SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

# The role of late sodium current in the electrophysiological properties of left ventricular myocytes

by Tamás Hézső, MD, DMD

Supervisor: Balázs Horváth, MD, PhD


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| Head of the **Defense Committee**: | György Panyi, PhD, DSc |
| Reviewers: | Péter Bencsik, PhD,<br>G. Tibor Szántó, PhD |
| Members of the Defense Committee: | Zsófia Nagy, PhD<br>Róbert Pórszász, PhD |

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# Introduction

*General consideration*

Voltage-dependent sodium channels ($Na_v$) play a key role in the activation of myocardial cells and the propagation of electrical signals within the myocardium. $Na_v$s in ventricular myocytes generate the upstroke of the action potential (AP). The positive feedback between the membrane potential and the $Na_v$s results in a transition from a closed to an open state of the channels within a few milliseconds, followed by a rapid inactivation. This results in 99% of the $Na_v$s entering an inactivated, nonconducting state 3-5 milliseconds after the AP upstroke is formed. This narrow spike in current is called the early component of the voltage-dependent sodium current ($I_{Na,early}$). Despite this, a small fraction of the channels do not inactivate and maintain a persistent current during the plateau phase of the AP, which we call the late sodium current ($I_{Na,late}$). The exact details of the functioning of the non-inactivated channels are still not known, and several mechanisms have been proposed in the literature to explain this phenomenon.

$I_{Na,late}$ has a low amplitude but it flows during the complete plateau phase, thus influencing the repolarization of cardiac APs and is also involved in the sodium and calcium homeostasis of myocardial cells. A lot of data demonstrates that increased $I_{Na,late}$ has a significant pathophysiological role in several acquired myocardial diseases, such as myocardial ischemia and heart failure (HF). Increased $I_{Na,late}$ prolongs repolarization and increases intracellular sodium ion concentration ($[Na^+]_i$) in myocardial cells, thus causing cardiac arrhythmias, reducing myocardial contractility and impairing myocyte energy balance.

Our current knowledge of $I_{Na,late}$ is mostly based on voltage-clamp data obtained under simplified conditions (using rectangular voltage impulses, under aphysiological ionic condition, using exogenous calcium buffers). The results obtained in this way have been used in model simulations in previous studies to reconstruct the dynamic profile of $I_{Na,late}$ during AP with the aim of understanding its role in AP formation. Although this approach has also contributed valuable insights to the study of $I_{Na,late}$, it is not by itself suitable for investigating the properties of $I_{Na,late}$ under physiological conditions. The main reason is a particular biophysical feature of $Na_v$s, the so-called non-equilibrium gating. Clancy and colleagues discovered a late increase in $I_{Na,late}$ during a repolarizing voltage ramp protocol, which they explained by the rapid return of $Na_v$ from an inactivated state and the so-called „nonequilibrium gating" during the repolarization ramp. The „nonequilibrium gating" may be responsible for a large fraction of $I_{Na,late}$.

*Voltage-gated sodium channels*

The different $I_{Na,late}$ profiles reported in the literature highlight the complexity of the gating of $Na_v$s. As far as we know so far, $I_{Na,early}$ is mainly shaped by Nav1.5. Since dynamic changes in membrane potential during AP significantly affect $I_{Na,late}$, we found it necessary to record the current directly under AP voltage-clamp (APVC) conditions.

$Na_v$ is also known to be regulated by $Ca^{2+}$, calmodulin (CaM) and $Ca^{2+}$/CaM-dependent protein kinase II (CaMKII); each of these molecules individually and collectively modulates $I_{Na,late}$. Previous studies exploring some elements of this complex regulation have reported

conflicting results. Some results have suggested that $Ca^{2+}$ modulates $Na^+$ current by direct binding to the EF-hand motif at the C-terminus, but further studies have provided evidence against this idea. The current consensus is that $Ca^{2+}$-CaM-CaMKII signaling increases cardiac $[Na^+]_i$. However, the simplified conditions used in conventional voltage-clamp experiments make it considerably more difficult to determine the integrated effects of the $Ca^{2+}$-CaM-CaMKII signaling pathway on sodium current during AP. Several previous studies have used a conventional voltage-clamp method with square-wave pulse protocols, but non-equilibrium gating of $Na_v$ was not investigated in these experiments.

Several of the antiarrhythmic drugs that act on $Na_v$, classified as class I in the Vaughan-Williams classification, also inhibit $I_{Na,late}$ in addition to $I_{Na,early}$. The latter effect was long thought to increase the risk of arrhythmias and consequently the incidence of sudden cardiac death. For this reason, the search for an agent to selectively inhibit $I_{Na,late}$ seemed to be a good development direction in recent decades. One such agent, known as GS-458967, 6-(4-(trifluoromethoxy)phenyl)-3-(trifluoromethyl)-[l,2,4]triazolo[4,3-a]pyridine (hereafter GS967), has been shown to be a particularly selective candidate when tested in rabbit ventricular cells.

It has also been shown that there is significant interspecies variation in the electrophysiological properties of individual myocardial preparations. Both the data presented in the present work and those previously reported suggest that canine ventricular myocytes are a relatively good model for human ventricular cells with respect to certain ionic currents.

The behavior of $Na^+$ current is not monotonic in time. Once the membrane potential reaches the threshold level for the voltage-gated $Na^+$ channels, a significant $Na^+$ influx depolarizes the membrane and creates the upstroke of the AP. However, this fast, early peak $Na^+$ current is rapidly inactivated causing the fast decay of the $I_{Na,early}$. Under certain conditions, $Na^+$ channels might recover from inactivation and reopen during the plateau phase of the AP, bringing a further depolarizing $Na^+$ influx, termed as the late $Na^+$ current ($I_{Na,late}$). As $I_{Na,early}$ increases the intracellular $Na^+$ concentration $[Na^+]^i$ at the upstroke of the AP, the $Na^+/Ca^{2+}$ exchange (NCX) switches to its reverse mode and removes $Na^+$ from the cell at the cost of intracellular $Ca^{2+}$ load. This reverse mode persists for only a very short period of time and NCX works in its forward mode at the rest of the AP, underlying the vast majority of sarcolemmal $Ca^{2+}$ extrusion. $I_{Na,late}$ is a minute, but persistent inward current which is much smaller in amplitude than $I_{Na,early}$ in healthy myocytes. However, under certain pathophysiological conditions, $I_{Na,late}$ can become much larger and might cause $Na^+$ and $Ca^{2+}$ overload leading to arrhythmogenesis.

*Structure of the voltage-gated sodium channels*

Voltage-gated $Na^+$ channels consist of a large pore-forming pseudotetrameric α subunit, accessory β subunits and scaffolding proteins. To date there are 9 different α subunits ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.4$, $Na_v1.5$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$, $Na_v1.9$) encoded by 9 different genes (SCN1A, SCN2A, SCN3A, SCN4A, SCN5A, SCN8A, SCN9A, SCN10A, SCN11A). As you can read, SCN6A and SCN7A are missing from the list. This is because the name SCN6A is no longer used, as this gene was previously found to be orthologous to the mouse SCN7A gene. And the SCN7A gene is called $Na_x$ in the literature because it is not voltage-dependent. It is an

atypical, non-inactivatable sodium channel whose expression in sensory circumventricular organs is essential for maintaining the body's fluid balance. $Na_x$ has also been detected in homeostatic effector neurons, such as vasopressin-secreting magnocellular neurosecretory cells, which secrete vasopressin into the bloodstream in response to hypertension and hyperthermia and play an important role in osmoregulation.

The $Na_v1.5$ is considered to be the dominant cardiac subtype. $Na_v1.5$ is relatively insensitive to the $Na^+$ channel blocker neurotoxin, tetrodotoxin (TTX). However, other TTX sensitive subtypes – such as $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.4$ and $Na_v1.6$ – are also reported to be expressed in the heart. An α subunit encoded by a specific gene determines not only the channel subtype itself, but also the receptor population of the particular channel. The 6 possible β subunits (β1, β1A, β1B, β2, β3, β4) are encoded by four genes (SCN1B, SCN2B, SCN3B, SCN4B). The α subunit alone is sufficient to form a functional channel; however, the auxiliary β subunits are required for regular channel kinetics and cell surface expression. In fact, as the β subunits modulate the number of the available channels on the cell surface, they play a role in regulating peak current density. Furthermore, β subunits control activation, inactivation, and recovery from inactivation by altering their voltage range.

Voltage-gated $Na^+$ channel α subunits consist of approximately 2000 amino acid residues, creating four domains (DI-DII-DIII-DIV). Each domain is formed by six transmembrane segments (S1-S6). Segments 1–4 (S1-S4) function as the voltage sensor domain of the channel. This domain senses membrane depolarization leading to channel activation. The S1-S4 connects to the channel's pore-forming domain S5-S6 via an intracellular linker. This structure encompasses the central aqueous pore domain. The selectivity filter is also located in the pore domain, recognizing the charge and radius of the ion. This is the narrowest part of the open pore, lined by protein ligands that interact specifically with the passing ion. It plays a key role in the regulation of channel selectivity, as altering its structure results in a significant or complete loss of selectivity. The structure, composition and pore size of several sodium channel selectivity filter oligomers have been revealed by crystallographic, mutagenesis and channel-blocking binding assays. The channel selectivity filter is formed by the pore loops between the S5 and S6 segments of the four domains together, with all domains contributing amino acid side chains to the DEKA sequence filter. The rest of the pore is surrounded by the S6 segments, which form the activation gate on the intracellular side of the pore.

At membrane potentials negative to the threshold of the $Na^+$ channel the channel's open probability is low. Upon depolarization, however, the α subunit undergoes a conformational change, the voltage sensor activates, the activation gate – and therefore the $Na^+$ channel – quickly opens, thereby conducting $Na^+$ current and resulting in the upstroke of the cardiac AP. A few milliseconds later the channel inactivates quickly as the inactivation gate closes into the channel's pore domain yielding a nonconducting state. The homologous domains are connected by intracellular interdomain loops (IDI/II, IDII/III and IDIII/IV). Inactivation gate incorporates the smallest interdomain cytoplasmic loop (IDIII/IV) and functions as a lid that locks the pore during inactivation. It closes the pore upon inactivation, as described in previous research. This rapid inactivation is brought about by the IFM motif, consisting of three amino acids, binding to the intracellular side of the pore and blocking the ionic flow pathway.

Normal inactivation is needed to prevent excess depolarization and to ensure timely repolarization. It has also been proposed that the inactivation gate is formed and stabilized as a molecular complex, formed by the IDIII/IV and the C-terminal loop of the α subunit. After depolarization and inactivation, during the repolarization phase, $Na^+$ channels recover from inactivation ready to be activated again.

*Electrophysiological Identification of $I_{Na,late}$*

Mammalian cardiac cells express a wide variety of $Na_v$ isoforms, differing in unit conductance, voltage sensitivity, kinetics, and drug sensitivity. In the majority of cardiac tissues, the dominant isoform of the pore-forming subunit is $Na_v1.5$, which is relatively insensitive to the sodium channel toxin tetrodotoxin (TTX). Many of the TTX-sensitive („non-cardiac") $Na_v$ channels ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.4$, and $Na_v1.6$) are also shown to be present in cardiac tissue. In nodal tissue $Na_v1.1$ and $Na_v1.5$ are expressed in the largest quantities. $Na_v1.5$ is mostly located in larger cells and their periphery, whereas $Na_v1.1$ is evenly distributed within the cell and is also found in smaller cells. Besides the pore-forming subunit, four auxiliary subunits (ß1, ß2, ß3, and ß4) and certain scaffolding proteins also participate in building up the whole complex, which also attaches to the cytoskeleton. These molecules can interact with each other and may modify the kinetics and voltage dependence of the channel.

The mechanisms discussed below may contribute to the profile of $I_{Na,late}$ during the AP. A better understanding of these mechanisms may be useful for the development of new antiarrhythmic therapeutic strategies targeting the $I_{Na,late}$.

*Properties of late sodium current*

$I_{Na,late}$ is normally a small but persistent current. It is active during the plateau phase of the cardiac AP; therefore, the current can play a significant role in determining the duration and the shape of the AP. A possible explanation of this discrepancy (i.e., a tiny current causing large effects on the AP) is given by the role of net membrane current. During the plateau phase of the AP the impedance of the cell membrane is high and according to Ohm's law, at this stage, small changes in net membrane current lead to relatively large changes in the membrane potential, and consequently, in AP duration (APD).

Three different gating modes of $Na_v1.5$ have been described in ventricular cells. The transient mode is the main gating mode for the $I_{Na,early}$. Burst mode and late scattered mode are responsible for $I_{Na,late}$; however, burst mode openings decline quickly, leaving the late scattered mode to be the main gating for $I_{Na,late}$ during the plateau phase. Furthermore, several inactivation processes have been proposed, each governing APD, $Na^+$ channel steady-state inactivation and $Na^+$ flux balance of the cell. Fast inactivation takes place only in the first milliseconds and the channel recovers rapidly at negative membrane potentials. This is followed by the intermediate inactivation which recovers slowly compared to the fast inactivation. Slow inactivation from the open state occurs over hundreds of milliseconds and finally, ultraslow inactivation can take seconds.

The window $Na^+$ current is a well-known phenomenon characterizing $Na^+$ channels. Due to an overlap between steady-state activation and inactivation curves („window of potentials"),

a fraction of $Na^+$ channels can recover from inactivation and might reopen. However, considering the voltage range of this window current (approximately – 70 mV), it is far below the physiological plateau potential of the AP, so it is unlikely to play a major role in the $I_{Na,late}$.

There are marked interspecies differences in the profiles of the $I_{Na,late}$. One practical difference is the shape and duration of the AP, resulting in distinct $I_{Na,late}$ profiles. Previously our team reported that the density of the $I_{Na,late}$ is comparable to the major repolarizing $K^+$ currents in guinea pig and rabbit myocytes. Also in rabbits, the atrial density of $I_{Na,late}$ was greater than measured in the ventricles.

$I_{Na,late}$ shows reverse rate-dependent properties, that is, the higher the pacing frequency (i.e. heart rate), the lower the density of the current. However, the early and late components behave differently. The higher $[Na^+]_i$, observed at high frequency, is mainly determined by the early component as $I_{Na,late}$ recovers slowly from inactivation at rapid pacing. Additionally, at higher frequencies, APD is usually shortened, allowing less time for activation of $I_{Na,late}$. Therefore the shorter the APD the smaller the $I_{Na,late}$ and $Na^+$ influx. In contrast, during bradycardia APD is longer and shows greater beat-to-beat variability. Therefore, bradycardia, associated with enhanced $I_{Na,late}$, may strongly be proarrhythmic. As there are marked interspecies differences in heart rate and APD, species having long APs (e.g. human, canine, guinea pig) are expected to manifest larger $I_{Na,late}$ and $Na^+$ influx than species with fundamentally shorter APs (e.g. rat, mouse).

### *$I_{Na,late}$ Is Underlain by Different Channel Gating Modes*

At the resting membrane potential, the vast majority of $Na_v1.5$ channels are in their closed state. Upon depolarization, $Na_v1.5$ channels open up within 1–2 ms after which they inactivate rapidly. This produces $I_{Na,early}$ and the upstroke of the non-pacemaker cardiac AP. During a sustained depolarization, $Na_v1.5$ channels can reopen with a small probability. In ventricular myocytes, three modes of $Na_v1.5$ channel activity have been characterized in single-channel experiments: transient mode (TM), burst mode (BM), and late scattered mode (LSM).

$I_{Na,early}$ is mainly the result of TM activity, while BM and LSM are responsible for the sustained sodium current ($I_{Na,late}$). The magnitude of the sustained current component is only about 0.5–1 % of $I_{Na,early}$ measured 50 ms after the onset of the depolarizing pulse . During a sustained depolarization BM openings rapidly decline in the first tens of milliseconds therefore leaving LSM as the gating mode being mainly responsible for $I_{Na,late}$ toward the end of the plateau phase.

Mutations of the channel protein and certain diseases can change the contribution of different $Na_v1.5$ channel activity patterns to the macroscopic current, therefore increasing $I_{Na,late}$. Apparently, each gating mode has a distinct drug sensitivity or drug affinity as well.

Based on this, selective pharmacological targeting of certain gating modes might have potential antiarrhythmic and/or cardioprotective effects. Characterising the real gating mechanism of $I_{Na,late}$ may also bring us closer to developing a more effective therapeutic protocol.

*Window Sodium Current*

The voltage dependence of the steady state activation and inactivation of most $Na_v$ channels overlaps with each other. This overlap provides a voltage range („window") where inactivated $Na_v$ channels are able to recover from inactivation and then might reopen. When the actual membrane potential falls within this „window" of overlap, a sustained current is evoked. Under physiological circumstances this „window current" mechanism likely plays a limited role in $I_{Na,late}$, because the $Na_v1.5$ voltage „window" is around −70 mV, falling quite far from the AP plateau. Additionally, in the window voltage range, the current density is less than 5 % of the maximum current density in healthy myocytes. Hence, the „window current" mechanism is unlikely to be a major determinant of $I_{Na,late}$ in healthy myocytes. Mutations of channel proteins or altered regulation in certain diseases may shift either the steady-state activation or inactivation curves of $Na_v$ channels to significantly change this voltage window, therefore increasing $I_{Na,late}$ under these pathological conditions. Three different mutations have been identified in long QT syndrome type 3 (LQTS). These include deletions of three amino acids (Lys-1505, Pro-1506 and Glu-1507, ΔKPQ) and two point mutations, N1325S (Asp-1325 converts to Serine) and R1644H (Arg-1644 converts to Histidine).

*Non-Equilibrium Channel Gating*

During the AP of cardiac myocytes, the membrane potential changes continuously. $Na_v$ channels are incorporated into this dynamic system. It has been proposed that the voltage „history" of the cell membrane can modulate the transition between $Na_v$ channel states, termed „non-equilibrium gating". As a result, recovery from inactivation is also modulated by the dynamics of voltage change. The theory is supported by experimental data showing that the application of repolarizing voltage ramps or AP shape voltage commands evoke a larger $I_{Na,late}$ compared to conventional square pulses or model simulations where „non-equilibrium gating" is not incorporated into the numerical model.

*Non-Cardiac Sodium Channel Isoforms in the Heart*

Epilepsy and certain skeletal muscle diseases has been associated with pathological ECG recordings. Therefore it seemed possible that non-cardiac sodium channel mutations might cause electrical alterations in the heart. Later, $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.4$, $Na_v1.6$, and $Na_v1.8$ isoforms have been identified in cardiac tissue. Based on the findings of Biet et al., as much as 44 % of $I_{Na,late}$ is due to non-cardiac sodium channels in canine ventricular cardiomyocytes. Furthermore, Yang et al. have shown that in mice and rabbit the TTX-resistant $Na_v1.8$ provides a substantial amount of $I_{Na,late}$. Based on these experimental data, isoform specific sodium channel modulators might provide a valid approach in pharmacological antiarrhythmic therapy.

*Role of $I_{Na,late}$ in Cardiac Electrical Activity*

However, the plateau phase of the cardiac AP is shaped by a delicate balance between minuscule inward and outward current fluxes. Therefore even a small change in these currents

may significantly alter the duration of the AP. Inhibition of $I_{Na,late}$ substantially shortens the cardiac AP in the conductive sys and in ventricular cells as well, indicating that $I_{Na,late}$ significantly contributes to determining the duration of the non-pacemaker AP in cardiac myocytes. Recent AP voltage clamp experiments show that the density of $I_{Na,late}$ is of similar magnitude as the major potassium currents in guinea pig and rabbit ventricular myocytes. There is a characteristic interspecies difference in the shape of $I_{Na,late}$ as shown in the case of guinea pig, canine, and human ventricular myocytes.

The sustained sodium current is also an important factor in determining electrophysiological properties of sinoatrial node cells. Tetrodotoxin, applied in lower than 1 μM concentrations, reduces the rate of spontaneous depolarization in sinoatrial node cells, clearly indicating that non-cardiac $Na_v$ isoforms also contribute to cardiac automaticity. However, we have not yet found a percentage distribution in the literature.

Cardiac Purkinje cells have the largest rate-dependence of their AP duration (APD) among cardiomyocytes with fast response APs. Purkinje cell APs are longer at lower stimulation rates, while shorter at higher rates than APs of ventricular cells. It has been shown that $I_{Na,late}$ contributes to this feature by possessing much slower decay and recovery kinetics in Purkinje cells than in ventricular cells. As a result Purkinje cell $I_{Na,late}$ is significantly larger at low heart rates, while smaller at high heart rates compared to ventricular cells. This unique feature predisposes Purkinje cells to serve as triggers in generating arrhythmias.

$I_{Na,late}$ plays a role in forming the atrial AP as well. $I_{Na,late}$ is expected to be larger in atria than in ventricles because $I_{Na,early}$ density is greater in atrial cells under similar conditions, suggesting a higher sodium channel expression in atrial cells. On the other hand, an overall more positive membrane potential, and a more negative steady-state inactivation voltage of the sodium current in the atrial cells reduce the availability of the sodium channels. Maximum $I_{Na,late}$ density has been reported to be greater in rabbit left atrial myocytes than in ventricular cells in one set of experiments by Luo et al. The two cell types seemed to be similar in this matter in a different investigation, so there is no clear explanation on this issue.

### *$I_{Na,late}$ Plays a Significant Role in the Sodium Homeostasis of Cardiomyocytes*

$[Na^+]_i$ is set by a dynamic equilibrium of the influx of $Na^+$ into the cell and efflux of $Na^+$ to the interstitial space. The $[Na^+]_i$ of non-paced ventricular myocytes is around 4–8 mM in guinea-pig, rabbit, and canine; and about twice as high in rat and mouse (9–14 mM). In non-paced human myocytes $[Na^+]_i$ is thought to be in the 4–10 mM range.

$Na^+$ can enter into the cell through $Na^+$ channels, $Na^+/Ca^{2+}$ exchanger (NCX) and $Na^+/H+$ exchanger (NHE). $Na^+$ leaves the cell mainly via the $Na^+/K^+$ pump (NKP), but the reverse mode NCX is also responsible for a moderate $Na^+$ efflux during the first few milliseconds of the cardiac AP. Furthermore, $Na^+/HCO3-$ cotransport, $Na^+/Mg^{2+}$ exchange, and $Na^+/K^+/2Cl^-$ cotransport can play a role in the sodium homeostasis of cardiomyocytes to a small extent. It also has to be mentioned that $Na^+$ concentrations between the cytosol and intracellular organelles are continuously balanced.

Upon pacing, $[Na^+]_i$ increases with increasing stimulation frequency, caused by the larger $Na^+$ entry through $Na^+$ channels and NCX. In paced, single cardiac cells approximately 25 % of the $Na^+$ entry is mediated by $Na_v$ channels. The $Na^+$ entry through $Na_v$ channels is about

equally distributed between $I_{Na,early}$ and $I_{Na,late}$, however this contribution can change at different heart rates. The higher $Na^+$ influx into paced cells is matched by an increased efflux through an elevated NKP activity. This is mainly caused by the increased $[Na^+]_i$ itself, but nitric oxide-, and phospholemman-dependent mechanisms can also add to this effect.

*Late sodium current in disease*

In normal, healthy myocytes, the amplitude of $I_{Na,late}$ is much smaller, less than 0.1% of the peak $I_{Na,early}$. However, as stated before, the current is persistent, lasting for 100–400 ms; therefore, the inward charge carried by $I_{Na,late}$ is comparable to $I_{Na,early}$ mediated within 1–2 ms. Some papers in the literature refer to this as endogenous $I_{Na,late}$ and is thought to be without any arrhythmic properties.

On the other hand, the density of $I_{Na,late}$ can be increased under many pathophysiological conditions, such as heart failure (HF), hypertrophic cardiomyopathy, inherited long QT syndrome 3 (LQTS-3), oxidative stress, or atrial fibrillation (AF) with intracellular $Ca^{2+}$ handling abnormalities. Moreover, even a low heart rate or pharmacological interventions can elevate $I_{Na,late}$. $I_{Na,late}$ is also augmented in myocardial ischemia/reperfusion injury and in the presence of characteristic components of ischemia (e.g. hypoxia, ischemic metabolites, hydrogen peroxide) as documented in voltage clamp experiments.

The AP lengthening effect of the augmented $I_{Na,late}$ can also be observed in HF. The increased $I_{Na,late}$ results in a $Na^+$ overload, which, in turn, leads to elevation of intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$. The concomitant abnormal conduction can cause sudden death in HF patients. Conduction velocity is determined also by the $Na^+$ channel function. In the ventricular conductive system (Purkinje fibers) – in contrast to ventricular myocardium – slow pacing generates a higher, while fast pacing results in a significantly lower $I_{Na,late}$. This transmural inhomogeneity may be a trigger for cardiac arrhythmias. All these mechanisms may lead to complex pathological electrical and mechanical performance, such as contractile dysfunction, disturbed myocardial energetics and arrhythmias. Increased $I_{Na,late}$ is most arrhythmogenic in those cases, where the repolarization reserve is already compromised, such as during treatment with $I_{Kr}$ inhibitors, or in the remodeled myocardium.

*The Role of Sodium Homeostasis and Elevated $I_{Na,late}$ in Cardiac Arrhythmias*

The pathophysiology of cardiac arrhythmias is based on the classical concept of „arrhythmic triad”; combination of a proarrhythmic substrate, a trigger, and the modulating effect of the autonomic nervous system. The exact combination depends on etiology, cardiac-, and extracardiac comorbidities. Abnormal $[Na^+]_i$ homeostasis can play a role in creating an arrhythmia-prone substrate as well as in generating a trigger for the rhythm disorder. Compared to non-failing myocytes, $[Na^+]_i$ is about 2–6 mM larger in myocytes from failing hearts. In a pressure- and volume-overload rabbit HF model, Despa et al. have found an increased TTX-sensitive $Na^+$ influx. Interestingly, this larger influx was present not only in electrically stimulated myocytes, but in non-paced cells as well. In paced cells the most plausible candidate of this increased TTX-sensitive $Na^+$ influx is $I_{Na,late}$. However, the underlying mechanism of this influx is not yet understood completely in resting myocytes.

*Clinical studies – Antiarrhythmic drug development*

It became clear that elevation of $I_{Na,late}$, $[Na^+]_i$, and $[Ca^{2+}]_i$ is arrhythmogenic; therefore, an effective antiarrhythmic treatment is necessary under these conditions. An obvious objective is the inhibition of voltage-gated $Na^+$ channels. However, the disappointing results of the CAST and SWORD studies clarified that blocking a single specific ion channel alone can lead to unexpected adverse effects. Recently, a need for selective inhibitors that are able to distinguish between $I_{Na,early}$ and $I_{Na,late}$ is rather emerging. The selectivity here is critically important since blocking the early component of the current can lead to a decrease in conduction velocity and might lead to conduction block and reentrant arrhythmias.

A number of inhibitors have been developed to date including ranolazine, eleclazine (GS-6615), lidocaine, GS-458967, GS-462808, and F15845, however, mainly ranolazine was used for excessive experimental and clinical studies. The main issue with most of these inhibitors is that they function in a voltage-dependent manner and exert their $I_{Na,late}$ selective effects mainly at lower than physiological membrane potentials. Coming closer to the physiologically relevant membrane voltage range, these inhibitors tend to block $I_{Na,early}$ more and more, thus losing their selectivity. Secondly, their blocking effect is rate-dependent, in other words, the inhibition of $Na^+$ current increases with the pacing frequency, exerting thus a lesser impact on $I_{Na,late}$ in bradycardia, when $I_{Na,late}$ is thought to be significantly greater .

# Aims

In spite of its relative importance, many aspects of $I_{Na,late}$ are still poorly understood. In contrast to the detailed data obtained in rabbit, guinea pig and porcine myocytes, we have only a limited number of recordings of native human and canine $I_{Na,late}$, since these experiments were typically performed using conventional voltage clamp arrangements and many of them at room temperature. Self action potential voltage clamp measurements, delivering the cell's own AP as a command signal, are not available in the literature either for canine or human ventricular cardiomyocytes.

1. Since canine ventricular cells are believed to be a good model for human ventricular myocytes in general regarding their cellular electrophysiological properties, our goal was to monitor and compare the profiles of $I_{Na,late}$ in ventricular cells obtained from canine, guinea pig and undiseased human hearts. In doing so, we wanted to take a step towards the goal of experimental cardiology to find an animal model that could replace the very limited human ventricular tissue samples available.
2. In these experiments we used the APVC technique to directly record the $I_{Na,late}$ profile during AP under normal cellular $Ca^{2+}$ homeostasis; during $Ca^{2+}$ overload; during reduction of intracellular calcium concentration ($[Ca^{2+}]_i$) by chelation and after CaMKII inhibition. Thus, we wanted to better understand the role of current in shaping the electrophysiological properties of left ventricular myocytes.
3. We also wanted to investigate how Anemone toxin II (ATX-II), which prevents the inactivation of $Na_v$s, alters the profile of $I_{Na,late}$ during AP.
4. We were interested in the role of $I_{Na,late}$ in the development of cardiac arrhythmias.
5. We wanted to investigate whether inhibition of $I_{Na,late}$ could be achieved completely independently of $I_{Na,early}$.
6. We sought to answer the question whether there is a sharp difference between frequency-dependent $I_{Na,late}$ inhibition described for $I_{Na,late}$ inhibitors that are said to be selective and that described for „general" class I/B $Na_v$ inhibitors.

# Materials and methods

*Preparations*

For our measurements we used hearts from experimental mixed-breed dogs and guinea pigs, and human hearts removed for transplantation. All interventions with experimental animals were performed in accordance with the European Parliament's guidelines on animal experiments (2010/63/EU) and the protocol approved by the University of Debrecen's Working Animal Care Committee (9/2015/DEMÁB). Human tissues were used in accordance with the Declaration of Helsinki and the protocols approved by the University of Szeged and the Scientific and Research Ethics Committee (4991-0/2010-1018EKU, 339/PI/010).

Experimentally bred, adult mixed-breed dogs (8-12 kg body weight) were anaesthetized with 10 mg/kg bw ketamine hydrochloride (Calypsol) and 1 mg/kg bw xylazine hydrochloride (Sedaxylan). Male Hartley guinea pigs, 3-5 months old, were anaesthetised with pentobarbital (100 mg/kg bw i.p.) after anticoagulation (heparin 800 IU i.p.). The depth of anaesthesia was monitored by bilateral eyelid closure reflex, pain sensation (painful pinching of toes) and jaw closure tone every two minutes. The surgical intervention was started after the absence of the bilateral eyelid closure reflex, the absence of limb protraction or respiratory response after the pain stimulation, and after dogs had had a marked decrease in jaw tone for at least 6 minutes (according to three consecutive examinations).

*Isolation of myocardial cells*

After opening the chest of the dogs, the heart was quickly removed and the left anterior descending coronary artery (LAD) was cannulated and perfused through it using the Langendorff apparatus. The so-called anterograde segment perfusion technique was also used for human cell isolation.

At the start of perfusion, a Joklik's modified solution (JMM, Minimum Essential Medium Eagle; Joklik's modification, product number M0518; Sigma) was used for 5 min to remove $Ca^{2+}$ and blood from the tissue, free of $Ca^{2+}$ (2.5 g/l), pyruvate (175 mg/l), ribose (750 mg/l), allopurinol (13.5 mg/l) and $NaH_2PO_4$ (200 mg/l).

The preparation was then perfused again with the JMM stock solution for approximately 30 minutes. This solution was also supplemented with collagenase (660 mg/l, 325 U/mg, Type II), calf albumin (2 g/l, Fraction V) and $CaCl_2$ (50 μM). During cell isolation, the temperature of the perfusion solution was maintained at 37 °C and the solutions were bubbled with carbogen throughout. After enzymatic digestion, a semicircular incision was made in the middle layer of the ventricular wall towards the cardiac apex, corresponding to the LAD supply area. The liquefied muscle tissue layer was cut into small pieces and these pieces were suspended in a modified JMM solution containing 50 μM calcium. This cell suspension was repeatedly sedimented, filtered and washed with a modified JMM solution of increasing calcium concentration. After isolation, about 30-60% of the cells in suspension were nearly rectangular in cross-section, with sharp edges, intact cytoplasm and regular ridge striations in solution containing 1.3 mM ionized calcium. These properties were monitored during the measurements to select the right cell, as they largely determine cell viability.

Measurements were started two to three hours after cell isolation was completed. Until use, cells were stored at 14-16 °C in Minimum Essential Medium Eagle solution (pH=7.4).

Guinea pig hearts were fixed by the aorta on a Langendorff apparatus and washed with Tyrode's solution (NaCl 135 mM, KCl 5.4 mM, CaCl2 1 mM, MgCl2 1 mM, NaH2PO4 0.3 mM, HEPES 10 mM, glucose 10 mM; pH=7.2 [NaOH]) for 3-5 min. The heart was then perfused with a calcium-free Tyrode's solution supplemented with 0.05 mM EGTA for 3 min. Finally, perfusion with calcium-free Tyrode's solution containing collagenase (600 mg/l, 298 U/mg, Type II) and protease (50 mg/l, Type XIV) for 25-30 min was followed by enzymatic digestion. At the end of this procedure, the left ventricle was cut into small pieces and incubated in the last described solution containing collagenase and protease for 40, 60 and 80 min at 37 °C to obtain isolated left ventricular myocytes. At the end of the incubation period, the cell suspensions were washed and placed in modified Tyrode's solution (121 mM NaCl, 4 mM KCl, 1.3 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 25 mM NaHCO3, 10 mM glucose; pH=7.35; hereafter BTy) containing bicarbonate. The osmolality of the BTy solution was 300±3 mOsm. The cell suspension was also stored at 14-16 °C until use.

*Electrophysiology*

At the start of each experiment, myocardial cells (or multicellular preparations) were placed in a suitable 0.5 ml volume Plexiglas measuring tube mounted in the stage of an inverted microscope (Nikon Eclipse TE2000-U or Diaphot 300). The microscope was used in combination with a vibration-free stage and a Faraday cage. The measuring bath was perfused with BTy solution at a continuous rate of 1-2 ml/min using a gravity-controlled perfusion system during measurements. Solutions with different compositions are mentioned separately in each chapter. The temperature of the solutions in the measuring bath was kept constant at 37 °C using a temperature controller (Cell MicroControls). The osmolality of this solution was 300 ± 3 mOsm. This was checked with a vapour pressure osmometer (Vapro 5520). The pH of the solutions was adjusted to the desired value by the addition of appropriate amounts of HCl and NaOH (for extracellular solutions) or KOH (for intracellular solutions) using an Orion 3 Star pH meter. For BTy the pH was set to 7.35, while for intracellular solutions the pH was set to 7.3 and 7.2.

For electrophysiological measurements, Multiclamp 700A and 700B amplifiers were mostly used. In other cases, the measuring instrument is mentioned separately in the text. For computer control and data acquisition we used pClamp 10.0 software. The connection between the amplifier and the computer software was established by a Digidata 1440A or 1332 analog-to-digital transducer.

*Patch-clamp technique*

For the measurements, borosilicate microelectrodes (patch pipettes) with a resistance of 2-3 MΩ were used. Both the patch pipettes and the pointed microelectrodes were prepared using a P-2000 type pipette puller. In terms of their operating principle, products using platinum or

nickel-chromium filaments or $CO_2$ lasers are available on the market. The P-2000 type is laser-operated and can therefore also machine borosilicate and quartz glass. It is one of the smallest tip producers on the market, which is why we chose it.

The internal solution in the commonly used pipette contained the following substances: 120 mM K-aspartate, 30 mM KCl, 3 mM MgATP, 10 mM HEPES, 3 mM $Na_2$-phosphocreatine, 0.01 mM EGTA, 0.002 mM cAMP, 10 mM KOH; pH=7.3. In experiments where a modification of the original solution was used, this is indicated separately in the text. In most experiments no or only minimal amounts of calcium chelator were used. We wanted to get as close to physiological conditions as possible and needed to ensure continuous cell contraction. We therefore used both ATP and phosphocreatine in the intracellular solution. We wanted to mimic cell function in vivo as closely as possible. ATP has a significant effect on the amplitude and kinetics of voltage-dependent currents, so we wanted to standardise this. In addition, the ATP-dependent $K^+$ current can also be affected by ATP.

A slight suction was applied with the tip of the pipette touching the cell surface, allowing a high-resistance contact (2-8 GΩ; so-called "gigaseal") to be formed. After the gigaseal was formed, additional suction or 1.5 V electrical pulses of 1-5 ms duration were used to break the membrane fragment under the pipette tip to achieve the whole-cell arrangement. The capacitance of the cell membrane surface was measured before each experiment by hyperpolarization from -10 mV to -20 mV for 10 ms and the ionic currents were related to the resulting cell capacitance. The total series resistance of the measurement set-up averaged 3-8 MΩ, which was compensated 50-80 % as needed.

For buffering $[Ca^{2+}]_i$ to approximately 100 nM (value calculated using MaxChelator), a pipette solution containing 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) $Ca^{2+}$ chelator was used: 85 mM K-aspartate, 30 mM KCl, 25 mM KOH, 3 mM Mg-ATP, 10 mM HEPES, 3 mM Na2-phosphocreatine, 0.002 mM cAMP, 10 mM BAPTA, 2.48 mM CaCl2; pH=7.2.

*Action potential voltage clamp*

In the whole-cell setup of the patch-clamp technique, cells were continuously stimulated in current-clamp mode by applying rectangular pulses of 2 ms width. The amplitude of the current pulses was set to 150 % of the threshold voltage to ensure that the excitation signal and the ascending stalk of the AP were well separated. The APs were digitized at a sampling frequency of 100 kHz and stored for later evaluation.

For experiments investigating the frequency dependence of AP shape, the excitation cycle length (CL) was first set to 5 s and then decreased to a final value of 0.5 s. Before performing the actual measurements, we waited at least 100 cycles to reach equilibrium. The frequency dependence was first recorded under control conditions (BTy solution) and then repeated after treatment with 10, 30 and 100 nM ATX-II.

*Action Potential Voltage Clamp*

We measured the patch-clamp technique in the whole cell configuration, using two versions of the voltage-clamp special method, called "action potential voltage-clamp" (APVC),

on guinea pig, dog and human cardiac myocytes. One uses the cell's own AP as a voltage command (so-called "self APVC" - sAPVC), while the other technique uses a previously recorded "canonical" midmyocardial AP as a command signal (so-called "canonical" APVC - cAPVC).

Action potentials were recorded in current-clamp mode using a patch pipette in BTy solution. Cells were continuously stimulated with a 700 ms CL (frequency 1.43 Hz; equivalent to a heart rate of 85 beats/min), 1 ms wide, suprathreshold, rectangular pulse so that the artificial product of the stimulus could be well separated from the ascending stalk of the action potential. In the case of sAPVC, APs were sampled from each cell for 5-10 min at a sampling frequency of 100 kHz, and then the AP most representative of the cell was selected. This signal was used as voltage command on the same cell and with the same excitation frequency, now in voltage-clamp mode. The resulting "background" current signal ($I_{background}$) was a horizontal curve running along the zero line, except for the initial few ms capacitive transient. In the cAPVC experiments, a previously recorded "canonical" midmyocardial AP was used as the command signal. In these experiments, the closer the background current fell to the zero line, the more similar the cell's own AP was to the canonical AP. The major advantage of cAPVC over sAPVC is that it eliminates the differences in current signals that arise in sAPVC from the unique voltage command applied only to that cell. At the same time, with sAPVC, we can study the current signals generated on the cells by the most physiological voltage command.

For both APVC techniques, the current signals were recorded using the so-called "sequential ion current isolation" method. The idea is to determine the ion currents from the same cell by applying specific inhibitors in the corresponding sequence. During continuous application of the voltage command, after recording the background current (Iháttér), the specific inhibitor of the first current to be tested is added to the perfusion solution, which excises the corresponding current from the background current ($I_{drug}$). From this, the profile of the first current flowing under the original AP ($I_1 = I_{background} - I_{drug1}$) can be determined. Then, using selective inhibitors of the subsequent ionic currents, the currents under the original AP can be determined (The n-th ionic current: $I_n = I_{drug(n-1)} - I_{drug(n)}$). Cells were perfused with each agent for at least 5 min to allow the effects to fully develop.

Our studies are a very good approximation of the physiological conditions because of three important factors. We use the cell's own AP, or a very similar "canonical" AP, as the voltage command. We used physiological or very similar fluid compositions in the intra- and extracellular solutions. Furthermore, we did not interfere with the cell's own calcium homeostasis by exogenous $Ca^{2+}$ buffers.

*Traditional voltage-clamp experiments*

We have also performed conventional voltage-clamp experiments on canine, guinea-pig and human isolated cardiac myocytes, using square pulses as command signals, at constant test potentials to determine the current density and inactivation kinetics of $I_{Na,late}$.

In this case, Tyrode's solution containing HEPES was used as an external solution (in mM), which contained (NaCl 144, $NaH_2PO_4$ 0.4, KCl 4.0, $CaCl_2$ 1.8, $MgSO_4$ 0.53, glucose 5.5 and HEPES 5.0; pH=7.4). This stock solution was supplemented with 1 μM nizol dipine, 0.5 μM HMR-1556 and 0.1 μM dofetilide to inhibit $Ca^{2+}$ and $K^+$ currents during the experiments. The

composition of the pipette solution was (in mM) CsCl 125, TEACl 20, MgATP 5, EGTA 10, HEPES 10, pH=7.2).

$I_{Na,late}$ was determined by depolarization from a holding voltage of -120 mV to -20 mV for 2 s. This voltage protocol was applied before and after the application of the agents. The current curves were then determined by subtraction. The $I_{Na,late}$ current density was read at 50 ms after the pulse onset. When determining the current integral, the initial 20 ms was excluded from the evaluation to minimize the contribution of $I_{Na,early}$ to the current curve under investigation.

To investigate the voltage dependence of $I_{Na,late}$ equilibrium inactivation, prepulses of 2 s duration between -130 and -40 mV were applied before the test potential of -20 mV. The peak currents ($I_{test}$) measured during these prepulses were normalized to the peak current ($I_{max}$) measured during the -130 mV prepulse and these $I_{test}/I_{max}$ ratios were plotted as a function of the corresponding prepulse potentials. The data were fitted with a Boltzmann function.

*Recording of action potentials from multicellular preparations*

Multicellular preparations (canine, guinea pig, and human left ventricular trabeculae and canine right ventricular papillary muscles) were chosen for AP recording to avoid the limitations of isolated myocardial cell assays (e.g., lack of cell-to-cell contacts or potential damage to channel proteins). In this experimental setup, we can better substitute in vivo conditions in some respects. The transmembrane potentials were recorded with glass microelectrodes filled with 3 M KCl, with a resistance between 10-20 MΩ. These electrodes were connected to the input of a high impedance electrometer (MDE GmbH, Heidelberg, Germany). The preparations were stimulated with a pair of platinum electrodes with rectangular pulses 1 ms wide and with an amplitude twice the threshold value at 37 °C. Before starting the experiments, the preparations were excited at 1 s CL for at least 60 min.

Following stimulation at 1 s CL, the CL of stimulation was varied sequentially between 0.3-5 s. At each CL, the 25th AP was recorded and then the CL was varied, so that overall an equilibrium frequency dependence was recorded in a relatively short time. The APs were digitized with an ADA 3300 data acquisition card (Real Time Devices Inc.) at 100 kHz and stored until later analysis. After data were recorded on all CLs under control conditions, preparations were perfused for 20 min with 2 μM TTX (canine, guinea pig and human left ventricular trabeculae) or 1 μM GS967 or 40 μM mexiletine (canine right ventricular papillary muscles), and then the CL switching protocol was repeated. Efforts were made to maintain the original electrode position during each experiment. If the electrode moved out of the preparation, we attempted to reposition the electrode. However, if the AP parameters recorded after repositioning were more than 5% different from the previous parameters, the experiment was discarded.

*Determination of beat-to-beat variability of APD in isolated myocytes*

Since beat-to-beat variability of APD is relatively small in multicellular preparations due to the balancing effect of the neighboring cells, these experiments were performed in isolated myocytes. Series of 50 consecutive action potentials were analyzed to estimate the

beat-to-beat variability of APD, defined as short term variability (SV), according to the following formula:

$$SV=\Sigma(|APD_{i+1}-APD_i|)/[n_{beats}*\sqrt{2}]$$

where SV is short term variability, APDi and APDi+1 indicate the durations of the ith and i + 1th APs, respectively, at 90% level of repolarization and nbeats denotes the number of consecutive beats analyzed32. Changes in SV were presented as Poincaré plots where 50 consecutive APD values are plotted, each against the duration of the previous AP.

*Statistics*

The results are expressed as arithmetic mean ± arithmetic mean standard error around the standard error of the mean (SEM). When comparing groups, one-way analysis of variance (ANOVA), Student's two-sample t-test and self-controlled t-test were used according to the statistical question. Linear regression was used to determine correlations between data. Correlations were expressed as Pearson's correlation coefficient (r) and slope of the line (m). The significance between correlation coefficients and slopes was also tested using Student's t-test. The n indicates the number of myocardial cells or multicellular preparations tested. Differences were considered significant when p was less than 0.05. Data were processed, analyzed and plotted using Excel (Microsoft Corp., Redmond, WA, USA) and Origin 2015.

# Results

*Determining the optimal TTX concentration*

Primarily the optimal TTX concentration for dissection $I_{Na,late}$ in canine ventricular myocytes had to be determined in order to find a TTX concentration that is suitable to excise a sufficiently large portion of $I_{Na,late}$ allowing visualization of the true profile of the current. Based on the concentration-response curves obtained by fitting these data to the Hill equation 10 μM TTX caused an approximately 87% inhibition of the current. Therefore 10 μM TTX was chosen to dissect $I_{Na,late}$ in the forthcoming voltage clamp experiments bearing in mind that this approach is likely to somewhat underestimate the density and integral of $I_{Na,late}$. Accordingly, the relative contribution of the high TTX-affinity „neuronal" $Na^+$channels were not monitored, i.e. the effects of lower (nanomolar) TTX concentrations were not studied. This may distort somewhat the EC50 values obtained for TTX. These measurements were performed in the presence of 1 μM nisoldipine to suppress L-type $Ca^{2+}$ current, since large concentrations of TTX have been shown to inhibit $Ca^{2+}$ current as well.

*True profiles of $I_{Na,late}$ in canine and guinea pig ventricular cells recorded under native APVC conditions*

Canine $I_{Na,late}$ displayed a „decrescendo" profile, i.e. its amplitude decreased monotonically during the time course of the AP, in contrast to guinea pig ventricular myocytes, where the amplitude of the current was increasing during the plateau and declined only on terminal repolarization. These differences are also reflected by the current voltage relations (phase-plane trajectories) obtained under the APs in canine and guinea pig cells. More quantitative approach to demonstrate the profile of $I_{Na,late}$ is to measure the density of the TTX-sensitive current at 20%, 50% and 100% of APD, where the APD at 90% repolarization (APD90) was considered as 100%. Accordingly, the density of $I_{Na,late}$ at 20% APD was −0.52 ± 0.09 pA/pF in dog versus the −0.21 ± 0.05 pA/pF in guinea pig ($p < .05$), while at 100% APD $I_{Na,late}$ had a greater density in guinea pig than dog (−0.78 ± 0.07 versus − 0.03 ± 0.02 pA/pF, $p < .05$). At 50% APD the densities were not significantly different in the two species. Although the charge carried by $I_{Na,late}$ was higher in guinea pig than in dog (86.2 ± 10.8 fC/pF, n = 20/9 versus 67.1 ± 9.2 fC/pF, n = 7/4, $p < .05$) these current integrals were in a similar range.

*Effect of command voltage shape on $I_{Na,late}$ profile in canine cardiomyocytes*

As already mentioned above, $I_{Na,late}$ current profiles in canine cardiomyocytes were determined under APVC conditions in two slightly different experimental groups. In the first group canonic APVC was used and $I_{Na,late}$ was determined in the presence of 1 μM nisoldipine. In the second group self APVC was used without nisoldipine. Although the values were somewhat smaller in the presence of nisoldipine, no significant differences were found between the two groups at 20%, 50% or 100% APD (−0.48 ± 0.08 versus − 0.52 ± 0.09 pA/pF, −0.30 ± 0.06 versus − 0.34 ± 0.06 pA/pF and − 0.06 ± 0.02 versus − 0.03 ± 0.02 pA/pF, respectively). The integrals were also similar (63.1 ± 9.2 fC/pF, n = 9/5 versus 67.1 ± 9.2 fC/pF, n = 7/4, respectively, N.S.). This comparison suggests that 10 μM concentration of TTX does not

interfere with the L-type $Ca^{2+}$ current. Since AP configuration has been shown to influence current profiles under APVC conditions, attempts were made to convert the decrescendo type canine $I_{Na,late}$ into a crescendo profile, characteristic of guinea pig myocytes. Therefore, duration-matched canonic guinea pig APs and voltage ramps resembling the ramp-like plateau of guinea pig cells were applied as command signals to canine cells. The ramp started from −80 mV, spent 10 ms at +40 mV and decayed to −20 mV during 200 ms. None of these interventions were capable to convert the canine decrescendo current profile to a crescendo guinea pig-like $I_{Na,late}$ profile. The current integrals were also similar in canine cells independently of the canine or guinea pig origin of the command AP (63.1 ± 9.2 fC/pF, n = 9/5 versus 65.4 ± 10.7 fC/pF, n = 6/5, N.S.).

*ATX-II-induced TTX-sensitive current profile in guinea pig and canine ventricular cells*

The sea-anemone toxin (ATX-II) has been shown to induce a current in cardiac tissues resembling $I_{Na,late}$ by removing the fast inactivation machinery of $Na^+$ channels. In guinea pig myocytes the profile of the TTX-sensitive current was similar in the absence and presence of 10 nM ATX-II, although its amplitude was significantly increased by ATX-II, i.e. the current followed crescendo kinetics in both cases. In contrast, canine myocytes usually produced early afterdepolarizations when exposed to 10 nM ATX-II for 3 min (not shown), therefore the canine cells were treated with a lower, 1 nM concentration of ATX-II. The TTX-sensitive current in these canine cells displayed a plateau-like shape, i.e. the current densities were largely similar at 20%, 50% and 100% APD in the presence of ATX-II. This current profile, containing the sum of baseline $I_{Na,late}$ plus the ATX-II-induced current component, was markedly different from that recorded in the absence of ATX-II. Therefore, in another series of experiments, where the ATX-II-induced current alone was visualized, the ATX-II-induced current showed moderate crescendo characteristics in canine myocytes. The TTX-sensitive current integrals were significantly larger in the presence of ATX-II (1 nM in canine and 10 nM in guinea pig cells) comparing to untreated cells: 102 ± 13 fC/pF, n = 7/4 versus 67 ± 9 fC/pF, n = 7/4 in canine; and 145 ± 19 fC/pF, n = 5/3 versus 86 ± 11 fC/pF, n = 20/9 in guinea pig myocytes, $p < .05$ for both. The duration of action potentials (APD90) was significantly increased by 1 nM ATX-II in canine cells from 214 ± 13 ms to 257 ± 17 ms (lengthening of 43.2 ms, n = 7/4, $p < .05$), in contrast to guinea pig myocytes, where the increase induced by 10 nM ATX-II (from 192 ± 15 to 212 ± 20 ms, lengthening of 19.5 ms, n = 5/3), was not significant statistically. These results clearly demonstrate that canine myocytes are much more sensitive to ATX-II that the guinea pig cells, and more importantly, the profile of the ATX-II-induced current is different from the native $I_{Na,late}$ in canine myocytes.

*Comparison of $I_{Na,late}$ properties in human, dog and guinea pig cardiac myocytes*

In the followings the profiles (under self APVC conditions) and the kinetic properties (using conventional voltage clamp techniques) of $I_{Na,late}$ were compared in human, canine and guinea pig ventricular cells. The 20 μM TTX-sensitive currents were similarly shaped in all types of myocytes when the membrane potential was switched from the holding potential of −120 mV to the test potential of −20 mV in the presence of 1 μM nisoldipine, 0.1 μM dofetilide and 0.5 μM HMR-1556. When the decay of TTXsensitive current was fitted to a

monoexponential function (excluding the initial 50 ms period) the inactivation time constants were 60 ± 3 ms (n = 13/7) in canine, 67 ± 5 ms (n = 5/3) in human, and 155 ± 16 ms (n = 5/3) in guinea pig cells. Thus the time course of inactivation of $I_{Na,late}$ was similar in human and canine myocytes, but was significantly ($p < .05$) slower in guinea pig. The corresponding current densities (obtained also by the monoexponential fitting procedure) were − 0.47 ± 0.05 pA/pF in human, −0.56 ± 0.04 pA/pF in canine and − 0.54 ± 0.14 pA/pF in guinea pig myocytes, which values were not significantly different. These current profiles were also very similar in canine and human cells (both sharing the decrescendo $I_{Na,late}$ profile), while markedly different from the crescendo type $I_{Na,late}$ in guinea pig.

*Effect of tetrodotoxin on APD90 in multicellular human, canine and guinea pig ventricular preparations*

The rate-dependent effects of TTX on AP morphology in multicellular ventricular preparations, obtained from the three species. All preparations were superfused with 2 μM TTX and the measurements were performed after equilibration. APD was shortened by TTX at each cycle length. The TTX-induced shortening, recorded at the cycle length of 1 s, was the largest (67 ± 16 ms, n = 4/3) in human, intermediate (21 ± 4 ms, n = 5/5) in canine and the smallest (11 ± 3, n = 7/7) in guinea pig preparations. The resting membrane potential and the amplitude of AP were not altered by TTX. The maximum velocity of depolarization (dV/dtmax) was moderately reduced by TTX from 310 ± 48 to 259 ± 24 V/s in human, from 231 ± 40 to 169 ± 33 V/s in canine, and from 226 ± 32 to 176 ± 21 V/s in guinea pig, however, these changes failed to reach the level of significance – except for the canine preparations.

*Effects of GS967 and mexiletine under action potential voltage clamp conditions*

Based on the non-equilibrium gating theory of Clancy et al., gating of the $Na^+$ channel is intimately influenced by the shape of the voltage protocol applied. Therefore, the effects of GS967 and mexiletine on $I_{Na,late}$ were studied also under action potential voltage clamp conditions using canonic mid-myocardial APs as command signals. 1 μM GS967 and 40 μM mexiletine dissected inward $I_{Na,late}$ profiles having current densities (measured at 50% of APD90) of $-0.37 \pm 0.07$ and $-0.28 \pm 0.03$ A/F, and current integrals of $-56.7 \pm 9.1$ and $-46.6 \pm 5.5$ mC/F, respectively. These differences, however, were not significant statistically. Since the $Ca^{2+}$ and $K^+$ currents were previously blocked in these experiments, the recorded current profiles are considered as $I_{Na,late}$ inhibited by GS967 and mexiletine during a regular ventricular AP.

The total density and integral of $I_{Na,late}$ in canine myocytes were estimated using concentration–response curves obtained with TTX, which was applied as a reference compound. The maximum value of the Hill-fit gave the total magnitude of $I_{Na,late}$. Comparing the respective current densities and integrals dissected by 1 μM GS967 and 40 μM mexiletine to these maximal values obtained with TTX, the inhibiting effects of GS967 and mexiletine could be expressed in a percentage form. Accordingly, GS967 and mexiletine blocked 88% and 67% of total $I_{Na,late}$ density, and 78% and 64% of total $I_{Na,late}$ integral, respectively. More importantly, the current profiles dissected by GS967 and mexiletine were different in shape. The GS967-sensitive current decreased monotonically during the AP, similarly to the TTX-

sensitive current (not shown), while the mexiletine-sensitive current displayed a saddle-like profile. This difference is likely attributable to the threefold faster dissociation of GS967 than mexiletine from the $Na^+$ channel.

*Effects of GS967 and mexiletine on beat-to-beat variability of APD*

Elevated beat-to-beat QT interval variability is a good predictor of ventricular arrhythmia in a wide variety of patients, while at a single cell level it translates into beat-to-beat variability of APD (also called short term variability, SV) — both parameters are considered as indicators of proarrhythmic risk. In contrast to multicellular preparations, where the neighboring cells may effectively balance the individual differences in APD, SV is more pronounced in single cells, therefore the effects of GS967 and mexiletine were studied on SV in isolated ventricular cells, paced at a constant cycle length of 1 s. Under these conditions SV was significantly decreased by 1 μM GS967 and 40 μM mexiletine (reduction of $1.01 \pm 0.19$ and $0.63 \pm 0.02$ ms, respectively, corresponding to $42.1 \pm 6.5\%$ and $24.6 \pm 12.8\%$ decrease), while APD was moderately shortened by $32.5 \pm 6.9$ and $41.4 \pm 4.1$ ms, respectively, (all $p < 0.05$ and $n = 8$). All these effects were largely reversible upon washout. Since APD itself is also known to affect the magnitude of SV, the term of relative SV (RSV) was introduced. Accordingly, RSV = dSV / dAPD, i.e. the change in SV is normalized to that of APD. The value of RSV was significantly higher for GS967 than mexiletine ($0.039 \pm 0.007$ versus $0.015 \pm 0.007$) predicting a better antiarrhythmic effectivity of GS967 than mexiletine.

# Discussion

An increased $I_{Na,late}$ is present in many heart diseases. The upregulated $I_{Na,late}$ lengthens the cardiac AP, increases $[Na^+]_i$, and causes $Ca^{2+}$ overload of cardiomyocytes by offsetting the forward mode NCX. The elevated $Ca^{2+}$, in turn, mainly through CaMKII, can further increase $I_{Na,late}$ in a vicious circle. These pathophysiological mechanisms together may result in impaired cardiac energetics and contractile dysfunction of the heart as well as cardiac arrhythmias. The prolonged AP can serve as a substrate that is prone to rhythm disorders, whereas $Ca^{2+}$ overload can be the trigger. $I_{Na,late}$ seems to possess a pathogenetic role especially in AF and in ventricular arrhythmias occurring under bradycardic conditions.

This is the first study to demonstrate the profiles of human and canine ventricular $I_{Na,late}$ under self APVC conditions. From this perspective our results are in line with those of Murphy et al. who have shown the profile of canine $I_{Na,late}$ using canonic APs. Regarding undiseased human ventricular cells, this is the first report using the APVC technique. Our most important finding was to demonstrate that canine myocytes can be used as a reasonably good model to study human $I_{Na,late}$ - in contrast to myocytes originating from other mammals including guinea pigs, rabbits and pigs, since all in these latter species the current displays a crescendo profile. Important implication of this interspecies difference is that the relative contribution of $I_{Na,late}$ to action potential morphology (with the concomitant $Na^+$ and $Ca^{2+}$ load resulting in increased arrhythmia propensity) increases with lengthening of APD in the „crescendo" group in contrast to the „decrescendo" situation, and vice versa, it will be relatively smaller at shorter APDs. More explicitly, $I_{Na,late}$ is the largest in amplitude at the time of terminal repolarization in the „crescendo" type species, consequently, a given prolongation of APD (eg. due to application of a HERG channel inhibitor) is expected to cause a greater extra inward $I_{Na,late}$ current (together with larger $Na^+$ and $Ca^{2+}$ load), which in turn, may magnify the APD lengthening effect of the $K^+$ channel inhibitor. Since $I_{Na,late}$ is practically inactivated at this time in the „decrescendo" type preparations, changes in APD will barely modify the magnitude of $Na^+$ entry via $I_{Na,late}$. Using similar argumentation, the therapeutic effects of $I_{Na,late}$ inhibitors (including the shortening of APD and reduction of cellular $Na^+$ and $Ca^{2+}$ content) is expected to be less pronounced in canine and human myocardium than in similar preparations from guinea pigs, rabbits and pigs. This should be taken into account when the results of pharmacological studies on $I_{Na,late}$ inhibitors or modifiers are interpreted or extrapolated to human, since some studies with such compounds have been performed in species displaying crescendo $I_{Na,late}$ profiles. Based on the present results, canine ventricular tissues or myocytes are suggested for pharmacological studies with drugs interacting with $I_{Na,late}$.

We have also revealed why the guinea pig $I_{Na,late}$ is growing under the AP plateau, while canine and human $I_{Na,late}$ decreases during the time course of the AP. In canine and human myocytes the inactivation time constant of $I_{Na,late}$ was in the range of 60–67 ms at −20 mV, consequently the current became practically fully inactivated by the time of terminal repolarization. In contrast, the decay time constant was much longer (156 ms) in guinea pig, thus the inactivation was slow enough to leave a significant portion of $Na^+$ cannels open by the time of terminal repolarization. Consequently, the increasing inward driving force acting on $Na^+$ ions could increase the amplitude of $I_{Na,late}$ during the monotonic slow repolarization in

guinea pig (and likely also in rabbit and pig). The well-known crescendo $I_{Na,late}$ profile in guinea pig was originally explained with the non-equilibrium gating theory of Clancy et al. This model predicts the accumulation of $I_{Na,late}$ during the plateau as a consequence of the ramp-like configuration of phase-2 repolarization of the guinea pig AP. However, application of guinea pig APs as well as repolarizing ramps as command signals failed to convert the decrescendo $I_{Na,late}$ profile of canine cells to crescendo, indicating that it is not the AP configuration, but rather the 2.5-fold slower inactivation kinetics, revealed by the conventional voltage clamp experiments, that accounts for the crescendo $I_{Na,late}$ profile in guinea pig. Since a portion of $I_{Na,late}$ is attributed to the function of noncardiac $Na^+$ channels in the canine heart, it is possible that the type or relative contribution of these TTX-sensitive channels or variances in their regulatory subunits are different in dogs (and humans) versus guinea pigs (and pigs or rabbits). However, elucidating the details behind the different inactivation kinetics of $I_{Na,late}$ among various species warrants further studies.

Here is to be mentioned that there is a wide variety of SCN5 mutations, responsible for LQT3 syndrome, and depending on the site and type of mutation many parameters of $Na^+$ channel gating, including the rate of inactivation and recovery as well as the voltage dependence of activation and inactivation, may be altered, however, augmentation of $I_{Na,late}$ is a critical and common feature in all cases. Since only a very small fraction of the total $Na^+$ cahnnel population may contribute to generation of $I_{Na,late}$ (compare the few tens of pA amplitude of $I_{Na,late}$ with the several nA amplitude of the peak $Na^+$ current), the macroscopic changes in $I_{Na}$ gating are less relevant from the pathophysiological point of view than the actual magnitude of $I_{Na,late}$.

Another important result of the present study was to show that the profile of the ATX-II-induced current recorded under APVC conditions in canine cells is markedly different from the shape of native canine $I_{Na,late}$, since the ATX-II-induced current displayed a crescendo profile in both species – similar to the native $I_{Na,late}$ of guinea pig, rabbit and porchine hearts, but different from the native $I_{Na,late}$ recorded from canine myocytes. This may be related to the well-known fact that ATXII slows inactivation of $Na^+$ channels. ATX-II is widely used for mimicking pharmacologically the consequences of an augmented $I_{Na,late}$, which is often seen under pathological conditions and ATX-II is a widely used pharmacological tool to model this. In canine myocytes (i.e. in the cells appearing to be the best model for studying human $I_{Na,late}$), however, this approach may be misleading due to the differences observed between the native $I_{Na,late}$ and ATX-II-induced current profiles. As a consequence, ATX-II modified $Na^+$ channels might also differ from the native channels in their in drug-sensitivity, making results obtained in the presence of ATX-II difficult to interpret.

APDs recorded from multicellular canine, human and guinea pig preparations were shortened by 2 μM TTX, which effect was the largest in human, intermediate in canine and the smallest in guinea pig preparations. This sequence can not be explained by the known (and presently described) properties of $I_{Na,late}$, since the current densities and integrals were largely similar in the three species. It is more likely therefore that the magnitude of the TTX-induced shortening depends on the actual repolarization reserve of the myocardium, which is the smallest in human, intermediate in canine, and with the strong IKs is the largest in guinea pig ventricular myocardium. This is a further argument in support of using canine myocytes for

modeling human $I_{Na,late}$. This interspecies difference (i.e. dog vs. guinea pig) in repolarization reserve may explain also the higher ATX-II-sensitivity of dog comparing to guinea pig. In canine myocytes 1 nM ATX-II caused twice greater lengthening of AP than 10 nM ATX-II in guinea pig, while 10 nM ATX-II initiated early afterdepolarizations in canine cells.

We studied and compared the effects of GS967 (1 μM) to those of the class I/B antiarrhythmic drug mexiletine (40 μM) on $I_{Na,late}$ and $I_{Na,early}$ in canine ventricular myocardium by combining the conventional microelectrode, voltage clamp and action potential voltage clamp techniques. It was found that GS967, which is generally considered as a selective blocker of $I_{Na,late}$, inhibits $I_{Na,early}$ as well, similarly to the class I/B antiarrhythmic drug mexiletine, but with higher potency. Based on the hypothetically selective $I_{Na,late}$ blocker nature of GS967, the drug was previously mentioned as a novel class VI antiarrhythmic agent. However, without questioning the theoretical possibility of the concept for selective $I_{Na,late}$ inhibition, an alternative approach should also be considered, since the concept whether $I_{Na,late}$ can be blocked selectively is an interesting and still unresolved issue. There is evidence that $Na^+$ current in cardiac tissues may also be conducted by $Na^+$ channels other than the cardiac specific $Na_v1.5$ channels, which were suggested to contribute to both $I_{Na,early}$ and $I_{Na,late}$. However, proper functional evidence for their role is still lacking while considerable interspecies differences were observed. If these channels play a role in $I_{Na,late}$ but not in $I_{Na,early}$, their pharmacological inhibition may result in selective $I_{Na,late}$ blockade. However, GS967 has not been shown to inhibit these types of sodium channels but it was reported to inhibit cardiac type $Na_v1.5$ channels. Therefore, it is difficult to estimate the relative contribution of neuronal type $Na^+$ channels to the GS967-sensitive current.

Taken together the present results and the literature, it is likely that a compound having kinetic properties similar to GS967 would be a very promising new antiarrhythmic agent, since several in vitro and in vivo studies support the potent antiarrhythmic activity of GS967. Its kinetic properties are better than those of mexiletine, as shown in this study, and also than those of ranolazine, agents known to suppress $I_{Na,late}$. GS967 had high brain penetration, which can be utilized as a potential new antiepileptic compound, and caused a profound use-dependent block on all sodium channel isoforms studied, making the compound prone for possible central nervous system side effects. It is unclear at present whether the effects of GS967 on the nervous system preclude the use of this drug as an antiarrhythmic therapeutic, however, a new agent exhibiting the same kinetic properties of GS967 without CNS side effects would represent a promising candidate for future development.

$Na_v1.5$ channels have complex and multiple open and closed states with different drug binding properties governing active, inactive and resting channel states. Accordingly, drugs interacting with the binding sites of the channels depending on their actual open or closed channel states may produce variable effects on $I_{Na,early}$ and $I_{Na,late}$. For example, when a drug binds rapidly and with high affinity to open and inactivated channel states, and it dissociates rapidly from closed resting channel states, it would not inhibit $I_{Na,early}$ but $I_{Na,late}$. This would be due to complete drug dissociation from its binding site in the resting closed states unless frequency was very high or at least the cycle length would be shorter than its dissociation from the channel. Consequently, whether a drug inhibits $I_{Na,early}$ or $I_{Na,late}$ selectively or both of them, largely depends on the stimulation protocol but not on existing specific $I_{Na,early}$ or $I_{Na,late}$ binding

sites. Present results and other recently published data are consistent with this suggestion and do not support a mechanism which is based on specific $I_{Na,late}$ inhibition that is distinctly different from class I/B antiarrhythmic actions described for drugs such as mexiletine, lidocaine, amiodarone and ranolazine. This approach is also in line with the reported high (38-fold) selectivity of ranolazine on $I_{Na,late}$ over $I_{Na,early}$, which is intermediate (13-fold) for amiodarone and much lower (only threefold) for flecainide.

On the basis of present and other results it is clear that GS967 affects $I_{Na,early}$ in a strongly rate- and moderately species-dependent fashion. GS967 shortened APD in a variety of preparations, including rat, murine, rabbit and human ventricular cells within a wide concentration range (0.1–1 μM) — similarly to the present observations in isolated canine ventricular cells. The reduction of $I_{Na,late}$ by GS967 may result the GS967-induced shortening of the elongated APD, reduction of the enhanced dispersion and short term variability of repolarization, changes often preceding torsade de pointes arrhythmias.

In summary, GS967 — similarly to mexiletine— inhibited both the peak and late components of $Na^+$ current and decreased beat-to-beat variability of APD. Based on its kinetic properties, GS967 should be classified as a new potent class I/B antiarrhythmic agent.

The results of the present study also suggest that investigations of „selective" $I_{Na,late}$ inhibitors should be carried out through a wide range of stimulation rates since the effect of drugs like GS967 or mexiletine, that possess fast offset kinetics of $I_{Na,early}$ inhibition, can be misinterpreted.

Multitude of pathophysiology studies have drawn the consequence that selective $I_{Na,late}$ inhibition is a favorable antiarrhythmic tool in many experimental settings. Despite all these studies, the one and only drug on the market that selectively inhibits $I_{Na,late}$ is ranolazine, although it significantly affects other ionic currents as well. Ranolazine has been a safe and effective anti$I_{Na,late}$ medication since 2006 based on large randomized studies. Some recent clinical evidence also proves that ranolazine shows favorable effects in AF and in ventricular arrhythmias. For stronger evidence, more phase 3 clinical investigations are necessary. Targeting $Na_v1.8$ with specific inhibitors is also an interesting novel approach in the future of antiarrhythmic drug therapy.

# New scientific results included in the dissertation

-This is the first study to present the $I_{Na,late}$ profile of human and canine ventricular in their self APVC conditions.

-For non-diseased human ventricular cells, this is the first report using the APVC technique.

-For the first time, we demonstrate that the therapeutic effects of $I_{Na,late}$ inhibitors (including shortening of APD and reduction in cell $Na^+$ and $Ca^{2+}$ content) are expected to be less pronounced in canine and human myocardium than in similar preparations from guinea pig, rabbit and porcine. This should be taken into account when interpreting or extrapolating the results of pharmacological studies with $I_{Na,late}$ inhibitors or modifiers to human populations, as some studies with such compounds have been performed in species that have shown a crescendo $I_{Na,late}$ profile.

-Based on the present results, canine ventricular tissue or myocardial cells are suggested for pharmacological studies with drugs interacting with $I_{Na,late}$.

-Our main finding was to demonstrate that canine cardiomyocytes can be used as a relatively good model for human $I_{Na,late}$ assays - in contrast to cardiomyocytes from other mammals, including guinea pig, rabbit and pig.

- We also first explored why guinea pig $I_{Na,late}$ increases during the AP plateau, while canine and human $I_{Na,late}$ decreases during the AP time course.

- Another important finding of the present studies was that we have shown that the ATX-II-induced current profile recorded in canine cells under APVC conditions differs significantly from the native canine $I_{Na,late}$ shape.

- ATX-II-induced currents in canine and guinea pig species showed a „crescendo" profile (similar to the native $I_{Na,late}$ of guinea pig, rabbit and porcine cardiac myocytes) , but differed from the native $I_{Na,late}$ recorded from canine cardiac myocytes. This may be related to the well-known fact that ATX-II slows down the inactivation of $Na_v$s.

- We found that GS967, considered to be a selective blocker of $I_{Na,late}$, also inhibits $I_{Na,early}$. This inhibition is similar to that of mexiletine but with greater potency.

-We first demonstrated that GS967 inhibited both the early and late components of the $Na^+$ current, similar to mexiletine, and also reduced the short-term variability of APD.

- It can be argued that the inhibition of $I_{Na,late}$ cannot be achieved completely independently of $I_{Na,early}$.

- There is no significant difference between the frequency-dependent $I_{Na,late}$ inhibition described for $I_{Na,late}$ inhibitors, which are said to be selective, and the frequency-dependent $I_{Na,late}$ inhibition described for "general" Class I/B $Na_v$ inhibitors.

# Summary

Although late sodium current ($I_{Na,late}$) has long been known to contribute to plateau formation of cardiac action potentials, lately it was considered as possible target for antiarrhythmic drugs. However, many aspects of this current are still poorly understood. The present work was designed to study the true profile of $I_{Na,late}$ in canine and guinea pig ventricular cells and compare them to $I_{Na,late}$ vrecorded in undiseased human hearts. $I_{Na,late}$ was defined as a tetrodotoxin-sensitive current, recorded under action potential voltage clamp conditions using either canonic- or self-action potentials as command signals. Under action potential voltage clamp conditions, the amplitude of canine and human $I_{Na,late}$ monotonically decreased during the plateau (decrescendo-profile), in contrast to guinea pig, where its amplitude increased during the plateau (crescendo profile). The decrescendo-profile of canine $I_{Na,late}$ could not be converted to a crescendo-morphology by application of ramp-like command voltages or command action potentials recorded from guinea pig cells. Conventional voltage clamp experiments revealed that the crescendo $I_{Na,late}$ profile in guinea pig was due to the slower decay of $I_{Na,late}$ in this species. When action potentials were recorded from multicellular ventricular preparations with sharp microelectrode, action potentials were shortened by tetrodotoxin, which effect was the largest in human, while smaller in canine, and the smallest in guinea pig preparations. It is concluded that important interspecies differences exist in the behavior of $I_{Na,late}$. At present canine myocytes seem to represent the best model of human ventricular cells regarding the properties of $I_{Na,late}$.

Another aim of the present work was to investigate the effects of GS967 on $I_{Na,late}$ and action potential (AP) morphology in canine ventricular myocytes by using action potential voltage clamp and sharp microelectrode techniques. The effects of GS967 (1 μM) were compared to those of the class I/B antiarrhythmic compound mexiletine (40 μM). It is concluded that the electrophysiological effects of GS967 are like those of mexiletine. In addition, beat-to-beat variability was significantly decreased by both GS967 and mexiletine.

These results should be considered when pharmacological studies with $I_{Na,late}$ are interpreted and extrapolated to human. Accordingly, canine ventricular tissues or myocytes are suggested for pharmacological studies with $I_{Na,late}$ inhibitors or modifiers. Incorporation of present data to human action potential models may yield a better understanding of the role of $I_{Na,late}$ in action potential morphology, arrhythmogenesis, and intracellular calcium dynamics.

In summary, we conclude that canine cardiomyocytes are a good model for human ventricular cells regarding the properties of $I_{Na,late}$ properties. However, further detailed studies are needed to describe gating kinetics of the $I_{Na,late.}$

The late sodium current plays an important role in the electrical and mechanical function of the heart. The medical relevance of the late sodium current in the pathophysiology and treatment of many cardiovascular diseases is prominent. This includes the previously mentioned cardiac arrhythmias in relation to prolonged action potentials and triggered activity. One can mention its role in heart failure in relation to calcium overload and energy consumption (more ATP is needed to maintain ionic balance). It is also important to mention the ischaemic reperfusion injury in ischaemic heart disease. At present, no other agent than ranolazine is used in clinical practice, so further research is ongoing to develop new drugs that

target late sodium current reduction, improving the prognosis of various cardiac diseases. Accordingly, inhibition of late sodium current may be a promising therapeutic strategy for the treatment of these conditions.