Role of automated patch-clamp systems in drug research and development

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Summary of Ph.D. Thesis

Szeged, Hungary

2016

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1. INTRODUCTION

1.1. Ion channels in drug research and discovery

Ion channels are a large and diverse family of transmembrane pore-forming proteins present in a large variety of cell types. They facilitate the rapid passive transport of specific inorganic ions through the plasma and organelle membranes.

Ion channels regulate a variety of physiological processes. Because of their pivotal role, ion channel dysfunction has adverse effects on physiology, and could lead to many severe chronic and acute diseases and disorders, so-called channelopathies. Most of these common diseases are primarily treated by drugs that pharmacological modulate ion channel functions; therefore ion channels represent a highly attractive class of molecular targets for both academic research and pharmaceutical industry.

Another important aspect of drug development programmes and ion channel drug screening is the safety evaluation of potential candidate molecules to assess possible adverse drug effects. Particularly important are the possibility of cardiovascular side effects, since several compounds exert their arrhythmogenic effects through the ion channel encoded by the *human ether-a-go-go-related gene* (hERG), expressed in cardiac ventricular myocytes. The inhibition of hERG channel may delay cardiac action potential repolarization and increase the risk of potentially fatal ventricular arrhythmias.

1.2. Methods of ion channel research

Despite their physiological importance and therapeutic relevance, ion channels remained an underexploited drug target class for a long time. Ion channels have historically been difficult to screen due to the limitations of methods. The available assay technologies cannot provide simultaneously the throughput and quality of data and put a trade-off between high-throughput and high-information content.

1.2.1. Conventional intracellular techniques

Conventional intracellular recordings involve a group of methods used to measure with accuracy the voltage across the cellular membranes by inserting a glass microelectrode filled with conductive medium to the interior of the cell. This makes it possible to record the potential difference between the intracellular milieu and an extracellular reference point.

1.2.2. Conventional manual patch-clamp

Today, ion channels are most precisely studied using the conventional patch-clamp technique. The method developed by Erwin Neher and Bert Sakmann in the late 1970s. A borosilicate glass microelectrode was patched onto the surface of a single cell and the ionic current passing through the enclosed ion channels in the plasma membrane was measured. The patch-clamp technique has rapidly become the 'gold standard' in studying ion channel behaviour, function, kinetics and pharmacology *in vitro*, both in native or cultured mammalian cells.
The patch-clamp is the only method which offers a direct, information-rich and real-time technology to study ion channel behaviour, function and regulation. Although providing excellent data quality, the very low data throughput excludes manual patch-clamp as a screening tool in early drug development and optimization, since in these drug screening phases require a much greater throughput.

1.2.3. Ion channel screening with high-throughput systems

Due to the limitations in throughput of manual patch-clamp, high-throughput techniques are also required and become integral components for primary drug screening and ion channel drug discovery programmes. These methodologies are based on ligand binding, radioactive flux and fluorescence (ion- or voltage-sensitive dyes) assays. Although these approaches are compatible with the throughput requirements of primary screening and have been useful in supporting the efforts to identify and profile compounds, the high data quality was sacrificed to gain high throughput. Most of these methods have low fidelity, low sensitivity and low temporal resolution.

1.2.4. Automated patch-clamp method

In recent years, numerous companies have developed and introduced automated patch-clamp platforms suitable for rapid and high quality screening and optimization of ion channel drug candidates. Attempts to automate glass electrode-based recordings started in the late 1990s and yielded limited popularity due to the low success rate and throughput of measurements. The breakthrough in automated electrophysiology came when planar patch-clamp technology without micromanipulation or visual control was launched and become commercially viable. In this method, by the application of negative pressure cells are moved towards the patch-clamp substrate with an array of microapertures for capturing from suspension followed by the formation of the seal with the substrate. Planar arrays replace glass pipette electrodes with

multi-well configurations either in a plate-based or chip-based format to enable higher throughput screening, currently this technology is used in the majority of automated systems.

1.3. Aims

Aim of the present work was to investigate the role of automated patch-clamp technique in drug research and development and compare it with the conventional cellular electrophysiological techniques. The two main scopes of our studies were:

1. To determine the value of results of screening processes obtained with automated patch-clamp methods using cell lines stably expressing ion channels. During this phase of work the main goals were to evaluate the usability of the studied automated patch-clamp equipment in different screening projects and to analyse the utility of the studied system with comparing the effect of the active compounds in cell lines used for screening and in native targets measured by the conventional electrophysiological methods.
2. To analyze the value of safety pharmacology data obtained with automated patch-clamp methods. During this phase of work the main goal was to evaluate the practical usability and the biological significance of the results obtained with the studied automated patch-clamp equipment in stable cell lines.

2. MATERIALS AND METHODS

2.1. Conventional microelectrode technique

New Zealand rabbits of either sex weighing 1-2 kg or adult mongrel dogs (8-14 kg) of either sex were used. Isolated muscle preparations obtained from the right ventricle were individually mounted in a tissue chamber with the volume of 50 ml. Each preparation was initially stimulated through a pair of platinum electrodes in contact with the preparation using rectangular current pulses of 2 ms duration. Transmembrane potentials were recorded at 37°C using conventional glass microelectrodes, filled with 3 M KCl and having tip resistances of 5-20 MΩ, connected to the input of a high impedance electrometer (Experimetria, type 309) which was coupled to a dual beam oscilloscope.

2.2. Manual patch-clamp method

Ventricular myocytes were enzymatically dissociated from hearts of New Zealand rabbits of either sex weighting 1-2 kg using the segment perfusion technique. Patch-clamp micropipettes

were fabricated from borosilicate glass capillaries (Harvard Apparatus) using a micropipette puller (Flaming/Brown, type P-97). These electrodes had resistances between 1.5 and 2.5 MΩ when filled with pipette solution. Membrane currents were recorded at 37°C with Axopatch 200B patch-clamp amplifiers (Molecular Devices) using the whole-cell configuration of the patch-clamp technique. . Membrane currents were digitized after low-pass filtering at 1 kHz using analog-to-digital converters (Digidata 1322A and 1440A, Molecular Devices) under software control (pClamp 8 and 10, Molecular Devices).

2.3. Automated patch-clamp experiments

The automated patch-clamp experiments were executed by using planar patch-clamp technology in the whole-cell configuration with a four channel medium throughput fully automated patch-clamp platform (Patchliner Quattro, Nanion, http://nanion.de/images/stories/pdf/patchliner.pdf) with integrated temperature control. Experiments were carried out at room temperature on suspension of native activated human lymphocytes, stable transfected HEK-GABA, HEK-HCN and CHO-Kv1.4 cell lines. Measurements were performed at room and/or physiological (37°C) temperature in case of HEK-GIRK and HEK-hERG cell lines.

2.4. Statistics

Data are expressed as arithmetic mean ± SEM values. Results were compared using Student's t-tests for paired data. Results were considered significant when P value was less than 0.05.

3. RESULTS

3.1. Evaluation and characterization of different cell lines with Patchliner

Native activated human lymphocytes: Characterization of Kv1.3 channel in human peripheral T lymphocytes was performed using tetraethylammonium (TEA) and two selective blockers of the channel, anuroctoxin (AnTx) and margatoxin (MgTx). TEA reduced the Kv1.3 current in dose-dependent manner showing a 28.14 ± 3.11 mM IC_{50} value. The selective Kv1.3 blocker peptid toxins originated from scorpion poison, anuroctoxin and margatoxin also inhibited the current in concentration-dependent manner. The IC_{50} values of both toxins found to be very low: 25.35 ± 1.64 nM (AnTx) and 68.59 ± 16.68 pM (MgTx).

HEK-GABA cell line: $GABA_A$ channels were investigated by using a stacked application for rapid administration of agonists to cells, in order to study concentration response relations.

The EC_{50} of GABA was determined to 32.20 ± 1.85 μM from the Hill plot. The effect of the $GABA_A$ channel antagonist bicuculline was investigated on $GABA_A$ current. The estimated IC_{50} value was 371.06 ± 3.43 nM.

HEK-HCN cell lines: The blocking action of ivabradine on HCN1 and HCN4 channels expressed in HEK cells was investigated. Ivabradine reduced both currents in 10 μM concentration by 64.52 ± 5.36% and 63.79 ± 1.07%, respectively.

HEK-GIRK1/4 cell line: Six compounds were studied on HEK-GIRK cells. These experiments were performed at room temperature and results grounded for the screening project performed (see Section 3.2.) also at room temperature. The IC_{50} of chloroquine, desipramine, JTV-519, NIP-142, propafenone and quinidine were 463.70 ± 34.61 nM, 1.47 ± 0.17 μM, 814.24 ± 109.34 nM, 175.75 ± 8.59 nM, 372.99 ± 27.69 nM and 5.04 ± 0.62 μM, respectively. Effect of the antiarrhythmic drug amiodarone and its main metabolite formed during chronic treatment (desethylamiodarone, DEA) was also investigated. These studies were performed as parts of the cardiac electrophysiological investigation of these compounds and were performed at 37°C. The IC_{50} values determined by the Patchliner were nearly identical (1.77 ± 0.18 and 1.82 ± 0.15 μM, respectively).

CHO-Kv1.4 cell line: The blocking effects of chromanol 293B and 4-aminopyridine on Kv1.4 ion channel were investigated at room temperature. Estimated IC_{50} values of these compounds found to be 85.69 ± 8.38 μM and 791.33 ± 26.84 μM, respectively.

HEK-hERG cell line: Two compounds, dofetilide and sotalol were tested in hERG assay with automated patch-clamp system. Results are shown in Section 3.3.1.

3.2. Screening projects: Investigation of ion channel modulator capability of compounds with automated patch-clamp equipment

The characterized cell lines were used in screening and safety pharmacology projects performed with the Patchliner. All screening projects were carried out at room temperature.

3.2.1. Screening of preselected chemical libraries on GIRK channel

In order to find agents for the treatment of atrial fibrillation, screening of preselected chemical libraries was performed on GIRK channel expressing cell line. During our screening project a set of 868 compounds was tested. Compounds were screened in two concentrations (1 and 10 μM). Approximately 13% of the preselected compounds showed a notable (i.e. at least 50% decrease in the GIRK current at 10 μM concentration) blocking effect. Compounds were ranked according to their GIRK inhibitory effect. Eleven compounds were chosen from the

group of most effective molecules upon their blocking effect and chemical structure. The dose-response curves of these compounds were determined in detailed experiments. Substrates were chosen for selectivity studies by the more precise results of these later studies: hERG inhibitory properties of 4 compounds with the most intense GIRK blocking effect (Ryt-143, Ryt-144, Ryt-230 and Ryt-243 – all having IC_{50} values under 0.5 μM) were investigated on HEK-hERG cells in 6 concentrations. One out of the 4 chosen compounds showed similar blocking activity on both GIRK and hERG channels: IC_{50} values of Ryt-243 were 100.14 ± 5.10 nM and 47.56 ± 5.31 nM, respectively. The other 3 compounds (Ryt-143, Ryt-144 and Ryt-230) showed an at least 10 times higher blocking activity on GIRK channels compared to the results obtained on hERG channel (IC_{50} values on GIRK and hERG channels were 281.29 ± 9.48 nM and 2.91 ± 0.29 μM for Ryt-144, 335.30 ± 23.81 nM and 8.05 ± 0.89 μM for Ryt-143, 495.43 ± 15.90 nM and 9.16 ± 1.30 μM for Ryt-230). As hERG blocking effect can be even useful in antiarrhythmic treatment Ryt-243 was subjected to further investigation: despite of its hERG blocking effect the compound lengthened the action potential in rabbit ventricular muscle neither in 5 μM nor in 10 μM concentrations. Ryt-243 was also studied in dog model of chronic atrial fibrillation. The compound had a strong antiarrhythmic effect in this investigational set-up: the incidence of atrial fibrillation was reduced by 65-70% by both 0.3 mg/kg and 1 mg/kg doses. Based on these antiarrhythmic results a patent was filed in which anti-atrial fibrillation effects of Ryt-243 are chartered. Compounds showing GIRK-selectivity (Ryt-143, Ryt-144 and Ryt-230) are also subjected to further investigation including antiarrhythmic effect evaluation and structure-effect analysis.

3.2.2. Screening of different substrates originated from plants on GIRK channel

3.2.2.1. Screening of natural plant compounds on GIRK channel

Compounds originated from natural sources were also investigated in our GIRK screen project. During this phase of screening project a set of 281 natural plant compounds was tested. These molecules were chosen randomly and were not selected. Compounds were screened also in two concentrations (1 and 10 μM). Approximately 9% of the investigated natural compounds showed a notable (i.e. at least 50% decrease in the GIRK current at 10 μM concentration) blocking effect. The 26 most effective molecules were selected for further evaluation following the primary screen. These compounds were also tested on Kv1.4 and hERG cell lines and selectivity of their GIRK blocking effect was evaluated with these experiments. Six natural plant compounds (Ryt-963, Ryt-964, Ryt-1009, Ryt-1103, Ryt-1187

and Ryt-1194) were chosen from the group of most effective ones upon their blocking effects and chemical structure. The dose-response curves of these 6 molecules were determined in detailed experiments. IC_{50} values on GIRK current were 524.25 ± 35.87 nM for Ryt-963, 2.25 ± 0.33 μM for Ryt-1009, 3.66 ± 0.30 μM for Ryt-964, 5.33 ± 0.23 μM for Ryt-1194, 9.68 ± 0.27 μM for Ryt-1187 and 12.20 ± 0.31 μM for Ryt-1103.

3.2.2.2. Screening of plant extracts on GIRK channel

Testing a new way of tracing biologically active compounds extracts of *Polygonum persicaria* were also investigated in our GIRK screen system, with the aim to identify natural sources of promising ion channel blocking compounds. For this purpose hexane, chloroform, methanol and aqueous extracts were prepared from the dried whole plant. Extracts of different polarity were investigated at two concentrations (0.01 mg/l and 0.1 mg/l). The chloroform extract exhibited considerable GIRK channel inhibitory activity. This extract was fractionated by vacuum liquid chromatography resulting six fractions (fractions 1-6), which were also evaluated for GIRK modulation activity. The most active fractions (fraction 4 and 5) were subjected then to RP-HPLC, affording the isolation of the main compounds 1-4 in pure form. The eluates containing the mixture of other (minor) compounds were also collected during the HPLC chromatography. Interestingly, either separated or combined application of the isolated main compounds of the active fractions (compound 1-4) possess only very moderate activity on GIRK channel, while the HPLC eluates of fraction 4 and 5 containing a mixture of minor compounds revealed to be active, indicating the presence of electrophysiologically active agents among the minor compounds. Further studies are on the way in order to identify the minor compounds responsible for the GIRK blocking effect. Nevertheless our studies confirm the usability of the applied method of tracing active compounds upon their biological activity.

3.3. Safety pharmacology studies

3.3.1. Assessment of hERG assay performed with automated patch-clamp system in the safety evaluation of investigational compounds

Two compounds (dofetilide and sotalol) were tested in hERG assay both at room temperature and at 37°C with automated patch-clamp system. The IC_{50} values of dofetilide were very similar (8.41 ± 0.19 nM at room temperature and 7.29 ± 0.16 nM at 37°C). Sotalol displayed different properties at room temperature versus physiological temperature (37°C). Sotalol blocked hERG channels at room temperature with an IC_{50} of 773.74 ± 9.28 μM. However, at

37°C sotalol was more potent (IC_{50} = 342.84 ± 24.82 μM). To evaluate the prognostic value of hERG assay these compounds were subjected for further investigations. The I_{Kr} current blocking capability of the compounds was tested on rabbit ventricular muscle with manual patch-clamp method. The IC_{50} values of dofetilide and sotalol were 13.02 ± 2.56 nM and 51.60 ± 9.82 μM, respectively. IC_{50} values obtained in hERG and I_{Kr} assays showed a good correlation in case of dofetilide while IC_{50} value obtained from I_{Kr} measurements was approximately 7 times lower in case of sotalol. To study the safety pharmacology consequences of the hERG and I_{Kr} inhibitory effect of dofetilide and sotalol the effect of these compounds on action potential configuration were studied in rabbit right ventricular muscle preparations. The prolongation of APD_{90} was 47.8 ± 12.9 % in case of 13 nM dofetilide while 52 μM sotalol extended the action potential duration with 56.0 ± 4.6 % (n = 5).

3.3.2. Effects of *Chelidonium majus* extracts and major alkaloids on hERG ion channel and on dog cardiac action potential

Chelidonium majus or greater celandine is spread throughout the world, and it is an important plant of the modern phytotherapy, used not only externally, but also internally. Safety pharmacology properties of this plant are not widely clarified. Therefore, effects of *C. majus* herb extracts and alkaloids on hERG K^+ current and on cardiac action potential were studied.

For the investigations of hERG blocking effects of *C. majus*, 25% and 45% ethanol extracts were prepared. Both extracts revealed significant hERG ion channel inhibitory activity at room temperature with estimated IC_{50} values of 8.31 ± 0.79 μg/ml and 5.09 ± 0.49 μg/ml.

The hERG blocking potencies of the major alkaloids of the plant (sanguinarine, chelidonine, berberine and coptisine) were also evaluated and found that all alkaloids showed considerable inhibitory effect apart from coptisine. Sanguinarine and chelidonine were to have the highest inhibitory effect with the IC_{50} value of 0.88 ± 0.08 μM and 1.00 ± 0.10 μM, respectively. The hERG channel modulatory activity of berberine could be characterized by the IC_{50} value of 6.46 ± 0.54 μM, whereas coptisine exhibited only marginal effect (IC_{50} = 90.08 ± 2.88 μM).

To investigate the safety pharmacology consequences of the hERG blocking effect of *C. majus* extracts and alkaloids the effect of these agents - with the exception of coptisine - on action potential configuration were studied in canine right ventricular muscle preparations. Both extracts moderately extended the action potential duration at 5 μg/ml concentration in statistically significant manner at basic stimulation cycle length of 1000 ms. The prolongation of APD_{90} was 10.5% in case of the 25% ethanol extract while the 45% ethanol extract lengthened the action potential duration with 6.7%. The effects of berberine, chelidonine and

sanguinarine on the action potential parameters at concentrations of 1 μM and 10 μM were also investigated in dog ventricular muscle at constant cycle length of 1000 ms. All compounds showed mild but statistically significant effect on APD_{90} (4.6%, 6.1% and 6.3% prolongation, respectively) at 1 μM concentration. At 10 μM concentration the action potential prolongation was more considerable (18.4%, 18.3% and 16.0%).

4. DISCUSSION

Measurement of the activity of individual ion channels became possible after the pioneering work of Erwin Neher and Bert Sakmann in the late 1970s. The manual patch-clamp method has rapidly become the 'gold standard' in investigating ion channels. The patch-clamp technique offers a direct, information-rich and real-time technology. On the other hand, traditional manual patch-clamp is too slow, technically demanding, and labor intensive, which excludes it as a screening tool in early drug development and optimisation. This does not mean that the conventional patch-clamp method is obsolete. Conventional patch-clamp is applied at the later stages of drug discovery and development and it still represents one of the primarily tools in ion channel research laboratories. In industry, the application of patch-clamp is limited to the end phases of drug research and ion channel screening and mainly used for final compound evaluation.

In recent years, many companies have developed and introduced automated patch-clamp equipments suitable for rapid and high quality screening and optimisation of ion channel drug candidates. Parallelisation of patch-clamp recordings permits higher throughput in characterisation of compounds and ion channels. By providing high quality, information rich assays, these appliances have the capability to bridge the gap between primary and secondary ion channel drug screening.

Automated patch-clamp has resided mainly in the pharmaceutical industry with focus on recombinant cell lines induced to overexpress ion channels, while investigations of endogenous ion channels in native cells remain common and important in academic research. Although automated patch-clamp systems are used mainly with recombinant cell lines we have found that Patchliner can produce high quality data from native cells and cells in primary culture, when the evaluation and characterization of the voltage-gated Kv1.3 K^+ channel was established in native activated human lymphocytes. Patchliner seems an appropriate tool for screening native lymphocytes, or even some other primary cells with acceptable success rate.

However, automated patch-clamp systems mainly use stably expressing cell lines where the quality of the cell suspensions is fundamental for acceptable success rates. A cell is trapped

randomly from the cell suspension; therefore a homogenous expression of ion channels among cells is required within the suspension with cells of good quality and health. This blind approach for catching cells is the major reason why most platforms on the market are not practicable to use primary or transiently transfected cells.

There are many advantages of the gene-centric approach in which a target protein is overexpressed in recombinant mammalian cell lines. These cell lines are easy to culture and are robust enough to be handled by automated screening platforms. The protein of interest can be overexpressed, elevating the current level and improving the signal-to-noise ratio. Host cells with null or very low background conductance can be chosen to enable very sensitive assays, and allows being certain of the molecular identity of the gene accountable for the observed signal. The major disadvantage of using non-native cell lines is that the cell is not the physiological target and the responses may differ from those that occur *in vivo*.

The evaluation and characterization of cell lines stably expressing GABA, HCN, GIRK, Kv1.4 and hERG channels were performed in our studies. $GABA_A$ chloride channels considered as important therapeutic region for drugs affecting anxiety, influencing sleep processes and treating epilepsy. Using the Patchliner platform, HEK-cells expressing $GABA_A$ ligand-gated ion channel were studied utilizing a stacked solution application for quick and precise administration of compounds to the patch-clamped cells. In this way, receptor desensitization can be minimized, and effects of co-applied drugs can be tested. Moreover, this method for stacked solution application, allowing short pulses of compound is convenient for the measurements not only $GABA_A$-receptor, but for most ligand-gated ion channels.

Among the investigated voltage-gated ion channels, HCN channels are the molecular determinants of the pacemaker or funny current (I_f) in the heart. We performed the evaluation of HCN1 and HCN4 cell lines on Patchliner by applying ivabradine, the most specific and selective I_f inhibitor, which reduced HCN1 and HCN4 currents, showing almost similar effects compared to data in literature about isolated myocytes.

GIRK1/4 channels are selectively expressed in the cardiac atrium and they are not present in the ventricle. Selective inhibition of GIRK channel might be a useful tool in the treatment of atrial fibrillation, devoid of serious ventricular side effects. After the characterization of this cell line, screening of preselected chemical libraries and compounds and extracts originated from natural sources was performed in order to discover new and effective medicaments for the treatment of atrial fibrillation.

The screening project of chemical libraries resulted in four promising compounds, which dose-response curves and IC_{50} values were determined in detailed experiments on both GIRK

and hERG channels. Ryt-243 showed similar inhibitory effect on GIRK and hERG channels. The other 3 compounds (Ryt-143, Ryt-144 and Ryt-230) revealed an at least 10 times higher blocking effect on GIRK channels compared to hERG. Ryt-243 was subjected to further investigations. Despite of its hERG blocking activity, Ryt-243 did not prolong the action potential in rabbit ventricular muscle. A potential explanation to this phenomenon is that this agent has multichannel effect and also blocks other ion channel(s) during cardiac action potential repolarization. Ryt-243 was also investigated in dog model, and the compound had a strong antiarrhythmic effect. Based on these antiarrhythmic results a patent was filed in which anti-atrial fibrillation effects of Ryt-243 are chartered. Compounds showing GIRK-selectivity (Ryt-143, Ryt-144 and Ryt-230) are also involved in further ongoing investigations including antiarrhythmic effect evaluation and structure-effect analysis.

Compounds originated from natural sources were also investigated in GIRK screen project. These natural plant compounds were chosen randomly and were not preselected. Our results reflect the high hit rate of the natural compounds. Testing a new way of tracing biologically active compounds extracts of *Polygonum persicaria* were also investigated in our GIRK screen system, with the aim to identify natural sources of promising ion channel blocking compounds. To the best of our knowledge, GIRK inhibitory assay was used for the first time for screening plant extracts for potassium ion channel blockers. In this study the GIRK channel inhibitory activity of the chloroform extract of *Polygonum persicaria* was demonstrated, and new natural flavonoids were identified from the extract. Unfortunately, the isolated compounds have only moderate activities on GIRK channel; therefore further studies are needed in order to identify minor compounds responsible for the potassium ion channel modulatory activity.

Kv1.4 is important in the regulation of the fast repolarizing phase of action potentials in heart and thus may influence the duration of cardiac action potential. This cell line had relatively high currents in the nanoamper range. Both tested reference compound (chromanol 293B and 4-aminopyridine) blocked the current. All these data are in good agreement with literature data in myocytes. Based on our results, several natural plant compounds were selected for further evaluation following the primary screen on GIRK channel. These agents were also tested on Kv1.4 cell line and selectivity of their GIRK blocking effect was evaluated with these experiments.

Besides the ion channel drug screening to discover and develop new and more effective drugs, another important aspect of the application of automated patch-clamp systems is the safety pharmacology. Life threatening cardiac arrhythmias and sudden cardiac death caused by

drugs are one of the major safety issues for pharmaceutical industry and regulatory agencies. This cardiac proarrhythmic effect of different compounds is attributed to their ability to block the rapid delayed rectifier K^+ current (I_{Kr}). Based on this concept investigation of I_{Kr} blocking and action potential duration lengthening capabilities became of required item of safety pharmacology profile of new compounds. However those tests are complicated and time consuming. Therefore analysis of I_{Kr} current in heart muscle cells was replaced by the examination of its recombinant equivalent hERG current (Kv11.1). Although hERG assay as a high-throughput technique is very powerful but sometimes gives false positive and negative results. The mechanism of the occasional failure of the hERG test is not well understood. Therefore we have compared the effect of dofetilide and sotalol on hERG current (at room temperature and 37°C) and on I_{Kr} current (37°C) and determined the value of hERG current measurements in evaluation of cardiac proarrhytmic risk of compounds and helping the understanding of mechanism of proarrhythmic pharmacological safety drug tests. Current responses of cells expressing the hERG channel at room temperature and physiological temperature were similar in case of dofetilide. Additionally, these data are in good agreement with our action potential measurements. Conversely, sotalol displays different potencies at room temperature and 37°C, and therefore, it is a desirable option to study ion channels at physiological temperature since compounds can exhibit different actions or potencies at 37°C. For safety screening, it may be particularly critical to test compounds at physiological temperature and this makes the Patchliner an ideal instrument for these experiments. Moreover, in case of sotalol, a significant difference was observed between the hERG IC_{50} values and the IC_{50} value on I_{Kr} current.

Based on our experiments, results obtained with automated patch-clamp equipment in HEK-hERG cells often show a reasonable conformity with outcomes of I_{Kr} current measurements. However there are important and not well understood differences between the evaluation of hERG-blocking ability with automated patch-clamp and other techniques such as the I_{Kr} or action potential experiments. Adhesion and precipitation have been identified as the major sources of potential right-shifted less accurate dose-response curves. Nevertheless, sotalol is among the least hydrophobic compounds, and numerous studies also reported extremely high IC_{50} values in hERG cell lines utilized for manual patch-clamp. Increasing evidence indicates that the composition of native I_{Kr} channels is very complex, and the α-subunit composition of the channel can affects its blocking sensitivity. The most of hERG screens have been conducted using recombinant cell lines expressing the hERG 1a subunit, although native ventricular I_{Kr} channels are heteromers containing both hERG 1a and 1b subunits. Although

the potency of most compounds (including sotalol) was similar for the two targets, some differences were observed. Thus the existing hERG 1a assays may underestimate the risk of some drugs and overestimate the risk of others. In addition, other studies have identified interacting proteins affecting hERG drug sensitivity. For example, MinK, MiRP1 and KCR1 coassembles with a pore-forming subunit to create stable complexes whose functional attributes resemble those of a native cardiac potassium channel, adding another level of complexity to the control mechanisms affecting cardiac electrophysiology. Since the results of hERG measurements - in spite of their beneficial time/cost value and popularity - are not as adequate as it was previously thought the estimation of proarrhytmic liability by hERG assays can be handled only with precaution.

Based on these experiences, safety pharmacology studies of *Chelidonium majus* were achieved, where the effects of extracts and alkaloids of the plant were investigated on hERG current as well as on ventricular action potential. In our electrophysiological study we investigated *C. majus* herb hydroalcoholic extracts and its alkaloids. The examined substances not only inhibit the hERG current, but also extend the duration of the action potential. In certain pathophysiological conditions where repolarization reserve is impaired all of these extracts and alkaloids may have additive effects further increasing the possibility of proarrhythmic risk and cardiac sudden death. Our results highlight the safety concerns regarding the internal use of *C. majus*: orally applied greater celandine products need cautious evaluation from the view of cardiovascular side effects.

5. CONCLUSIONS

1. The fully automated patch-clamp platform Patchliner provides high data quality and robust recordings. The Patchliner is compatible with many different cell lines, most often showing little discrepancy between the results obtained with automated and manual patch-clamp methods. Because of the elevated throughput and high success rate obtained in our experiments the Patchliner proved to be an excellent tool in cellular electrophysiological experiments and we positively suggest its use both in academic and pharmaceutical industrial research.
2. Several cell lines were characterized and used in screening projects of preselected chemical libraries performed with Patchliner. Based on these screening results a patent was filed in which anti-atrial fibrillation effects of Ryt-243 are chartered.

3. Compounds originated from natural (plant) sources were also involved in GIRK screen project. Our studies reflect the outstandingly high hit rate of compounds originated from natural sources.
4. To the best of our knowledge, GIRK inhibitory assay was used for the first time for screening of plant extracts for inhibitors, with the aim to identify natural sources of promising ion channel blocking compounds.
5. In safety pharmacology studies, we showed important and not well understood differences between the hERG-blocking ability of different compounds evaluated with automated patch-clamp method or other techniques such as I_{Kr} experiments with manual patch-clamp or action potential studies. We demonstrated that the estimation of proarrhytmic liability by hERG assays is not as adequate as it was previously thought and can be handled only with precaution.
6. Our electrophysiological studies on extracts and alkaloids of *Chelidonium majus* herb revealed that the plant possess hERG channel inhibition and prolong the duration of cardiac action potential. These results may contribute to the understanding of the toxic effects of *C. majus* and highlight the potential cardiac risk of the usage of this herb.

6. ACKNOWLEDGEMENTS

First of all I greatly acknowledge to late **Prof. Dr. Ádám Vas**. He supported my work with all his efforts and without his help the completion of this work would not be possible.

I am deeply indebted to **Prof. Dr. András Varró** who has guided and supported me on many ways. I am very grateful for his continuous advices. I am especially grateful to **Prof. Dr. Judit Hohmann** for her valuable advices and continuous support during my experimental work.

I wish to thank **Dr. László Virág** for being my tutor and helping me both with advices and technical help since the beginning of my Ph.D. studies. I am grateful to **Dr. László Tálosi** for his support and for providing me the technical background and the working facilities.

I am also grateful to **Imréné Molnár**, **Zsófia Kohajda** and **Amir Geramipour** for their help in my experimental work and for their excellent technical assistance.

I pleased to express my thanks to all co-authors of my publications for the valuable cooperation.

Finally, I acknowledge the financial support of the **Richter Gedeon Talentum Foundation** and the continuous technical assistance of the **Nanion Technologies GmbH**.

LIST OF STUDIES RELATED TO THE SUBJECT OF THE DISSERTATION

I. **Effects of Chelidonium majus extracts and major alkaloids on hERG potassium channels and on dog cardiac action potential - a safety approach.**

Orvos P, Virág L, Tálosi L, Hajdú Z, Csupor D, Jedlinszki N, Szél T, Varró A, Hohmann J.

Fitoterapia. 2015 Jan; 100:156-65.

IF: 2.345 [2014]

II. **Inhibition of G protein-activated inwardly rectifying K^+ channels by extracts of Polygonum persicaria and isolation of new flavonoids from the chloroform extract of the herb.**

Lajter I, Vasas A, Orvos P, Bánsághi S, Tálosi L, Jakab G, Béni Z, Háda V, Forgo P, Hohmann J.

Planta Med. 2013 Dec; 79(18):1736-41.

IF: 2.339

III. **Identification of diterpene alkaloids from Aconitum napellus subsp. firmum and GIRK channel activities of some Aconitum alkaloids.**

Kiss T, Orvos P, Bánsághi S, Forgo P, Jedlinszki N, Tálosi L, Hohmann J, Csupor D.

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OTHER STUDIES

I. **Electrophysiological effects of ivabradine in dog and human cardiac preparations: potential antiarrhythmic actions.**

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