EFFECTS OF VOLTAGE SENSITIVE DYE di-4-ANEPPS ON GUINEA PIG MYOCARDIUM

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Abstract: Voltage-sensitive dyes are used for recording monophasic action potentials (MAPs) in various heart preparations. Aim of this study was to prove that dye loading has no pharmacological or toxic effects to the preparation, e.g. to prove the viability and quality of the heart at the beginning of MAP recording. The hearts of fifteen guinea pigs were perfused according to Langendorf, loaded with the dye (di-4-ANEPPS) and washed out. During the experiment electrogram and mean coronary flow was recorded. High performance liquid chromatography (HPLC) was used to test on possible ischemic changes of the heart during loading and electron microscopy to check the microscopic changes in the dye exposed tissue. We observed direct effect of di-4-ANEPPS on conductive system and working myocardium of guinea pig heart (changes in heart rate and shape of electrogram). The mean coronary flow does not significantly change during the period of loading with the dye, however during washout decreases. Since no increased production of oxygen free radicals was found and no ultrastructural changes observed, we conclude that the dye application causes vasoconstriction in coronary system without functional or morphological consequences and that loading procedure does not damage the heart muscle and its viability.

Introduction

Voltage-sensitive dyes have been employed to record monophasic action potentials (MAPs) in a variety of heart preparations with results comparable to classical microelectrode method [2], [7]. However, still little data are available on the response of the heart muscle to the loading with the dye. One of the most consistently used dyes – di-4-ANEPPS - is employed in our laboratory to record MAPs from isolated guinea pig hearts [3]. It is of great importance to prove that loading of the heart muscle does not result in pharmacological or toxic effects to the preparation, since the experiments are of long duration, e.g. to prove the viability and quality of the heart at the beginning of the experiment.

Materials and Methods

Isolated perfused heart according to Langendorff

A perfusion apparatus for small animal hearts based on Langendorff technique has been modified in our laboratory for employing in pharmacological experiments [4]. At any rate, more than one reservoir is needed. The common glass bath keeps the desired temperature in all reservoirs at the same moment. Each reservoir is oxygenated separately. A system of four-way stop-cocks allows rapid switching of solutions between respective reservoirs. Small diameter of the connecting tubes accounts for a small dead space. This is very important for minimizing variations in temperature when switching from one solution to another. A special system keeps the perfusion pressure constant and equal in all reservoirs in spite of different amount of solution in each of them (Figure 1).

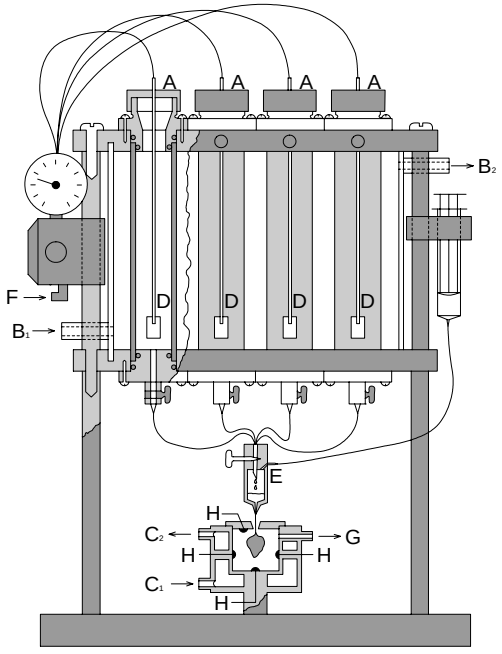

Figure 1: Modified perfusion set according to Langendorf. Four reservoirs (A) filled with perfusion solution are placed in a common bath (connected to a thermostat – B1, B2). The heart is placed in a small double-walled bath connected to a thermostat as well

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(C1, C2). The solution in every reservoir is separately oxygenated (D). Warmed and oxygenated perfusate is driven to the heart fixed on the cannula at the tip of a bubble-trapper (E) under the perfusion pressure kept by a special system (F), connected to all reservoirs and to balloon with O_2 and CO_2. The perfusate leaving the heart drops into the solution in the bath. The overflow-tube (G) is placed in the wall of the bath close to proximal margin. Six silver-silver chloride disc electrodes are placed on the inner surface of the bath (H).

The experiment proceeds in several steps: obtaining the heart, control perfusion period, loading with the dye, and measurement of optical APs under various conditions (ischemia, after pharmacological interventions, etc.).

Briefly, the guinea pig is deeply anaesthetized by Ketamine and Xylasine, artificially ventilated and its chest is opened. Then the heart is excised with a sufficiently long segment of ascending aorta. Next, the aorta is cannulated, the heart mounted on a Langendorff apparatus and placed in a thermostat-controlled bath (37°C) filled with the 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 has to be equilibrated with 95% O_2 and 5% CO_2. The isolated organ is then perfused with the same solution at the constant perfusion pressure (90 mmHg) for 25 - 30 minutes – control period. All hearts exhibiting any dysrhytmias during this period are excluded from next experiment.

Recording of electrogram and mean coronary flow

During the whole experiment electrogram is recorded and mean coronary flow monitored. The recording of electrogram is carried out 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 subject. The mean coronary flow is measured every fifth minute during the whole experiment.

Loading the heart

The heart is then exposed to voltage-sensitive dye diluted in Krebs-Henseleit solution to the concentration of 2mM (stock solution in DMSO, 2μM). The tissue is perfused with this mixture for 22 - 27 minutes (according to the respective coronary flow). Again, the electrogram and mean coronary flow are monitored.

Then the period of washout follows. Its length is the same as period of loading with dye for each respective heart. After the washout, the heart is ready for recording of optical APs.

High performance liquid chromatography (HPLC-ECD)

Isolated hearts were perfused with Krebs-Henseleit solution containing 1 mmol/l salicylate during control period and then with Krebs-Henseleit buffer containing 1 mmol/l salicylate and di-4-ANEPPS for loading. Hydroxylated products of salicylic acid were analysed in the coronary effluents. Determination of 2,5-DHBA rather than 2,3-DHBA was used because of its greater sensitivity for the quantification of hydroxyl radicals productions. The effluents were collected at the end of the both perfusion periods (with and without di-4-ANEPPS) and stored at –80°C until assayed by HPLC – ECD. Retention times for the peak 2,5-DHBA and salicylic acid were verified by injecting standards.

The HPLC apparatus consisted of high pressure LCP 4000.1 pump, electrochemical detector Coulochem II with analytical cell 5010 (ESA, Chelmsford, MA, USA) and Rheodyne 7125 syringe loading sample injector (Cotati, CA USA), sample loop 20 μL. The Chromatography Station for Windows ver.1.5 (Data Apex, Prague, CZ) was used for the quantification. All analyses were performed on a reversed–phase column (LiChrospher 100 RP-18,5 μm, 100x3 mm I.D, Merck) with precolumn (Separon SGX, 5 μm). Detection of 2,5-DHBA and salicylic acid (SA) was performed by isocratic elution with the mobile phase containing 20% (v/v) MeOH in the buffer pH 3.6 (0.03 M citric acid and 0.06 M NaH_2PO_4), flow rate 0,5 ml/min. The mobile phase was filtered through Nylon membrane filter (0,2 μm pore size, SolVac, USA). Analytes were detected on a dual electrode analytical cell with the first electrode (E_1) set to oxidize the 2,5-DHBA at +250mV and the second electrode (E_2) set to oxidize SA at +750mV. A guard cell (model 5020) was placed between the pump and sampler at a potential of +775mV to oxidise contaminants in mobile phase.

Electron microscopy

In case the viability of preparation was checked by electron microscopy, the tissue was prepared by following procedure. The heart at the end of experiment was perfused with 300mmol/l solution of glutaraldehyde for 10 minutes, removed from Langendorff apparatus and strips 1x1x3 mm in size were cut from both atria and ventricles. The samples were immediately fixed in a 400mmol/l solution of glutaraldehyde in 0.1M phosphate buffer at pH 7.4. Fixation was carried out in two bath of 40mmol/l OsO_4 solution in phosphate buffer at pH 7.4.

Dehydration, immersion and embedding in Durcupan ACM followed the standard procedure. Ultrathin sections were made on an LKB Nova ultramicrotome and stained with lead citrate or with uranyl acetate and lead citrate. The sections were viewed and photographed in a Tesla BS 500 electron microscope and Morgagni 286 D transmission electron microscope.

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Results

Perfusion of isolated guinea pig heart with voltage-sensitive dye di-4-ANEPPS caused prominent changes of electrograms in all examined hearts. The most noticeable is marked prolongation of R-R intervals already in the very first minutes of application (Figure 2).

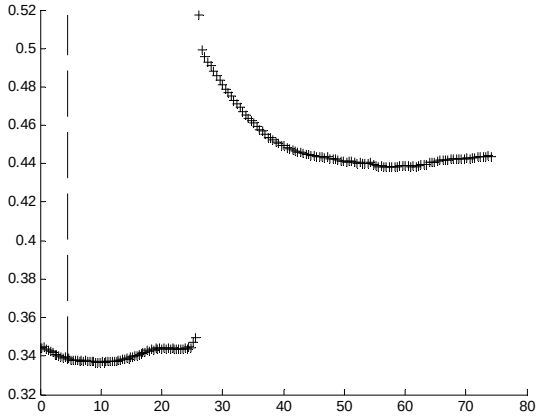

Figure 2: Evolution of R-R intervals during the first 70 seconds of dye loading. Discrete line marks application of the dye.

R-R interval responses to voltage-sensitive dye application with a delay. However, the delay is affected by coronary flow that is individual even if using hearts of the same species, sex, age, and similar weight. Long-term analysis of the R-R intervals reveals further gradual slowing of heart frequency during loading. The heart frequency is partly restored during wash-out after loading period [1].

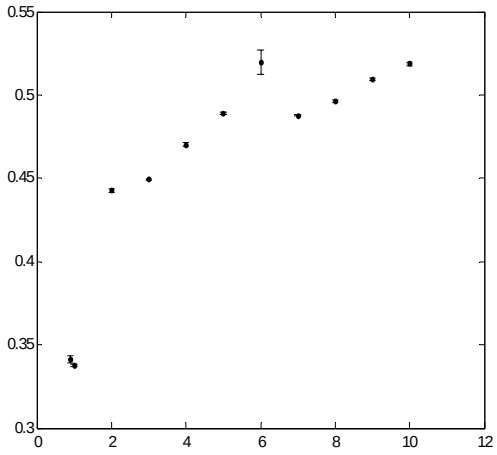

Figure 3: Evolution of R-R intervals during 10 minutes of VSD application

Various changes in the shape of electrogram were observed – mainly the shape of QRS complex and T wave are often impaired by perfusion with the dye. Changes in the shape of QRS complexes during VSD application were observed in all experiments. To prove this observation, time domain analysis was employed. The observations were confirmed [1].

Among other changes of ECG, the most prominent one is "floating" of P wave or partial block in AV node.

All these changes are partially reversible during the washout period.

An example of the effect of perfusion with di-4-ANEPPS on electrogram is given in Figure 4.

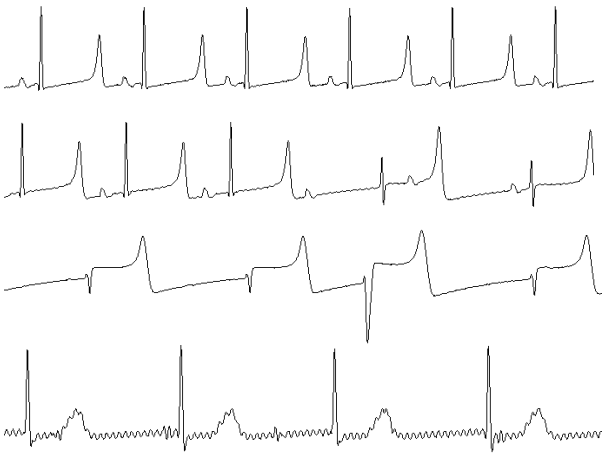

Figure 4: Electrogram of isolated perfused guinea pig heart (from top to bottom) in control, immediately after introduction of the dye, in the 10th minute of perfusion with the dye, and in washout period. Note the prominent change of heart rate, partially reversible in washout.

Coronary flow changes are summarized in Figure 5. The mean coronary flow does not significantly change during the first half of loading with the dye. During the second half of loading and during the entire washout period, a decrease in the mean coronary flow can be observed in most of the hearts. However, this decrease is insignificant (see SD in Figure 5).

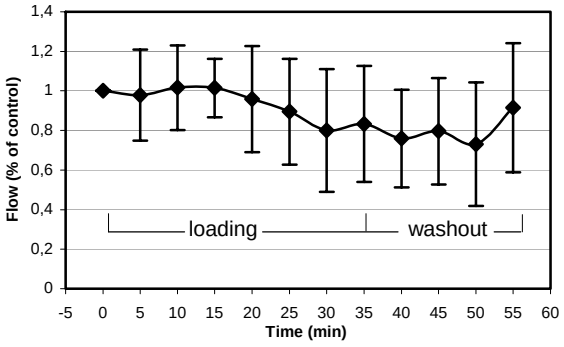

Figure 5: Mean coronary flow as measured during loading and washout period (0 min – flow at the end of control, 5 – 30 mins loading, 35 – 55 mins washout).

In our previous experiments (unpublished results) we hade proved the literature data that hydroxylation products of salicylic acid are formed during ischemia-reperfusion conditions and 2,5-DHBA can be used as a marker of OH radicals formation [6]. We tested whether hydroxyl radicals were formed during control period

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and if the presence of di-4-ANEPPS can affect their formation. Isolated hearts were perfused in the presence 1mmol/l salicylic acid. We analysed the production of 2,5-DHBA in coronary effluents and the influence of di-4-ANEPPS on its formation using HPLC with the electrochemical detection. We didn't find any peak of 2,5-DHBA in the chromatogram during perfusion period as well as in the presence of di-4-ANEPPS (Figures 6, 7).

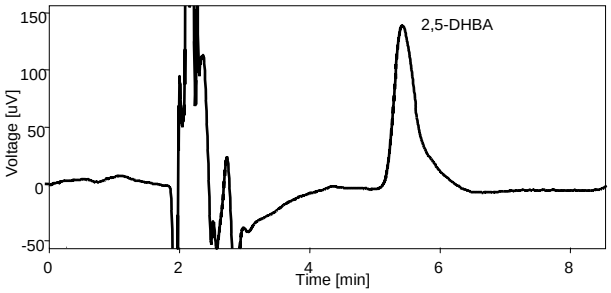

Figure 6: Standard of 2,5-DHBA

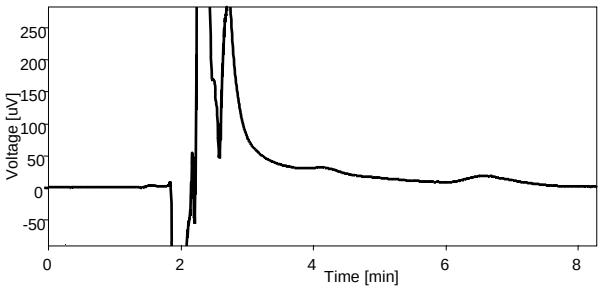

Figure 7: The effect of di-4-ANEPPS. Note the missing peak of produced radicals, which is present when the standard of 2,5-DHBA is used.

The last part of our study was focused on examination of possible ultrastructural changes caused by long-lasting exposure to voltage-sensitive dye. A typical structure of cardiac muscle was observed in controls sections. The tissue was composed of interconnected mono-nucleated cells imbedded in a weave of collagen. The picture contains a large number of myofibrils, striated like in skeletal muscle. A large fraction of the cell volume is occupied by mitochondria. Myofibrils and mitochondria occupy about 85% of the heart cell volume, the rest contains the sarcolemma, T-tubules, sarcoplasmic reticulum, the intercalated disks, and gap junctions (nexus). Examination of the cardiac tissue loaded with di-4-ANEPPS did not reveal any ultrastructural changes detectable by electron microscopy.

Discussion

Although the intracellular microelectrode technique still represents the golden standard for recording transmembrane action potentials, the optical method approached comparable signal-to-noise ratio recently and thus may spread more widely in experimental and clinical cardiology. Voltage-sensitive dyes provide a powerful new technique for measuring membrane potential in systems where – for reasons of scale, topology, or complexity – the use of electrodes is inconvenient or impossible. It is mainly the recording in the presence of external electric fields - uninterrupted and artefact-free recording during pacing stimuli and defibrillation shocks and recording of high-resolution maps of cardiac repolarization.

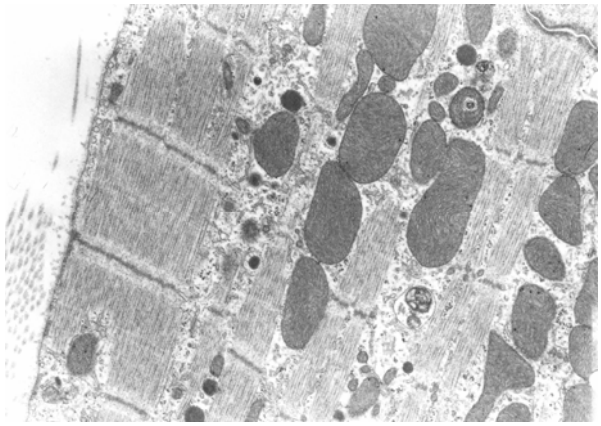

Figure 8: Electronoptic picture of guinea pig heart muscle loaded with di-4-ANEPPS.

The possibility of recording dynamic changes in the transmembrane potential of excitable cells by optical means was first suggested in 1968. The first cardiac application was then introduced in 1981 – the localization of pacemaker activity in embryonic heart preparation. Since then the method has been improved and many new voltage-sensitive dyes from various chemical groups tested. Several problems have to be solved before voltage-sensitive dyes were introduced to everyday laboratory practice. One of the most important tasks was to minimize side effects of the dye on the preparation in the absence and presence of light. Most prominent pharmacological effect of voltage-sensitive dyes on cardiac tissue is so-called photodynamic or phototoxic damage. The exact mechanism of these effects remains unknown. One of possible explanations is formation of free radicals or direct interaction with voltage-gated calcium and/or potassium channels, which may alter the conductivity and time-dependent gating of them.

Our data support the idea of direct effect of voltage-sensitive dye di-4-ANEPPS on conductive system and working myocardium of guinea pig heart, published recently [5]. Sharp slow-down of heart rate can be caused by a block of excitation transfer from the sinoatrial node to the atrioventricular (AV) node. AV node then undertakes the task of a pacemaker at lower frequency. Another possible explanation is a partial block in AV node. The shape changes of electrogram support the idea of direct effect of the dye on cardiac ionic channels. Since T wave is often changing its shape and amplitude during the loading and washout periods,

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we assume that predominantly potassium channels are affected.

Decrease of mean coronary flow observed during loading but more marked mainly during reperfusion period outlasted in various extend till the end of experiment. The explanation of this phenomenon is very difficult, since we did not find any production of free oxygen radicals in consequence to loading the cardiac muscle with voltage-sensitive dye. Also morphological examination did not bring any light into this problem. It seems that application of voltage-sensitive dye di-4-ANEPPS causes vasoconstriction in coronary system in isolated guinea pig heart perfused according to Langendorf without any functional or morphological consequences.

Conclusions

Although the guinea pig hearts responded to loading procedure with voltage-sensitive dye di-4-ANEPPS with certain electrophysiological changes and mean coronary flow remained changed after finishing the whole procedure, e.g. at the beginning of MAPs measurement, we conclude that it is possible to consider the heart tissue viable and not damaged. This conclusion is based on the facts that there were no increased production of oxygen free radicals found and no ultrastructural changes observed.

Acknowledgement

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References

[1] BLAHA M., PROVAZNÍK I., VESELÝ Z., and NOVÁKOVÁ M. (2004): 'Influence of voltage-sensitive dye on ECG parameters', Proc. of Advances in Medical Signal and Information Processing, Sliema, Malta, 2004, p. 58-61

[2] LOEW L.M. (1996): 'Potentiometric dyes: Imaging electrical activity of cell membranes', *Pure Appl. Chem.,* **68**, pp.1405-1409

[3] Molecular Probes: Product Information 'Potential-Sensitive ANEP Dyes'. Revised: 15-February-2001.

[4] NOVÁKOVÁ M., MOUDR J. and BRAVENÝ P. (2000): 'A modified perfusion system for pharmacological studies in isolated hearts', Proc. of Analysis of Biomedical Signals and Images - 15th Biennial International Eurasip Conference Biosignal 2000, Brno, Czech Republic, 2000, p. 162-164

[5] NYGREN A., KONDO C., CLARK R.B. and GILES W.R. (2003) 'Voltage-sensitive dye mapping in Langendorff-perfused rat hearts', *Am J Physiol Heart Circ Physiol,* **284**, pp. H892-H902

[6] ONODERA T. and ASHRAF M. (1991): 'Detection of hydroxyl radicals in the post-ischemic reperfused heart using salicylate as a trapping agent', *J Mol Cell Cardiol,* **23,** pp. 365-370

[7] SALAMA G. (2001): 'Optical mapping: Background and historical perspective', in ROSENBAUM D.S. and JALIFE J. (Ed): 'Optical Mapping of Cardiac Excitation and Arrhythmias', (Futura Publishing Company, Inc., New York), pp. 9-31

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