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Short communication

Di-4-ANEPPS causes photodynamic damage to isolated cardiomyocytes

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Abstract. Action potential recordings from isolated guinea pig ventricular cells in the whole-cell recording mode were used to study the toxic and photodynamic properties of the voltage-sensitive fluorescent dye di-4-ANEPPS. Staining of the cardiomyocytes with di-4-ANEPPS (30 or 60 μM; 10 min) did not alter the action potential shape. When the stained cells were illuminated (1W/cm^2) severe effects on the action potential were observed. There was a prolongation of the action potential duration, occurrence of early afterdepolarizations, reduction of the membrane resting potential and eventually inexcitability. Addition of the antioxidant catalase (100 IU/ml) to the extracellular solution delayed the onset of these effects, suggesting that reactive-oxygen-intermediates take part in di-4-ANEPPS induced photodynamic damage. Since di-4-ANEPPS is a very important tool for optical membrane potential recordings in heart tissue and single cardiomyocytes catalase might be useful in suppressing photodynamic damage during optical potential recordings.

Key words: voltage-sensitive dye, di-4-ANEPPS, photodynamic damage, ventricular cells, catalase

Introduction.

Optical methods for monitoring of membrane potential allows one to study heart electrophysiology in several situations in which conventional intracellular recording techniques are unsuitable. These methods are non invasive compared to microelectrode techniques, capable of simultaneous recording from numerous sites within the specimen with high temporal and spatial resolution, and measure the "real" membrane potential timecourse (provided that the "optical measurement" is temporally separated from the contraction [1,6,18] or contraction is suppressed [5,8,9,13,15]) without stimulation artefacts caused by e.g. field stimulation.

Despite these advantages of optical potentiometric methods the toxic and photodynamic effects of the voltage-sensitive dye have to be considered. The dye molecules themselves may disturb membrane properties like fluidity and may influence ionic currents by interacting with ion channels (light-independent "toxic" effects). Furthermore the intense illumination of the stained specimen necessary for optical recordings from isolated cells may induce photodynamic damage via the formation of reactive-oxygen-intermediates and/or free radicals [7].

The fluorescent potentiometric dye di-4-ANEPPS has been used in several studies for optical potential mapping in heart muscle, including, most recently, study of arrhythmia [5,8,15]. This dye was first used in whole hearts [10,15] and heart tissue preparations [1,5,8,10,12,13] and more recently it has been used by us [1,18] and others [9,14] for recording from isolated cardiomyocytes. In the whole heart and isolated heart tissue preparations no or negligible toxic or photodynamic effects during optical recordings with di-4-ANEPPS have been reported [5,12,13,15], whereas severe damage to isolated cardiomyocytes was recognized by several authors [9,14,18]. Elimination of spontaneous activity in neonatal rat heart cells [14] as well as shrinking and irreversible contracture of rabbit [9] and guinea pig [9,18] ventricular cells have been seen after illumination of the di-4-ANEPPS stained cells. Therefore di-4-ANEPPS may change the electrical properties of the single cardiomyocytes previous to morphologic changes thus causing unphysiological conditions in optical membrane potential measurements.

In this paper we use conventional patch-electrode techniques to get a first insight into possible di-4-ANEPPS induced toxic and photodynamic effects on the action potential in single guinea pig ventricular cardiomyocytes.

Materials and Methods.

Single isolated ventricular cardiomyocytes were prepared by collagenase perfusion of guinea pig hearts. Action potential recordings were performed with the giga-seal technique using pipettes with a resistance of 2-3 MΩ. The pipette solution consisted of (in mM): KCl 140; $MgCl_2$ 2; $CaCl_2$ 1; EGTA/K^+ 11; Hepes/K^+ 10; ATP/K^+ 4.3; pH 7.4. During the experiments the cells were superfused with a physiological salt solution (composition

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in mM: NaCl 137; KCl 2.7; $NaHCO_3$ 11.9; $MgCl_2$ 1.1; NaH_2PO_4 0.4; Hepes/Na^+ 5, $CaCl_2$ 1.8; glucose 5.6) at a temperature of 35-36°C. An amplifier (Dagan, model 8100, Minneapolis, USA) was used for action potential recordings. Voltage signals were stored on video tapes using a PCM audioprocessor (Sony, model 501, Tokyo, Japan). For data evaluation the signals were transferred to an HP 1000/A-900 minicomputer (Hewlett-Packard, Sunnyvale, USA). All experiments were performed on rod-shaped cells showing clear cross striations. After establishing whole-cell recording configuration the cardiomyocyte was stimulated throughout the experiment with a superthreshold pulse (5 ms) at a frequency of 1 Hz, the microscope light was turned off and all of the following procedures (except illumination) were performed under very dim room light. One minute after the onset of electrical stimulation the flow of extracellular solution was stopped and the dye was added to the experimental chamber (chamber volume ~1.4 ml) via a micropipette. The dye used for staining was taken from an ethanol stock solution containing ~6 mM di-4-ANEPPS (Molecular Probes, Eugene, USA) and was diluted to a final concentration of ~60 or ~30 µM. Ten minutes after adding the dye the flow of the extracellular solution was restarted and the dye was washed out. The cells were illuminated from minutes nine to eleven and fourteen to sixteen after start of washout via the standard illumination unit (12 V, 100 W, halogen-lamp, broad band light without additional filters) of the microscope (Axiomat, Zeiss, Oberkochen, Germany) with an illumination intensity of about 1 W/cm^2 at the level of the bath chamber. To test the contribution of reactive oxygen intermediates in di-4-ANEPPS (60 µM) induced photodynamic damage, several experiments were performed with 100 IU/ml of the antioxidant catalase (Sigma Chemical, St. Louis, USA) added to the bathing medium when washout was started. All data were expressed as mean ± SEM. Error bars in figs. 2,3 represent SEM. Statistical analysis was performed using Student's *t*-test (level of significance $p < 0.05$).

Results.

Fig. 1 shows representative (one out of >30 cells in which similar effects were seen) di-4-ANEPPS (60 µM) induced action potential alterations at different experimental stages.

Fig.1A shows a control recording (before staining). Staining of the cardiomyocyte did not alter the action potential shape: fig. 1B shows the recording taken 5 min after washout was started. In contrast, illumination of the stained cell (starting nine minutes after washout) resulted in severe action potential alterations (fig. 1 C, D). The general course of these effects (seen at both 60 and 30 µM di-4-ANEPPS) was: first a prolongation of the action potential duration with appearance of early afterdepolarizations (EADs; fig. 1C), followed by depolarization of the resting membrane potential, spontaneous activity and finally inexcitability with a resting membrane potential of about -30 mV (fig.1 D).

The di-4-ANEPPS/illumination induced action potential prolongations occurred without visible changes of the cell shape whereas the state of inexcitability was accompanied with shrinking and hypercontracture.

Although illumination caused a progressive prolongation of the action potential individual action potentials showed large variabilities in duration (fig. 1C). The timecourse of the photodynamically induced action potential parameter alterations (di-4-ANEPPS 30 µM) are shown in fig.2 (n=4). The first period of illumination as shown in fig.2 induced negligible action potential alterations compared to the alterations (mainly APD90) induced by the second illumination period. The period of darkness in-between both illuminations (minute 11 to 14) caused an intermission in the increase in APD90 whereas the period of darkness from minute 16 to 19 had almost no effect on the increase in APD90.

Illumination (4 min) of unstained cardiomyocytes (n=6) and cardiomyocytes that were exposed for ten minutes to ethanol

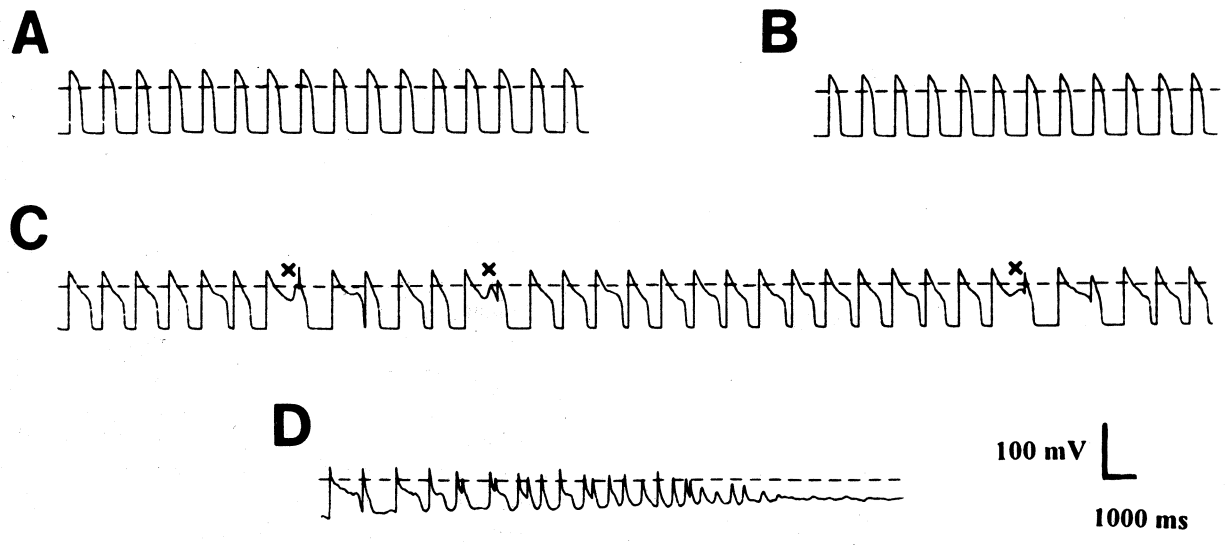

Fig. 1. Action potential recordings. **A:** control action potentials before staining, **B:** action potentials after 5 minutes of washout (di-4-ANEPPS 60 µM), **C:** action potentials after 3 minutes of illumination showing alterations of action potential duration and EADs (indicated by **x;** fast spikes are caused by the electrical stimulation) whereas action potential amplitude and $\dot{V}_{max}$ (maximum upstroke velocity) remained essentially unchanged, **D:** depolarization of membrane resting potential, spontaneous activity and state of inexcitability after 4 minutes of illumination. The zero-potential level is indicated by the dashed line.

(ethanol absolute diluted 1:100) (n=4) did not result in action potential prolongations (not shown) indicating that the effects described above are due to the illumination of the di-4-ANEPPS stained cells. Figure 3 shows the influence of staining concentration (60 and 30 µM di-4-ANEPPS) and the statistically significant beneficial effect of catalase (100 IU/ml) on the photodynamically induced action potential prolongations evaluated 14 and 19 minutes after start of washout. The addition of 100 IU/ml catalase (n=4) to the extracellular medium (catalase exposure started five minutes prior to illumination) delayed the onset of di-4-ANEPPS (60 µM) induced photodynamic action potential alterations (see fig. 3) but did not entirely suppress these effects. Catalase itself (100 IU/ml; n=6) when administrated for 15 min did not cause action potential alterations (not shown).

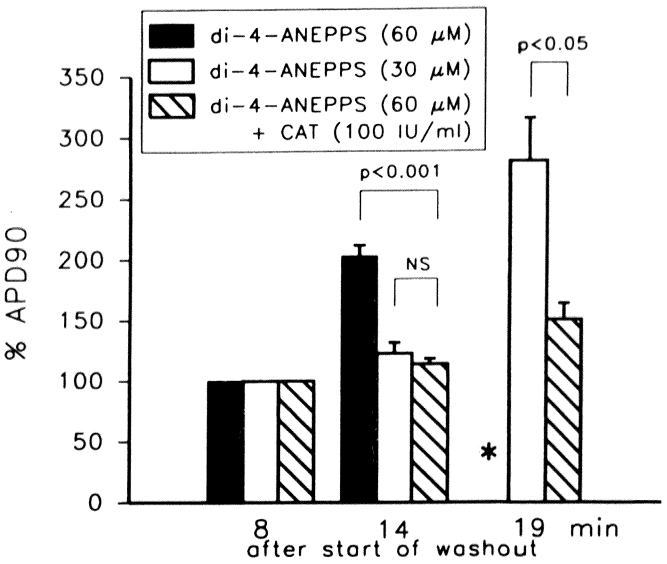

Fig. 3. Percentage change of action potential duration APD90 (at 90% of repolarization) in di-4-ANEPPS stained and illuminated cardiomyocytes at minutes 8, 14 and 19 after start of washout. For each cell the average of 10 consecutive action potentials was taken as APD90. The APD90 previous to illumination (8^{th} min) was taken as 100%. The cardiomyocytes were illuminated from minute 9 to 11 and 14 to 16 after washout. The increase in staining concentration enhanced and accelerated the action potential alterations whereas addition of catalase delayed the onset of alterations.
* All cardiomyocytes stained with 60 µM di-4-ANEPPS were in the state of hypercontracture and inexcitability.
For each column number of cells = 4. Data are mean ± SEM. The corresponding p-values of a statistical comparison between catalase and catalase free groups are shown. NS= not significant.

Discussion.

This study demonstrates that: i) di-4-ANEPPS staining of guinea pig ventricular cells did not cause action potential alterations; ii) the di-4-ANEPPS stained cardiomyocytes were sensitive to illumination (low power ($1W/cm^2$) in comparison with laser (up to 200 W/cm^2) [1,9,18]); iii) these photodynamic effects altered the action potential primarily in the repolarization phase; iv) the antioxidant catalase delayed the onset of photodynamic effects indicating the contribution of reactive-oxygen-intermediates to di-4-ANEPPS induced photodynamic damage. This

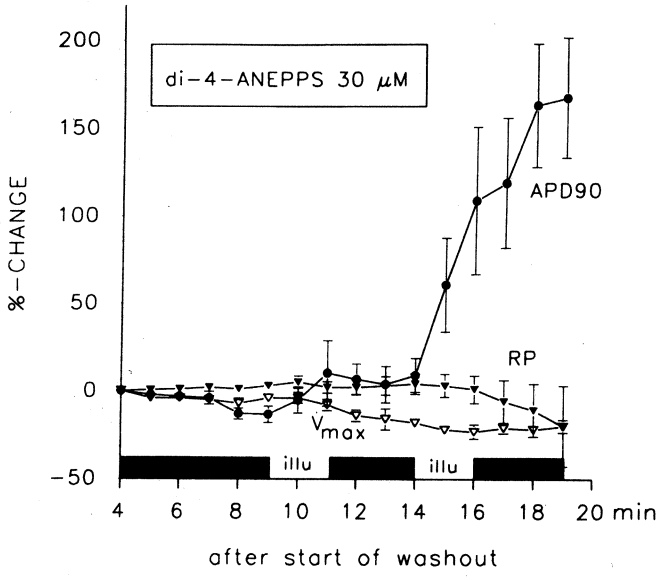

Fig. 2. Timecourse of photodynamically (di-4-ANEPPS 30 µM) induced action potential parameter alterations (maximum upstroke velocity $\dot{V}_{max}$, action potential duration at 90% of repolarization APD90, resting potential RP) during washout and intermittent light exposure. Changes in the action potential parameters are given as percentage changes with respect to the parameter values 4 min after start of washout ($\dot{V}_{max}$ = 141 ± 6.6 V/s, APD90 = 424 ± 44.4 ms, RP = -88.2 ± 1.7 mV). Data are mean ± SEM, number of cells = 4. To rule out errors due to beat to beat alterations the parameters of 10 consecutive action potentials were averaged. Error bars are shown when larger than symbols. illu = illumination

conclusion is reinforced by the finding that the pattern of action potential alterations seen under di-4-ANEPPS induced photodynamic conditions was very similar to effects seen in cardiomyocytes under various "oxidant stress conditions". Action potential prolongations and EADs were found in canine cardiomyocytes exposed to free radical generation systems [2] and in rat and guinea pig ventricular cells superfused with H_2O_2 [3] or dihydroxyfumarate [4]. Similar action potential prolongations were found in rabbit and frog cardiomyocytes after "oxidant stress" caused by photoactivation of the fluorescent dye rose bengal [11,16]. The antioxidant catalase (even inactivated) has been shown to protect heart myocytes against rose bengal/photoactivation generated reactive oxygen intermediates [17]. The beneficial effect of the antioxidant catalase in the present study suggests that catalase might be useful in suppressing photodynamic damage in optical potential recordings. However the usefulness of catalase (or other antioxidants) for suppressing photodynamic damage in optical potential measurements remains to be clarified because it requires that these compounds do not cause electrophysiological alterations themselves and that they do not disturb the potential dependent optical signal (e.g. by decreasing the signal to noise ratio) [7]. Preliminary experiments (unpublished observation) using catalase in

"optical voltage recordings" (for a description of the "optical method" used see: [1,18]) showed no major change in the quality of the voltage dependent optical signal but a considerable cell-protective effect. A screening for the usefulness of antioxidants in "optical recordings" might yield agents even more effective than catalase and provide information about the species of reactive oxygen intermediates involved. The ionic mechanisms that are responsible for di-4-ANEPPS photodynamic action potential alterations in cardiomyocytes are at present unknown but might be similar to what was seen in heart cells exposed to free radicals [3,4,11].

Although our study has several limitations (e.g. use of dye concentrations that were higher than usual [1,5,8,9,10,12,14,15,18], use of broadband low power light instead of monochromatic laser light [1,8,9,12,13,18]), we consider our data to be representative of optical membrane potential monitoring in single heart cells. The "total light exposure" (illumination intensity (W/cm^2) x duration of illumination) that led to hypercontracture in the experiments presented in this paper is comparable to values seen during optical recordings using an argon ion laser (514 nm) as excitation light source [1,18]. Nevertheless the extent and the timecourse of photodynamic action potential alterations might mainly depend on the type of preparation and experimental conditions (e.g. light source, dye concentration) used. Further our results indicate that even the light necessary for manipulating the stained preparation might damage the cardiomyocytes prior to optical recordings. Therefore it is essential to minimize "non-excitation-light" generated photodynamic damage (using "proper wavelengths" apart from the absorption range of the dye e.g. $\lambda > 630$ nm for di-4-ANEPPS and keeping the illumination of the stained cells as short as possible).

The fact that di-4-ANEPPS induced photodynamic damage initially alters the repolarization phase with occurrence of EADs emphasizes that care has to be taken in interpreting optical recordings from isolated cardiac cells representing the repolarization phase [9]. Although di-4-ANEPPS induced photodynamic damage has not been reported in multicellular heart preparations, the photodynamic properties of di-4-ANEPPS might enhance reentry conditions in multicellular preparations after "excessive" light exposure because EADs and inhomogeneities in action potential duration are considered as arrhythmogenic mechanisms [4]. Although di-4-ANEPPS causes photodynamic damage we regard this dye as a very important tool for studying the electrical properties of isolated cardiomyocytes provided that one knows the limitations of its use.

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