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Confocal microscopic detection of potential-sensitive dyes used to reveal loss of voltage control during patch-clamp experiments

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Abstract We used a fast, fluorescent, potential-sensitive indicator (Di-8-ANEPPS) in combination with laser-scanning confocal microscopy in the line-scan mode (temporal resolution 500 Hz) to independently determine the transmembrane potential in voltage-clamped cells. While a linear relation between command voltage and Di-8-ANEPPS fluorescence was found in unexcitable Sf9 cells, pronounced nonlinearities were observed in cardiac myocytes. Comparison of the fluorescence records and current traces indicated that most of the observed nonlinearities could be attributed to voltage-escape during flow of membrane current. Voltage-escape during large membrane currents may lead to various experimental difficulties during voltage-clamp experiments. The voltage recording technique based on fluorescence was then used to compare the voltage-escape during flow of Na^+ and Li^+ ions via voltage-dependent (TTX sensitive) Na^+ channels in cardiac myocytes. In these experiments, no significant differences in the degree of voltage-escape was found, suggesting that the two currents were similar in amplitude. In addition to the application presented in this paper, confocal microscopic detection of transmembrane potential with fluorescent dyes may be a useful technique for experiments in preparations that are difficult to impale with microelectrodes because of their small size.

Key words Confocal microscopy · Voltage clamp · Voltage-sensitive fluorescent indicators · Cardiac muscle · Di-8-ANEPPS

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Introduction

During the last few years a range of fluorescent potential-sensitive probes has been developed for measuring the transmembrane potential in various preparations, particularly in very small specimens in which voltage measurements with conventional microelectrode techniques are impossible or very difficult [13]. Signals from fluorescent, potential-sensitive probes are usually detected with photodiodes at a single site or with an array of photodiodes from multiple sites within a preparation [2, 4, 11]. Recently, laser-scanning confocal microscopes have become commercially available and have been successfully used for fluorescent indicator imaging studies. Confocal microscopy offers several potential advantages over conventional video systems for fluorescence imaging. Firstly, images can be recorded without the blurring effect of out-of-focus light encountered in conventional fluorescence imaging. This is possible by virtue of a pinhole in front of the light detector that blocks out-of-focus light and allows confocal optical sectioning. Secondly, the scanning process is very flexible in most confocal instruments. For example, instead of recording two-dimensional images, a single line can be repeatedly scanned at high frequency. The resulting line-scan images have a high temporal resolution (up to 500 Hz on our instrument) while still maintaining the spatial information in one dimension.

In the present study we used the confocal microscope in the line-scan mode to record independently the membrane potential with the fluorescent potential sensitive probe Di-8-ANEPPS, while simultaneously controlling the membrane voltage with the patch-clamp technique in the whole-cell mode. Signals recorded from unexcitable cells were compared with those measured in isolated cardiac ventricular cells. Voltage-clamp protocols designed to elicit and measure Ca^{2+} current (I_{Ca}) and Na^+ current (I_{Na}) were applied to both types of cells. Unlike the voltage-clamp technique, the potential sensitive indicators do not sense the transmembrane voltage between the extracellular space and the bulk cytosol and are also not af-

fected by the series resistance of the recording electrode. Instead, the real transmembrane potential (including the surface charges) can be monitored directly. We were therefore able to compare the voltage-clamp command voltage with the real transmembrane potential and to resolve directly any voltage escape due to membrane current flow. We also found that voltage escape induced by the current via Na^+ channels is identical for the charge carriers Na^+ and Li^+, suggesting that both currents are of similar amplitude. Preliminary results have been presented to the Biophysical Society [10].

Materials and Methods

Cell isolation

Cells derived from the insect *Spodoptera frugiperda* (Sf9 cells) were grown in Grace's medium at 28°C in culture flasks according to standard techniques [12]. Cells were suspended and a small aliquot of cell suspension transferred into a superfusion chamber on the stage of an inverted microscope immediately before an experiment. Ventricular heart muscle cells were isolated from guinea-pig ventricles by established enzymatic methods [6].

Solutions

Cells were superfused with a solution containing (mM): NaCl 140; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)/NaOH 5; glucose 10; pH 7.4. For Sf9 cells glucose 20 mM was added to match the osmolarity of the culture medium (350 mosmol/l). Preincubation in ryanodine (10 μM) was used to eliminate Ca^{2+} release from the SR and to minimize cell movement of cardiac myocytes. Pipettes were pulled from borosilicate glass tubing (Clark Electromedical Instruments, Reading, UK) to resistances of 0.8–1.2 MΩ on a horizontal puller engineered for high tip-diameter reproducibility (Zeitz, Augsburg, Germany). The pipette filling solution contained (mM): Cs-aspartate 120; adenosine triphosphate, Mg salt (Mg-ATP) 5; HEPES/CsOH 20; ethylenebis(oxonitrilo)tetraacetic acid (EGTA) 0.1; pH 7.2. Di-8-ANEPPS 5 μM (Molecular Probes, Eugene, Ore., USA) was added from concentrated 7.5 mM stocks in dimethylsulphoxide and 25% w/w Pluronic. Cells were superfused with dye-containing solution for about 5 minutes after the whole-cell configuration had been established. All experiments were performed at room temperature (20–22°C).

Voltage measurements

The transmembrane potential in voltage-clamped cells as reported by fast potential sensitive dyes was recorded with a laser-scanning confocal microscope (MRC600, Bio-Rad, Glattbrugg, Switzerland) in the line-scan mode at scanning intervals of either 6 ms/line or 2 ms/line (i.e. much slower than the response time of the dye [11]). The setup and line-scan mode have been described in detail elsewhere [5]. To resolve the small fractional fluorescence change of these indicators with the limited dynamic range of the confocal microscope (8 bit), a large, but constant, negative offset was applied. The dyes were excited at 514 nm and the emission was recorded at wavelengths greater than 600 nm. The membrane surface from which fluorescence emission was collected corresponded to less than 50 μm^2. An Axopatch 200 (Axon Instruments, Foster City, Calif., USA) voltage-clamp amplifier was used to deliver the voltage-protocol to the cells and to record membrane currents. The pipette series resistance was not compensated electronically.

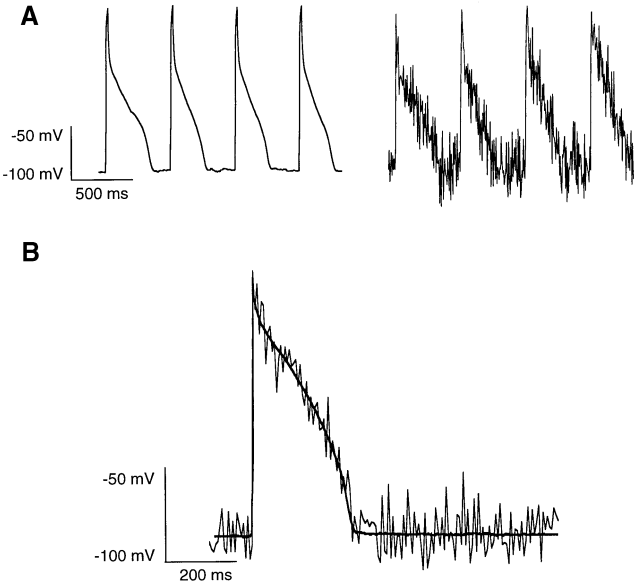

Fig. 1A, B Action potentials recorded in a ventricular myocyte. The membrane potential was simultaneously recorded as the fluorescence from the potential-sensitive indicator Di-8-ANEPPS and with a voltage-clamp amplifier in the current-clamp mode. Current pulses (5 ms) were applied to trigger a train of action potentials. An action potential recorded with the confocal microscope is superimposed on the identical signal measured with the current-clamp in **B**

Results

The fractional fluorescence change of the most sensitive potential-sensitive dyes available is in the range of about 10% per 100 mV [11]. This compares very unfavorably to a more than 40-fold dynamic range for fluorescent Ca^{2+} indicators like fluo-3 [8]. Since the maximal dynamic range of most confocal systems, including the Bio-Rad MRC600, is limited to 256 levels of grey (8 bit), one might expect considerable difficulties in detecting these small fluorescence changes. We therefore first carried out some experiments in freshly isolated ventricular myocytes using the current-clamp mode of the voltage-clamp amplifier. Series of action-potentials were triggered by brief current injections (5 ms) while the fluorescence signals were monitored in the line-scan mode for high temporal resolution. With this mode of the confocal microscope, we can increase the temporal resolution up to 2 ms, but we need to sacrifice one spatial dimension (for a detailed description of the method see [5]). Surprisingly, we could recognize readily the cardiac action potentials in the line-scan images. To achieve better matching of the dynamic range of the confocal microscope with the small fractional fluorescence change we introduced a large, but constant, negative offset to the photomultiplier current. Figure 1 shows a series of action potentials recorded simultaneously with both techniques, patch-clamp and fluorescence. For clarity, photobleaching has already been removed mathematically from the fluorescence records (see below for a description of this

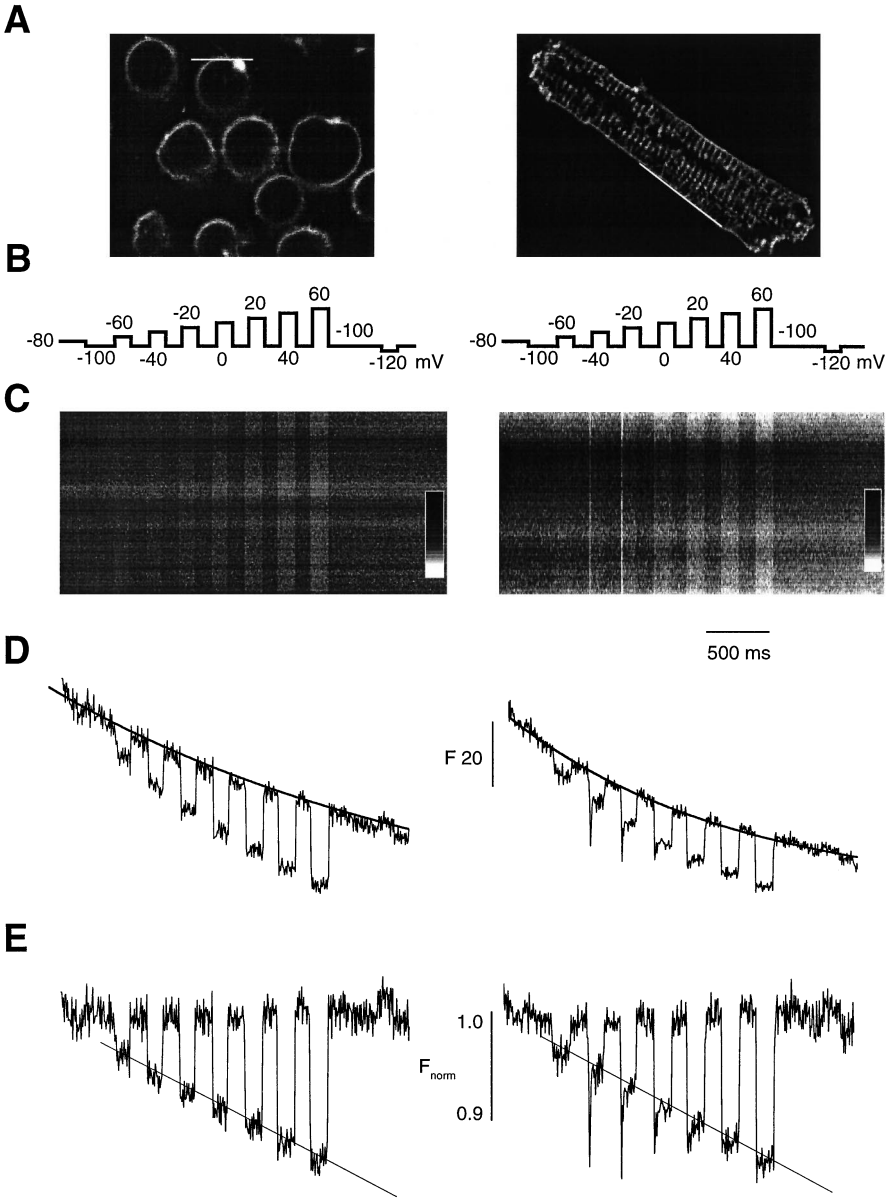

Fig. 2A–E Membrane potential changes in Sf9 cells and cardiac myocytes during voltage-clamp depolarizations. Confocal optical sections of Sf9 cells (**A** *left*) and a cardiac ventricular myocyte stained with Di-8-ANEPPS (**A** *right*). While the Sf9 cells are almost perfectly spherical, the T-tubular network is clearly visible in the ventricular cell. The *white lines* indicate position of line-scans (after zooming in and adjusting the cell direction). **B** A voltage protocol was applied to an Sf9 cell (**B–E**, *left panels*) and an isolated heart muscle cell (**B–E**, *right panels*). **C** The membrane potential was recorded with the confocal microscope in the line-scan mode (6 ms per line). A non-linear look-up table was chosen to emphasize the small fluorescence changes. The *wedge* corresponds to grey-scale values from 0 to 255. The raw fluorescence traces (**D**) were corrected for bleaching by normalizing (**E**) with an exponential function fitted to the resting values. While the fluorescence change was linearly related to the clamp potential in the unexcitable Sf9 cells, spikes and non-linearities were obvious in the raw line-scan image (**C**) as well as in the fluorescence trace recorded from the ventricular myocyte (**D** and **E**, *right panel*)

procedure). To allow a direct comparison, we have superimposed an action potential recorded by either method in Figure 1B. All fluorescence signals are original records without averaging.

In the next series of experiments we recorded the fluorescence changes associated with voltage-clamp depolarizations from unexcitable Sf9 insect cells and compared these signals with those recorded from cardiac ventricular myocytes. Figure 2 summarizes the results. Confocal optical sections of either cell type are shown in Fig. 2A. It is evident that the Sf9 cells have a spherical cell body with a diameter of approximately 15 μm. In the cardiac myocyte, all membranes accessible from the extracellular space are stained (i.e. also the T-tubules). Note that intracellular membranes (e.g. the nuclear envelopes) are not fluorescent, suggesting that the lipophilic Di-8-ANEPPS does not readily permeate into the cell. Figure 2B and C illustrates the applied voltage-clamp protocol and the resulting line-scan image, where time runs horizontally and the remaining spatial dimension vertically. Depolarizations of increasing amplitude resulted in corresponding changes in fluorescence, shown as bright bands in Fig. 2C. The line-scan images have been inverted and contrast enhanced for better reproduction in print. The grey-scale wedge was linear (from 0 to 255) before the images were processed and is included to reveal the degree of image processing. While the fluorescence changes appeared to be linearly related to the applied voltage in the Sf9 cell, a different behavior was observed in the cardiac myocyte. Early during depolarizations to –40 mV and –20 mV a much larger change in fluorescence was observed than later during the same voltage-clamp step. This is clearly visible as two bright vertical bands in the line scan image (Fig. 2C) and as corresponding voltage spikes in the line plots derived from this image (Fig. 2D and E). Figure 2E represents

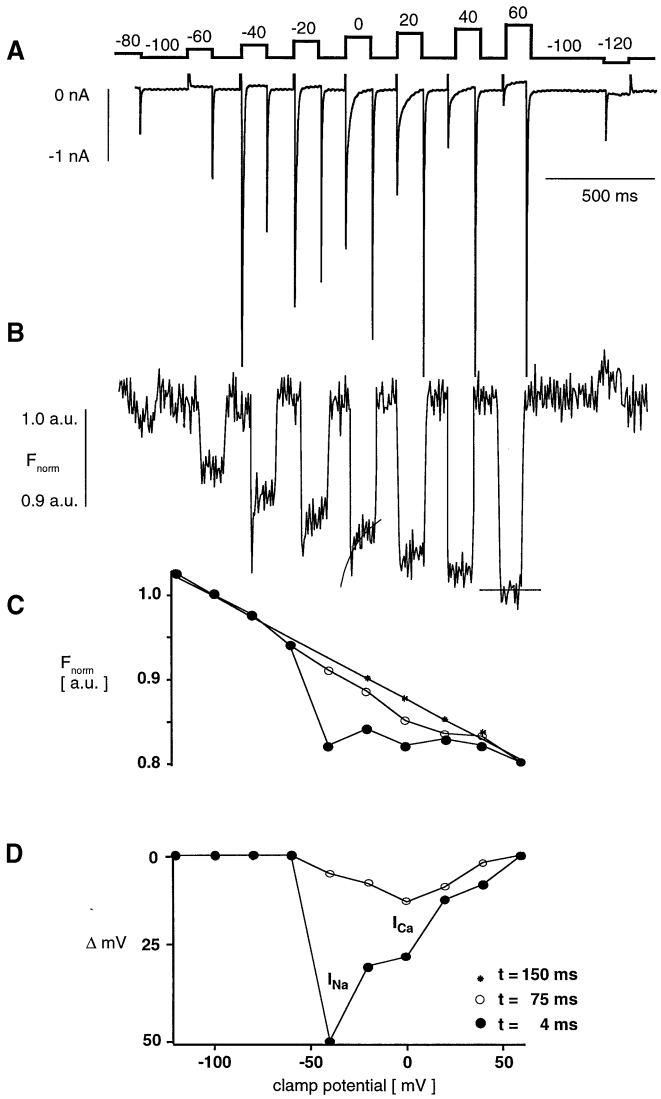

Fig. 3A–D Membrane currents and transmembrane potentials recorded in ventricular myocytes. **A** The membrane potential was controlled with a voltage-clamp amplifier to measure membrane current in the whole-cell mode while the transmembrane potential was simultaneously recorded with the fluorescent indicator Di-8-ANEPPS. **B** The spikes in the fluorescence signal were related to inward sodium current (I_{Na}) while the later nonlinearities in the fluorescence/voltage relationship appeared to be more correlated with the L-type calcium current. This is summarized in **C** where the fluorescence/voltage relationship of the peak-current (●), the current after 75 ms (○) and the steady-state current after 150 ms (*) are plotted. The fluorescence was converted into membrane potential to construct panel **D**. The voltage dependence of the escape voltage (ΔmV) at different times during the pulse closely resembled the current/voltage relationships of the sodium and calcium currents (I_{Na} and I_{Ca}), respectively

the fluorescence trace after correcting the signal for photobleaching. This was realized by first fitting a monoexponential function to the –100-mV segments of the trace and then normalizing with this exponential function. The actual rate of photobleaching is difficult to determine from these traces because of the negative offset present in the raw data. However, assuming a 10% fractional fluorescence change per 100 mV we can estimate the degree of photobleaching to be about 25% after 3 s.

The voltage spikes observed in cardiac myocytes during the depolarizations to intermediate voltages could reflect loss of voltage-clamp control during I_{Na} and I_{Ca} [1]. This possibility was investigated with the combined recordings of membrane currents and fluorescence changes shown in Figure 3 A and B. The spikes and nonlinearities clearly followed the corresponding current flow via I_{Na} and I_{Ca}. The phenomena were further analysed by determining the voltage dependence of the divergence between clamp-potential and transmembrane potential recorded with Di-8-ANEPPS. For this purpose, the fluorescence was determined at the peak of the spike (●), after 75 ms (○) and at the end of the pulse (150 ms, *). The conversion of fluorescence to potential assumes that the voltage error due to current flow was minimal at the end of the voltage-clamp pulse. Using this procedure, the deviations from linearity were found to be largest immediately after the depolarization (●), somewhat smaller after 75 ms (○) and almost absent after 150 ms (*). In Fig. 3D the differences between the steady-state values at 150 ms and the fluorescence signals at 4 and 75 ms were converted into voltages and are shown as a function of the clamp potential. The two plots closely resemble the current/voltage relationship for the I_{Na} (●) and the I_{Ca} (○), suggesting that the deviation is proportional to the flow of current. The most likely explanation for the fluorescence and voltage spikes observed in cardiac myocytes is thus loss of voltage control and consequently voltage escape during the large currents. Indeed, voltage escape of similar amplitude and duration has also been detected with a two-electrode voltage-clamp approach [1, 3]. Although I_{Ca} may contribute to the I_{Na} spike, it is unlikely to contaminate it significantly, given that I_{Na} is many times larger than I_{Ca} and that I_{Ca} is not yet fully activated after 4 ms.

In a previous study, we replaced Na^+ with Li^+ to produce comparable voltage escape under both conditions during I_{Na} [6]. The purpose of these experiments was to exclude the activation of Ca^{2+} channels by voltage-escape during I_{Na} as a source for triggering Ca^{2+} entry into the cell. In this previous study we measured similar current amplitudes for I_{Na} and the Li^+ current I_{Li} and thus assumed that the escape was most likely also similar. In the present study we now have the possibility to determine directly voltage escape with the voltage-sensitive dyes. In this series of experiments we depolarized cardiac myocytes from –100 mV to –40 mV in the presence of Na^+ or Li^+ (Fig. 4A). Brief and large inward currents of almost identical amplitude and duration could be measured under both conditions. Averaged fluorescence measurements again revealed the expected escape early during the depolarization (Fig. 4B). Superimposing the two averaged fluorescence signals indicates that the fluorescence change during I_{Li} is not different from that during I_{Na} (Fig. 4C). This provides direct evidence for our earlier assumption that voltage escape is similar during I_{Li} and I_{Na}.

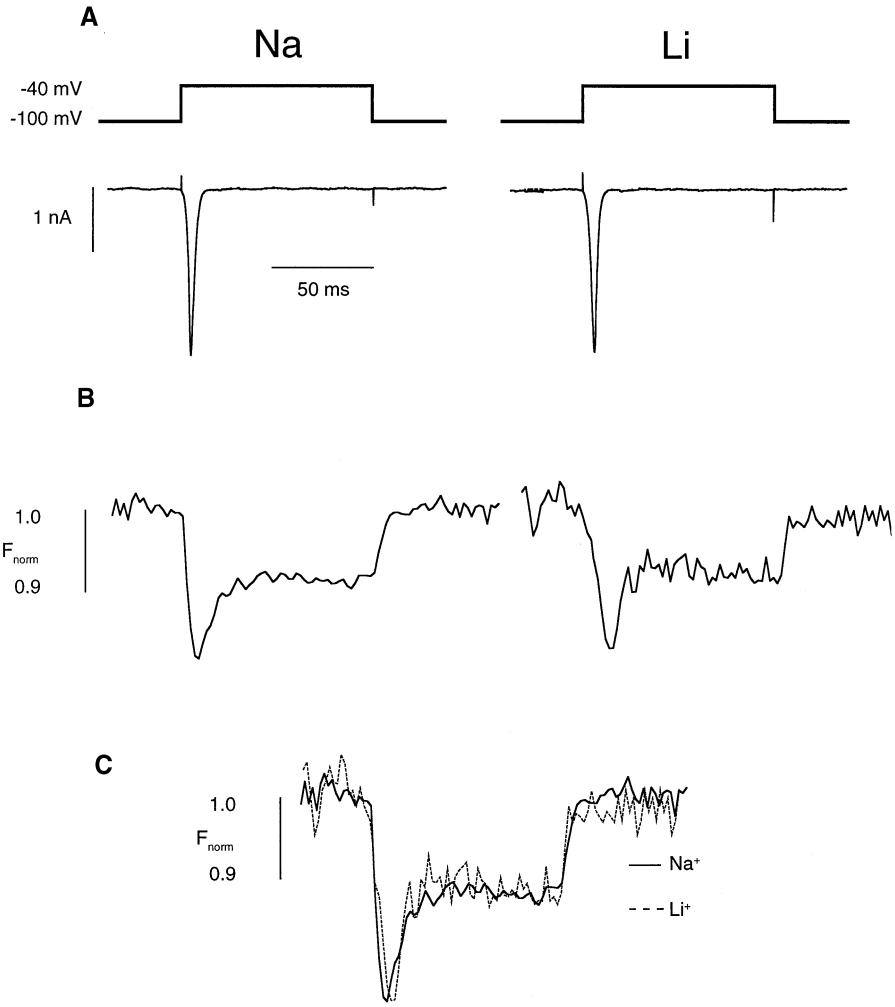

Fig. 4A–C Transmembrane potentials recorded in ventricular myocytes during I_{Na} and Li^+ current (I_{Li}). **A** Shows whole-cell currents in Na^+-containing and in Na^+-free solutions (substitution by Li^+) after changing the membrane potential from –100 mV to –40 mV. An average of 16 traces is shown for the fluorescence signal during I_{Na} (**B** *left panel*), while eight sweeps were averaged for I_{Na} in zero Na^+ (**B** *right panel*). No significant difference was revealed when the two traces were superimposed (**C**). This finding suggests that the voltage escape during I_{Li} is not significantly different from that during I_{Na}

Discussion

While confocal imaging has already been used to record the fluorescence from "slow" voltage-sensitive dyes with temporal resolution in the range of seconds [7], this is the first application of this technique on a millisecond time scale. The increased temporal resolution made possible by the line-scan mode of confocal microscopes greatly broadens the suitability of this approach since it enables recording voltage signals from excitable cells that typically show responses in the millisecond time domain. However, before applying the technique presented in this paper a number of technical points need to be considered and it is worthwhile comparing this approach with the conventional photodiode technique. Since the confocal microscope records only from a thin optical section, only a small patch of the cell membrane can be used for a given line scan. This peculiarity results in disadvantages as well as advantages. As a disadvantage, the small useful membrane area puts constraints on the number of fluorescence photons that can be collected and on the signal-to-noise ratio that can be achieved. In confocal microscopes, the signal quality depends on photon statistics and thus ultimately on the number of detected photons per time, which in turn depends on the illuminated membrane area. The vertical thickness of the confocal optical section is less than 1 μm for a high numerical aperture lens, also depending on the pin-hole diameter [9]. To record the fluorescence from Sf9 cells we usually selected a line tangential to the spherical cell, in cardiac myocytes a line parallel to the cell boundary was chosen. On average we had a membrane area of around 5 μm^2 available. From this area we could record fluorescence signals with a temporal resolution of ≈500 Hz. Should an experiment require much higher temporal resolution, confocal microscopic detection may not be the ideal method. However, the small illuminated membrane area also has advantages. Only a small fraction of the total cell surface was illuminated and bleached. Because the photo degradation products of voltage-sensitive dyes are known to be very toxic this turned out to be very favorable [11]. In a conventional setup the duration of an experiment is typically limited to a total illumination period of only a few seconds. However, we were able to perform experiments lasting some 30 min (total illumination duration about 5 min). In conclusion, if experiments over extended periods of time at moderate temporal resolution are intended, confocal microscopy may be the imaging

method of choice for voltage-sensitive fluorescent indicators.

Di-8-ANEPPS is believed to partition preferentially into the outer leaflet of the the cell membrane and to permeate only very slowly into the cell interior. Consistent with this, we found that only Di-4-ANEPPS stained the nuclear membranes in Sf9 cells and cardiac myocytes (not shown), while Di-8-ANEPPS, with the longer lipophilic tail, did not (see Fig. 2A).

Di-8-ANEPPS has also been used in a dual-wavelength excitation mode [4] to measure the membrane potential ratiometrically. This approach, widely used with Ca^{2+} indicators like Fura-2, reduces problems with fluorescence changes (e.g. due to photobleaching) and generally improves the signals. While not impossible, such measurements may be more difficult with laser-scanning confocal microscopes than with wide-field illumination. It requires the rapid switching of two excitation wavelengths from a multi-line laser or the perfect alignment of two laser light sources.

Changes of cytosolic [Ca^{2+}] may also be expected to modify the transmembrane potential by screening surface charges. These voltage changes, although small, could be sensed by the voltage-sensitive dyes located within the membrane. However, with the present approach we were not able to detect such signals during Ca^{2+} transients, presumably these surface charge screening effects are too small.

In summary, we used a combination of laser-scanning confocal microscopy and potential-sensitive dyes to analyse the membrane potential of cardiac myocytes. This approach may be useful also for determining the membrane potential in other excitable cells with good temporal and spatial resolution, particularly in cells posing experimental difficulties due to their small size.

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