Comparison of Two Procedures of Loading with Voltage-Sensitive Dye Di-4 ANEPPS in Rabbit Isolated Heart

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Abstract

Recording of monophasic action potentials (MAPs) by optical method using voltage sensitive dyes has been established as possibility to measure membrane potential changes in certain models. We studied two procedures of isolated rabbit hearts loading with di-4-ANEPPS - "slow" method (perfusion with low concentration of dye for longer time) or "fast" method (single microinjection of highly concentrated bolus of the dye in the aorta). Signal-to-noise ratio was evaluated in each minute of continuous exposure to excitatory light and persistence of dye in the tissue was estimated. We conclude that both methods enable us to record MAPs, but during long exposure to light the persistence of the dye in the tissue is insufficient in "fast" method. Although the "slow" loading affects electrophysiological properties of the heart, in case of rabbit heart it is negligible since its relative resistance to the side-effects of di-4-ANEPPS.

1. Introduction

Voltage-sensitive dyes (VSD's) undergo changes in their electronic structure, and as a consequence also in their fluorescence spectra. These changes result from changes in the surrounding electric field, for instance in excitable tissues such as myocardium or neurons. Various VSDs have been introduced into everyday laboratory practice (merocyanine, ANEPPS, etc.). Dyes from ANEPPS group (amino-naphthyl-ethenyl-pyridinium) are the most constantly used in cardiac preparations [1,2]. One of them, di-4-ANEPPS is utilized in our laboratory for recording of monophasic action potentials (MAPs) by optical method in various animal models. Most often employed experimental model in our projects is isolated rabbit heart perfused according to Langendorff. In our experimental set-up, the heart is exposed to VSD diluted in Krebs-Henseleit (K-H) solution to the concentration of 2microM. The tissue is perfused with this mixture for 20-25 minutes and then washed out for the same time. In other laboratories, different way of loading is preferred – direct application of small amount of concentrated dye into the bubble trapper of Langendorff perfusion set by means of a microsyringe or a pump followed by short washout.

In this paper, these two ways of loading of rabbit myocardium with di-4-ANEPPS are compared.

2. Methods

The animals in both groups were treated in the same way and the hearts prepared by the same procedure. Each experiment consisted of five phases: isolation of the heart, control perfusion, loading with the dye, washout, and MAPs recording under control conditions (37°C, spontaneously beating heart).

Briefly, the animal was deeply anaesthetized by ketamin (60mg/kg of body mass) and xylazin (2mg/kg of b.m.), artificially ventilated by the respiratory pump and the chest was opened. Then the heart was excised with a sufficiently long segment of ascending aorta. The aorta was cannulated, the heart mounted on the Langendorff apparatus and placed in thermostat-controlled bath (37°C) filled with Krebs-Henseleit solution of following composition (in mM): NaCl 118, $NaHCO_3$ 24, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 5.5, Taurine 10, and $CaCl_2$ 1.2. The solution was oxygenated with 95% O_2 and 5% CO_2. The isolated heart was then perfused with the same solution at the constant perfusion pressure (80 mmHg) for 25 - 30 minutes – control period. All hearts exhibiting any dysrrhythmias during this period were discarded.

During the whole experiment, electrogram was recorded by the touch-free method [3]. Six silver-silver chloride disc electrodes (4 mm in diameter) were placed on the inner surface of the bath in which the heart was placed during the experiment. The bath was filled with perfusion solution. Electrical signals were recorded from three orthogonal bipolar leads (X, Y, and Z). The signals

were then amplified and digitized at a sampling rate of 2kHz by a three-channel, 16-bit AD converter. The maximum amplitude of recorded signals varied between 100 μV and 500 μV, depending on the heart.

Monophasic action potentials were recorded by the optical system [4]. It consists of a flexible bifurcated fiber cable with seven optical fibers (six illumination fibers positioned in a circle and a detection fiber positioned in the center of the cable). The fiber optics together with micromanipulator makes the system flexible so the user is able to scan action potentials from various places on the heart surface with almost no mechanical constraint. The optical probe is softly attached to the preparation to suppress motion artifacts without a need of focusing. The "input" end of the cable with six illumination fibers is connected to a light source. The "output" (detection) fiber is connected to a light detector that senses the beam of emitted light. The optical fibers are protected by a silicon inner tube and a flexible chrome plated brass outer tubing. The tubing also gives stress relieve.

The cold light source with high intensity light output is used for excitation of the dye (150W halogen). It contains a built-in IR filter which prevents a preparation from heating, and a band-pass filter (560 nm +/- 30 nm), which selects light at excitation maxims of the used dye. The light intensity can be adjusted by a crescent shaped diaphragm and by controlling the lamp voltage.

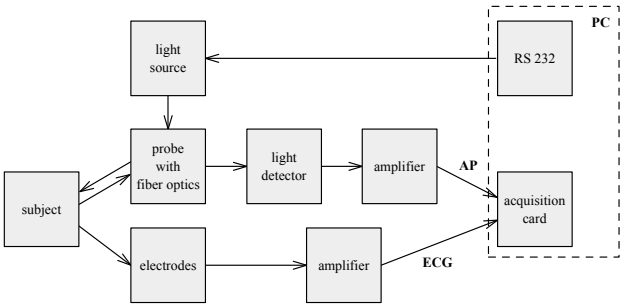

Figure 1. The block diagram of the acquisition system. The excitation light is generated by a light source Intralux DC-1100 with a 150W tungsten-halogen lamp. The light is led by flexible fiber optics to the sample. Fluorescent light is emitted by voltage-sensitive dye present in the sample and led back by the parallel fiber optics. The emitted light hits a photodiode detector. An electrical signal from the detector is amplified and digitized.

The changes in dynamics of transmembrane potential result in amplitude modulation of the emitted light. This is detected by a photodiode detector with a high-pass (>610 nm) filter. The output signal of the photodiode detector is preamplified so that the two stage amplifier adjusts the signal to input range of data acquisition card (±1 V). The electrical circuits include also an analogue anti-aliasing filter (lowpass filter fc=2 kHz) and a high-pass filter (fc=0.05 Hz) to suppress DC offset.

The data acquisition card processes the preamplified and filtered signal. The card digitizes the signal with 12 bits dynamic range and at rate of 4000 samples/sec. The digital signal is stored on a hard disk for further off-line processing (noise suppression, visualization and analysis). Data acquisition is controlled by subroutines of a software package LabView.

The block diagram of the acquisition system is depicted in Figure 1.

An example of synchronous recording of electrogram and MAPs is given in Figure 2.

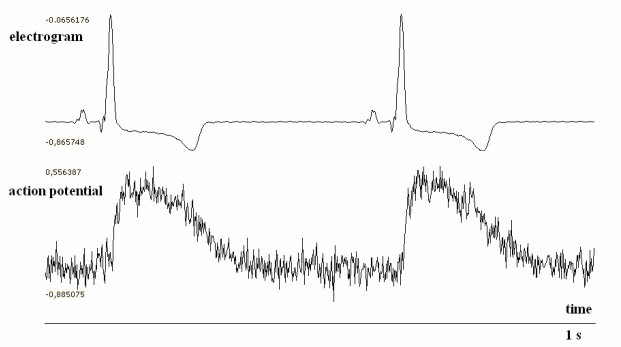

Figure 2. Synchronous recording of electrogram by touch-free method (top, one bipolar lead) and corresponding MAPs by optical method (bottom) from isolated rabbit heart perfused according to Langendorff. Recording at control conditions (37°C, spontaneously beating heart), unfiltered signal.

VSD di-4-ANEPPS (Molecular Probes, Eugene, OR, USA) was prepared as a stock solution by diluting in DMF (dimethylformamide, Sigma Aldrich, CZ) to the final concentration of 2mmol/l.

We studied two procedures of loading in the model of isolated rabbit heart. The hearts of the first group were loaded by “slow” procedure – the dye was diluted from stock in Krebs–Henseleit solution to the final concentration of 2μmol/l. The tissue was then perfused with this mixture for approximately 20-25 min. The speed of perfusion was given by coronary flow (the modification of perfusion at constant pressure is used). The dye was then washed out for the same period of time as was the loading.

The hearts in the second group were loaded by “fast” method - instant application of 20micro of stock solution into the bubble trapper above the aortic cannula by means of microsyringe, followed by short, approximately 5min washout.

After the heart was loaded by either procedure and the

excess of VSD washed out from its coronary system, continuous exposure to light source and recording of MAPs started. Recording was terminated at the moment when signal was too small to measure or noise too big to suppress.

In each minute of recording, MAPs were manually marked in their maximum and signal-to-noise ratio (SNR) was evaluated according to formula:

SNR = 20 x log (signal/noise)

Figure 3 shows representative recording of monophasic action potential with example of signal-to-noise ratio obtaining.

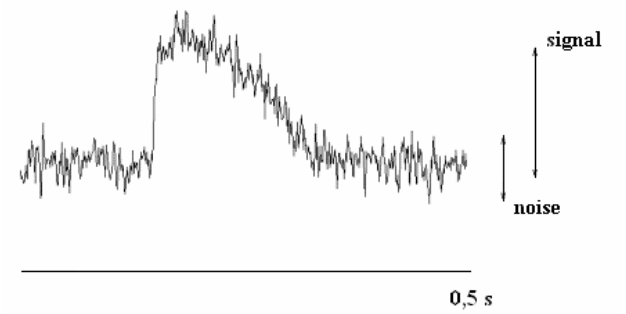

Figure 3. Monophasic action potential recorded by optical method at control situation (37°C, spontaneously beating heart), unfiltered signal. The way of manual acquiring signal-to-noise ratio is indicated.

3. Results

By "slow" loading with relatively low concentration of the dye applied into the coronary system together with perfusion solution tissue with higher signal-to-noise ratio was obtained. Also the recorded signals were smoother. Persistence of the dye in the myocardium in this case was quite high. This can be seen in Figure 4 where data from one such experiment are summarized. The heart was exposed to the excitatory light continuously for more than two hours and SNR was measured in each minute. Clear declining trend is present in this picture, however SNR at the end of this long-lasting continuous exposure to light is still quite high and recorded MAPs are still of reasonably good quality.

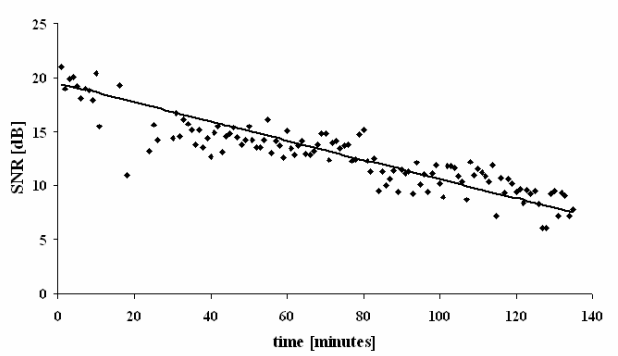

Figure 4. Signal-to-noise ratio of MAPs optically recorded in the rabbit isolated heart perfused according to Langendorff at control conditions (37°C, spontaneously beating heart).

On the contrary to previous results, the rabbit hearts loaded by so-called "fast" procedure exhibited lower signal-to-noise ratio and moreover the recorded signals were not as smooth as in case of slow loading with low concentration of the dye. Also fading of the signal during continuous illumination of the hearts loaded in this way was quite fast (in other words – the persistence of the dye in the tissue is lower in comparison with the first procedure). Changes of SNR during long-lasting continuous illumination of rabbit hearts loaded by both compared methods are presented in Figure 5. Note that the declination of trends is more less the same in the hearts loaded by either method but absolute values of SNRs differ significantly already at the beginning of experiment.

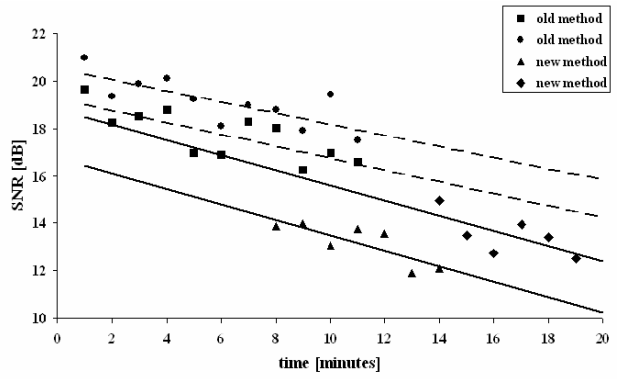

Figure 5. Comparison of signal-to-noise ratios of MAPs recorded in four isolated rabbit hearts perfused according to Langendorff at control conditions (37°C, spontaneously beating heart). Always two hearts were loaded by the same method. Old method = "slow" loading with low concentration of the heart, new method = "fast" loading with a bolus of highly concentrated dye.

4. Discussion and conclusions

The first information about recording of the dynamic changes of the transmembrane potential of excitable cells by optical means was reported in late sixtieths. The first cardiac application of this method was reported in 1981 – the localization of pacemaker activity in embryonic heart preparation. Since then recording of MAPs by optical systems using VSDs developed into a method which represents sophisticated, up-to-date approach to a measurement of fine voltage changes on the membrane of excitable cells.

Voltage-sensitive dyes are mainly used for measuring membrane potential changes in systems where – because of scale, topology, or complexity – the use of classical electrodes is inconvenient or impossible (e.g. in the presence of external electric fields – uninterrupted and artifact-free recording during pacing stimuli and defibrillation shocks or recording of high-resolution maps, e.g. of cardiac repolarization) [1].

Of course certain technical troubles have to be solved in order to be able to record settled MAPs. What is somewhat problematic from biological point of view is the quality of preparation which may be changed by application of VSD. The dyes have been extensively employed by numerous research groups to record optical action potentials in a wide variety of heart preparations from single cardiomyocyte to isolated heart. In many cases, various side effects of the dye on the preparation in the absence and presence of light were reported [2,5]. Most prominent pharmacological effect of VSDs on cardiac tissue is so-called photodynamic or phototoxic damage. The exact mechanism of these effects remains unexplained but there is no doubt that it is very complex. Formation of free radicals or direct interaction with the voltage-gated calcium and/or potassium channels have been suggested, which may result in altered conductivity and the time-dependent gating.

In our previous study [5] on the effect of loading of guinea pig and rabbit isolated hearts with di-4-ANEPPS, we observed slowing of the heart rate and a partial block in AV node. Accompanying changes of the shape of electrogram curve favored the idea of direct effect of the dye on cardiac ionic channels – and because T wave was often impaired in its shape and amplitude during the loading and washout periods, we concluded that predominantly potassium channels were affected.

Most of laboratories load the myocardium by concentrated dye as a bolus. During short period of loading in this case hardly any electrophysiological changes can be detected. The loading procedure used in our laboratory gives us the opportunity to obtain preparation with quite high persistence of the dye in the heart muscle. We have proved that although certain electrophysiological changes are present in the hearts of both species, at least in rabbit these changes are mostly reversible and thus its myocardium can be loaded with di-4-ANEPPS by "slow" method and be considered a reliable model for electrophysiological studies.

In conclusion, both methods of loading of rabbit myocardium with VSD di-4-ANEPPS result in loaded heart where recording of MAPs was possible. However, in case of long-lasting exposure to light it is necessary to pay attention to persistence of the dye in the heart muscle. Although the slow procedure might affect electrophysiological properties of the myocardium, in case of rabbit heart it is in fact negligible since the heart muscle of this species is quite resistant to the side-effects of di-4-ANEPPS.

Acknowledgements

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