Cellular cardiac electrophysiological effects of HCN channel blockers

Summary of Ph.D. Thesis

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ACRONYMS AND ABBREVIATIONS

APA	action potential amplitude
APD_{50}	action potential duration at 50% of repolarization
APD_{90}	action potential duration at 90% of repolarization
ASSOCIATE	evaluation of the Antianginal efficacy and Safety of the aSsociation Of the I_f Current Inhibitor ivAbradine with a beTa-blockEr
BEAUTIFUL	morBidity-mortality EvAlUaTion of the If inhibitor ivabradine in patients with coronary disease and left ventricULar dysfunction
CHF	congestive heart failure
ERG	electroretinogram
HCN	hyperpolarization-activated and cyclic nucleotide-gated
INITIATIVE	INternatIonal TrIal of the Antianginal effecT of IVabradinE compared with atenolol
MDP	maximum diastolic potential
MiRP1	MinK-related protein
SAN	sino-atrial node
SBAs	specific bradycardic agents
SHIFT	Systolic Heart failure treatment with the IF inhibitor ivabradine Trial
SNI	sinus node inhibitors
STEMI	ST-segment elevation myocardial infarction
V_{max}	maximal rate of depolarization.

1. INTRODUCTION

1.1 HCN channels. General overview.

The hyperpolarization-activated cyclic nucleotide-gated (HCN) channel family implies four members (HCN1-4) which are expressed in the heart and the nervous system. The current flowing through HCN channels was termed as I_f or I_h or I_q. It was demonstrated that the heterogeneity of native I_h currents is attributable to, at least partly, the tissue-specific expression of HCN channel genes. The hyperpolarization–activated current was named "funny" current (I_f) in the heart because of its unusual electrophysiological characteristics, while this current in the nervous system was designated I_h (for "hyperpolarization") or I_q (for "queer" current). I_f/I_h current plays a substantial role in the initiation and control of the heart rate. I_f/I_h generates rhythmic activities particularly in thalamocortical circuits, has a function in regulating synaptic integration (e.g. in prefrontal pyramidal neurons), in neuronal cells takes part in the determination of resting membrane potential (I_h is inward, and the others (Kir2 and K_{leak}) are outward, there is dynamic equilibrium between them), probably plays an important role in olfactory information processing, generates and controls oscillations of the membrane potential. The increased expression level of I_h and HCN isoforms in sensory nerves indicates that they might play a prominent role in sensory function (e.g. by modulating pain thresholds) and could be targets in analgesic therapy in special conditions e.g. nerve injury based neuropathic pain. Interestingly, it turned out that some volatile anaethetic agents are able to influence the operation of HCN channels, opening an other significant field in drug research. HCN isoforms have also been found in pancreatic beta-cells and kidney, where they regulate important physiological functions. HCN channels are expressed in the retina. Ivabradine is a novel heart rate-lowering antianginal agent which inhibits the pacemaker current in the heart with high selectivity. According to the BEAUTIFUL study, ivabradine can be used to reduce the incidence of coronary artery disease outcomes in a subgroup of patients who have heart rates of 70 bpm or greater. A large proportion of patients suffering from heart failure die of sudden cardiac death caused by arrhythmia. It emerged from experiments analyzing the expression pattern of HCN channel isoforms in failing heart that suppressing the activity of the upregulated HCN channel subtypes can probably preclude the development of that type of arrhythmias which are triggered by abnormal pacemaker activity in the working myocardium, as it was found that both mRNA and protein levels of HCN2 and HCN4 isoforms were significantly augmented in failing ventricles. Therefore, selective HCN blockers might serve as effective agents of this profile. HCN4 is the predominant isoform in undiseased human heart, and its expression is increased by disease (e.g. heart failure). The classic antiarrhythmic compounds might exert little or insignificant effect on this isoform as recently it turned out that the blocking effects of them /quinidine, disopyramide, cibenzoline, lidocaine, mexiletine, aprindine, propafenone, flecainide, propranolol, and verapamil/ on the HCN4 channel current were weak, D,L-sotalol hardly influenced the HCN4 channel current in clinical concentrations in HEK293 cells.

1.2 HCN channels in the heart

Heart rate is regulated by spontaneous electrical pacemaker activity in the sino-atrial node mainly controlled by the I_f current. The I_f current determines the slope of diastolic depolarization and cardiac frequency, and its inhibition causes heart rate reduction. The ´funny´ (I_f) current is so termed because of its unusual characteristics, including that of being an inward current that is activated on hyperpolarization and not on depolarization like other known currents. The role of currents other than I_f and cellular mechanisms (I_{CaL}, I_{CaT}, I_{Na}, I_{Kr}, I_{st}, I_{NCX}, intracellular Ca^{2+} cycling = "calcium clock") in pacemaker activity is also important and it is still argued. Nevertheless, it can be stated that I_f plays a principal role in regulating

sinoatrial node pacemaker activity. It is generally agreed that it has modulatory effect on pacemaker rate. HCN channels (hyperpolarization-activated cyclic nucleotid-gated) are responsible for generating the I_f pacemaker current in the heart. The channel relating to the cardiac I_f current is known to be a member of a family of channels denominated the hyperpolarization-activated cyclic nucleotid-gated (HCN) channels. The I_f current is controlled by opposite processes, i.e. the beta adrenergic stimulatory and muscarinic cholinergic inhibitory actions occur via adenylate cyclase and cAMP. The I_f is a mixed Na^+ and K^+ current that is activated on hyperpolarization to voltages within the pacemaker range. Recently, Ca^{2+} influx through HCN channels has indirectly been postulated and then directly the Ca^{2+} permeability of native rat and human I_f has been shown.
Heart rate has a prominent role in the development and pathophysiology of myocardial ischaemia. In patients with coronary artery disease, ischaemic episodes could be generated by an elevation in heart rate that elicits imbalance between myocardial oxygen supply and demand. Therefore, lowering the heart rate is regarded as an important therapeutic target in preventing ischaemia by reducing myocardial oxygen consumption. Therefore, reducing heart rate is one of the most important therapeutic possibilities in the treatment of stable angina pectoris. Additionally, resting heart rate is associated with the severity and rate of progression of coronary atherosclerosis and is an independent predictor of plaque rupture in coronary arteries, therefore, heart rate lowering e.g. by ivabradine the specific HCN blocker should prevent atherosclerosis and cardiovascular events.
The current pharmacological treatment of stable angina pectoris is based on beta adrenoceptor blockers, calcium channel antagonists, nitrates and trimetazidine with beneficial metabolic effect on the heart. The antiischemic effect of beta blockers is attributable, at least in part, to heart rate reduction. Beta blockers apart from the numerous, proven, valuable effects in the treatment of cardiovascular diseases (angina pectoris, myocardial infarction, heart failure, arrhythmias) have several side effects including fatigue, depression, change in lipid status, sexual dysfunction, bronchospasm, atrioventricular block, possible increase in blood sugar level, rebound phenomena. They can cause paradoxical vasoconstriction of large epicardial arteries during exercise or in patients with resting angina associated to coronary vasospams. Beta blockers (except carvedilol and nebivolol) may be contraindicated in patients with peripheral vascular disease. Concerning the nitrates headache, regarding the calcium channel blockers pedal edema are potential side effects. The hemodynamic effects might be also limiting factors at application e.g. the negative inotropic effect of the beta blockers, the considerable blood pressure lowering property of the calcium channel blockers. In ST-segment elevation myocardial infarction (STEMI) early i.v. use of beta blockers is clearly contraindicated in patients with clinical signs of hypotension or congestive heart failure. Therefore, highly selective HCN channel blockers, producing solely heart rate lowering, e.g. ivabradine, lacking the negative inotropic effect, may offer a new therapeutic perspective in the treatment of ischaemic heart diseases e.g. in stable angina pectoris, particularly in patients with left ventricular dysfunction. Based on the recent clinical trials and experiments, widening the indication of ivabradine for the treatment of heart failure or myocardial infarction is expected. Thus, to seek for specific blockers of the I_f current is in the focus of cardiovascular pharmacological research.

1.3 Characteristics of the I_f current and its role in the adrenergic and cholinergic modulation of heart rate

The sino-atrial node (SAN) is a small, specialized structure located in the right atrium. The nodal cells (specialized myocytes) generate pacemaker activity, i.e. their diastolic membrane potential is unstable, a gradual depolarization to a threshold occurs. I_f current is deactivated during the upstroke of an action potential. Upon repolarization when membrane potential

reaches the I_f activation voltage (below -40 mV), I_f becomes activated, and its slow activation counteracts the repolarizing I_K and slowly depolarizes the membrane voltage. Reaching the threshold a new action potential is elicited. The potential ionic currents and mechanism responsible for the pacemaker activity are as follows: I_f, I_{CaL}, I_{CaT}, I_{Na}, I_{Kr}, I_{st}, I_{NCX} and probably the pacemaker function is dependent on complex interactions between localized, subsarcolemmal Ca^{2+} releases and action potentials. The role of I_f in the control of the firing rate of the sino-atrial node and therefore determining the heart rate is heavily argued. The clinical studies might give proof of the considerable influence of I_f on the pacemaker function, as the highly specific I_f current blocker ivabradine used in the trials reduced heart rate by 10 to 15 beats/min. The heart, regarding its control by the autonomic nervous system, receives both sympathetic (responsible for increasing the heart rate, via stimulation of β_1 adrenoceptors) and parasympathetic (liable for decreasing the heart frequency, through activation of M_2 cholinergic receptors) innervation. The predominant tone is the parasympathetic, mediated by acetylcholine. At least in rabbits, functional inhomogeneity of the sinus node is possibly responsible for the predominance of the effect of acetylcholine over that of adrenaline. Cyclic AMP is a second messenger in the acetylcholine inhibitory action on the I_f pacemaker current, the parasympathetic cholinergic-stimulated decrease of intracellular cyclic AMP concentration occurs via inhibition of a high basal adenylate cyclase activity. Sympathetic stimulation by noradrenaline (via increasing the activity of adenylate-cyclase resulting in a higher intracellular cAMP level) increases the I_f current during hyperpolarizations and accelerates its activation rate. The second messenger cAMP promotes HCN (I_f) channel opening by direct binding to the C-terminal channel domain. Therefore, compounds that increase I_f current (e.g. $beta_1$ adrenoceptor agonists) accelerate heart rate and conversely, substances that decrease I_f current (e.g. acetylcholine) slow heart rate. In 1979, the I_f pacemaker current was first described in the sino-atrial node cells by Brown, DiFrancesco and Noble in Oxford. According to its special property, i.e. being activated on hyperpolarization, this inward current was nominated as the "funny" current. Later, I_f was identified in atrial and ventricular myocytes. A similar current, denominated I_h, was identified in several different types of neurons (responsible for controlling excitability, integrating synaptic activity) and in retina cells. The I_f pacemaker current is an inward current activated by both voltage hyperpolarization and increase in intracellular cAMP. Cyclic AMP activates I_f current by a mechanism other than phosphorylation, implying a direct interaction with the channels at their cytoplasmic side. Cyclic AMP stimulates I_f by shifting its activation curve to more positive voltages. According to an allosteric model cAMP has a higher binding affinity to open than to closed channels. I_f is enhanced by $beta_1$ adrenoceptor activation, and is decreased by acetylcholine. I_f current possesses slow kinetics of activation and deactivation (i.e. most I_f/I_h channels activate quite slowly with time constants ranging between hundreds of milliseconds and seconds). I_f is carried by a mixture of Na^+ (the major contributor) and K^+ ions. Recently, Ca^{2+} entry through HCN channels has indirectly been postulated and then directly the Ca^{2+} permeability of native rat and, more importantly human I_f in the presence of physiological extracellular Ca^{2+} concentrations at the physiological resting membrane potential has been shown. Representative values of mid-activation voltages of I_f current measured in the sino-atrial node cells are in the range of -60 to -70 mV. In human sino-atrial cells activation threshold has been reported to be between -50 and -60 mV. I_f kinetics and activation range are modulated by cAMP (see above); auxiliary subunits e.g. MinK-related protein (MiRP1) co-assembling with HCN2/ acting as a β subunit for HCN2 pacemaker channel subunits resulting in larger I_f current with more rapid activation and deactivation kinetics. Other processes contributing to adjustment of the current activation range and kinetics are phosphorylation-dependent mechanisms (possible candidates for the phosphorylation events are protein kinase A, C, tyrosine kinases) as phosphorylation increases

I_f conductance, potentiates the action of isoproterenol on I_f ; interaction with structural proteins e.g. binding to filamin A slows HCN1 channel kinetics and reduces the density of channel expression and whole-cell conductance. Recently, evidence for a major role of other regulatory elements of HCN current has been indicated. Among these are phosphatidylinositol 4,5 bisphosphate (PI(4,5)P2), and caveolin 3. PI(4,5)P2, can induce positive shifts in voltage dependence. Disorganization of caveolin-rich microdomains in the membrane is able to shift the activation curve of HCN channels toward more positive voltages. HCN channels are also regulated by intracellular pH, intracellular increase in protons shifts the voltage dependence of activation to more negative potentials leading to inhibition of channel operation.

1.4 I_f current inhibitor heart rate-reducing agents

In accordance with the prominent role of I_f current in the generation and autonomic control of spontaneous activity in the sino-atrial node, selective inhibition of the pacemaker current is expected to be able to modify the slope of diastolic depolarization, therefore to modulate cardiac chronotropy without influencing other cardiovascular parameters e.g. inotropy, impulse conduction or repolarization. At the end of 1980s, relatively selective inhibitors of the I_f current, e.g. the first described alinidine and falipamil, were termed as "specific bradycardic agents, SBAs". Later, compounds acting by the same mechanism have been denominated as "heart rate-lowering" agents. They perform cardiac slowing without the negative inotropic side effect and ideally without slowing down atrioventricular (AV) conduction, characteristic for the classic, currently used drugs (beta receptor blockers and Ca^{2+} antagonists) also aiming to reduce heart rate. Specific bradycardic agents can be grouped into three different chemical classes: phenylalkylamines such as zatebradine, imidazolines such as alinidine, and ZD7288, which is the only diaminopyrimidine whose activity on HCN channel has been reported. Alinidine was the first bradycardic agent with known sino-atrial pacemaker current (I_f) inhibitory property which is an imidazoline compound derived from clonidine. Further bradycardic substances (also termed as newer "sinus node inhibitors" SNIs) have been derived from the calcium channel inhibitor verapamil, e.g.: falipamil, cilobradine, zatebradine and ivabradine. Alinidine (ST567), (N-allyl-clonidine), reduced the spontaneous frequency and the slope of diastolic depolarization dose-dependently and prolonged repolarization in rabbit and guinea pig sino-atrial node cells. Due to the voltage clamp experiments, the underlying ionic currents responsible for these changes in configuration of the action potentials turned out to be –concerning the repolarization lengthening- the reduced outward current (I_k) and –regarding the frequency and slope- the decreased hyperpolarization-activated inward current (I_f). Regarding I_f current block no use or frequency dependence were reported indicating that alinidine binds equally to open and closed channel states. Lack of specificity e.g. the ability of alinidine to block of K^+ channels in similar concentrations exerting bradycardic effect and the consequent action potential prolongation and negative inotropism and visual side-effects resulted that its development was discontinued. The verapamil analogue falipamil (AQ-A39), (2-(3-{[2-(3,4-dimethoxyphenyl)ethyl]methyl-amino}propyl)-5,6-dimethoxy-2,3-dihydroisoindol-1-one hydrochloride) apart from reducing sino-atrial frequency by blocking I_f , possesses several unwanted receptorial actions e.g. it exhibits direct vagolytic effect, via a competitive action of the drug with acetylcholine at the muscarinic receptor, reduces (the time-dependent) potassium current (I_K), blocks calcium channels. Similarly to zatebradine (below), the block of I_f conductance presents use-dependence with falipamil. The proarrhythmic effects attributable to excessive potassium and calcium channel blockade resulted in discontinuing the development of this drug.

The benzazepinone derivative zatebradine (UL-FS49; /1,3,4,5-tetrahydro-7,8-dimethoxy-3-[3-[[2-(3,4-dimethoxy- phenyl)ethyl]methylimino]propyl]-2H -3-benzazepin-2-one hydrochloride/), is a bradycardic compound that blocks the hyperpolarization-activated

current (I_f) in the rabbit sinoatrial node. Zatebradine behaves as an open channel blocker of (I_f) channels, block occurs within the pore, at a distance of about 39% of the membrane thickness from its internal side. Zatebradine-induced block is use dependent. Zatebradine is able to block hKv1.5, prolongs AP duration in concentrations exerting bradycardic effect. Zatebradine exerted positive inotropic actions in guinea pig papillary muscles. A phase III clinical trial ascertained that zatebradine seems to provide no additional antianginal benefit to patients already receiving nifedipine. Moreover, patients taking zatebradine experienced visual disturbances. Patch-clamp recordings from newt rod photoreceptor cells showed that zatebradine caused use- and concentration-dependent (0.1-100 μM) inhibition of I_h , which may explain its effects on the electroretinogram (ERG) in vivo and its adverse effects on vision in clinical studies. Cilobradine (DK-AH269), a newer congener of zatebradine induced a more effective (2-fold higher) and faster block of I_f in sino-atrial node myocytes than zatebradine. Similarly to falipamil and zatebradine, cilobradine produced a use-dependent block of I_f. Cilobradine requires open channels to access the binding site and acts intracellularly. Block by cilobradine is voltage-dependent and is relieved by strong hyperpolarization. Cilobradine performed considerable (~22%) I_K reduction in mouse sino-atrial node cells at 5 μM, only preclinical studies were accomplished. Zeneca ZD 7288 (also known as ICI-D7288), which is a lipophilic quaternary cation, is a distant analogue of alinidine. The block of ZD7288 (4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride) on I_f current is not use-dependent, whereas zatebradine (UL-FS 49) displays use-dependent I_f current inhibition. The lack of use-dependence in the effect of ZD7288 on I_f current might reflect an affinity of the drug for the closed or resting state of the channel, resulting in the development of inhibition even in the absence of activation. Distinctly, zatebradine (UL-FS 49) performs small or no inhibition of the current in the absence of activation. ZD7288 had no significant effect on the delayed rectifier potassium current (I_K) in guinea-pig dissociated sinoatrial node cells, but both alinidine and zatebradine (UL-FS 49) significantly inhibited I_K at the same concentrations which decreased I_f . Although ZD7288 is usually represented as pyridinium ion, it is likely that at physiological pH the non-ionic structure is present in sufficient amount to allow the penetration of the compound through the cell membrane. In different regions of the central nervous system (rat hippocampal CA1 neurons, guinea pig substantia nigra neurons, thalamocortical neurons of the cat ventrobasal thalamus) ZD7288 considerably blocked the hyperpolarization-activated current (I_h). The compound YM758 (Fig.2), [(-)-N-{2-[(R)-3-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)piperidin-1-yl]ethyl}-4-fluorobenzamide monophosphate], a novel I_f current blocker, was being developed as a treatment for stable angina and atrial fibrillation by Astellas Pharma and has recently been discontinued.

1.5 Heart rate reduction with a highly selective I_f inhibitor: ivabradine (S16257)

The resolution of the benzocyclobutane derivative S 15544 (a racemate compound) into its two isomers S 16257 (*S* configuration) and S 16260 (*R* configuration) provided the compound S 16257, ivabradine, an optically pure molecule. Both isomers reduced the spontaneous firing of rabbit sinus node preparations. S 16260 lengthened ventricular repolarization, which is a potential proarrhythmic effect. The electrophysiological selectivity of S 16257 made it possible its further preclinical evaluation. The chemical formula of ivabradine is [3-(3-{[((7S)-3,4-dimethoxybicyclo-[4,2,0]-octa-1,3,5-trien-7-yl)methyl]-methylamino}-propyl)-1,3,4,5-tetrahydro-7,8-dimethoxy-2*H*-3-benzazepin-2-one hydrochloride].

Ivabradine is a novel antianginal agent, a potent blocker of the pacemaker current, which has significantly weaker ability to block other ion currents than its predecessors. Ivabradine (Procoralan®, Coralan®, Corlantor®) is an anti-ischaemic agent, which inhibits the I_f current with high selectivity. Ivabradine induces a marked exponential use-dependent blockade of the

hyperpolarization activated I_f current. The IC_{50} for the block of I_f is 2.8 μM. It has been shown that a high concentration of ivabradine (10 μM) has no detectable effect on T-type calcium current and slightly decreases L-type calcium current without significant use-dependent block. Also it moderately decreases the delayed outward potassium current in rabbit sino-atrial node cells. The effect of ivabradine on the action potential repolarization was studied in guinea pig papillary muscle and rabbit sinus node pacemaker cells. In these preparations ivabradine did not show considerable effect on action potential duration at therapeutically relevant concentrations. Ivabradine blocks ion flow (through HCN channels) from the cytoplasmic side of the membrane through competing with permeating ions in their binding to specific ion binding sites in the single file permeation pathway of the HCN channels. Ivabradine blocks HCN channels from the cytoplasmic side requiring drug molecules to enter the cell before acting ("open channel" block). Use dependence appears as a slowly progressing block accumulation during repetitive channel activation/deactivation cycles. The I_f inhibition by ivabradine is highly voltage-dependent, being stronger at depolarized voltages, and current dependent, since the direction of current influences the stability of its binding in the pore of the channel. The study investigated the blocking action of ivabradine on mouse (m) HCN1 and human (h) HCN4 channels heterologously expressed in HEK293 cells revealed IC_{50s} of 0.94 μM for mHCN1 and 2.0 μM for hHCN4.

1.6 Therapeutical advantages of ivabradine in coronary artery disease and heart failure patients

Ivabradine is the only clinically available selective heart rate-reducing drug, and it exerts anti-ischaemic effects in patients with chronic stable angina. Ivabradine has been associated with a good safety profile during its clinical development. In a trial, mild, tolerable visual symptoms (e.g. abrupt changes in light intensity) were reported as side effects. Contrary to several heart rate-reducing drugs, ivabradine lowers heart rate both at rest and during exercise without producing any negative inotropic or vasoconstrictor effect. The bradycardic effect of ivabradine is proportional to the resting heart rate, the effect tends to plateau. Therefore, severe sinus bradycardia is not common. In a study with healthy volunteers it has been demonstrated that for a similar decrease in heart rate at rest and during sympathetic stimulation, acute administration of ivabradine decreased myocardial oxygen demand to the same extent as a reference beta-blocker, propranolol, but withot proof of depressant action on cardiac function. The advantages of heart rate-lowering by beta-blockers are in part counterbalanced by unmasked alpha-adrenergic coronary vasoconstriction, therefore selective heart-reducing compounds might be able to decrease heart rate without unmasking alpha-adrenergic coronary vasoconstriction. The INITIATIVE study compared the anti-anginal and anti-ischaemic effects of ivabradine and the beta receptor blocker atenolol and revealed that ivabradine is as effective as atenolol in patients with stable angina. The ASSOCIATE study evaluated the anti-anginal and anti-ischaemic efficacy of ivabradine in patients with chronic stable angina pectoris receiving beta-blocker therapy and found that the combination of ivabradine 7.5 mg b.i.d. and atenolol at the commonly used dosage in clinical practice in patients with chronic stable angina pectoris produced additional efficacy with no untoward effect on safety or tolerability. According to the BEAUTIFUL trial, in patients with coronary artery disease and left-ventricular systolic dysfunction ivabradine can be used to reduce the incidence of coronary artery disease outcomes (e.g. hospitalization for myocardial infarction and coronary revascularization) in a subgroup of patients who have heart rates of 70 bpm or greater. The placebo arm of this trial which was a large cohort of patients with stable coronary artery disease and left-ventricular dysfunction showed that elevated heart rate was associated with an increased risk of adverse fatal and non-fatal cardiac events. The results of the recent SHIFT study (also) support the significance of heart-rate reduction with ivabradine for

improvement of clinical outcomes in heart failure and confirm the important role of heart rate in the pathophysiology of this disorder. Moreover, an increased heart rate has been demonstrated to be associated with more pronounced atherosclerosis progression and a higher risk of coronary plaque rupture. Heart rate lowering has slowed atherosclerosis progression and improved endothelial function in experimental models.

1.7 Future possibilities: ivabradine as a reasonable alternative to beta receptor blockers in myocardial infarction, results with ivabradine in experimental models of myocardial infarction

In acute myocardial infarction, the negative inotropic and hypotensive effects of beta receptor blockers contraindicate their use in patients with borderline blood pressure, pulmonary congestion, manifest pulmonary edema, or cardiogenic shock. After myocardial infarction, for chronic therapy beta receptor blockers are considered as a first-line therapy but side effects e.g. fatigue, change in lipid status, sexual dysfunction, bronchospasm, atrioventricular block may limit their use. Ivabradine is devoid of negative inotropic effect and does not influence coronary vasomotion, and it has no impact on blood pressure and AV node conduction. These favourable properties might put ivabradine in a status to lower heart rate in unstable patients. Ivabradine improves myocardial stunning following myocardial ischaemia better than beta receptor blockade. In a recent study performed in a rabbit model of myocardial infarction, ivabradine improved global and regional systolic function of the reperfused heart. Previously, improvement of regional myocardial blood flow and function and reduction of infarct size by ivabradine have also been described. Also, chronic treatment with ivabradine positively affected global cardiac remodelling in post-myocardial infarcted rats.

1.8 HCN channels and arrhythmogenesis

It has been shown that cardiac HCN4 isoforms are fundamental for physiological heart impulse generation and conduction in adult mice supporting that dysfunctional HCN4 channels might be a direct cause of arrhythmias. HCN4 is the main isoform (based on mRNA analysis) expressed in human sino-atrial node (hSAN) and ivabradine blocks this channel in a use-dependent way. In rabbit atrioventricular node (AVN) and human Purkinje fibres HCN4 is the main isoform expressed. In human, the main HCN isoforms both in atria and ventricles are HCN4 and HCN2. HCN upregulation probably contributes to increased I_f and may play a role in ventricular and atrial arrhythmogenesis in heart failure. I_f overexpression has been reported in failing human hearts. In diseased human hearts, current density is higher in ischemic than in dilated cardiomyopathy, indicating that functional I_f overexpression is related to the etiology of the disease. At the mRNA and protein level, it has been documented that HCN2 and HCN4 isoforms are upregulated in failing human ventricles. Single-channel, cell-attached recordings carried out in human atrial myocytes, showed that activation potentials were more positive than previously suggested in whole-cell measurements, supporting the idea that I_f might contribute to arrhythmogenesis in disease states described by I_f overexpression e.g. heart failure, cardiac hypertrophy (also HCN2 and HCN4 are upregulated). Altered expression of HCN channels as a result of electrophysiological remodeling may represent an arrhythmogenic mechanism in heart failure, therefore HCN4 or HCN2 specific or HCN4/2 preferring I_f blockers might be considered as potential therapeutic agents which may modulate abnormal automaticity.

1.9 Recently synthesized HCN channel blockers

In order to design isoform-selective phenylalkylamines, Melchiorre *et al.* tested compound EC4, and its *cis* isomer EC32, on HEK cells stably expressing mHCN1, mHCN2 and hHCN4

channel isoforms. It was found that these molecules show different ability to block I_h current: in particular, while EC4 shows a preference for the HCN1 channel over HCN2 and HCN4, EC32 blocks the HCN2 channel more potently than HCN1 and HCN4. These findings lead to the discovery of analogs with improved selectivity, allowing to derive some preliminary structure-activity relationships. In particular: a *R* absolute configuration is important for a strong interaction with the HCN2 channel (MEL55A), and therefore to gain selectivity for one isoform over the others; the increase of steric bulkiness on the basic nitrogen seems to drive the activity only on the HCN1 subtype, as suggested by the high selectivity of compound **MEL57A**, *R* isomer. Moreover, the incorporation of spacer B into a cyclohexane ring gave compound **EC18**, which interacts selectively with the HCN4 isoform with respect to HCN1 and HCN2, suggesting that cyclic constraints seem necessary to achieve selectivity for the HCN4 isoform.

1.10 Aims of the study

The main goal of the present study was to describe in detail the cellular cardiac electrophysiological effects of compounds ivabradine and EC18 and MEL57A in dog and human heart preparations using standard microelectrode technique. The effect of ivabradine on the action potential repolarization was only studied in guinea pig papillary muscle and rabbit sinus node pacemaker cells, i.e. in species having relatively small hearts with high frequencies. In these preparations ivabradine did not show considerable effect on action potential duration at therapeutically relevant concentrations, and at high concentration (50 μM) it produced frequency-dependent V_{max} block in guinea-pig papillary muscle. The effects of ivabradine on V_{max}, repolarization and spontaneous depolarization have not yet been reported in human isolated cardiac preparations and in large animals close to human in heart size and spontaneous frequency. We investigated the important question whether these substances might possess subsidiary anti –and/or proarrhythmic actions.

2. MATERIALS AND METHODS

2.1 Whole cell configuration of the patch-clamp technique

Ventricular myocytes were enzymatically dissociated from hearts of mongrel dogs or rabbits of either sex weighing 10 – 15 kg and 1-2 kg, respectively, as described earlier in detail. One drop of cell suspension was placed within a transparent recording chamber mounted on the stage of an inverted microscope, and individual myocytes were allowed to settle and adhere to the chamber bottom for at least 5 minutes before superfusion was initiated. Only rod shaped cells with clear cross striations were used. HEPES buffered Tyrode's solution served as the normal superfusate. This solution contained (in mM): NaCl 144, NaH_2PO_4 0.33, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 0.53, Glucose 5.5, and HEPES 5.0 at pH of 7.4. Patch-clamp micropipettes were fabricated from borosilicate glass capillaries using a P-97 Flaming/Brown micropipette puller (Sutter Co, Novato, CA, USA). These electrodes had resistances between 1.5 and 2.5 MΩ when filled with pipette solution containing (in mM): KOH 100, KCl 40, K_2ATP 5, $MgCl_2$ 5, EGTA 4, $CaCl_2$ 1.5 and HEPES 10. The pH of this solution was adjusted to 7.2 by aspartic acid. Measuring K^+ currents, nisoldipine (1 μM) (gift from Bayer AG, Leverkusen, Germany) was added to the external solution to eliminate inward L-type Ca^{2+} current (I_{Ca}). The rapid delayed rectifier potassium current (I_{Kr}) was separated from slow I_{Ks} component by using the selective I_{Ks} blocker HMR-1556 (0.5 μM). Membrane currents were recorded with Axopatch-200B patch-clamp amplifiers (Molecular Devices –Axon Instruments, Union City, CA, USA) using the whole-cell configuration of the patch-clamp technique. After establishing a high (1-10 Gohm) resistance seal by gentle suction, the cell membrane beneath the tip of the

electrode was disrupted by suction or by application of 1.5 V electrical pulses for 1 - 5 ms. The series resistance was typically 4 - 8 MΩ before compensation (50 - 80%, depending on the voltage protocols). Experiments where the series resistance was high, or substantially increased during measurement, were discarded. Membrane currents were digitized using analog-to-digital converters (Digidata 1200 and Digidata 1322, Molecular Devices –Axon Instruments, Union City, CA, USA) after low-pass filtering at 1 kHz under software control (pClamp 8.0 Molecular Devices –Axon Instruments, Union City, CA, USA). The same software was used for off-line analysis. All patch-clamp data were collected at 37 °C.

2.2 Conventional microelectrode technique

2.2.1 Dog tissue

All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication No 85-23, revised 1985). The protocols were approved by the Review Board of the Committee on Animal Research of the University of Szeged (54/1999 OEj). Ventricular muscles were obtained from the right ventricle, and free-running (false tendons of) Purkinje fibres were isolated from both ventricles of hearts removed through a right lateral thoracotomy from anesthetized (thiopental 30 mg/kg *i.v.*) mongrel dogs of either sex weighing 10 - 15 kg. The preparations were placed in a tissue bath and allowed to equilibrate for at least 2 h while superfused (flow rate 4-5 ml/min) with Locke's solution containing (in mM): NaCl 120, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 22, and glucose 11. The pH of this solution was 7.40 to 7.45 when gassed with 95% O_2 and 5% CO_2 at 37°C. During the equilibration period, the ventricular muscle tissues were stimulated at a basic cycle length of 1000 ms, Purkinje fibres were stimulated at a basic cycle length of 500 ms. Electrical pulses of 2 ms in duration and twice diastolic threshold in intensity (S_1) were delivered to the preparations through bipolar platinum electrodes. Transmembrane potentials were recorded with the use of glass capillary microelectrodes filled with 3 M KCl (tip resistance: 5 to 15 MΩ). The microelectrodes were coupled through an Ag-AgCl junction to the input of a high-impedance, capacitance-neutralizing amplifier (Experimetria 2004). Intracellular recordings were displayed on a storage oscilloscope (Hitachi V-555) and led to a computer system (APES) designed for on-line determination of the following parameters: resting membrane potential, action potettial amplitude, action potential duration at 50% and 90% repolarization and the maximum rate of rise of the action potential upstroke (V_{max}). The following types of stimulation were applied in the course of the experiments: stimulation with a constant cycle length of 1000 ms (ventricular muscles); stimulation with a constant cycle length of 500 ms (Purkinje fibres); stimulation with different constant cycle lengths ranging from 300 to 5000 ms (or to 2000 ms in the case of Purkinje fibres to prevent spontaneous diastolic depolarizations at cycle lengths longer than 2000 ms). To determine the recovery kinetics of V_{max}, extra test action potentials were elicited by using single test pulses (S_2) in a preparation driven at a basic cycle length of 400 ms. The S_1-S_2 coupling interval was increased progressively from the end of the refractory period. The effective refractory period was defined as the longest S_1-S_2 interval at which S_2 failed to elicit a propagated response. The diastolic intervals preceding the test action potential were measured from the point corresponding to 90% of repolarization of the preceding basic beat to the upstroke of the test action potential and were increased progressively. To determine the onset kinetics the preparations were continuously stimulated at cycle length of 1000 ms, the stimulation was interrupted for 1 min, and then a train of 40-beat stimuli was applied with a cycle length of 400 ms. Control recordings were obtained after equilibrium period. The effects of ivabradine were determined at the given concentrations, with recordings started 25 minutes after the addition of each concentration of the drug in a cumulative manner. For all experiments ivabradine (Sequoia Research Products Ltd, Pangbourne, United Kingdom) was dissolved in

distilled water at stock solution concentration of 1 or 10 mM. Compounds EC18 and MEL57A were kindly provided in the frame of collaboration with Prof. Elisabetta Cerbai's research group, University of Florence; compounds EC18 and MEL57A were dissolved in DMSO.

2.2.2 Human tissue

Non-diseased human hearts that were technically unusable for transplantation (based on logistical, not patient-related, considerations) were obtained from organ donors. Before cardiac explantation, organ donor patients did not receive medication except dobutamine, furosemide and plasma expanders. The investigations conform to the principles outlined in the *Declaration of Helsinki* of the World Medical Association. All experimental protocols were approved by the Scientific and Research Ethical Committee of the Medical Scientific Board at the Hungarian Ministry of Health (ETT-TUKEB), under ethical approval No 4991-0/2010-1018EKU (339/PI/010). Human cardiac tissue was stored in cardioplegic solution at 4°C for 4–8 hour. Papillary muscles were obtained from the right ventricle. Preparations were initially stimulated at a basic cycle length of 1000 ms and allowed at least 1 hour to equilibrate while they were continuously superfused with Locke's solution. Temperature of the superfusate was kept constant at 37°C.

2.3 Statistical analysis

Results were analyzed by using Student's t-test for paired and unpaired data. Differences were considered significant when $p < 0.05$. Data are expressed as mean ± S.E.M. (standard error of the mean).

3. RESULTS

3.1 Effects of ivabradine on ionic currents

The effects of the drug on rapid delayed rectifier (I_{Kr}), the transient outward (I_{to}) and the inward rectifier (I_{K1}) potassium currents were measured in rabbit and dog ventricular myocytes. 10 µM ivabradine did not influence I_{K1} (control: 365±73 pA, drug: 361±83 pA, at -60 mV test potential, n=5) in rabbit ventricular myocytes. The drug did not affect I_{to} in rabbit (control: 496±101 pA, drug: 479±76 pA, at 50 mV test potential, n=5), and in dog ventricular myocytes (control: 2592±577 pA, drug: 2573±516 pA, at 50 mV test potential, n=4). In rabbit ventricular myocytes the amplitude of I_{Kr} was decreased concentration-dependently by ivabradine with an estimated IC_{50} value of 3.5 µM.

3.2 Effects of ivabradine on transmembrane action potentials

In dog Purkinje strands, stimulation (2 Hz) was terminated to allow development of spontaneous activity. The developed spontaneous frequency in the control preparations was 0.471 ± 0.06 Hz. Ivabradine concentration-dependently decreased the amplitude of spontaneous diastolic depolarization (n=6) and slowed spontaneous rate of firing (n=5) of the Purkinje fibres. Spontaneous activity was completely abolished by 10 µM ivabradine in all preparations. In isolated dog Purkinje fibres ivabradine concentration– and rate-dependently decreased the maximum rate of rise of the action potential upstroke (V_{max} , n=11), and action potential amplitude while action potential duration measured at 50% of repolarization was shortened in a concentration-dependent manner (n=8) at pacing with a constant cycle length of 500 ms. The depression of V_{max} evoked by 0.1 and 1 and 10 µM ivabradine was strongly dependent upon stimulation frequency ("use-dependent"); i.e., as pacing cycle length was decreased, the depression of V_{max} was increased. In dog ventricular muscles (n=7) stimulated at the cycle length of 400 ms the onset kinetics of V_{max} block induced by 10 µM ivabradine

was fitted to a single exponential, resulting in the onset rate kinetic constant of 13.9 ± 3.2 beat $^{-1}$. In dog ventricular muscles (n=8) at the basic cycle length of 400 ms, the recovery of V_{max} during control was best fit to a single exponential relation. The time constant for recovery of V_{max} during control was fast (τ_{fast} = 46.2 ± 4.3 ms) and before final repolarization of the basic action potential, it was almost complete. In the presence of 10 μM ivabradine the recovery kinetics of V_{max} was best fit with a two exponential relation. In addition to a fast component (τ_{fast} = 41.2 ± 8.2 ms) which reflects recovery of the drug-free sodium channels, a slow component (τ_{slow} = 8.76 ± 1.34 s) of recovery of V_{max} was revealed following exposure to ivabradine. This second slow component for recovery of V_{max} may reflect effects on drug-affected sodium channels. In dog ventricular muscle (n=8) at a stimulation cycle length of 1000 ms the drug moderately and in a concentration dependent manner prolonged the action potential duration, while after attenuation of the repolarization reserve by inhibition of the inward rectifier potassium current (n=5) by adding 30 μM $BaCl_2$ it further lengthened the ventricular repolarization at concentrations of 1 and 10 μM. In human ventricular muscle preparations (n=4) at a stimulation cycle length of 1000 ms 1 μM ivabradine did not change ventricular repolarization and a small, but significant prolongation of the action potential repolarization was observed only in the presence of high (10 μM) concentration of ivabradine.

3.3 Effects of compounds EC18 and MEL57A on transmembrane action potentials

We investigated the effects of compounds EC18 and Mel 57A on the action potential parameters of dog papillary muscle preparations and Purkinje fibers. EC18 has similar action on the spontaneous diastolic depolarization to ivabradine (i.e. dose-dependently reduced the amplitude of diastolic depolarization phase) and at 10 μM, decreased the AP plateau in dog Purkinje fibre. In dog ventricular muscle preparations, EC 18 did not influence the repolarization. MEL57A did not affect spontaneous diastolic depolarization in dog Purkinje fibres, but at 1 and 10 μM dose-dependently decreased the AP plateau in dog Purkinje fibre. In dog ventricular muscle preparations, MEL57A did not influence the repolarization.

4. DISCUSSION

Some of the results is confirmation of previous findings in small animals at high concentration i.e. ivabradine (S16257) at 50 μM induces frequency-dependent V_{max} block and at 3 and 10 μM prolongs repolarization in guinea-pig papillary muscles. The main new finding of the present study is that ivabradine in large animal (dog) and human cardiac preparations, in addition to decreasing heart rate, inhibits V_{max} , I_{Kr} , and at a concentration higher than 1 μM moderately prolongs APD i.e. exerts combined Class I, Class III and Class V antiarrhythmic actions.

4.1 Possible ionic mechanisms

We have examined the specific ionic mechanisms by which ivabradine affects action potential configuration. Repolarization lengthening could be related to the I_{Kr} block found in rabbit isolated ventricular myocytes. Dose- and frequency dependent V_{max} block in dog Purkinje-fibres could be attributed to the inhibitory effect of ivabradine on the fast Na^+ current. V_{max} measurements are indicative for I_{Na} function, but can not be used for quantitative estimation of sodium channel availability, which could be underestimated by such measurements, since V_{max} can be regarded as a nonlinear indicator of the fast inward sodium current. Block of sodium current has been demonstrated to be more pronounced when the resting membrane potential is partly depolarized e.g. in ischaemic tissues. The effects of ivabradine on recovery of V_{max} observed in isolated ventricular muscle indicate that ivabradine resembles kinetically slow antiarrhythmic agents. According to reported data at 10 μM concentration ivabradine

does not exhibit a 100% blockade of the I_f current, therefore additional factors such as inhibitory effect on sodium channels can not be excluded as possible contributor. A slowly activating sodium current, I_{Na3}, has been described by patch-clamp analysis in the late diastolic depolarization of dog single Purkinje cells. This current is sensitive to lidocaine and tetrodotoxin, and contributes to attainment of the threshold for the upstroke in the oscillary range of late diastolic depolarization. Another possible explanation might be the functional subunit interspecies and tissue specific difference of hyperpolarization-activated cyclic nucleotide gated (HCN) channels. Inhibition of I_{Kr} by ivabradine at higher concentrations might also influence the spontaneous diastolic depolarization in Purkinje fibres owing to the „K^+ current decay hypothesis". Effect on the plateau slope might be related to the inhibitory effect of ivabradine on the persistent, 'window' Na^+ current. Block of this Na^+ current might also have an additional therapeutic value and would also limit action potential prolongation at slow rate due to the I_{Kr} inhibition of the drug at higher concentration. In this context it has to be mentioned that similar relatively high (2.8 μM) IC_{50} of ivabradine was reported on I_f in rabbit sinus node cells. Therefore, the possible decrease of I_{Na} by the drug may contribute to the inhibition of the pacemaker function.

4.2 Possible clinical implications

As the present study shows, ivabradine, in addition to blocking the pacemaker current, at micromolar concentration ranges also inhibits the rapid component of the delayed rectifier potassium current, and presumably, the sodium channels. Though therapeutic plasma concentrations of ivabradine are about 0.04-0.07 μM, the drug has been tested at higher (0.17 μM) concentration in healthy volunteers, therefore the (0.1-1-10 μM) concentrations applied in our experiments may be relevant, since the tissue concentration can be expected to be higher than that of the plasma, in accordance with the high volume of distribution (close to 100 L) value of the drug. In this context, the relatively low therapeutic plasma concentration might be the result of possibly meaningful tissue appearance of ivabradine, therefore, we can not underestimate the probable I_{Na} and the proven I_{Kr} blocking ability of ivabradine at higher concentrations. Mostly, because CHF (congestive heart failure) -induced impairments (e.g. deterioration in V_{max}) in dog Purkinje tissue conduction have been lately reported. It has to be emphasized that the drug so far has proved to be safe and free from any proarrhythmic events in clinical trials. However, considering the bradycardic action of the drug, possible very rare proarrhythmic episodes can not be completely ruled out in certain, special cases during application of ivabradine, especially in some pathological conditions, e.g. in case of drug accumulation or intoxication, or in case of attenuated repolarization reserve like in heart failure, diabetes or in LQT. On the other hand, the mild sodium channel blocking ability on Purkinje fibres might be considered as an *antiarrhythmic* property and might suppress the initiation of an extrasystole, limit any repolarization lengthening and most importantly, it could decrease dispersion of repolarization. The frequency-dependent V_{max} block might diminish conduction of early extrasystoles or bursts with fast rates which potentially elicit cardiac arrhythmias in the presence of an ischaemic cardiac substrate. Therefore, the most conspicious finding was that this bradycardic drug might possess subsidiary antiarrhythmic actions, which can be utilized in the therapy.

4.3 Electrophysiological effects of compounds EC18 and MEL57A

We also investigated compounds EC18 and MEL57A on dog papillary muscles where they did not influence repolarization and on cardiac Purkinje fibers, a cardiac subsidiary pacemaker exhibiting spontaneous diastolic depolarization, where isoforms HCN4 and HCN2 have been suggested to be the prevalent f-channel isoforms at variance with isoform HCN1 which is no – or poorly – expressed in mammalian heart, including humans. EC18 strongly

decreased the amplitude of Purkinje fibers diastolic depolarization phase. On the contrary, MEL57A did not change the diastolic depolarization phase. This latter observation suggests that HCN1 selective inhibitors may be devoid of – or with less pronounced - cardiac adverse effects while HCN4 blockers may be active also at ventricular level. Given HCN4 overexpression in cardiomyopathies such as in case of heart failure, such a property might be advantageous possessing potential antiarrhythmic effects. Effect of EC18 and MEL57A on Purkinje fibre AP plateau might indicate subsidiary sodium channel or L-type calcium channel inhibition.

5. CONCLUSIONS

Based on the cellular cardiac electrophysiological properties of ivabradine it can be concluded that the drug, in addition to its well established bradycardic effect, also shows Class I and III antiarrhythmic properties which can be advantageous to treat patients with ischaemic heart disease, heart failure liable to disturbances of cardiac rhythm. Concerning –in general- the bradycardic agents the HCN4 isoform selectivity is desirable according to its prominent role in cardiac pacemaking. Reaching the lack of visual side effects (attributable to the retinal I_h block) would be advantageous in future development. As to the arrhythmogenic potency of HCN4 and HCN2 upregulation in the working myocardium of the failing heart, simultaneous block of them seems to be a feasible opportunity. As it was seen in the history of development of beta blocker generations, valuable subsidiary effects (alfa-blocker, antioxidant, very high $beta_1$ selectivity) were discovered at some relatively new representatives of them. In case of I_f blockers, similar phenomen might happen, adding extra beneficial property to future bradycardic compounds. HCN1 isoform selective I_h inhibitors (MEL57A) might be appropriate for the treatment of neuropathic pain associated with nerve injury. The HCN1 isoform also play an important role in the effect of general anaesthetic agents. Regarding the potential adverse effects, and considering that HCN channels are also expressed in liver, testis and pancreas it can be stated that synthetizing a well tolerable, safe agent is very important, and therefore the isoform selective approach might be the basis of future developments.

6. ACKNOWLEDGEMENTS

I am very grateful to my supervisors **Prof. Dr. András Varró** (**Director of the Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Szeged**) and **Dr. László Virág (associate professor)** for their professional mentorship, scientific guidance for my research. I would like to express my gratitude to **Prof. Dr. Julius Gy. Papp** (**academician**) for his continuous scientific and moral support to me. I would like to express my thanks to my **Family**.

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