Experimental and theoretical work on excitation and excitation-contraction coupling in the heart

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Summary. A combination of experimental and theoretical work has been used to investigate the movements of calcium during cardiac excitation. In addition to calcium entry through several types of calcium channel, calcium efflux occurs to balance the entry during each cycle of activity. Measurements of net membrane calcium movements have been made with the right time resolution by Don Hilgemann in Los Angeles by investigating fast extracellular calcium transients[10,11]. This work shows that, in mammalian cardiac cells, net calcium exit occurs quite early during repolarization and is nearly complete by the time the resting potential is re-established. These results correlate very well indeed with measurements made in the Oxford laboratory of calcium-activated inward current in single cardiac myocytes[6–8]. Both approaches are consistent with the view that calcium efflux occurs largely through the sodium-calcium exchange process. Modelling of this process in equations developed recently with Dario DiFrancesco, Susan Noble and Don Hilgemann[4,12,13,24] succeeds in reproducing both the ionic current changes and the fast extracellular calcium transients.

Key words. Sodium-calcium exchange; excitation-contraction coupling; cardiac modelling.

Introduction

One of Silvio Weidmann's major interests has been in the influence of small perturbations of electric current on the properties of cardiac muscle. It was this interest that led to his seminal work on the variation of membrane slope conductance in Purkinje fibres and the phenomenon of all-or-nothing repolarization[30,31]. It also led to an important paper, with Wood and Heppner[32], on the influence of current-induced changes in electrical activity on subsequent mechanical activity. We now know that the causal sequence of events involved flows not only in the direction 'electrical activity→mechanical activity' but also in the opposite direction. This implies not only that small changes in action potential shape can influence the storage of calcium for subsequent activation of the contractile proteins but also that this liberation of calcium can itself influence the electrical event, presumably by Ca_i-activated membrane channel or carrier mechanisms.

Until recently, this degree of complexity was beyond the reach of mathematical models of electrical activity. The McAllister-Noble-Tsien (MNT)[17] model and the Beeler-Reuter (BR)[1] model followed in the same footsteps as the original Hodgkin-Huxley model of the nerve impulse, i.e. they described surface membrane events with no reference to intracellular processes. Meanwhile, mechanical models of the heart described possible movements of calcium but were not linked to excitation (see Hilgemann and Noble[12] for references).

When Dario DiFrancesco and I set out to reconstruct an electrical model of the heart some six years ago, we did not initially have in mind the need to link the two approaches. We were motivated rather by the then urgent need to explain how the process of extracellular potassium depletion could have so successfully masked the properties of the hyperpolarizing-activated pacemaker current, i_f, as to make it behave like a K^+ specific current, i_{K2}[3]. But, in making the move to reconstruct the 'i_{K2}' results from the processes of i_f, i_{K1} and K^+ depletion, we had to introduce a Na^+-K^+ pump to maintain the model in ionic balance, and a description of intracellular sodium changes as a consequence of the pump activity. It is never easy to decide where to draw the boundaries of useful mathematical models. Sometimes, the process of modelling for one purpose acquires its own momentum and this was certainly true in this case. Once the Na-K pump, $[Na]_i$ and $[K]_o$ changes were introduced it was only a small step to also adding the sodium-calcium exchange mechanism and a simple description of intracellular calcium changes.

And so, without initially intending to do so, we were on the road to constructing the kind of model that would be applicable to problems in excitation-contraction coupling and in the influence of inotropic on electrical state[4]. We were also confronted immediately by the need to assess the validity of Mullins's[20,21] hypothesis that ionic current carried by the sodium-calcium exchange process might contribute to the 'slow inward current' in the heart.

More recently, these questions have been taken even further by work done in collaboration with Don Hilgemann in order to correlate the ionic current analysis with net membrane calcium fluxes[12,13], and by experimental work on isolated cardiac myocytes designed to record the predicted ionic current changes[6–8].

My purpose in this symposium article is not to repeat in detail the material to be found in the papers quoted above describing the modelling and experimental work involved, but rather to highlight some of the important conclusions and to point the way to further work that remains to be done.

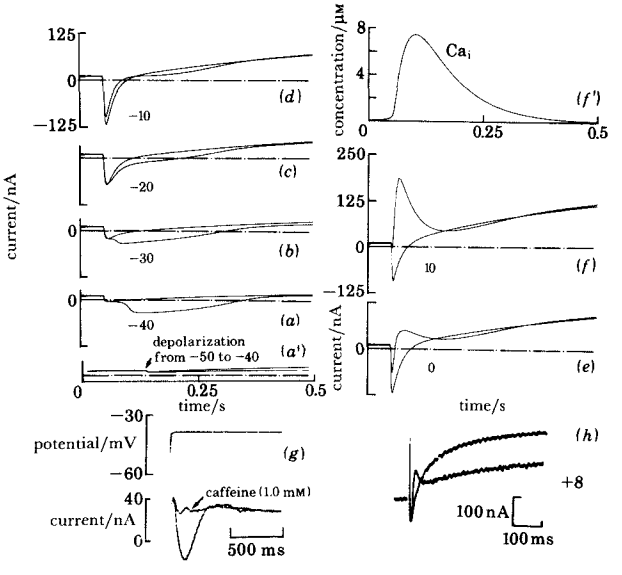

Figure 1. Voltage clamp currents computed from the DiFrancesco-Noble model. In records (*a*) to (*f*), the voltage was stepped from −80 mV to the potentials indicated, first using the equations with g_{Na} set to zero and then with i_{to} and i_{NaCa} also set to zero to mimic the expected result of eliminating $[Ca]_i$-dependent currents. Record (*f'*) shows the intracellular calcium transient computed during record (*f*). Record (*a*) shows the effect of changing the holding potential to −50 mV. The very slow inward current seen on clamping to −40 or −30 mV from −80 mV is then no longer seen. (*h*) shows superimposed experimental records from Siegelbaum and Tsien[29]. They clamped from −45 mV to +8 mV. Record (*g*) shows experimental records from Eisner, Lederer and Noble[5]. Note that the time scales for the experimental records are not the same as those for the computed traces. (From DiFrancesco and Noble[4])

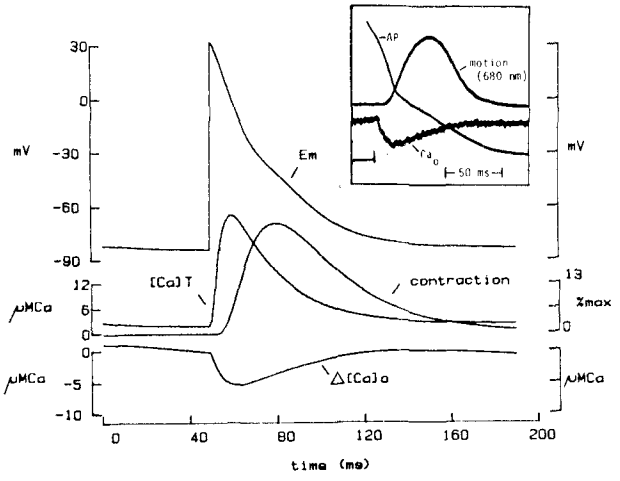

Figure 2. Behaviour of the Hilgemann-Noble rabbit atrial model during steady state simulation at 0.5 Hz. The inset shows experimental results. The computed action potential (E_m), like the experimental one (AP), shows two phases of repolarization. The curve labelled [Ca]T is the total cytosol calcium, including that bound to fast buffers. The free $[Ca]_i$ transient is similar in time course but is about 50 times smaller. The computed contraction curve compares with the motion record in the experimental result. Finally, the computed extracellular calcium transient compares well with the experimental record of changes in $[Ca]_o$. (From Hilgemann and Noble[12])

Sodium-calcium exchange: its stoichiometry

When we started our work, estimates for the stoichiometry of the exchange process included 2:1 (Na:Ca), which was the original Reuter and Seitz[26] hypothesis, implying an electroneutral exchange; 2.5:1 (see Fozzard[9]); 3:1 (see e.g. Reeves[27]) and 4:1 (see Mullins[21]). Our first task therefore was to see what values would be consistent with the data on intracellular Na and Ca ion activities. It was easy to satisfy ourselves that 4:1 would generate either too low a level of Ca_i or would require a more substantial membrane Ca leak. Equally, a ratio of 2:1 was inadequate to produce a low enough level of Ca_i without supposing that the latter was primarily determined by a sarcolemmal calcium pump. The evidence favoured the view that the latter, while showing a high Ca_i affinity, has a relatively low capacity compared to the exchange process.

So, we were left with the choice between 3:1 and 2.5:1 or some other non-integer value. Our approach was to start off with the suspicion that the real ratio was an integer and then see how the model might explain an apparent non-integer value. We therefore used a real stoichiometry of 3:1 and it was then easy to show that, with a surface calcium leak, this leads to non-integer values less than 3 for the apparent stoichiometry using steady state calculations of Na_i and Ca_i (DiFrancesco, Hart and Noble, unpublished). The reason is very simple. The calcium leak keeps $[Ca]_i$ higher than the exchange would produce at strict thermodynamic equilibrium, and this effect weakens the influence of the sodium gradient on the calcium gradient, particularly at very low values of $[Ca]_i$.

The current-voltage relation for the exchange process

Having settled on a suibtable stoichiometry, the voltage dependence of the exchange process was required. The elegant patch clamp work of Kimura, Noma and Irisawa[15] was not then available. Our approach therefore was to see whether we could simplify the Mullins[20] model to produce a plausible description of the voltage dependence that would satisfy one of our key experimental requirements. This was that slow inward currents of the kind that might be attributable to the exchange current had been found at relatively negative voltages but not, normally, at positive voltages[22]. This implied that the mode of the exchange activated by internal calcium and moving sodium inwards was strongly voltage dependent and that the process will carry most current at negative potentials.

It turned out to be easy to make some simplifying assumptions that made the Mullins model give this type of voltage dependence. The equation we used was

$$i_{NaCa} = \frac{k_{NaCa}\left(\exp\left(\gamma\frac{EF}{RT}\right)[Na]_i^3[Ca]_o - \exp\left(-(1-\gamma)\frac{EF}{RT}\right)[Na]_o^3[Ca]_i\right)}{1+d_{NaCa}([Ca]_i[Na]_o^3 + [Ca]_o[Na]_i^3)}$$

where k_{NaCa} and d_{NaCa} are scaling factors and γ is a partition parameter that we introduced that divides the influence of the electrical field between the inward and outward movements of the bound carrier across the membrane. We usually used 0.5 for the value of γ. It turns out that this equation is quite a good fit to the experimental results of Kimura et al.[15] (though their results suggest a value of γ nearer 0.65) but I should emphasize that we did not know of those results when we formulated our equation. Nor would we defend the equation other than on practical grounds. There are certainly other simplifications, for example of the Johnson-Kootsey[14] model, that give equally good or even better results (Hilgemann, pers. comm.).

Nevertheless, for many purposes, the simplification we chose is clearly close to the actual experimental situation, which is that inward movement of sodium in exchange for outflow of calcium shows a nearly exponential dependence on membrane potential.

This means that the conditions for detecting the flow of exchange current during electrical activity in cardiac cells have to be chosen with care. Strong depolarizations will produce a large calcium current and a relatively small exchange current in response to the intracellular calcium transient. Very weak depolarizations may fail to initiate calcium

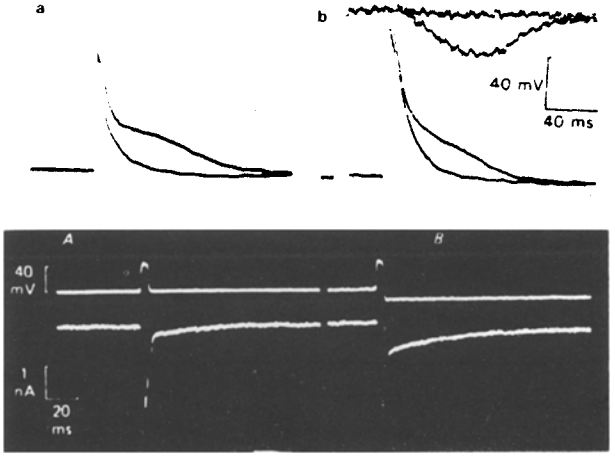

Figure 3. The late phase of repolarization in rat ventricle and the slow inward current underlying it. Upper traces: (*a*) Superimposed action potentials in normal solution and in a solution in which 89% of sodium ions have been replaced by lithium ions (shortened action potential). (*b*) Abolition of late phase of action potential (lower traces) and of contraction (upper traces) by 3-min exposure to 1 μM ryanodine. (From Mitchell et al.[18]) Lower traces: Slow current tails recorded on depolarization from −40 to 0 mV followed by repolarization to −40 mV (A) or −60 mV (B). (From Mitchell et al.[19])

release. The critical area therefore is close to, and even below, the calcium current threshold. Calcium release from the SR may still occur but the calcium current itself will be relatively small.

Figure 1 shows the results obtained during voltage clamp depolarizations using the DiFrancesco-Noble[4] model. As expected, a slow inward flow of exchange current dominates the computed total ionic current near the calcium current threshold but not at other ranges of potential. It is in just this range that slow current dependent on intracellular calcium release (and therefore eliminated when this release is blocked by tetracaine) can be observed experimentally in Purkinje fibres[5].

Functional role of the exchange process during normal electrical activity

The question that then arises is whether the flow of such a current is of any functional importance during normal electrical activity. It was in order to answer this question that Hilgemann and I decided to use the modelling approach in combination with electrical and flux measurements to see whether the current flow and net membrane calcium flux movements could be correlated and, if so, whether we could predict the relative magnitudes of the calcium current and the sodium-calcium exchange current. The impetus for this project came in a large part from his own experimental work measuring the net membrane Ca^{2+} movements by recording extracellular Ca^{2+} depletion and accumulation using the extracellular Ca^{2+} marker tetramethylmurexide[10, 11]. The other experimental point of departure was work in the Oxford laboratory designed to measure the quantitiy of sodium-calcium exchange current flowing during the action potential[6, 8]. I have to confess that, initially, I had not noticed the obvious connection between these two experimental approaches. The reason is that Hilgemann first drew my attention to his own work at a time (January 1985) when that attention was diverted by the financial crisis that has had such damaging effects on scientific work in Britain today[23]. Fortunately, he persisted in insisting that we should look at the problem and came to my laboratory to see whether the DiFrancesco-Noble model would predict the net Ca^{2+} movements he had been measuring. It did in a semiquantitative way, and that was sufficient encouragement for us to embark on a more ambitious project, which was to extend that model to refine its analysis of calcium movements inside the cell. This was done by making the following major changes:

1) The buffering of calcium by calmodulin and by troponin was included.

2) The calcium sequestration and release equations for the sarcoplasmic reticulum were completely reformulated. The aims here were (a) to reproduce the much faster $[Ca]_i$ transients observed experimentally (time to peak near 10–15 ms – rather then 50 ms as in the DiFrancesco-Noble model), and (b) to reproduce, at least qualitatively many of the properties of the SR described in Fabiato's work (see Hilgemann and Noble[12] for further explanation of these refinements).

3) The equations for calcium dependent inactivation of the calcium channel were improved. In particular, Ca and voltage dependent inactivation processes were arranged in series rather than in parallel. This avoids some of the defects of the DiFrancesco-Noble formulation.

4) Since calcium binding to troponin was represented, it was not too difficult to add equations for the activation of contraction. Much of Hilgemann's[10, 11] own work was done on rabbit atrium, so we started from the rabbit sinus mode model[24] and, by eliminating i_f and adding i_{K1}, converted it in to a rabbit atrial model.

Figure 2 shows one of our results. The inset shows the atrial action potential, together with contraction and the extracellular calcium transient $[Ca]_o$. The main panel shows our reconstructed action potential, the intracellular and extracellular calcium transients and the computed contraction. Clearly, the extracellular calcium transient is well reproduced. During the first 20–30 ms the calcium current produces depletion of $[Ca]_o$, after which there is net calcium exit from the cell. In the model, this calcium efflux is attributable to the sodium-calcium exchange process.

The functional role of the exchange is therefore to drive calcium out of the cell during the action potential, not after it, as has often been assumed[21]. At least, this seems to be the situation in mammalian heart muscle. The situation in amphibian heart, where reticulum calcium release is not important, is different[2].

Relative magnitudes of i_{Ca} and i_{NaCa}

From this reconstruction, we can derive the relative amplitudes of the current carried by the calcium exchange and the calcium channels. The result is that the peak value of the inward exchange current would be about 7–10% of the peak value of the calcium current and that the time integral of i_{NaCa} would be 50% of the time integral of i_{Ca}. The ratio of time integrals is, of course, determined by two major assumptions made in the model: 1) The Na:Ca stoichiometry is 3:1, and 2) the exchange is the major route for calcium exit during the action potential.

Since the charge carried inwards to move calcium out of the cell is 1, whereas each calcium moving through the calcium channel will carry two charges, the integrals are then necessarily in the ratio 0.5:1. This result would be fairly insecure if it depended only on the net calcium fluxes and the modelling work since it is by no means necessary that the exchange process should be the major route for calcium exit during the action potential. A sarcolemmal calcium pump is an obvious alternative, and one which we have also explored[12]. But there is a way of testing the conclusion and that is to compare the predicted relative amplitudes of i_{Ca} and i_{NaCa} with those measured experimentally. Fedida, Noble, Shimoni and Spindler[8] have measured calcium current and Ca_i-activated inward current in single guinea pig ventricular myocytes and the ratio between the peak amplitudes is indeed around 10:1.

Influence of inotropic state on electrical activity

The flow of a current activated by Ca_i will necessarily perturb the electrical potential change during the course of the action potential. Trevor Powell and his colleagues have already demonstrated the importance of this phenomenon in rat ventricular cells[18, 19]. Their conclusion is that the late phase of the rat action potential is almost entirely attributable to Ca_i-activated current. Schouten and Ter Keurs[28] reached a similar conclusion from their work.

Figure 3 shows the evidence from Mitchell et al.[18, 19]. The recordings of action potential contraction and current were taken from Mitchell et al.'s papers and rearranged by Noble and Powell[25]. The late plateau is in this case virtually abolished when extracellular sodium is reduced (left) or when the contraction is abolished by ryanodine (right). Figure 4 shows a reconstruction of the low sodium experiment using the model. The inset shows experimental results from Schouten and Ter Keurs[28]. The main panel shows the reconstructed result from Hilgemann and Noble[12].

Conclusions

Reconstruction of the flow of sodium-calcium exchange current has provided a pivot in this work around which to construct a model which, for the first time, integrates the surface membrane processes with descriptions of intracellular calcium movements and, in turn, their influence on surface membrane currents. It should therefore be possible to see whether the results of Wood, Heppner and Weidmann[32] can be accounted for.

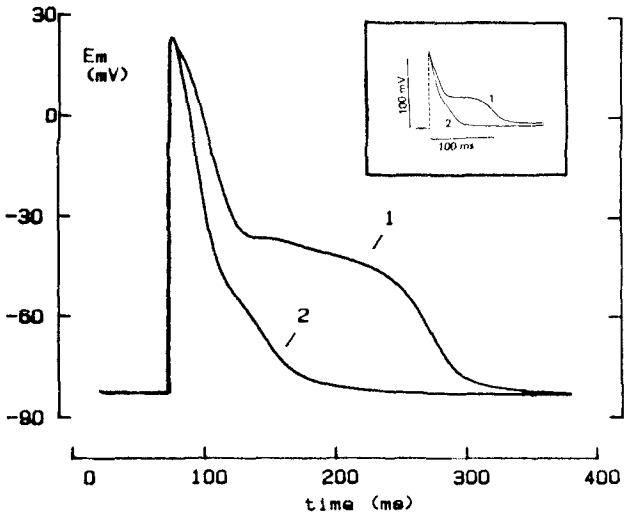

Figure 4. The inset (top right) shows experimental results of Schouten and Ter Keurs[28] from rat ventricle. The simulated results using a model developed from the Hilgemann-Noble atrial model are shown as the main figure. Curves marked 1 correspond to low frequency stimulation which gives larger contractions and a markedly enhanced final low plateau phase. The curves marked 2 are after substitution of lithium for extracellular sodium. In both experimental and computed results the late slow plateau is almost completely abolished by this substitution. (From Hilgemann and Noble[12])

In principle, it is clear that small changes in membrane potential during electrical activity will have an effect on calcium storage. For example, repolarization must cause calcium to leave the cell more rapidly since the inward exchange current is then larger. Pulses applied during one action potential might for this reason alone influence the inotropic state during subsequent contractions by altering the ratio of calcium moved out of the cell to calcium pumped into the reticulum. I had intended, therefore, for this symposium, to see whether the model might reproduce the Wood, Heppner and Weidmann results. In the event, I decided against doing this since there are still some very major uncertainties. For, although we clearly do now have plausible models of electrical activity of the kind shown in the atrium, and the very similar responses seen in rat ventricle, it has to be admitted that we do not yet have a plausible model for ventricular action potentials of the kind seen in most mammalian hearts, and in particular for the species used by Wood, Heppner and Weidmann.

The uncertainties here arise from the fact that we do not know whether the plateau in these cases is maintained entirely by the main calcium channel (the L channel generating what in our reconstruction work we have called $i_{Ca,fast}$), together with any exchange current that may flow, or whether there is also significant activation of a separate maintained calcium channel, which we call $i_{Ca,s}$, of the kind described by Lee, Noble, Lee and Spindler[16].

This uncertainty is important not only to the reconstruction of the ventricular action potential but is also crucial to the reconstruction of the effect of current perturbations, since $i_{Ca,s}$ may have a crucial role as a 'loading' current, i.e. it may serve not only to help maintain the plateau but also to load the sarcoplasmic reticulum, as suggested by Fabiato's work. I have also to draw attention to a second major uncertainty. If $i_{Ca,s}$ is a loading current, $i_{Ca,fast}$ (i.e. L channel current, plus perhaps T channel current) has usually been thought of as a 'triggering' current, whose role is primarily to activate calcium-induced calicum release from the SR. This would explain the well known close correlation between i_{Ca} and contraction, despite the fact that the majority of activation of contraction in the mammal arises from SR Ca^{2+} release. This close correlation though might merely be a *steady state* correlation. We have found recently[6] that, by making very rapid application of calcium current blockers to single cells, the majority of i_{Ca} can be blocked without initially altering Ca_i-dependent inward current. Since the appearance of that abstract we have confirmed that the contraction also is initially unaffected. Conversely, catecholamines first increase i_{Ca} without altering the slow Ca_i-dependent current[7].

Clearly there is still much to be learnt about excitation contraction coupling before we are fully ready to reconstruct Wood, Heppner and Weidmann's[32] results.

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Mechanical parameters determined in dispersed ventricular heart cells

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Summary. A high-resolution laser diffraction system suitable for studying the basic mechanical properties of small contractile single cells has been developed. This method was used to establish the mechanical behavior of 95 ventricular cells isolated from adult guinea pig hearts. During contraction, the sarcomere length shortened from 1828 ± 43 nm (mean ± SD) to 1518 ± 99 nm. The maximal velocities were 1.98 ± 0.64 μm/s for shortening and 1.93 ± 0.54 μm/s for re-lengthening. The twitch duration from 20% shortening to 80% re-lengthening was 622 ± 120 ms.
Key words. Myocardial contraction; myocytes; sarcomere length; laser diffraction.

Introduction

Single cells isolated enzymatically from adult hearts represent a useful model for electrophysiological studies. They offer several advantages over multicellular preparations[5]. They have been used successfully to investigate the electrical behavior of the cell membrane as well as the function of individual ionic channels[4]. However, to date little is known about the mechanical properties of unloaded isolated myocytes[6, 9, 12]. This situation appears to be mainly due to technical limitations. Since no reliable and easy method for studying the mechanical properties was readily available, an opto-electronic system based on the principles of laser diffraction was developed. This system enabled the measurement of sarcomere length at rest as well as during contraction. The aim of this study was to assess the basic mechanical properties of enzymatically isolated mammalian myocytes under control conditions.

Methods

Cell isolation and experimental conditions

Cells were isolated from adult guinea pig ventricles by means of an enzymatic procedure as described by Kao[7] and modified for guinea pigs[11]. Briefly, adult guinea pigs were anesthetized with ether, killed by cervical dislocation, and the hearts excised quickly. The aorta was mounted on a plastic cannula and the heart perfused in a retrograde fashion with a cardioplegic solution containing 50 μmol/l Ca^{2+}. After this period, the perfusate was replaced with a solution containing collagenase (100 mg/100 ml) and hyaluronidase (100 mg/100 ml). Following isolation, the cells were allowed to recover for an hour suspended in standard bathing solution (see below). To perform an experiment, a small aliquot containing about 100 cells was transferred to the experimental chamber (volume: 1 ml). Once the cells had settled down, superfusion was started (flow rate: 3 ml/min). The chamber consisted of a circular Perspex frame with a glued-on glass bottom, fixed to a revolving device, and mounted on the stage of an inverted phase-contrast microscope (Diavert, Leitz, Wetzlar, FRG). An air isolated table-top system (Technical Manufacturing Corp., Peabody, MA, USA) prevented interferences from ground vibrations.

The composition of the bathing solution was as follows (in mmol/l): Na^+, 137; K^+, 5.4; Ca^{2+}, 1.8; Mg^{2+}, 0.5; Cl^-, 147; Glucose, 5.5; HEPES, 5; pH 7.4 (adjusted with NaOH). Temperature: 22–23 °C.

Laser diffraction measurements

To measure the sarcomere length of isolated myocytes at rest and during contraction the principle of laser diffraction was used. In order to do so, an opto-electronic system was developed which was suitable for small cells. Technical details and performance of the system have been described elsewhere[13] and will only be summarized briefly here. The beam diameter of a polarized 5 mW He-Ne laser (Melles-Griot, Irvine, CA, USA) was reduced to 50 μm using the modified fluorescence adaptor of the inverted microscope. After intensity adjustment by means of a polarizing filter, the beam passed through the cell under investigation, which acted as a diffraction grating. The resulting diffraction pattern was projected on to a high resolution linear image sensor (CCD-Camera. Texas Instruments, Dallas, TX, USA). A custom built electronic circuit determined the position of the first order diffraction maximum every 2 ms and delivered an analog voltage signal proportional to the sarcomere length. This signal was digitized by means of a transient recorder (W.+W., Basel, Switzerland) and stored by microcomputer for later analysis. Calibration of the opto-electronic device was performed by means of two standardized diffraction gratings