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Fluorescent styryl dyes applied as fast optical probes of cardiac action potential

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Abstract. Several styryl dyes were tested as fast optical probes of membrane action potentials in mammalian heart muscle tissue. After staining, atrial specimens were superfused in physiological salt solution, and fluorescence was excited by an argon ion laser. Excitation spot size on the surface of the preparation was 60 µm in diameter. Dyes RH 160, RH 237, and RH 421 performed excellently as fast fluorescent probes of cardiac membrane potential. Fractional fluorescence changes, $\Delta F/F$, due to the action potential were in the range 2 to 6% at 514.5 nm excitation. Rise times of the action potential onset detected with each of the dyes were less than 0.5 ms, which is as fast or even faster than microelectrode measurements (atria of the rat). Thus membrane potential changes could be monitored with high resolution in both time and space. Emission spectra from heart muscle preparations stained with these dyes were shifted to shorter wavelengths by 70 nm and more as compared to spectra of the dyes in ethanol solution. The fluorescence spectrum of RH 160 at resting potential and the spectrum recorded during the plateau phases of the action potential were measured and showed no difference within the spectral resolution. As can be concluded from measurements of fluorescence changes at different excitation wavelengths, electrochromism cannot be the only mechanism causing the potential response.

Key words: Potential sensitive dyes, myocardium, action potential

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Introduction

A large number of dyes respond to changes in electrical field strength by alteration of their fluorescence light intensity. This effect has been applied to detect membrane potential changes in various excitable tissues in vitro (for reviews see: Cohen and Salzberg 1978; Waggoner 1979).

Depending on the dye and on the specific membrane, many different mechanisms underlying this fluorescence change are conceivable, for instance: aggregation, orientation and movement of dye molecules, or electrochromism of the molecules; the electric field can also influence the orientation and conformation of membrane structures which bind dye molecules and thereby cause fluorescence changes (Ross et al. 1974; Warashina and Tasaki 1975; Conti 1975; Waggoner et al. 1975; Tasaki et al. 1975; Tasaki and Warashina 1976; Waggoner 1976; Waggoner and Grinvald 1977; Waggoner et al. 1977; Loew et al. 1978; Loew et al. 1979; Waggoner 1979; Krasne 1980a, b; Loew 1982; Loew et al. 1985).

Optical methods for monitoring electrical activity show important advantages when compared to the microelectrode technique:

1) No impalement artefacts
2) Precise selection of both the size and location of the measuring spot
3) Many cells can be monitored simultaneously.

The first successful application of a potential sensitive probe (Merocyanine 540) to heart muscle tissue (heart of the bullfrog, *Rana cartesbeiana*) was reported in 1976 (Salama and Morad 1976a, b). The fractional fluorescence change due to the action potential was measured there as 1.2% at signal-to-noise ratios of 10/1 to 40/1. However, the rise time of the action potential was slow (40 ms) and preparations were kept in a low Ca (50 µ*M*) environment to suppress mechanical motion. The dye used was

found to be biologically inert on this preparation, but other studies established its high toxicity in nerve tissue and mammalian heart muscle as well (Ross et al. 1977; Windisch and Müller 1983).

Since 1976 several new dyes (for instance merocyanine-rhodamine, merocyanine-oxazolone, WW 781) have been investigated as potential sensitive probes on various heart muscle tissues and improved signal-to-noise ratios and minor toxic and photodynamic damage were reported (Morad and Salama 1979a, b; Hirota et al. 1979; Fujii et al. 1981a, b; Kamino et al. 1981; Hill and Courtney 1982; Hill et al. 1982; Sakai et al. 1983; Sawanobori et al. 1984).

Grinvald et al. (1982) reported the synthesis of improved fluorescent probes for the measurement of rapid changes in membrane potential. 20 potential sensitive styryl dyes were tested on cultured mouse neuroblastoma cells and fractional fluorescence changes (%/100 mV potential change) of 12% (RH 160), 14% (RH 237), 7% (RH 270), and 6% with RH 376 were obtained. With RH 421 fractional fluorescence changes up to 21% during action potential were found (Grinvald 1984). However, measurements with RH 237 on invertebrate ganglia on the other hand (segmental ganglia of the leech) showed fractional fluorescence changes of only 0.8%/100 mV (Grinvald et al. 1982).

We tested RH 237 on mammalian heart muscle preparations (Windisch et al. 1985). On atrial and ventricular sheets of the rat this dye performed excellently as a fast fluorescent probe of membrane potential. Rise times of action potential onsets detected in fluorescence mode were as fast as, or even faster than, microelectrode measurements in the same preparations. The mean value of optically measured risetimes (10% to 90% of action potential amplitude) was 0.45 ms vs. 0.59 ms obtained using microelectrode techniques (atrial sheets of the rat). The mean value of fractional fluorescence changes was about 3%.

The aims of the investigations reported here were to select the most efficient styryl dyes for electrophysiological experiments with mammalian heart muscle tissue and to determine the spectral properties of the dyes when they are bound to living tissue. Data presented here are necessary to optimize the optical system (choice of light source, filters and beam splitters, photodetectors) for any experiment using these potential sensitive dyes on heart muscle preparations. The results also contribute to the discussion of the electrochromic mechanism involved in the action of these dyes when they bind to physiological membranes instead of to artificial bilayers (Platt 1960; Loew et al. 1978, 1979, 1985; Loew 1982).

Materials and methods

Heart muscle preparations

Rats and guinea pigs were killed by a blow to the neck. The hearts were rapidly removed and placed in O_2-saturated and pH-balanced Tyrode solution. Atrial sheets, typically 5 mm in diameter and about 0.5 mm thick (about 50 cell layers) were excised and transferred to the staining solution. Specimens were not transparent and therefore optical measurements were made in the fluorescence mode. After staining, the sheets were placed in an experimental chamber and superfused with dye-free Tyrode solution. Specimens displayed constant electrical and mechanical activity for more than 2 h.

Staining with voltage-sensitive dyes

Fresh staining solutions were prepared for each experiment from a stock solution of ethanol saturated with dye. Dyes used here were: RH 160, RH 237, RH 270, RH 376 and RH 421, all of which were first synthesized by Grinvald et al. (1982, 1983). This stock solution was diluted with O_2-saturated Tyrode solution by 100 to 200 fold (about 25 to 50 μM final dye concentration) and immediately thereafter the atrial sheets were stained for 10 to 12 min at room temperature.

Tyrode solution

In all experiments the preparations were continously superfused with Tyrode solution at 36.5 $\pm$ 0.5 °C. The Tyrode solution was composed of: 132.1 mM NaCl, 2.7 mM KCl, 2.5 mM $CaCl_2$, 1.15 mM $MgCl_2$, 24 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 5.6 mM D-Glucose saturated with 95% O_2 and 5% CO_2, the pH was 7.2.

Lipid vesicles

Lipid vesicles were prepared from a mixture of soybean lecithin (Sigma GmbH, Munich, W. Germany) purified according to Kagawa and Racker (1971) and cholesterol (Fluka, puriss.) in a 6:1 weight ratio (Schindler and Quast 1980). 10 mg of the lipid mixture were dissolved in 10 ml of hexane, dried under nitrogen to a thin surface film in a 2-litre-flask, and resuspended in 25 ml of KCl solution (120 mM KCl, 10 mM HEPES).

Apparatus

Figure 1 shows the schematic diagram of the apparatus used for measurements of both membrane potential changes and spectra. The argon ion laser (Coherent, INNOVA 90-5, Neu Isenburg, W. Germany) was used as light source. The advantages of the argon ion laser compared with conventional light sources are: Precise and easy adjustment of light power, of monochromatic excitation wavelength and of excitation light path as well as the possibility of using an optical shutter (Pockel's cell) to switch the beam. The laser beam passed the Pockel's cell, PC, (Laser Optronic, Munich, W. Germany) and was expanded by the telecentric system, TS. The light intensity was reduced by a filter FI (Neutral Density Glass, 0.1, Schott & Gen., Mainz, W. Germany) because laser stability was best at the highest laser output power (optical noise then was about 0.2% rms). Illumination light power of 1 to 3 mW at a spot size of 60 µm diameter (excitation light intensity of 40 to 120 W/cm^2) was normally used.

The fluorescent light was detected by diode 1, the laser reference beam by diode 2, and light, backscattered by the specimen, by diode 3. Total intensity of the fluorescent light (cut-off filter KV 550, Schott) at an excitation intensity of 3 mW was typically 200 to 500 nW (current of about 50 to 100 nA) measured with preparations, stained as usual and a mirror instead of the beamsplitter in the light path. Excitation duration (10 to 40 ms) was adjusted by the Pockel's Cell (PC). To minimize thermal instability of the cell the laser was powered just 100 ms before opening of the PC. The ratio of excitation light power on the surface of the tissue to the fluorescence light power detected by photodiode 1 was in the range 6,000:1 to 15,000:1 for all three dyes, depending on the staining efficiency. This overall efficiency is determined by extinction and reflexion effects of the optics (roughly a factor of 0.25), the geometrical factor given by the working distance and diameter of the objective (0.20), and the quantum efficiency of the membrane-bound dye multiplied by the ratio of photons absorbed by dye molecules to the total number of incoming photons. The latter product was found to be 0.0033 to 0.0013, and is a function of staining efficiency. Therefore, about 300 to 750 excitation photons are necessary to yield one fluorescent photon and every 20th fluorescent photon is detected by the photodiode.

For spectral measurements part of the fluorescent light passed through the beam splitter and was focused on the entrance slit of the spectrograph (Spex Minimate, Mod. 1670, 150 grooves/mm, slits 0.25 mm, band width 8 nm, Spex Ind., Munich, W. Germany). Spectra were stored on the Silicon

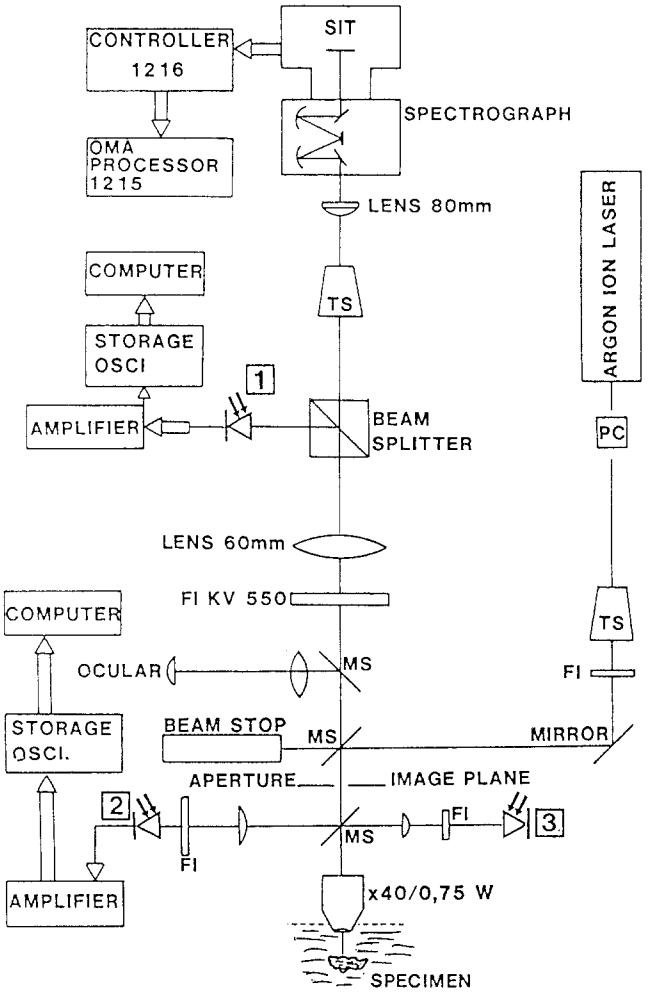

Fig. 1. Schematic diagram of the apparatus used for measurements of both membrane potential changes and spectra.

The argon ion laser was used as light source for both spectral measurements and optical recordings of membrane potential changes. The laser beam passed the Pockel's cell PC (optical shutter) and two apertures and was expanded by the telecentric system TS (focal lengths of lenses: 40 and 100 mm). The light intensity was reduced by a filter FI (Neutral Density Glass, 0.1) and the beam directed to the objective by a standard microscope coverslip MS placed at 45° to the beam path (about 10% of the light was reflected, 90% absorbed by the beam stop). By means of an adjustable aperture and the water immersion objective (× 40) measuring spots of 30 to 70 µm diameter could be illuminated on the preparation. The ratio of laser output to illumination power was 650:1 (laser light stabilisation was best at highest laser power). A second microscope coverslip was used to guide the laser light to photodiode 2, which was used to detect the intensity of the exciting beam. The backscattered light was partially reflected by the same coverslip to photodiode 3 and coupled to the ocular by a third one. Via this ocular it was possible to observe and adjust the measuring spot location and size. Backscattered light was cut off by the filter KV 550 (Schott, Mainz, W. Germany), fluorescence light passed through the beam splitter to both the photodiode 1 and to the entrance slit of the spectrograph. For optical action potential measurements only, the beam splitter was replaced by a mirror. Spectral data were read from the Silicon Intensified Target (SIT) by the Detector Controller 1216 and the signals were then processed by the Optical Multichannel Analyzer (OMA)

Intensified Target (SIT, EG & G Instruments, Munich, W. Germany) and read out within a few milliseconds. The data were transferred by the Detector Controller 1216 (EG & G) to the Optical Multichannel Analyzer (OMA, EG & G), and then calibrated and smoothed by the OMA-System. The spectral characteristic of the instrumentation (SIT and Spectrograph) was corrected by means of a thermopile detector (Oriel, 7104, 6 mm dia., Oriel Corp., Darmstadt, W. Germany) within the range 580 to 710 nm. Each measured spectrum was multiplied by the correction function, which was stored in the OMA memory on floppy disk.

Optical measurements on heart muscle preparations

Immediately after staining, the preparations were mounted onto the rubber floor of a superfusion bath which was moveable in all directions and located under the objective. Two electrodes were fixed to the bottom and used for the electrical stimulation of the preparation at 0.5 to 2 Hz frequency. At low laser light intensities a measuring spot was selected on the surface of the tissue. Either the stimulus or the "experimental start trigger" (from the OMA) initiated a variable 3 channel delay unit to co-ordinate the timing of the laser power and the Pockel's cell and to trigger the storage oscilloscope (Explorer III, Nicolet Instr. Corp., Madison, Wisconsin, USA). The settings of the delay unit depended on the type of measurement being performed. For measurements of fluorescence spectra during the action potential the Pockel's cell was switched on for 13 ms, triggered with the rising phase of the action potential which was optically monitored. In the other experiments the duration of illumination was 20 to 40 ms.

Spectra of solutions

Fluorescence spectra of solutions or stained vesicle suspensions were also detected by the apparatus described above. For these experiments the objective dipped into the fluid. Transmission spectra were obtained with a Hitachi Photometer (Hitachi 220, Inula, Vienna, Austria).

Results

Styryl dyes used as probes of membrane potential on atrial sheets of the rat

All the dyes used (RH 160, RH 237, RH 270, RH 376 and RH 421) were successfully employed as fast molecular probes to measure changes in membrane potential. The depolarizing phases of optically detected action potentials in atrial sheets of the rat were all remarkably fast, compared to those measured in the same preparations via glass microelectrodes. The mean values of the duration of the fast parts of the rising phases (10% to 90% of the action potential amplitude) were 411 ± 112 µs for RH 160 ($n = 93$), 411 ± 165 µs for RH 237 ($n = 121$) and 381 ± 172 µs for RH 421 ($n = 134$). The mean values do not differ significantly (t-test, $\alpha < 0.05$).

The shape of the rising phase of the action potential is shown in Fig. 2. The relative fluorescence change, $\Delta F/F$, due to the potential change during the rising phase of the action potential was typically smaller than 2% for RH 270 and RH 376

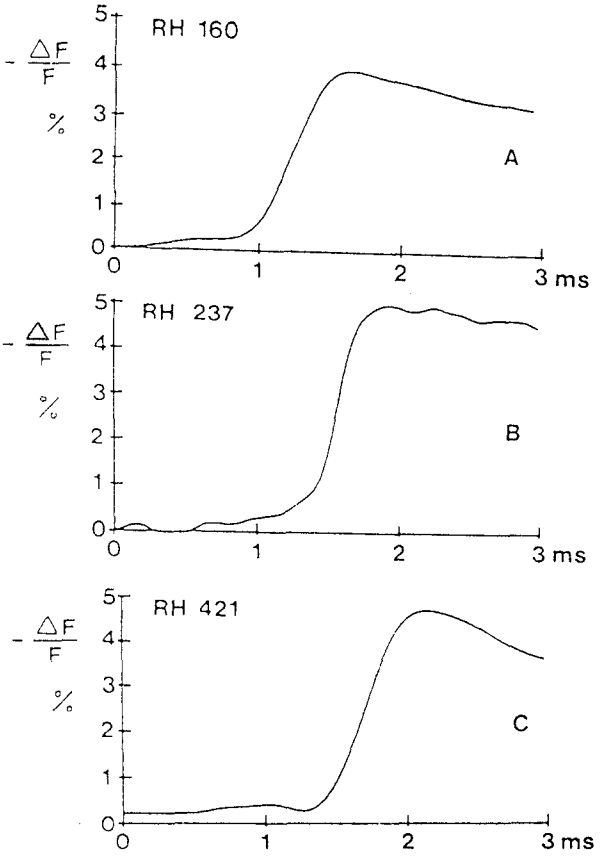

Fig. 2. Depolarizing phases of atrial action potentials, detected with fluorescent potential-sensitive styryl dyes RH 160, RH 237 and RH 421, first synthesized by Grinvald.

Smoothed traces, obtained by digital filtering (Windisch et al. 1985), of fractional fluorescence changes $\Delta F/F$ from the rising phases of action potentials are displayed. Diameter of illuminated measuring spot was 60 µm, excitation light power was 3 mW, overall response time (10% to 90%) of the detection system was 0.12 ms and duration of laser light pulse was 25 ms (3 ms evaluated here). Traces were triggered electrically, some milliseconds before preparations were stimulated. Filter KV 550 was used to cut off backscattered light

and between 2% and 6% for RH 160, RH 237 and RH 421 (λ_{ex} was 514.5 nm).

The photodiode current due to the fluorescent light was characteristically 50 nA to 100 nA at an excitation intensity of 3 mW on the surface of the specimen (diameter of illuminated spot was 60 µm).

Variation of excitation light power from 1 to 6 mW (λ_{ex} was 514.5 nm) at the given spot size did not alter the relative fluorescence response, $\Delta F/F$.

The signal-to-noise ratio (peak to peak) was typically 20:1 (S/N 60:1 rms) or better for RH 160, RH 237 and RH 421 and about 10:1 (peak to peak) for RH 270 and RH 376.

The rising phase of the action potential was not influenced by the motion of the tissue. Mechanical artefacts appeared 10 to 15 ms after the rising phase of the action potential.

Fluorescence spectra

The normalized fluorescence spectra of the dyes RH 160, RH 237, RH 421 bound to atrial sheets of the rat are shown in Fig. 3. The fluorescence spectra of membrane-bound dyes differ markedly from those measured for the same dyes dissolved in ethanol. The peak emission of all of the potential sensitive styryl dyes used (RH 160, RH 237, RH 270, RH 376, RH 421) was shifted to shorter wavelengths when they were bound to heart muscle tissue or to vesicles (Table 1).

For all the dyes the actual shape of the spectra appeared to be very similar when attached either to vesicle membrane, to cells of heart muscle tissue of the rat, or the guinea pig.

Dyes in Tyrode solution were much less fluorescent than in either ethanol or vesicle suspension. With RH 160 and RH 237 a decrease of two orders of magnitude in fluorescent light intensity was registered when 10 µl of saturated ethanol stock solution was diluted with 1 ml of Tyrode solution (or with water) as compared with 1 ml of vesicle suspension or ethanol.

Table 1. Peak emission of potential sensitive dyes in ethanol, in vesicle-suspension, and bound to heart muscle tissue

Dye	Ethanol	Vesicles	Atrium rat	Atrium guinea-pig
RH 160	692	619	622	624
RH 237	> 710[a]	647	645	653
RH 270	685	617	627	621
RH 376	688	617	627	621
RH 421	697	618	624	629

[a] Beyond calibration range

Fluorescence spectra of RH 160 bound to atrial sheets of the rat were taken at different excitation wavelengths (514.5, 496.5, 488.0, 476.5, 457.9 nm) and no detectable spectral differences were found.

Two spectra, one measured during the first 13 ms of the plateau phases of the action potential, the other one registered several seconds before, at

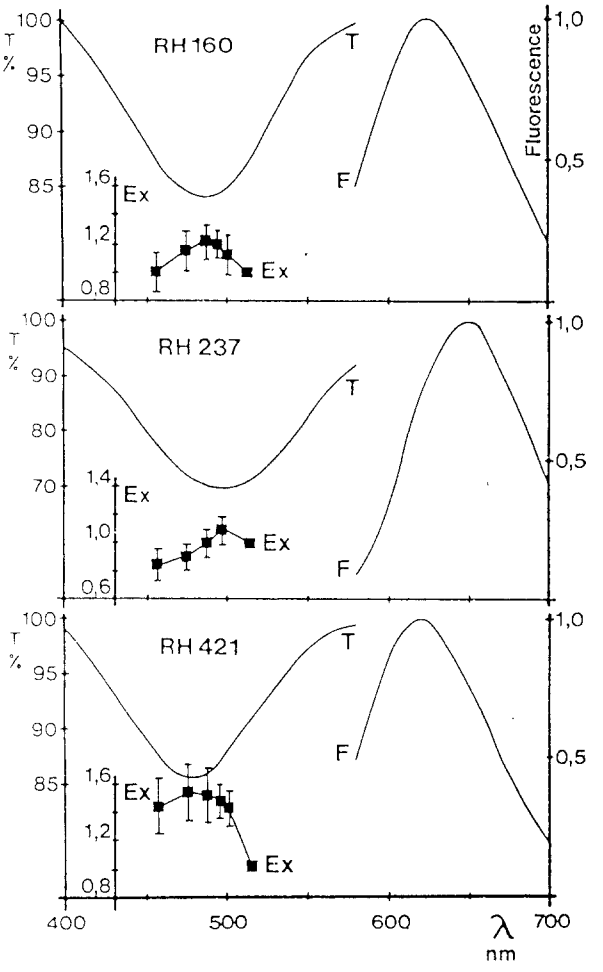

Fig. 3. Spectral properties of membrane-bound dyes RH 160, RH 237 and RH 421. *T*: Transmission spectra of dyes bound to bilayer lipid vesicles (Hitachi photometer, halfwidth 4 nm); 1 µl of saturated ethanol stock solution diluted with 1 ml of vesicle suspension. *F*: Fluorescence spectra of dyes bound to atrial sheets of the rat, measured with the apparatus shown in Fig. 1. Duration of illuminating laser pulse was 20 ms, excitation wavelength 514.5 nm, excitation light power 3 mW, halfwidth of spectrograph was 8 nm and the wavelength dependence of the whole system (spectrograph + SIT) was corrected by means of a thermophile detector. *Ex*: Relative excitation spectra from stained atrium of the rat. Ratios of fluorescent light intensity F to excitation intensity I_{ex} were taken at different excitation wavelengths of the argon ion laser. Before and after each measurement, the ratio of F/I_{ex} at 514.5 nm was determined and the ratio at the given wavelength was divided by the mean of these two values. The excitation efficiency at 514.5 nm was normalized to 1. Means of six measurements at each wavelength are shown (*squares*)

resting potential (about -100 mV in rat atrial tissue), were compared and no spectral shifts could be found within the spectral detection resolution (± 4 nm). The signal-to-noise ratio in these measurements was better than 100 : 1.

Transmission spectra

Transmission Spectra of RH 160, RH 237 and RH 421 dissolved in vesicle suspension are also shown in Fig. 3 (labelled "T"). These spectra are noticeably different from those found with the dyes dissolved in ethanol (Grinvald et al. 1982; not shown in Fig. 3). The peak absorption (minimum transmission) in vesicle suspension is shifted to shorter wavelengths as compared to the peaks in ethanol; maximum absorption in vesicle suspension was found at 488 nm for RH 160, at 497 nm for RH 237, and at 480 nm for RH 421 vs. 526 nm, 544 nm (Grinvald et al. 1982) and 528 nm (R. Hildesheim, personal communication) respectively. The transmission spectra were measured with the dyes in a vesicle suspension containing 1 µl of saturated ethanol stock solution per ml of vesicles.

Relative excitation spectra of RH 160, RH 237, and RH 421

During optical action potential measurements at different excitation wavelengths the intensity of the exciting laser beam, I_{ex}, was also measured and the excitation efficiency, F/I_{ex}, of the bound dye was determined. Before and after each measurement at a selected wavelength, the excitation efficiency, F/I_{ex}, at 514.5 nm was determined and the mean of these two values was compared to the ratio F/I_{ex} at the selected wavelength. With this procedure, bleaching effects could be eliminated. The excitation efficiency was then normalized to 1 at 514.5 nm. Means of 6 measurements at each of the laser wavelengths (496.5, 488.0, 476.5, 457.9) are shown in Fig. 3 ("Ex", squares).

With unstained atrial sheets the level of the total light intensity, which reached photodiode 1 at 514.5 nm excitation was less than 1% and at 457.9 nm less than 3% of the intensity found with the stained preparation.

All dyes, when bound to atrial sheets of the rat, showed excitation maxima between 457.9 and 514.5 nm.

Wavelength dependence of the fluorescence response

The dependence of the relative fluorescence change of the whole fluorescence spectra, $\Delta F/F$ (measured during the rising phase of the action potential) on the wavelength of the excitation light was also determined for RH 160, RH 237, and RH 421. The values of the fractional fluorescence changes (all wavelengths of the spectra were collected) at different excitation wavelengths were obtained simultaneously with the measurements of the relative excitation spectra described above. Here too, before and after each measurement at the given wavelength, the value of $\Delta F/F$ at 514.5 nm excitation was used as reference. The ratio of the mean of the two fractional responses at 514.5 nm and the fluorescence change at the given wavelength is shown in Fig. 4, with values normalized to 1 for 514.5 nm. The mean fractional response at 514.5 nm was $-2.6 \pm 0.7\%$ for RH 160 ($n = 63$), $-2.9 \pm 0.8\%$ for RH 237 ($n = 88$), and $-2.8 \pm 0.7\%$ for RH 421 ($n = 74$).

At all excitation wavelengths with all three dyes the fluorescent light intensity decreased during the upstroke of the action potential.

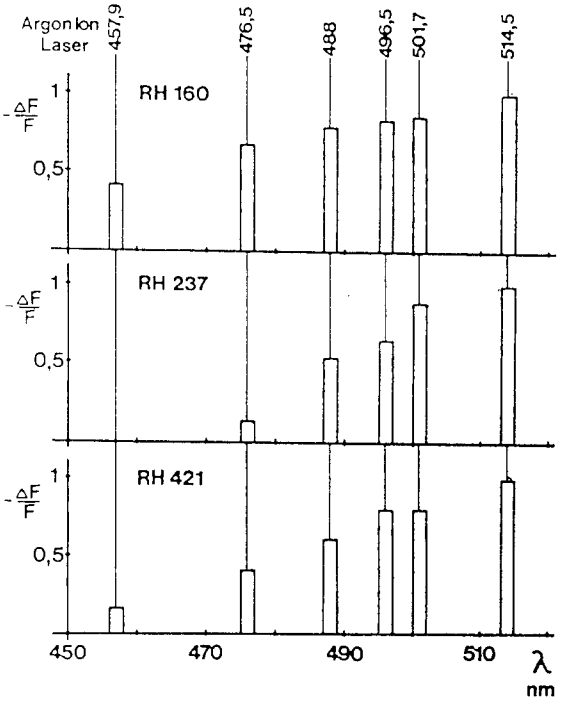

Fig. 4. Dependence of fractional fluorescence change $\Delta F/F$ amplitude on the excitation wavelength. All values were referred to the mean value of readings at 514.5 nm excitation taken directly before and after each measurement at the given wavelength. Atrial sheets of the rat were stained as usual and fluorescence changes during depolarizing phases of action potentials were registered. Values for 514.5 nm were then normalized to 1. Spot size was 60 µm, laser power at all lines was 200 mW (neutral filter 0.1 was removed and the exciting light power was 3 mW). Means of 6 measurements at each wavelength are shown

All of the dyes investigated in this manner showed a continuous rise in the absolute value of fractional fluorescence change with increasing excitation wavelength. At 457.9 nm, though the intensity of the fluorescence light, F, was large with all 3 dyes, $\Delta F/F$ with RH 160 and RH 421 were small, in some of the measurements even undetectable, and no change at all could be found with RH 237.

Lack of toxicity of the dyes on heart muscle tissue

Action potentials were measured by means of glass microelectrodes during the staining procedures of atrial sheets of the rat which lasted from 10 to 12 min, and every five minutes afterwards for 30 min. No significant differences to action potentials of unstained atrial tissue could be found.

Discussion

Spectral properties

Maxima of fluorescence emission spectra from heart muscle preparations stained with RH 160, RH 237, RH 270, RH 376, and RH 421 were shifted to shorter wavelengths by 70 nm and more when compared to spectra of the dyes in ethanol solution. Excitation and transmission spectra of membrane-bound dyes are also different to those from the dyes in ethanol. The knowledge of the dye's spectra when bound to the surface of the preparation of interest is indispensable for the optimization of the optical system, particularly for the choice of the cut-off filter (Grinvald 1982) and the light source. The dyes used here are well matched to the argon ion laser excitation as can be seen in Fig. 4 (fluorescence response for various laser wavelengths) and Fig. 3 (excitation and transmission spectra of the dyes).

Fluorescence emission spectra were very similar in both atrial sheets of the rat and of the guinea pig to those of dyes in vesicle suspension (Table 1). The slight shifts (about 10 nm) in the spectra may be caused by different quenching and scattering of the fluorescent light when detected from the surface of the stained preparation as compared to stained vesicles in a suspension, and, possibly, by differences in the chemical environment.

Fluorescence spectra on myocardium were measured within a few milliseconds both at resting potential and during the action potential (a change in electric field strength of about 10^7 V/m). These spectra were compared and no differences could be found (spectral resolution: $\pm$ 4 nm).

Application of RH 160, RH 237, and RH 421 as optical probes of membrane potential on myocardium

Optical methods to record the overall time-course of electrical events in heart muscle are complicated by mechanical contractions of the tissue and experiments were therefore often carried out either at low Ca concentrations (Salama and Morad 1976a, b; Morad and Salama 1978a, b; Sawanobori et al. 1984) or with the Ca-antagonist Diltiazem (Dillon and Morad 1981) to prevent electromechanical coupling, i.e. to stop mechanical activity. We chose not to pursue this course because of the disadvantages of this low Ca environment (Tritthart et al. 1973; Windisch and Tritthart 1981) and of the use of the Ca-entry blocker, both of which influence electrical activities of cardiac membranes. Cleemann et al. (1984) used transmitted light at different wavelengths to correct distortions caused by mechanical contraction of the preparation when measuring depletion of Ca^{++} by optical means. For these measurements, however, additional methods had to be used (low Ca^{++}, no Mg^{++}, compression of the specimen) and experiments were performed only with those preparations in which the uncompensated contraction artefacts were small.

Attempts to suppress mechanical artefacts by using the light backscattered by the surface of the specimen as a reference were, in general, inefficient, because the backscattered light did not in all cases mirror exactly the motion-induced changes in the fluorescence signal (Windisch and Müller 1983). For efficient compensation of artefacts caused by the tissue's motion a more refined optical reference system would be necessary. The spatial intensity distribution of the light within the measuring spot used for the reference must be identical to that of the light collected from the measuring spot which contains the information on transmembrane electrical changes. This condition could be realized by utilizing the fluorescent light as a reference itself at excitation wavelengths where no fluorescence response, $\Delta F/F$, due to the potential change is detected (for instance $\lambda_{ex} = 457.9$ nm with RH 237, Fig. 4). For this application two excitation wavelengths of the laser (multiline operation) could be chopped, resulting in two different fluorescence signals, one containing the information on the mechanical activity only, the other both the mechanical signal and the fluorescence change due to the potential. By refining this method a calibration of the optical signal could be possible and thus absolute values of the potential could be calculated.

Registrations of depolarizing phases of cardiac action potentials are usually not influenced by me-

chanical motions of the tissue, due to their delayed onset (10 to 15 ms after the rising phase). With all the dyes used, the rise times of the depolarizing phases were strikingly fast (means: 411 µs for RH 160, 411 µs for RH 237, 381 µs for RH 421). A method commonly used to establish linearity of the dye's response involves simultaneous microelectrode measurements (e.g. Grinvald et al. 1982). A comparison of results with RH 237 with glass microelectrode measurements was done in a previous publication (Windisch et al. 1985). Our recordings with all of the dyes used could be differentiated after digital filtering. This allows the measurement of upstroke velocity and the generation of phase plane plots.

With preparations containing many cell layers (up to about hundred in our preparations) photometric action potential measurements in fluorescence mode are more suitable then measurements in transmittance mode because of the low light intensity transmitted and because of the impossibility of detecting fast action potential upstrokes in transmittance mode due to the superposition of the activities of many cells which do not occur at the same time.

Mechanisms of potential dependence of RH 160, RH 237, and RH 421 on mammalian heart muscle tissue

With the assumption of a purely electrochromic mechanism, as proposed by Loew (1982), to explain the potential sensitivity of the dyes, a biphasic shape of the fluorescence response as a function of excitation wavelength, crossing the axis near the value of maximum excitation (Fig. 3), was expected. An electrochromic mechanism should, furthermore, provide a transmittance response spectrum with the shape of the first derivative of the resting absorption spectrum (Loew 1982). The different absorption values due to a change in potential would also cause fluorescence changes proportional to these absorption changes. A comparison of these expectations with experimental data (Fig. 4; filter KV 550, cutoff wavelength: 550 nm was used as usual, therefore all wavelengths of the fluorescence spectra were detected and thus shifts of the fluorescence spectra did not influence the results) shows that these dyes do not work as pure electrochromic potential sensitive probes on heart muscle tissue. The mean values of the fractional fluorescence changes were -2.6% with RH 160, -2.9% with RH 237, and -2.8% with RH 421 at 514.5 nm and diminished monotonously with shorter excitation wavelength. There was not a single measurement with any of the dyes which produced a positive value for $\Delta F/F$ at excitation wavelengths either above or below the value of maximum excitation. At excitation wavelengths of 546 and 578 nm (beyond the range of our laser lines) negative fractional fluorescence changes were also reported for these dyes (Grinvald et al. 1982; preparation: cultured neuroblastoma cells and leech ganglia).

To explain these discrepancies it is necessary to include one or more additional mechanisms superimposed onto the electrochromic one. For RH 160 the transmittance response spectrum $\Delta T/T$, detected on the hemispherical bilayer was published by Loew (1982). The measured fluorescence change spectrum ($\Delta F/F$) agrees qualitatively with the calculated $\Delta F/F$-spectrum, based on this $\Delta T/T$-spectrum, if an additional mechanism, generating a negative contribution to ΔF which is independent of the excitation wavelength, is assumed. The value of this additional contribution can be determined at the wavelength of maximum excitation efficiency (Figs. 3 and 4).

With RH 160 a fractional fluorescence change due to this additional mechanism of approximately 0.7 times the value of the total change at 514.5 nm is found, i.e. a fractional change of 1.8% on the average. With RH 237 or RH 421 a mean value of 2% or 1.4% respectively are found. These findings are not surprising: Fluhler et al. (1985), expected for RH 421 and analogues, that potential-dependent changes in the structure of these more flexible probes could contribute to the signals and either reinforce or detract from the electrochromic response at a given set of wavelengths.

Experimental data demonstrate clearly that responses calibrated on a model membrane cannot be generalized without modification to heart muscle preparations. Comparison of theoretical predictions (Platt 1961; Loew et al. 1978, 1979; Loew 1982) and experimental results from various physiological preparations (Grinvald et al. 1982, 1983, 1984; Grinvald 1984; Loew 1985) and data obtained from measurements on mammalian heart muscle presented here, emphasize the requirement of testing the dyes separately on every physiological preparation of interest.

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