High-precision recording of the action potential in isolated cardiomyocytes using the near-infrared fluorescent dye di-4-ANBDQBS

Mark Warren,1 Kenneth W. Spitzer,1 Bruce W. Steadman,1 Tyler D. Rees,1 Paul Venable,1 Tyson Taylor,1 Junko Shibayama,1 Ping Yan,2 Joseph P. Wuskell,2 Leslie M. Loew,2 and Alexey V. Zaitsev1

1Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, Salt Lake City, Utah; and 2R. D. Berlin Center for Cell Analysis and Modeling, University of Connecticut Health Center, Farmington, Connecticut

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The cardiac action potential (AP) is a propagating wave of sarcodlemmal depolarization that controls adequate timing and modulates force of cardiac contraction. Abnormalities of the AP shape can cause disturbances of the contractile function and life-threatening arrhythmias.

The standard approach for AP recording using micropipettes requires aggressive perturbation of the system by perforation of the cell membrane. This procedure leads to ionic and metabolite exchange between the intracellular medium and the pipette solution (47). Alteration of the intracellular chemical environment may thus preclude studies in which preservation of the native intracellular medium is imperative. In addition, perforation of the membrane generally limits the time interval available for the recording of AP due to undesired side effects, such as leak of the micropipette seal and run down of the preparation. From the industrial perspective, a major limitation of the microelectrode technique is its low throughput, which is determined by the overall complexity of the approach requiring, among others, high-precision positioning of the microelectrode. Various automated platforms based on the basic patch-clamp operation have recently been developed and commercialized with the objective of increasing the throughput of the technique (16).

The development of electrochromic voltage-sensitive fluorescent dyes (VSDs), characterized by very linear and fast response to membrane potential (20, 36), has led to their widespread use for imaging of cellular AP in multicellular preparations (18, 45a, 54). Commonly used VSDs include 1-(3-sulfonatopropyl)-4-[β-[2-(di-n-butylamino)-6-naphthyl]vinyl] pyridinium betaine (di-4-ANEPPS) (20) and 1-(3-sulfonatopropyl)-4-[β-[2-(di-n-octylamino)-6-naphthyl]vinyl] pyridinium betaine (di-8-ANEPPS) (24), which are excited by photons in the blue-green range of the spectrum. The downside of this dye class is that the fluorescent signals consist of a large background component over which a small voltage-dependent change in fluorescence is superimposed upon cellular depolarization. This fluorescent property is a direct result of the spatial shift of charge occurring during photo-excitation and the ensuing wavelength shift in the absorption and emission spectra elicited by membrane depolarization. Thus a feature that characterizes the efficiency of an electrochromic dye for detecting voltage changes is the ratio of ∆F/ Frest, namely the change in fluorescence on depolarization (∆F) over the level of fluorescence at resting potential (Frest). To date, the ∆F/ Frest of the most commonly used dyes reach values of up to 10% (35), which is low if compared with the sensitivity of other fluorescent reporters, such as modern calcium dyes (10, 51). Despite this low ∆F/ Frest value, in whole tissue preparations, where the signal is integrated over a volume comprising numerous cells (29), VSDs such as di-4-ANEPPS or di-8-ANEPPS have proved highly successful for the measurement of cellular excitation and have been widely used (18, 45a, 54).

Unfortunately, in isolated cardiomyocytes, the source of fluorescent photons is limited to only the outer cell membrane of a single cell, resulting in a very low voltage-dependent signal. In addition, single cells exhibit an increased susceptibility to photodynamic damage (48), especially at high dye concentrations (28), which manifests as an AP duration (APD) prolongation and instability. Limiting the dye concentration to avoid toxicity results in an extremely low signal-to-noise ratio (SNR), thus requiring that the fluorescent signals be averaged over various excitation cycles to obtain meaningful data (28). This, in turn, requires prolonging the exposure to the excitation light, which further prompts the development of photodynamic damage. Altogether, the combination of these limiting factors...
leads to a vicious circle, which, to date, has hindered the development of the technique for practical usage.

In view of the aforementioned, our challenge was to create the appropriate conditions for recording AP in isolated cells with fluorescence by overcoming one or more of the limiting factors, which are ultimately responsible for the impossibility to establish the technique.

METHODS

Quantum Mechanical Calculations

Semiempirical quantum mechanical calculations of electron redistributions in β-[2-(dimethylamino)-6-naphthyl]ethenyl-1-methylpyridinium cation (di-1-ANPEM) and β-[2-(dimethylamino)-6-naphthyl]butadienyl-1-methylquinolinium cation (di-1-ANBDQM) upon photoexcitation were carried out using ArgusLab (Thompson M. A. ArgusLab 4.0, Planaria Software, Seattle, WA, 2004). For ground state geometry optimization, the PM3 method (31) was used; for excitation related properties, the ZINDO-CI method (53) was used. Solvent effects were not included in these calculations.

Myocyte Isolation

Adult ventricular myocytes were isolated from guinea pig (300 – 400 g) hearts, as previously described (52). All procedures involving animals were approved by the Animal Care and Use Committee of the University of Utah and complied with the American Physiological Society’s Guiding Principles in the Care and Use of Animals. In brief, animals were anesthetized with pentobarbital sodium (50 mg/kg ip), then the excised heart was attached to an aortic cannula and perfused with solutions gassed with 100% O2 and held at 37°C, pH 7.3. Perfusion with a 0 mM Ca2+ solution for 5 min was followed by 7 min of perfusion with the same solution containing 0.05 mg/ml proteinase K (type XIV, Sigma Chemical, St. Louis, MO). The heart was then perfused for 5 min with the same solution containing no enzymes and 0.1 mM CaCl2. The left ventricle was minced and shaken for 10 min and then filtered through a nylon mesh. Cells were stored at room temperature in normal N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered solution. All myocytes used in this study were rod-shaped, had well-defined striations, and did not spontaneously contract. Experiments were performed within 5 h of isolation.

Cell Chamber and Solution

Myocytes were placed in a 1-ml flow-through chamber mounted on the stage of an inverted microscope (see below for more details). The coverslip on the bottom of the chamber was coated with laminin to enhance cellular adhesion. Bathing solutions flowed continuously through the bath at ~4–5 ml/min, and solution depth was held at ~2–3 mm. Exchange of the bath solution required ~10 s. The control bathing solution for myocytes during imaging of voltage-sensitive fluorescence was a HEPES-buffered Tyrode solution containing (in mM) 126 NaCl, 4.4 KCl, 1.0 MgCl2, 1.1 CaCl2, 11 dextrose, and 24.0 HEPES (free acid), titrated to pH 7.40 with NaOH. To test the efficiency of the antioxidants in preventing the photodynamic damage, we added the following chemicals to the control solution: 32.0 mM 1-(4-sulfanatobutyl)-4-[β(2-di-n-butylamino)-6-naphthyl]butadienyl-1-methylquinolinium betaine (di-4-ANBDQBS) (39) (in powder form) was prepared by dissolving the powder in pure ethanol at a ratio of 32 mg/1.5 ml. Myocytes were loaded with dye according to one of the two procedures, as follows. In one procedure, cells were loaded with di-4-ANBDQBS by incubation of 0.5–2 µl of dye stock solution delivered to a 2-ml volume of cell-containing solution. The effective concentration of dye in the myocyte-containing solution during incubation was 18.4, 36.8, or 73.6 µM. Incubation times were 6–7 min, and the procedure was carried out at room temperature. In the other procedure, cells were loaded with the dye after attachment to the bath bottom by continuous perfusion (4 ml/min during 0.25–0.5 min) with a 18.4 µM dye solution (dissolved in control solution) delivered at a flow rate of 4 ml/min either at 32.0 ± 1.0 or 37.0 ± 1.0°C, as indicated in the text. This procedure was followed by washout with control solution free of dye.

Optical Setup

Imaging and electrophysiological studies were performed after placing the cells in a perfusion bath. We used a 200-mW red solid-state laser (660 nm), coupled to a flexible fiber-optics light guide (Edmund Optics) to deliver excitation photons to the myocyte via a 692-nm dichroic mirror (Omega Optical) placed beneath the microscope stage. Backscattered fluorescence was collected through a ×40 oil-immersion objective lens (numerical aperture = 1.3). The collected light was divided in a ratio 80%/20% between the video port and the eyepiece. The fluorescence was recorded by an electro multiplier (EM) charge-coupled device (CCD) camera (iXon 860, Andor Technology, Belfast, UK) connected to the video port and equipped with either a 750- or 700-nm long-pass filter (Omega Optical). Movies of fluorescence were recorded with the camera using EM gain of 300 at spatial and temporal resolutions of 64 × 64 pixels and 860 frames/s, respectively.

Excitation power density. Power density of the incident excitation light was determined by means of a power meter (LaserMate-Q, Coherent) and a pinhole (radius, 300 µm) placed above the coverslip at an identical location to that of the myocytes used for imaging. After switching the laser on and ensuring that the excitation light followed the same pathway as during the imaging experiment, we placed the power meter sensor directly above the pinhole (to collect all transmitted light), and a value of the total power crossing the pinhole was measured. The power density of the incident excitation photons was determined by dividing the total recorded power by the area delimited by the pinhole. By adjusting the laser power, the power density of the excitation source was set to 38 mW/cm².

Data Analysis

Recordings of di-4-ANBDQBS fluorescence using the EM CCD camera yielded a series of frames with resolution of 64 × 64 pixels. From these frames, a computer algorithm selected a fluorescent image of the myocyte using a threshold criterion, which reliably separated the myocyte from the background (Supplemental Fig. 1; the online version of this article contains supplemental data). Occasional bright pixels corresponding to other fluorescent objects in the field of view,
such as dead cells or debris, were manually discarded. The fluorescence was averaged over the cell image yielding an optical AP (OAP), which showed a decrease in the amplitude upon depolarization, consistent with a shift of the absorbance or emission spectrum to a shorter wavelength with cellular depolarization (39). di-4-ANBDQBS fluorescence signal (F) was corrected by subtracting the extrinsic background (Fex) measured under exactly the same conditions (i.e., in the presence of laser illumination, laminin, dye, and perfusion flow), but in the absence of myocytes (Supplemental Fig. 2A). To correct for the decreasing drift in fluorescence caused by photobleaching (Supplemental Fig. 2B), we used a detrending algorithm implemented as follows. We first determined the OAP activation time (OAPAT), defined as the time at which the fluorescence derivative dF/dt (where t is time) achieved a minimum value (Supplemental Fig. 2C). After that, we computed the average values of fluorescence of a k-frame-long box n frames before OAPAT and of a k-frame-long box m frames after OAPAT. The values of n and m were selected to obtain points reliably outside the OAP and were 50 and 600, respectively. The value of k was set to 10. From these values, we calculated the slope and the y-intercept of a straight line crossing the two points determined by the aforementioned mean fluorescence values and the time points n and m, respectively. These two points were an accurate representation of the fluorescence values of the diastolic interval before the depolarization and after the repolarization, respectively. After setting the y-intercept value to zero, the set of values for each frame of the straight line were subtracted from the set of values constituting the recorded OAP, thus eliminating the drift (Supplemental Fig. 2D).

After correcting for Fex and detrending (Supplemental Fig. 2D), we computed the Frest by averaging the fluorescence values over a 10-frame-long box 20 frames before OAPAT. We computed the peak value of fluorescence (Fpeak) by averaging the fluorescence of the five frames following the frame with the minimum value of fluorescence. The OAP amplitude (∆F) was determined as the absolute difference between Fpeak and Frest. The time of 90% repolarization (REP90) was determined as the frame at which repolarization of the OAP achieved 90% of the ∆F value. The APD at REP90 (APD90) was computed as REP90 − OAPAT. In some cases, we calculated the normalized APD0 by dividing each APD90 value by the APD90 value of the first recorded OAP and expressed this ratio as a percentage. Estimation of the level of noise in the fluorescent signal was determined by calculating the root-mean-square value of a 20-frame-long box located n frames before the OAPAT. The SNR was determined by calculating the ratio of ∆F and root mean square. The voltage sensitivity was estimated by calculating the ratio ∆F/Frest.

**Illumination Protocols**

Practical implementation of the technique requires determining AP via fluorescence for varying periods of time, depending on the application. We utilized three different illumination protocols (“minimal”, “moderate”, and “intense”) with varying degrees of exposure to the excitation light, as explained in Supplemental Fig. 3. Throughout each illumination/recording protocol, myocytes were continuously field-stimulated at the cycle length of 1 s. The minimal protocol utilizes a series of short laser exposures (814 ms), just enough for recording a single AP, separated by relatively long (1–5 min) intervals of no exposure. Such protocol may be sufficient for a variety of applications, such as pharmacological tests. Other applications, such as the study of AP alternans (11), require a continuous monitoring of the AP, and we, therefore, investigated the recording of fluorescence under conditions of longer continuous exposure to the excitation light (10 s) applied every minute for the total of 5 min (moderate protocol). Finally, our intense illumination protocol is similar to one described previously by Schaffer et al. (48) and consists of two episodes of 130- to 150-s-long laser exposure, separated by a 210- to 230-s-long pause. We used this protocol primarily to investigate strategies directed to eliminate the development of marked photodynamic damage elicited by prolonged exposure to excitation light.

**RESULTS**

Our approach to create the appropriate conditions for non-invasive optical measurement of the AP in single cardiac myocytes was threefold. First, following an approach for the rational design of charge shift potentiometric probes based on molecular orbital calculations (34, 37), we synthesized the di-4-ANBDQBS probe, so as to shift both the absorption and emission spectra toward higher wavelengths, as well as to increase the ∆F/Frest. To compare the chromophores of new molecules to that of di-4-ANEPPS, we used two different semiempirical quantum mechanical methods optimized, respectively, for calculation of ground state molecular structure and the vertical excited state electronic configuration (see METHODS). To limit the computational load, we did not include side chains in the calculations, but just used methyl substituents on the flanking nitrogens in the chromophores. di-1-ANEPM and di-1-ANBDQQM contain the same chromophores as di-4-ANEPPS and di-4-ANBDQBS, respectively. The chromophore is the essential feature of the dye molecules that determine their spectra and electrochromic sensitivity. Therefore, it is unnecessary to run quantum mechanical calculations that explicitly include the full side chains. The most stable ground state structures for both the aminophenyldi-pyrindinium (ANEPI) and the ANBDQ chromophores was found to be planar; the highly favorable conjugation of the π-system in both chromophores was thus able to overcome some unfavorable steric interactions in the planar conformation. The calculations predict an absorbance maximum of 491 and 541 nm, respectively, for the ANEPI and the ANBDQ chromophores. Given the approximations inherent in these calculations, including the omission of solvent effects, this compares favorably with the absorbance spectra in ethanol for di-4-ANEPPS (495 nm) and di-4-ANBDQBS (561 nm). The key finding of a significant red shift in the absorbance spectrum of the new dye can be readily explained by the increased size of the π-system, achieved by increasing the length of the linker region from a single double bond in di-4-ANEPPS to a dienyl linkage in di-4-ANBDQBS and by incorporating a quinolinium group in the latter compared with the pyridinium moiety in the ANEPI dyes. More importantly, the increased length of the π-system results in an increased distance over which the chromophore positive charge shifts in going from the ground state to the excited state (Fig. 1). This charge redistribution is the source of the electrochromic mechanism (20, 34, 37), whereby the size of the fluorescence response is determined by the coupling of the membrane electric field with the excitation-induced movement of positive charge. Therefore, the larger charge shift of the ANBDQ chromophore (Fig. 1) explains its larger potentiometric sensitivity.

Second, based on the knowledge that photodynamic damage is generated via the formation of reactive oxygen species (ROS) (48), we supplemented the cell perfusion solution with a cocktail of antioxidant chemicals to restore the impaired ROS scavenging capabilities of the myocyte and thus limit the extent of the photodynamic damage.

Third, as a sensor, we used a CCD video camera with on-chip EM stage (iXon 860, Andor Technology). The ultrahigh sensitivity, high frame rate, and good quantum efficiency
in the range of di-4-ANBDQBS emission, combined with the capability of analyzing the cell image, made the iXon 860 camera a powerful tool for the AP recording in isolated cardiomyocytes.

**Correlation Between Electrical and Fluorescent Recordings of AP**

Simultaneous recording of AP with suction pipettes and di-4-ANBDQBS fluorescence demonstrated that, after rescaling OAP, the fluorescent and electrical recordings closely tracked each other (Fig. 2A). This similarity was conserved throughout prolonged time intervals, as indicated by the overlapping values of APD\(_{90}\) depicted in Fig. 2B. The same result was obtained in seven other cells subject to similar experimental conditions, including temperature (32.0 ± 1.0°C). The Pearson product-moment correlation coefficient between APD\(_{90}\) values calculated from both types of AP recordings was \( R^2 = 0.99954 \) (\( n = 58 \) simultaneous recordings). The mean percent difference between optically and electrically derived values of APD\(_{90}\) was 0.57 ± 0.34% (\( n = 58 \); mean ± SD). Dual recordings of electrical AP and OAP carried out at 37.0 ± 1.0°C in an additional group of six cells showed a similar degree of correlation between both types of APD\(_{90}\) measures (\( R^2 = 0.99816 \); mean percentage difference: 0.90 ± 0.56%; \( n = 11 \) simultaneous recordings; mean ± SD).

**Individual Effects of the Dye or Laser Excitation on Electrical AP**

Di-4-ANBDQBS could be loaded to the cell membrane without significantly altering the cellular AP. Figure 3A shows that APD\(_{90}\) and AP amplitude were quite stable throughout the control, perfusion with dye, and washout periods. Note that some degree of beat-to-beat variability of APD is an inherent characteristic of isolated cardiomyocytes (52). Figure 3B shows electrical APs (traces 1–3) and OAP (trace 2) recorded at respective time points 1–4 indicated in Fig. 3A. The OAP shown in Fig. 3B demonstrates that the amount of dye loaded to the cell membrane was sufficient to obtain a good OAP signal. Similar results were obtained in five other myocytes loaded with dye following the same procedure and under the same experimental conditions, including the perfusate temperature (32.0 ± 1.0°C). Further experiments designed to load the dye at 37.0 ± 1.0°C showed that a higher perfusate temperature during loading could, in some instances, lead to a slight transient or persistent reduction of APD following the dye loading and washout procedures, as shown in Supplemental Fig. 4. Nonetheless, comparison between the APD\(_{90}\) of electrically recorded AP in unstained myocytes to the APD\(_{90}\) of OAP recordings from non-microelectrode patched myocytes loaded with di-4-ANBDQBS via incubation at room temperature showed that there were no significant differences between
AOP. This operation permitted alignment of the resting membrane potential (OAP) and determined the resting membrane potential for the electrical AP (in arbitrary units. Supplemental Fig. 3). Recordings were performed at 32.0 °C, 30 myocyte paced at 1 Hz and exposed to “minimal” illumination conditions (see Fig. 4, B–F). There was no significant change in APD90 throughout the acquisition interval (P = 0.966), whereas both Frest and ΔF exhibited a small but significant reduction with time (P = 1.514 × 10⁻⁵ and P = 0.00475, respectively), which is consistent with a reduction in the pool of dye molecules responding to excitation. There was also a slight decrease in SNR and ΔF/Frest with time, but the latter did not reach statistical significance (P = 0.017 and 0.273, respectively).

In two additional cells subjected to the minimal illumination protocol, the dye concentration was either doubled (36.8 μM, squares in Fig. 4, B–D) or quadrupled (73.6 μM, triangles in Fig. 4, B–D). In these cells, the measured parameters also remained stable over 30 min. Note that there was no consistent relationship between the dye concentration and the values of Frest, ΔF, and ΔF/Frest for reasons not known to us. For example, the highest values of Frest, ΔF, and ΔF/Frest were obtained in a cell loaded with the intermediate concentration of the dye (36.8 μM, squares in Fig. 4, B–D). In fact, the ΔF/Frest value recorded in this cell (26.1% in the first OAP) was the highest among all cells tested in this study. Measurements in the first OAP obtained in all myocytes subjected to various illumination protocols (n = 28) yielded the following statistics: SNR, mean ± SD 40 ± 13.2, range 22.0–74.8; ΔF/Frest, mean ± SD 19.2 ± 4.1%, range 11.5–26.1%. Measurements of fluorescence were performed at 37.0 ± 1.0°C (n = 19 myocytes) or 32.0 ± 1.0°C (n = 9 myocytes). Supplemental Fig. 5A depicts a subset of typical OAPs recorded under minimal illumination conditions.

Measurement of OAP Using Moderate Illumination Protocol

Whereas during the minimal illumination protocol, dye concentrations between 18.4 and 73.6 μM did not cause toxic effects (see Fig. 4), under the moderate illumination protocol (Supplemental Fig. 3), dye concentrations >18.4 μM induced marked fluctuations of APD similar to those reported previously by Schaffer et al. using di-4-ANEPPS (48). Representative examples are shown in Fig. 5. The cell incubated with 18.4 μM of the dye (solid asterisks) show fluctuations of APD not exceeding 10% of the initial value, which is within the range of beat-to-beat APD variability observed in isolated cardiomyocytes (52). Importantly, there was no overall progressive prolongation of APD throughout consecutive laser exposure episodes, with a possible exception of the sixth episode, where there was a slight trend for APD increase in the last two OAPs (Fig. 5B). In contrast, in the cell incubated with 36.8 μM of the dye (shaded asterisks), the APD prolongation progressed in each laser exposure episode (Fig. 5C). Notably, the first episode induced a 9% APD increase, and the total APD...
prolongation after the fifth episode was ~50%. Furthermore, during the sixth episode, we observed marked beat-to-beat fluctuations in the APD (Fig. 5C, cross symbols). Notably, during the time separating each laser exposure episode, there was a trend for recovery of APD prolongation, but this was only partially accomplished (Fig. 5A, inset). The higher dye concentration also resulted in an increased SNR (31 for the cell loaded with 36.8 μM vs. 23 for cell loaded with 18.4 μM in Fig. 5). However, it is clear that, in this case, the trade-off is unacceptable, because the higher dye concentration precludes accurate measurements of APD. A sequence of OAPs recorded using the moderate illumination protocol can be viewed in Supplemental Fig. 5B. All myocytes subjected to moderate illumination for recording of OAP were maintained at 37.0 ± 1.0°C.

**DISCUSSION**

The recording of APs from isolated cells via fluorescence has been impeded by a combination of unfavorable factors, including low-photon yield, dye toxicity, and marked photodynamic damage (27, 28, 48). Thus, to date, a stark contrast exists between the use of VSDs for AP recording in multicellular systems (which has been widespread and largely successful) (18, 45a, 46, 54) vs. their use in isolated cells, which has been minimal, despite the considerable time elapsed since their initial development (27, 28, 43, 48).

In this study, we have developed a method that overcomes these limitations via the use of a recently synthesized fluorescent dye (di-4-ANBDQBS), in combination with high-sensitivity EM CCD technology (12). The fluorescent signals recorded from isolated cardiac myocytes by means of this technique exhibit high ΔF/F<sub>rest</sub> and unprecedented level of SNR.
Using the minimal illumination protocol, optical APD90 is within a 1% margin of error compared with electrical APD90 and is stable for at least 30 min (see Fig. 4). This amount of time is usually sufficient for testing effects of drugs. Following the recognition by the Food and Drug Administration and other international health agencies that a number of cardiovascular and noncardiovascular drugs can lead to the development of the potentially fatal arrhythmia known as torsade de pointes (TdP) (45), new guidelines directed to pharmaceutical companies have emerged for the early detection of the proarrhythmic potential of new drugs (50a). The guidelines are based on the current knowledge of the mechanisms that lead to the initiation of TdP and include an overall evaluation of the effects of potential new drugs on the electrophysiological events underlying TdP, such as their effect on the ion channel function, on the cardiac AP, and on the whole tissue electrophysiology (8, 19, 45). Thus we believe that the presented technique using the minimal illumination protocol is immediately applicable for testing the proarrhythmic potential of new drugs.

Fig. 4. OAP parameters during minimal illumination protocol. A: stacked OAP traces acquired every 5 min during 30 min from a single myocyte loaded with dye by incubation in solution containing di-4-ANBDQBS (18.4 μM). Different colors indicate specific time points as specified in the legend. B–F: time course of various OAP parameters (APD90, B), change in fluorescence on depolarization (ΔF; C), Frest (D), signal-to-noise ratio (SNR; E), and ΔF/Frest (F). Different symbols represent different dye concentrations, as indicated in the common legend in B. Open symbols indicate average values from five cells loaded with 18.4 μM dye. Solid symbols indicate parameters calculated from two additional cells, one loaded with 36.8 μM dye (squares) and the other loaded with 73.6 μM dye (inverted triangles). All of the OAP recordings were performed at 37.0 ± 1.0°C.
the purposes of drug testing with respect to their effects on the AP repolarization, which is critical for assessment of potential arrhythmogenic effects (7, 8, 19).

The key advantages of the presented technique include the improved fluorescent properties of di-4-ANBDQBS compared with its predecessor and most widely used VSD, di-4-ANEPPS (39). This new VSD has a longer chromophore than di-4-ANEPPS; as confirmed by quantum mechanical calculations, the longer chromophore produces higher wavelength spectra and also permits a larger electronic redistribution upon excitation. Based on an electrochromic mechanism (34), the size of this charge shift imparts a higher potentiometric sensitivity to the fluorescence of the newer dye. In whole tissue preparations using epifluorescence mode, di-4-ANBDQBS showed 50–100% increase in ΔF/F_red over the level of di-4-ANEPPS (12–15 vs. 7–9%) (39). Our estimates of ΔF/F_red in single cardiac myocytes are considerably higher, 19% on average and reaching 26% in the best case. The most likely reason for the difference is the fact that, in intact heart tissues, di-4-ANBDQBS stains other cell types, including vascular endothelium. Fluorescence emitted by dye molecules bound to endothelium would contribute to F_red, but not to AP-dependent ΔF, thus decreasing the ratio of ΔF/F_red.

Another important factor is the use of an EM CCD recording device, which combines the advantages of CCD sensor technology with the potential of devices implementing EM to

Fig. 5. Concentration-dependent photodynamic damage during moderate illumination. A: the dynamics of optical APD90 (normalized to the value of the first recorded OAP) under “moderate” illumination protocol. Measurements were taken from a cell loaded with 18.4 μM di-4-ANBDQBS (solid asterisks) and from a cell loaded with 36.8 μM di-4-ANBDQBS (shaded asterisks). Shaded and solid bars represent timing of charge-coupled device (CCD) recordings and laser exposure, respectively (see also Supplemental Fig. 3). Inset: time-expanded evolution of APD90 during minutes 2 and 3 in the cell loaded with 36.8 μM dye. Arrows point to the APD90 of the first OAP recorded in minutes 2 and 3. B and C: detailed dynamics of APD90 during each of the six consecutive 10-s laser exposure episodes occurring from minute 0 to minute 5 (the same data as in A). B: cell loaded with 18.4 μM di-4-ANBDQBS. C: cell loaded with 36.8 μM di-4-ANBDQBS. Each laser exposure episode is denoted with a different symbol, as indicated in B. All of the OAP recordings were performed at 37.0 ± 1.0°C.
incubation at room temperature presents an opportunity for subsequent recording of AP optically at physiological temperatures without a significant effect of the dye on the AP profile.

While minimal illumination conditions preserved the AP of stained myocytes, longer exposures to laser illumination often led to the development of photodynamic damage, as previously reported for di-4-ANEPPS (48). This effect is clearly concentration dependent, and thus bringing the concentration of di-4-ANBDQBS below the threshold level may fully abhish this effect. In our case, a concentration of 18.4 μM still allowed for maintaining stable APDs during the moderate illumination protocol. In contrast, 36.8 μM caused significant APD instabilities (see Fig. 5). Importantly, a dye concentration of 18.4 μM was sufficient to achieve high SNR and permitted accurate estimates of APD (see Fig. 4).

Photodynamic damage caused by excitation of VSDs is most likely due to formation of ROS and can be alleviated by adding enzyme catalase into the cell-dialyzing solution (48). Moreover, the lack of such damage in a significant amount in whole tissue preparations likely indicates that the cellular ROS scavenging system is somehow affected by the cell isolation procedure. Indeed, in cardiac tissue preparations, dye concentrations up to 500 μM were used before toxic effects were observed (38). Redox system deficiency in isolated cardiac myocytes or other cell types may be a general complication of the cell isolation procedures, although it is only rarely appreciated (9). The approach to modify cell-dialyzing solution via a micropipette suggested by Schafer et al. (48) would defeat the goal of achieving a noninvasive method of AP recording. Therefore, we explored a possibility that the apparent deficiency of the redox system in isolated myocytes can be remedied by bathing the cells in a solution complemented with a mixture of antioxidants. A variety of substances has been shown to be involved in the biochemical regulation of ROS in organisms (6, 26, 41). We, therefore, used the lipid-soluble and water-soluble dietary antioxidants α-tocopherol and ascorbic acid, as previously indicated (44). Additionally, we used the extracellular precursor of the intracellular redox regulator glutathione, N-acetyl cysteine (41), as previously detailed (1). Finally, we used uric acid, which is a ubiquitous metabolite present in plasma with antioxidant capacity (6). This treatment appears to be quite effective in alleviating the photodynamic damage during the intense illumination protocol (see Fig. 6). Indeed, in the cells treated with the antioxidant mixture, the severe abnormalities of repolarization leading to AP alternans and loss of one-to-one capture were fully abolished, and the AP prolongation was limited to 10–15% of the control value. For practical purposes, even this amount of APD prolongation may be too high, but the degree of reduction in photodynamic damage is striking. It is likely that the effectiveness of the antioxidant treatment may be further improved by longer incubation with antioxidants and optimization of the antioxidant mixture. These possibilities should be explored in future studies.

Previous studies have shown multiple mechanisms by which ROS may modulate the AP. These mechanisms are dependent on the time of ROS exposure, animal species, cellular compartment, tissue type, and ROS type and ROS-generating system (32). ROS-induced modification of the AP includes depolarization or hyperpolarization of the resting membrane potential, prolongation or shortening of APD, formation of...
afterdepolarizations, and promotion of cellular inexcitability (3, 30). These AP changes are mediated by the effect of ROS on various ion channels and transporters, including the inward rectifying potassium channel (14, 42), the glibenclamide-sensitive channel (23, 50), the Shaker channels (Kv1.3, Kv1.4, and Kv1.5) and the Shaw channel (Kv3.4) (17). The L-type voltage-sensitive Ca\(^{2+}\) channels (14, 21, 25, 50), and the Na\(^+/Ca\(^{2+}\) exchanger (13, 22). A comprehensive review of literature addressing the effect of ROS on ion transport mechanisms was provided by Kourie (32). The mechanisms leading to altered ion channel function may be via the oxidation of the cysteine residues lining the channel proteins and/or the membrane phospholipids, as well as via the effect of ROS on second messengers such as intracellular calcium. Further studies are required to address the specific effect of ROS generated during photo-excitation of the near-infrared VSD on ionic channel function.

It is also noteworthy that the present system does not fully capture all of the photons emitted by the dye. A large fraction of the fluorescent signal is lost through the optical system. The development of approaches based on the use of microfiber optics (49), which enable recording of fluorescence almost directly from the source, may further improve quality of signals and/or permit longer exposure times without causing the photodynamic damage.

Combination of fluorescent AP recording technique with flow cytometry (2) holds a promising chance to be used in high-throughput screening assays of new drugs with respect to their cardiotoxic effects. Furthermore, the red excitation spectrum of di-4-ANBDQBS renders this dye optimal for recording of fluorescence from blood-perfused tissue, given that blood has an absorption minimum at 650 –700 nm (39, 40). This property, in addition to the described high resolution of fluorescence recordings at the single-cell level, raises the possibility for implementing this technology into recording systems utilizing microfiber optics (49) in combination with procedures that enable local delivery of dyes (33). Such a system could have important implications for use as a diagnostic tool in the clinical setting.

In conclusion, the technique presented in this study sets a new standard for the noninvasive optical recording of AP in cardiomyocytes, which, to date, had remained unattainable for all practical purposes. di-4-ANBDQBS fluorescence generates signals of exceptionally high ΔF/F\(_{\text{rest}}\) (∼19%) and SNR (∼40) values, which closely track the cellular AP and permit determination of APD with high precision and without toxic consequences, provided the conditions of dye loading and illumination are carefully selected. The possibility to record high-resolution AP in single myocytes via fluorescence could have a strong impact, both for basic research and for the pharmaceutical industry.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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