-system.)

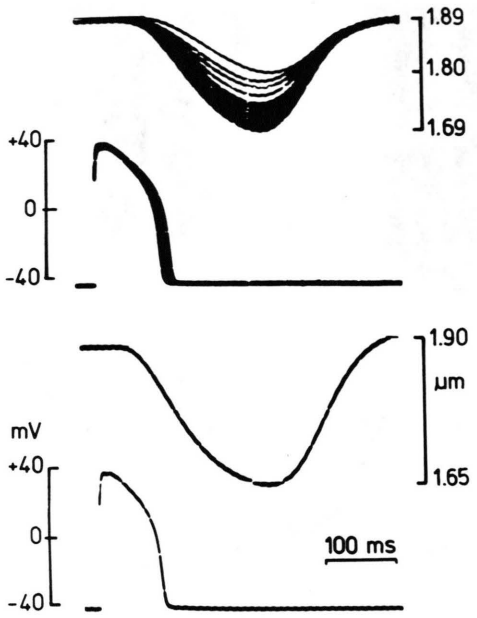

Fig. 3. Contractions in response to slow AP's; the influence of stimulation frequency. The superfusate contained 20 mM KCl and 3.6 mM $CaCl_2$. The cell had a resting potential of −44 mV and responded with slow AP's which are shorter than "normal" AP's (elevated $[K]_o$ speeds up the repolarization, [15]). The SL signals have been displaced from the photodiode array through a 4 Hz low pass filter directly to a storage oscilloscope; the filter prolonged the "latency" of the signal artificially. Top: after a 2 min rest period, stimuli were applied at 0.5 Hz. The first or "rest" response is the longest slow AP and the weakest contraction. From beat to beat, the rate and the extent of shortening increased, the time to peak declined. After 20 beats a new steady state was reached. In regard to the rest contraction, the extent of shortening had doubled from 5% to 10%. Below: the same cell at steady 1 Hz stimulation (extent of shortening 13%).

After latency, the rate of shortening increases steeply and reaches a maximum within 50 ms (indicated as downward deflection to −2.2 µm/s in Fig. 2). Later, when the SL becomes shorter than 1.8 µm, the rate of shortening declines (Fig. 2: phase plane). Minimum SL is recorded with a TTP (time to peak) of 250–300 ms.

Relaxation re-lengthens the sarcomeres without any external load. The rate of the re-lengthening process comes within 100 ms to a maximum of 1.9 µm/s (Fig. 2B) which is about 80% of the maximum rate of shortening. The rate of re-lengthening declined when the SL had increased over 1.65 µm. The final approach of diastolic SL occurred with very low rate (0.3 µm/s in Fig. 2), this phase appeared as a separate component of re-lengthening.

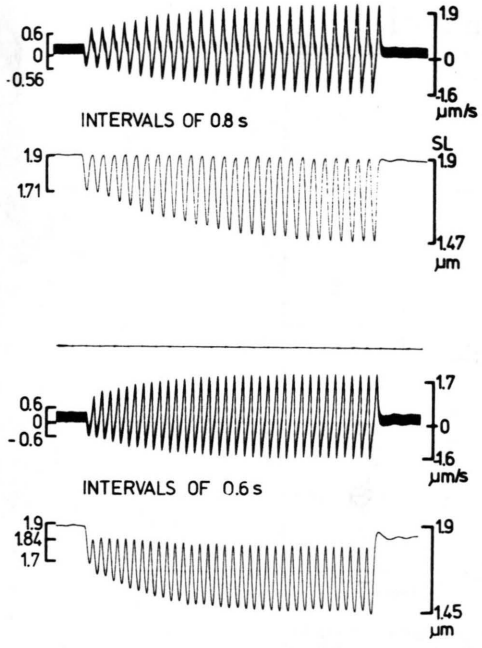

Fig. 4. The "positive Herztreppe" (signals of a pen recorder, filtered at 4 Hz). The myocyte remained unstimulated for 2 min. Following this rest period, stimulation at 1.25 Hz (intervals of 0.8 s) or 1.67 Hz (intervals of 0.6 s) started. Shortening and re-lengthening of SL are shown together with their first derivative. Note that 0.6 s long intervals are too short for complete re-lengthening ("diastolic shortening" to 1.84 µm), and that the last beat is followed by small after-concentrations.

Inotropic interventions. Contractility increased with the *frequency of stimulation* [23], ("positive Herztreppe", Figs. 3, 4). The first contraction following a 2 min rest period was weak: the extent of shortening was less than 0.1, the rate of shortening was smaller than 0.6 µm/s and the TTP was long. Afterwards, in a beat to beat fashion, rate and extent of shortening increased, latency and TTP declined. Also, and most pronounced, the rate of relengthening was enhanced which slightly shortened the contraction. Despite this shortening, at frequencies greater than 1.4 Hz the interval between the beats was too short for complete relaxation (lower part of Fig. 4).

When the myocytes were exposed to 0.1 µM *adrenaline* both extent and rate of shortening increased, sometimes minimum systolic SL's of 1.3 µm were measured. The rate of re-lengthening responded most sensitive, especially, when it had been low before [24].

Doubling $[Ca]_0$ from 1.8 to 3.6 mM enhances contractility both in extent (factor 1.6) and rate of shortening (factor 1.7), TTP shortened moderately. The rate of re-lengthening was most sensitive [25], its maximum became usually faster than the maximum rate of shortening (Fig. 5). However, the sarcomeres did not approach diastolic *SL* before excitation had finished. Most often, an after-contraction shortened the sarcomeres a second time (Fig. 5). When the APD was shortened by anodal current flow, the after-contractions disappeared, in addition, extent and rate of shortening as well as rate of re-lengthening were reduced. A strong reduction of APD was also recorded from KCl-depolarized cells (Fig. 3, compare [16] for bovine trabeculae). Reduction of the APD to 50% reduced the extent of shortening to 70% (compare [17]). The rate of re-lengthening remained large, its maximum was often twice as large as the maximum rate of shortening.

Conclusion. The results show that the isolated unloaded bovine ventricular myocyte follows a time course of contraction that is similar to the isometric twitch of a bovine papillary muscle [17]. In comparison with multicellular preparations, all sarcomeres shorten homogeneously (compare [26]); distortions by intercellular sharing forces are missing.The minimal systolic SL of the isolated cell can easily overcome the limiting 1.65 µm which are known from multicellular tissue [27]. Therefore, one may ask if shortening of non-dissociated tissue is really limited by the length of the myosin-filaments or, perhaps in addition, by the encapsulating collagene network [28]. On the other hand, the isolated cells shortened with a unusually low rate. In part, this is a peculiarity of bovine ventricular myocytes, preliminary experiments with guinea pig ventricular myocytes indicated for TTP 90–100 ms and for the maximum rate of shortening 6–8 µm/s. (For isolated rat ventricular myocytes, a value of 9 µm/s has been described [26].) In addition, a reduced rate of shortening has to be expected from diastolic SL = 1.88 µm (*in vivo* diastolic SL = 2.05 µm, [28]): low diastolic SL are known to depress both the release of activator Ca [29] and myosin-actin interactions (*cf.* rev. [23, 30]).

It remains the possibility that during isotonic shortening the myofibrils contract against a length-dependent restoring force acting in opposite to contractile force [27]. Though externally unloaded, the cell would contract against an internal load.

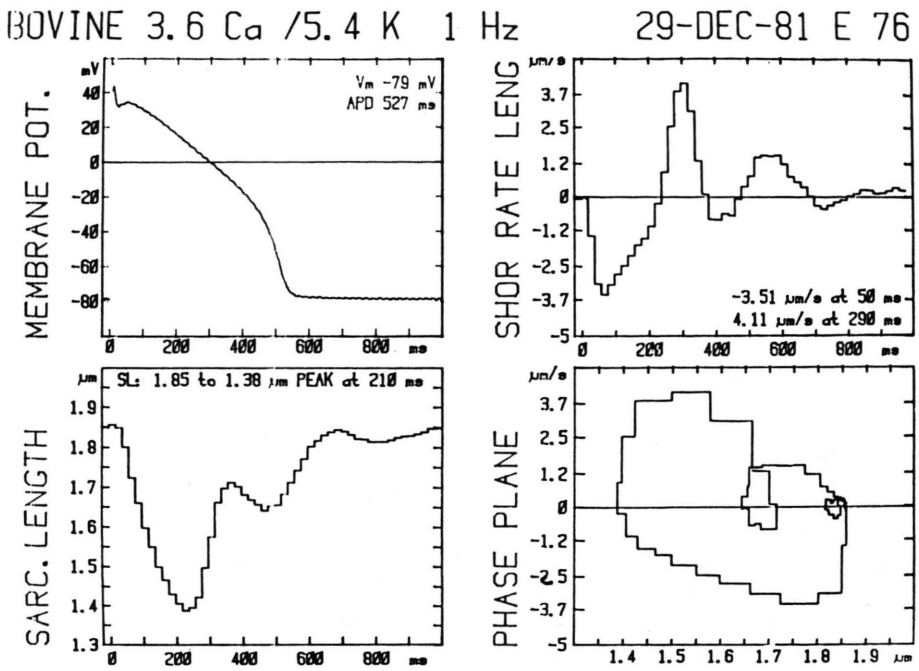

Fig. 5. Excitation and concentration of an isolated myocyte superfused with Tyrode solution containing 3.6 mM $CaCl_2$. Note that an after-contraction is superimposed on the re-lengthening.

The load could be described with a simple spring model, since the rate of shortening declined with SL. (This influence of SL on rate of shortening was recently described for lightly loaded single frog atrial cells [31, 32].)

The internal load could give the force for re-lengthening of the sarcomeres. Kinetic energy stored in a "bending" of the myosin filaments probably speeds re-lengthening up to the maximum rate observed at a SL of 1.65 µm. About the force restoring the SL from 1.65 to 1.9 µm not very much is known (see [29]).

In addition to this "load-dependent" relaxation a second "activation-dependent" relaxation process [33] could have been indicated by the final, very slow phase of re-lengthening (Fig. 2); as the restoring force was small (weak contraction) the disappearance of activator calcium may have become rate-limiting. At elevated $[Ca]_0$ (Fig. 5) sustained Ca entry may have overloaded the SR (p. 9), and regenerative Ca release could have activated the after-contractions [34].

The inotropic interventions enlarged the isotonic shortening both in extent and rate, but not by the same amount. Therefore, (and because the properties of the "internal spring" are not known), both parameters should be used to characterize the contractility. The positive inotropic effect of doubled $[Ca]_0$ can be primarily attributed to enhanced Ca-entry. Enhanced Ca-filling of and Ca-release from the SR is connected with the "positive Herztreppe". Both mechanisms contribute probably to the adrenaline effect. Since the isolated ventricular cell responds with a inotropic response similar to that known from isometric contractions of multicellular tissue (see rev. [1–4]), one may conclude that the underlying mechanisms were not seriously damaged by the cell isolation procedure, and that the isolated cells can be used as a model for EC studies.

I_{Ca} and Ca entry

Ca enters through the heart cell membrane via Ca channels. Excitation opens ("activates") and closes ("inactivates") the channels. When the channels are open, the electrochemical gradient drives Ca from the extra- to the intracellular space. The net charge transfer through the membrane generates the current I_{Ca} which can be measured by voltage-clamp techniques [19]. By definition I_{Ca} appears negative

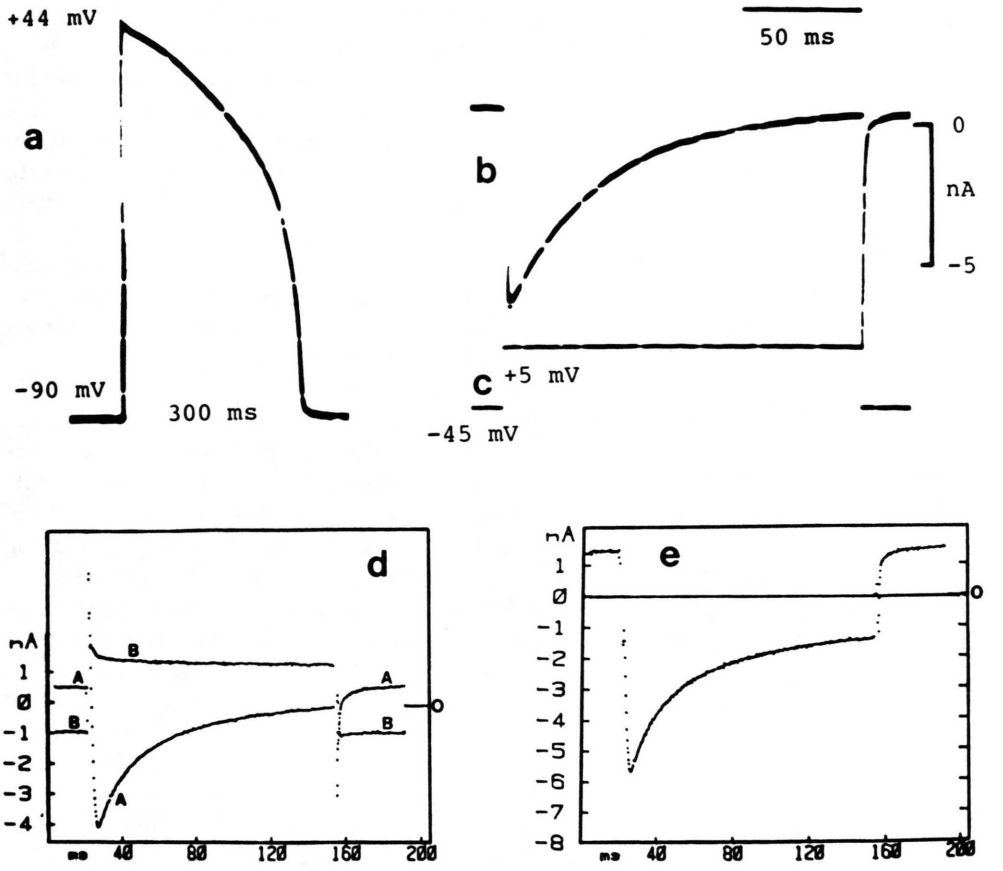

Fig. 6. Evaluation of I_{Ca}. AP's are carried by sodium and by calcium inward currents, contributions of I_{Na} have to be eliminated. For record (b) I_{Na} has been inactivated by a conditioning prepulse (−45 mV) which preceded the clamp step used for analysis (c: 150 ms to + 5 mV). I_{Ca} contributes to the net current (b) a large negative current wave. Repolarization to −45 mV deactivates I_{Ca}, and an inward directed tail current results. Experiment (d) was performed in a sodium free solution (substitution by choline for 30 min). The cell was depolarized from −80 mV to + 5 mV for 130 ms and repolarized to −80 mV afterwards. Finally, the clamp circuit was switched off (zero current 0). Depolarization evokes a large negative net current wave (A) contributed by I_{Ca}. Exposure to 5 mM Ni^+ blocks this contribution. From the difference trace A minus trace B, I_{Ca} is evaluated as Ni-sensitive current (e). The positive difference current seen at −80 mV suggests contributions of a Ca-activated potassium current.

(inward flow of positive ions). As shown in Figs. 6 and 7, I_{Ca} peaks quickly to −6 nA and turns off slowly afterwards. The area between zero line and current trace is a measure of charge from which the amount of Ca entry can be computed. Such a procedure suffers from several problems:

The voltage clamp. In the unclamped mode, the charge transported by I_{Ca} generates the plateau and (if I_{Na} is inactivated) the upstroke of the slow AP. Since the membrane has properties as a capacitance (C_m), changes in membrane potential induce a current $i_C = -C_m\, dV/dt$ which provides electroneutrality. Using the voltage clamp, external current impresses an artificial steplike excitation, dV/dt and i_C should have finished before I_{Ca} starts activating. For an adequate analysis, the artificial change in potential should be the same for all membranes that bear Ca channels: because activation and inactivation of the channels are graded non-linear functions of membrane voltage (*e.g.* [22]), any escape from voltage homogeneity will change the records.

In multicellular trabeculae or papillary muscles, the requirements suffer from the multicellular nature of those preparations. Cell-to-cell junctions and the narrow extracellular cleft space form a resistance (R_s^d) in series with the clamped membrane.

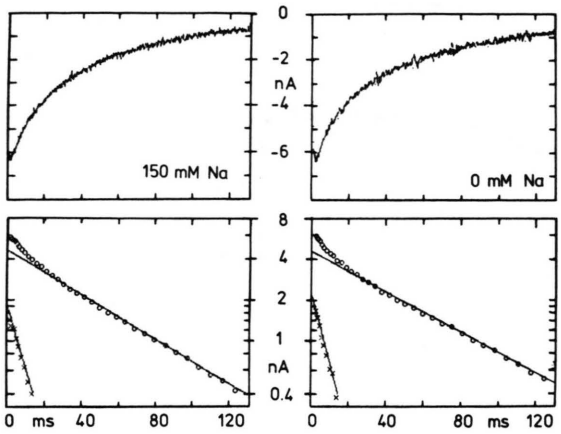

Fig. 7. I_{Ca} was evaluated as Ni-sensitive current in the presence and absence of sodium (NaCl substituted by Tris-Cl for 25 min); the clamp step went from −44 to −3 mV. Inactivation was fitted in semilog coordinated according to $I_{Ca} = I^0_{Ca} + I^1_{Ca} \cdot \exp(-t/\tau_1) + I^2_{Ca} \cdot \exp(-t/\tau_2)$. The non-inactivating component I^0_{Ca} is 0.72 nA in the presence and 0.78 nA in the absence of sodium. For the slowly inactivating component (circles) the amplitudes are 4.6 and 4.5 nA, and the time constants 26 and 29 ms. For the fast inactivating component (x's), the values are 1.8 nA or 2.2 nA and 4.5 or 4.2 ms, respectively. Both in presence and absence of sodium, I_{Ca} peaks within 3 ms to 6.2 nA, and the inactivation is fitted with nearly identical parameters.

$R^d_s \times C_m$ limits the rate dV/dt, and therefore, long lasting capacitive currents can superimpose and mask I_{Ca} [11]. Further, the voltage drop $R_s \cdot I_{Ca}$ prevents voltage homogeneity. Therefore, the interpretation of the records has been doubted (*cf.* [11, 13]).

Most of those technical problems can be avoided when the voltage-clamp experiments are performed on isolated ventricular myocytes ($R_s < 25\,\text{Ohm} \cdot \text{cm}^2$, [35] in comparison with $R_s > 500\,\text{Ohm} \cdot \text{cm}^2$, [12]). Comparing I_{Ca} of Fig. 5 with tracings published for bovine ventricular trabeculae [36], I_{Ca} appears with greater amplitude (34 μA/cm² vs. 8 μA/cm²) and faster kinetics (*e.g.* time to peak 3 ms vs. 25 ms). In the unclamped mode no differences were seen (especially not in regard to dV/dt of the slow AP [18]). Therefore, it is unlikely that the differences in I_{Ca} result from an alteration of the Ca channels. Addition of an artificial R_s gave the tracings recorded from the single cell the appearance of those of multicellular tissue, therefore it has been concluded that the great R_s of multicellular tissue hinders the adequate evaluation of I_{Ca} [35]. Correspondingly, the I_{Ca} records from multicellular tissue displayed a shorter time to peak when R_s was smaller in preparations of smaller size: 10 ms have been reported for cat papillary muscles [16], 6 ms for embryonic heart cell aggregates [8], and 3 ms for very small preparations of the rabbit SA node [37]. Because I_{Ca} has a larger amplitude and faster kinetics than thought previously it might be worth to re-consider the question of how much Ca enters via I_{Ca}.

Analysis of I_{Ca}. The voltage-clamp method measures a net membrane current which is composed of several components. Within this mixture the contribution of I_{Ca} has to be isolated.

Pharmacological dissection. Drugs like verapramil, D600, nifedipine or ions like Co, Mn, Ni "block" the Ca channels [38, 39]. Provided that no other channels are modified, I_{Ca} can be defined as difference current: net current in the absence minus net current in the presence of the Ca channel blocking agent. As an example, in Fig. 6d subtraction of trace B from A results in the Ni-sensitive current of Fig. 6e. – At least in bovine ventricular tissue the procedure is not without problems: block of Ca entry reduces the "Ca-activated" potassium current [40, 41], which may contribute the positive difference current at −80 mV of Fig. 6e. If a "Ca-activated" potassium current would contribute at +5 mV, the pharmacological dissection of Fig. 6 would underestimate I_{Ca} and Ca entry.

Kinetics (at 0 mV). Depolarization opens the Ca channels with an activation time constant of 0.6 ms [35]. Repolarization closes the channels by deactivation (time constants less than 0.5 ms for potentials more negative than −40 mV). During sustained depolarization about 85% of the channels close by inactivation, the other fraction of 15% non-inactivating channels close only when repolarization induces deactivation. (Incomplete inactivation is known for Purkinje fibers from ungulate hearts [42, 43].) Inactivation diminishes I_{Ca} with a half decay time of 40 ms. Formally, the time course can be described with 2 exponentials (see legend of Fig. 7).

Does I_{Ca} *transport* Na^+ *ions?* When the 150 mM NaCl of the superfusing Tyrode-solution are isoosmotically replaced by choline-CL, peak I_{Ca} falls to about 75% of the control. (A similar reduction is seen if NaCl is substituted by Tris-Cl, TMA-Cl or by taurine). Reuter and Scholz [36] observed a similar reduction and they concluded that a significant fraction of I_{Ca} is carried not by Ca but by Na ions. However, sodium removal induces in the

isolated heart cell a strong contracture (SL about 1.25 μM) indicating an increase in $[Ca]_i$ (Na-Ca exchange mechanism [44, 45], rev. [1]). When the cells remained in sodium-free medium for more than 15 min, the contracture disappeared, and in parallel to the re-lengthening of the sarcomeres, complete recovery of I_{Ca} was observed (Fig. 7, right). The results suggest that removal of sodium ions reduced I_{Ca} via an increase in $[Ca]_i$. They further suggest that sodium ions carry only an insignificant fraction of I_{Ca} and that I_{Ca} can stay for Ca entry.

Net Ca entry via I_{Ca} can now be estimated according to

$$\mathrm{mCa}(t) = \frac{1}{z \cdot F} \int_{t}^{t'} I_{Ca}(t)\, dt\,.$$

Using $z = 2$ for the equivalence charge and $F = 96400$ for the Faraday constant one calculates a Ca entry of 3×10^{-17} mol/ms (3×10^{6} ions/ms) when I_{Ca} peaks, of 1×10^{-17} mol/ms 50 ms later and of 0.4×10^{-7} mol/ms when only the non-inactivating I_{Ca} fraction remained ($t > 150$ ms). Ca entry is finished as repolarization deactivates the Ca channels (see p. 508).

Formally, the amount of Ca entry can be attributed to the cell volume (V) of 50 pl (estimated by light microscopy [15]). The expression

$$\Delta[Ca]_i^T = \frac{1}{z \cdot F \cdot V} \int_{t=t_0}^{t} I_{Ca}(t)\, dt$$

gives the increment in total intracellular Ca concentration as a function of the duration of depolarization (Fig. 8). At start of depolarization (when I_{Ca} is maximal) $\Delta[Ca]_i^T$ rises steeply (rate 0.6 μM/ms), it comes to 10 μM within 25 ms. Later $\Delta[Ca]_i^T$ increases with attenuated rate. Due to the non-inactivating I_{Ca}, $\Delta[Ca]_i^T$ does not saturate but increases linearly with time (rate of 0.1 μM/ms). In Fig. 8 $\Delta[Ca]_i^T$ is 25 μM for 100 ms after start of depolarization, and it would be 35 μM after another 100 ms period. – The results can be generalized in a sense that the large increase in $\Delta[Ca]_i^T$ is not a peculiarity of the bovine ventricular myocyte. Preliminary results of I_{Ca} recorded from isolated ventricular myocytes of rats, cats or guinea pigs are similar as those shown in Figs. 6 and 7. However, differences between the species may exist when the non-inactivating I_{Ca} is concerned or when the contamination of I_{Ca} with "Ca-activated I_K" plays a role (both seem to be absent in the cat papillary muscle).

3. Ca entry and activation of contraction

In the literature [3] $[Ca]_i^T$ has been used to calculate the fraction of mechanical tension that might be activated. The data have been referred to Tab. 2 in Solaro *et al.* [46] who measured on "skinned" cardiac tissue the relationship between $\Delta[Ca]_i^T$ (atomic absorption) and percentage of maximum isometric tension. Solaro *et al.* found that

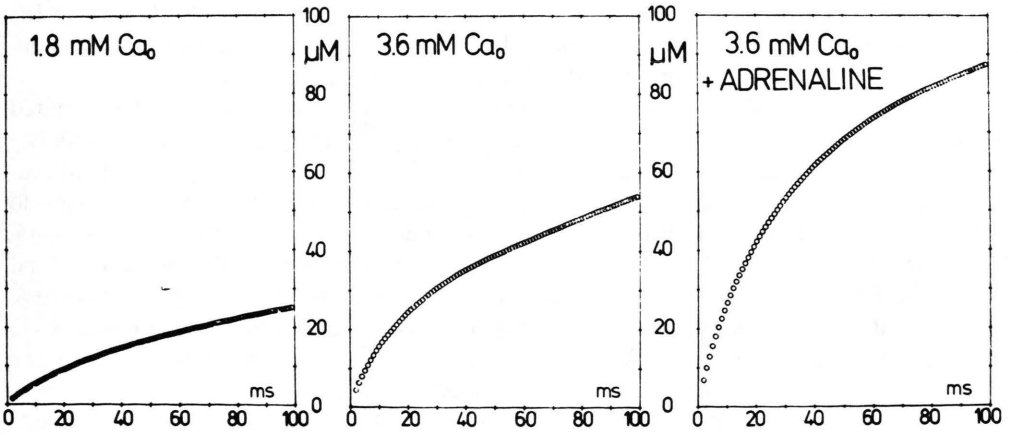

Fig. 8. Increment in total intracellular calcium concentration $\Delta[Ca]_i^T$ as function of the duration of depolarization. $\Delta[Ca]_i^T$ was calculated by integrating the Ni-sensitive current over time. The calculations are based on clamp depolarizations to + 5 mV which were performed on cells superfused with normal (sodium containing) Tyrode solution, Tyrode solution with doubled calcium content, or with additional 0.1 μM adrenaline. For the inotropic interventions different cells were used because the blocking effect of 5 mM Ni only reserved slowly. The results are representative for 5 other cells.

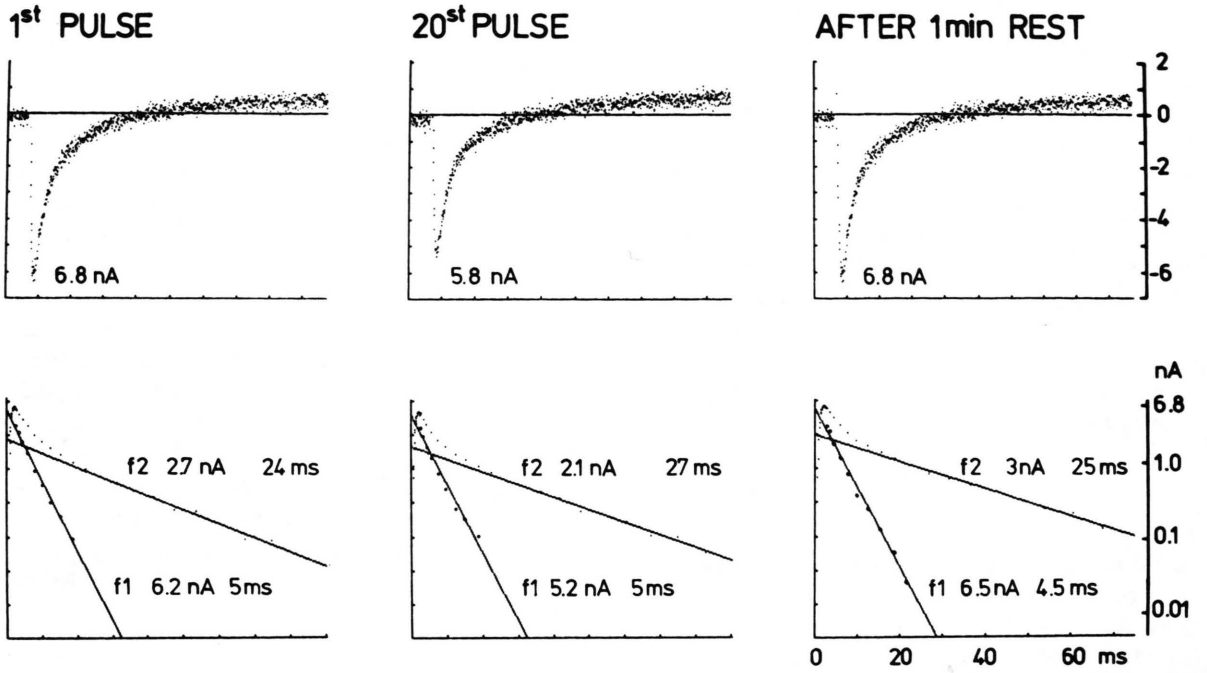

Fig. 9. I_{Ca} does not increase during the "positive Herztreppe". I_{Ca} is approximated by net membrane currents elicited by clamp steps depolarizing for 120 ms from −45 to +3 mV. The first pulse followed a 2 min rest period, afterwards the pulses were applied at 1.25 Hz. Repetitive pulsing increased contractility very similar as described in Fig. 4 (0.8 s intervals). Repetitive pulsing reversibly decreased the current by 15%. When the current is split in a fast (f_1) and slow (f_2) component of inactivation, the amplitudes of f_1 and f_2 are reduced by about the same degree, the time constants are nearly unchanged. (Data analysis by Dr. George Christé.)

maximum tension is achieved with about 90 μM $\Delta[Ca]_i^T$, and that 50% of maximum tension require about 40 μM $\Delta[Ca]_i^T$. On the basis of the present results and Tab. 2 of Solaro *et al.* [46] one estimates that Ca entering via I_{Ca} activates 40% of maximum tension (100 ms long depolarization to +5 mV, see also [47]). During positive inotropic interventions which enhance I_{Ca}, 80% ($[Ca]_0$ doubled to 3.6 mM) or nearly 100% (exposure to adrenaline) of maximum tension should be activated by Ca entry. Thus, the amount of Ca entering via I_{Ca} is sufficient for activating the contraction directly, *i.e.*, the required concentrations of activator Ca could be achieved without Ca release from the SR.

Despite such an argument, the new data do not proof a simple and direct relationship between Ca entry and activation of contraction. It might be doubtful whether $\Delta[Ca]_i^T$ can be referred to the $[Ca]_i^T$-tension curve [46] measured at equilibrium conditions. $\Delta[Ca]_i^T$ is a rapid Ca transient where Ca distribution is far from equilibrium. Furthermore, $\Delta[Ca]_i^T$ is not a real concentration because those factors that reduce $\Delta[Ca]_i^T$ have not been considered. It seems to be impossible to calculate which fraction of $\Delta[Ca]_i^T$ appears at a given time as freely ionized $[Ca]_i$ between the myofilaments because the largest parts of $\Delta[Ca]_i^T$ are "buffered" inside the cell (concentration- and, therefore, time-dependent Ca-binding and -sequestration).

Contraction of heart muscle cannot be initiated without foregoing Ca entry and often contractility runs in parallel to I_{Ca} and $\Delta[Ca]_i^T$ [3, 22]. But as contractility is changing with time those changes do not always reflect a change in $\Delta[Ca]_i^T$ [37, 42–45, 48–50]. Dissociation between Ca entry and contractility is demonstrated during the "positive Herztreppe": following a 2 min rest period, 1 Hz stimulation increases extent and rate of shortening by more than 200% (Fig. 4), but I_{Ca} and Ca entry decline by 15%. The dissociation strongly suggests that Ca entering via I_{Ca} does not "directly" activate the myofilaments, and that the necessity still exists to have the Ca stored in and released from the SR (see models of [2, 4, 51]). In such a model, the SR

becomes depleted of Ca during the 2 min rest period and it is filled up by the Ca entering with I_{Ca} in a beat to beat fashion. (According to Fig. 8, 15–20 excitations should load the SR up to 400 μM.) If contractility is mostly attributed to Ca release (which increases with the Ca load) the myofilaments must be effectively shielded from the 25 μM $\Delta[Ca]_i^T$. (The longitudinal SR could do so since it surrounds the myofibrils like a filter net and has sites with high Ca affinity.)

The non-inactivating Ca-entry sustains as long as the cell is depolarized, it is terminated by repolarization (p. 508). In the above EC model, non-inactivating Ca entry loads the stores the better the longer the depolarization lasts which explains that abbreviated AP's go along with reduced contractility [17, 52–54]. In Fig. 2, re-lengthening occurs before excitation has finished and the non-inactivating Ca entry has been switched off. Obviously, the rate of "Ca-redistribution" can (transiently) exceed the rate of non-inactivating Ca entry (in the order of 0.5 nA or 5×10^{-15} mol/s and cell). Re-lengthening was incomplete and superimposed by after-contractions when $[Ca]_0$ was elevated to 3.6 mM. Thus, it seems that the cell cannot handle $\Delta[Ca]_i^T$ adequately if inactivating Ca-entry is in the order of 1–2 nA. With the above model one would postulate that the SR became overloaded with Ca (maximum storage capacity of dog heart SR 400 μM [55], see also [56]), and the mechanism of "Ca-induced Ca release" [2, 34, 47] may trigger contraction without excitation and Ca entry.

Finally, regarding the new numbers of Ca entry, one wonders how the large amount of $\Delta[Ca]_i^T$ can disappear, especially when 20 beats can completely load the SR [56]. Instead of an answer only 2 speculations can be offered: (a) Ca can diffuse along an electrochemical gradient from the junctional SR into the extracellular space. (b) One of the I_{Ca} components is a Ca-release signal describing circulation of Ca between SR and cytosol but not increment in total intracellular Ca content. For both ideas one has to postulate that the junctional SR communicates with the extracellular space, either in an electrical and/or in a diffusional sense. The experimental support for such a hypothesis is still weak up to now.

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