Tissue Response during Staining and Illumination of Voltage-Sensitive Dye in Rabbit Myocardium

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Abstract

Voltage-sensitive dyes (VSD's) undergo changes in their electronic structure, and consequently their fluorescence spectra, in response to changes in the surrounding electric field and are therefore used for recording of monophasic action potentials (MAPs) by optical method. S.-c. phototoxic effects of VSDs appear under intense illumination and include alterations in heart electrical signals. To examine putative side effects of VSD di-4-ANEPPS in rabbit hearts, we studied electrogram, mean coronary flow and formation of hydroxyl radicals in the control, staining and washout periods and during illumination. Possible ultrastructural changes were examined by electron microscopy. Rabbit myocardium is resistant to di-4-ANEPPS side effects during staining and also resistant to bleaching during intense illumination since only minor changes in ECG and no change in other parameters were observed.

1. Introduction

Voltage-sensitive dyes are used for recording of monophasic action potentials (MAPs) by optical method in a range of heart preparations (from isolated cardiomyocytes to multicellular preparations such as papillary muscles, atria and trabeculae to isolated hearts). Optical method of MAP recording represents sophisticated, up-to-date approach to the measurement of fine voltage changes on the membrane of cardiac cell. Simultaneous recordings of transmembrane potential by optical and microelectrode techniques have validated the high conformity of optical MAPs compared with microelectrode recordings and demonstrated that optical MAPs detected the classic features of MAPs from various parts of the conductive system and myocardium [1].

Recording of the dynamic changes of the transmembrane potential of excitable cells by optical method was introduced in late sixtieths. The first cardiac application was reported in 1981 – the localization of pacemaker activity in embryonic heart preparation. Since then the method has been noticeably improved and various new voltage-sensitive dyes from numerous chemical groups have been tested. Among all troubles which had to be solved before the dye was introduced to everyday laboratory practice, a prominent one was to minimize side effects of the dye on the preparation in the absence and presence of light. Most disturbing pharmacological effect of voltage-sensitive dyes on cardiac tissue is so-called photodynamic or phototoxic damage. The exact mechanisms of these side effects remain unknown, although for instance formation of free radicals or direct interaction with the voltage-gated calcium and/or potassium channels has been discussed. The latter may results in altered conductivity and the time-dependent gating of specific ionic channels and thus in diverse electrophysiological disturbances of the cardiac preparation [2].

2. Methods

VSD di-4-ANEPPS (di-4-amino-naphthyl-ethenyl-pyridinium) is used in our laboratory to record MAPs in isolated rabbit hearts [3]. The animal is deeply anaesthetized by ketamin (60mg/kg of body mass) and xylazin (2mg/kg of b.m.), artificially ventilated and the chest is opened. Then the heart is excised with a sufficiently long segment of ascending aorta. The aorta is cannulated, the heart fixed on the Langendorff setup [4] and placed in thermostat-controlled bath (37°C) filled with Krebs-Henseleit solution of following composition (in mM): NaCl 118, NaHCO3 24, KCl 4.2, KH2PO4 1.2, MgCl2 1.2, glucose 5.5, Taurine 10 and CaCl2 1.2. The solution is oxygenated with 95% O_2 and 5% CO_2. The isolated heart is then perfused with the same solution at the constant perfusion pressure (80 mmHg) for 25 - 30 minutes – control period. All hearts exhibiting any

dysrhytmias during this period are discarded.

During the whole experiment, electrogram is recorded and mean coronary flow monitored. The electrogram is recorded by the touch-free method. Six silver-silver chloride disc electrodes (4 mm in diameter) are placed on the inner surface of the bath. ECG signals are recorded from three orthogonal bipolar leads (X, Y, and Z). The signals are amplified and digitised at a sampling rate of 500 Hz by a three-channel, 16-bit AD converter. The maximum amplitude of recorded signals varies between 100 μV and 500 μV, depending on the heart.

The mean coronary flow (ml/min) is measured every fifth minute during the whole experiment.

The heart is then exposed to voltage-sensitive dye di-4-ANEPPS (amino-naphthyl-ethenyl-pyridinium) diluted in Krebs-Henseleit solution to the concentration of 2microM (2mM stock solution in DMF). The tissue is perfused with this mixture for 20 - 25 minutes. Again, the electrogram and mean coronary flow are monitored.

The dye is washed out for the same period of time as the period of staining. After the washout, the heart is ready for recording of optical APs.

Because a decrease in coronary flow has been observed in our previous experiments (mainly in isolated guinea pig hearts, but to some extent also in rabbit hearts), the idea of putative ischemic damage has been brought in discussion. Therefore we tested whether hydroxyl radicals were formed during control period and if the presence of di-4-ANEPPS can affect their formation. The isolated hearts were perfused in the presence of 1mM salicylic acid and we analysed the production of 2,5-DHBA (2,5-dihydrobenzoic acid) in coronary effluents and the influence of di-4-ANEPPS on its formation using high performance liquid chromatography (HPLC) [5].

Finally, the stained hearts were examined by electron microscopy technique in order to search for potential signs of ultrastructural damage – to test the viability of the preparations. The tissue was prepared by the usual procedure (perfusion with 3% solution of glutaraldehyde for 10 minutes at the end of experiment, preparation of the strips 1x1x3 mm in size from both atria and ventricles, immediate fixation in a 400mmol/l solution of glutaraldehyde in 0.1M phosphate buffer at pH 7.4, and then dehydration, immersion and embedding in Durcupan ACM). Ultra thin sections were made and stained with lead citrate or with uranyl acetate and lead citrate and then viewed and photographed.

Then, the tissue was illuminated for two hours and the recorded monophasic action potentials were measured.

3. Results

In our study, 15 New Zealand rabbits (both sexes, average weight 1500 ± 250 gr) were employed.

The heart rate of isolated rabbit hearts decreased slightly in staining, partially restored in washout in some hearts or remained slowed down and then did not change subsequently. For example of original recording from a representative experiment see Figure 1.

Only minor changes of electrogram shape and the time intervals (other than R-R) were observed during staining and washout – namely these were changes in T wave morphology and episodic atrio-ventricular dissociation. An example of original recording with A-V dissociation may be seen in Figure 2.

No such changes were detected under focal illumination and the optical signal did not show any signs of fading.

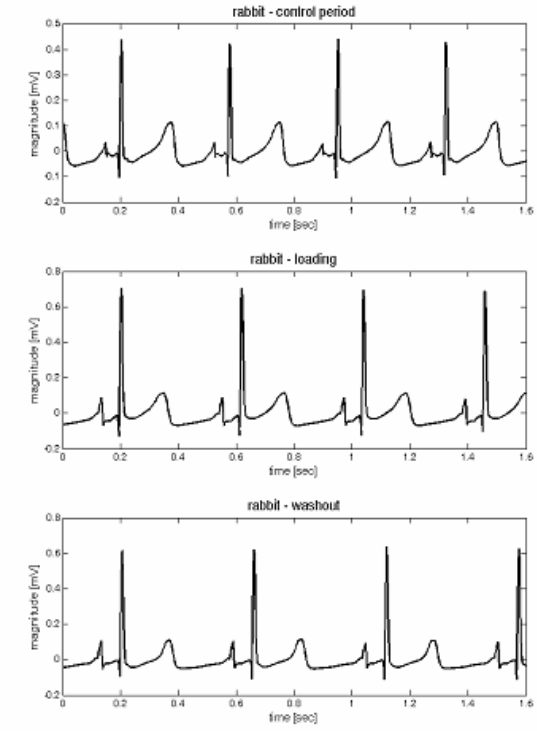

Figure 1. Original recording of electrogram from rabbit isolated heart during the control, staining (loading) and washout period. Illumination period is not shown. Note the R-R intervals prolongation during staining which in this case slightly increased even in washout period.

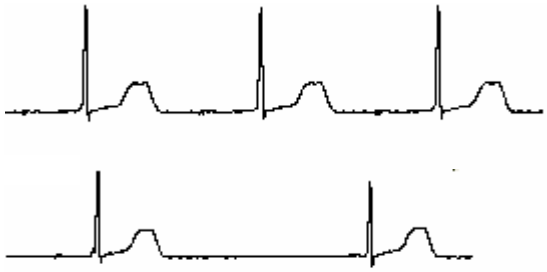

Figure 2. Original recording of electrogram from rabbit isolated heart during the control (top) and staining (bottom) period. Note the A-V block prolongation during staining.

The mean coronary flow measured by collecting the perfusate from the outlet of Langendorff set chamber at the end of each fifth minute underwent only minor changes during the course of loading and washout (see Figure 3).

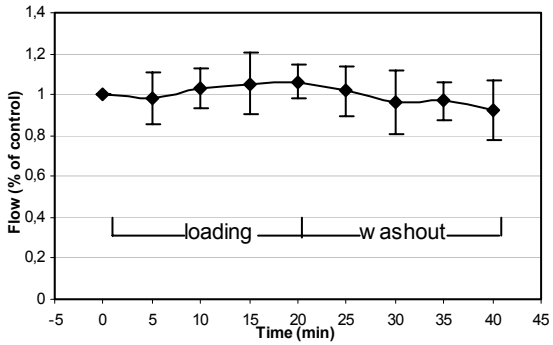

Figure 3. Mean coronary flow in isolated rabbit hearts during loading and washout period (0 min – flow at the end of control, 5 – 20 min loading, 25 – 40 min washout).

The production of hydroxyl radicals was observed neither in the control nor in the washout periods. For details see Figure 4.

In next part of our study, we examined whether long lasting exposition of isolated hearts to VSD is accompanied by ultrastructural changes as examined by electron microscopy. In comparison with control hearts, those loaded with di-4-ANEPPS did not reveal any ultrastructural changes detectable by electron microscopy.

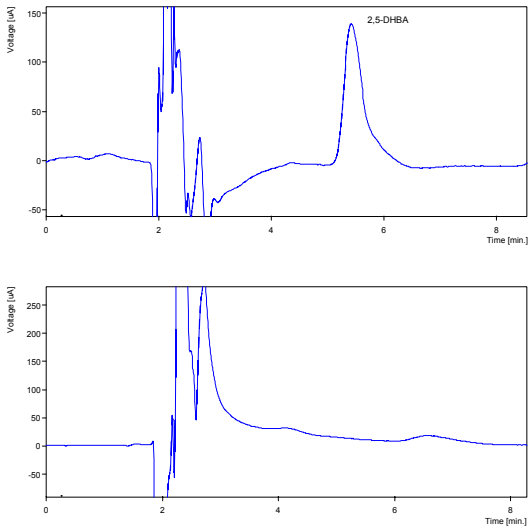

Figure 4. Control HPLC chromatogram of the Krebs-Henseleit solution containing standard of 2,5-DHBA (top). Peak of 2,5-DHBA has retention time 335 s. HPLC chromatogram of coronary effluent containing 1 mM salicylate and di-4-ANEPPS (bottom). No peak of 2,5-DHBA is seen.

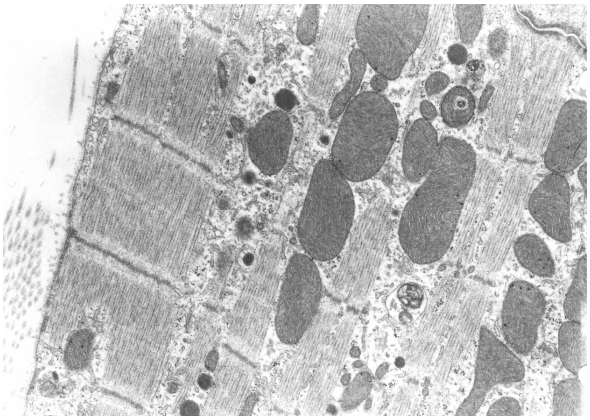

Figure 5. Electronoptic picture of rabbit myocardium stained with VSD di-4-ANEPPS. The picture contains a large number of myofibrils, striated like in skeletal muscle. A large fraction of the cell volume is occupied by mitochondria, the rest contains sarcolemma, T-tubules, and sarcoplasmic reticulum.

No fading in recorded optical signals was observed even after illumination lasting as long as two hours.

4. Discussion and conclusions

Although the intracellular microelectrode or whole-cell patch clamp techniques are still considered to be the golden standard for recording of transmembrane action potentials, the optical method has approached comparable signal-to-noise ratio recently and thus may become a candidate for applications in experimental and clinical cardiology. Voltage-sensitive dyes provide a powerful technique for recording of membrane potential in situations and systems where – because of scale, topology, or complexity – the use of classical electrodes is problematic or impracticable, e.g. in the presence of external electric fields - uninterrupted and artefact-free recording during pacing stimuli and defibrillation shocks or recording of high-resolution maps of cardiac repolarization.

Side effects of VSDs are the biggest problem of biological part of experiments with these substances. Our data presented in this paper support the idea of direct effect of voltage-sensitive dye di-4-ANEPPS on the conductive system and working myocardium of the rabbit heart.

Firstly, prolongation of R-R intervals (decrease in heart rate), although slight and insignificant and occasional A-V dissociation in our experiments points to direct effects of di-4-ANEPPS on conductive system. However, these effects are of much lower extent in rabbit myocardium when compared with guinea pig isolated hearts [6].

The shape changes of electrogram, found mainly in T wave area, support the idea of direct effect of the dye on cardiac ionic channels, presumably potassium channels which are operating during repolarization of the heart.

A slight decrease of the mean coronary flow observed during staining and the reperfusion period outlasted in various degree till the end of the experiment. However, this decrease in perfusion is insignificant when compared with our results from previous study on isolated guinea pig hearts [7]. The explanation of this phenomenon is somewhat complicated, since we do not have any direct evidence of the putative effect of di-4-ANEPPS on coronary arteries. But on the other hand, we did not find increased production of hydroxyl radicals in consequence to loading the cardiac muscle with voltage-sensitive dye and thus we may assume that the tissue is probably not ischemic. Also morphological examination did not bring any light into this problem. It seems that application of voltage-sensitive dye di-4-ANEPPS causes very slight vasoconstriction in coronary system in isolated rabbit heart perfused according to Langendorff without any functional or morphological consequences.

We can conclude that rabbit myocardium is resistant to di-4-ANEPPS side effects during staining and also resistant to bleaching during intense illumination.

Acknowledgements

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All experiments followed the guidelines for animal treatment approved by local authorities and followed the EU law.

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