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Tetrodotoxin-sensitivity of cardiac L-type calcium channels

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The Examination takes place at the Department of Medical Chemistry, Faculty of Medicine, University of Debrecen
at 11 am, on December 1, 2014

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
at 1 pm, on December 1, 2014

INTRODUCTION

It is generally believed that the marine tetrodotoxin (TTX), is a highly selective inhibitor of voltage-gated Na^+ channels in various excitable tissues. This is really the case in skeletal muscle and neural tissues, where sodium current (I_{Na}) is blocked by TTX in the nanomolar range (the half maximal inhibitory concentration (IC_{50}) ~ 10 nM). In mammalian cardiac muscle, however, micromolar concentrations of TTX are required to suppress Na^+ current effectively. As a consequence, TTX concentrations as high as 30 μM had to be applied in order to achieve a full suppression of I_{Na} in mammalian cardiomyocytes.

TTX-sensitive Ca^{2+} current components have been identified in cardiac tissues under pathological conditions, such as in hypertrophied guinea pig or infarcted rat hearts. Furthermore, a TTX-sensitive fraction of a current, carried by Ca^{2+} ions in the absence of external Na^+, has been described in a variety of cardiac tissues, including human, rat, and guinea pig myocardium. From these experiments, one may conclude that Ca^{2+} is the charge carrier of this current likely flowing through Na^+ channels in the absence of external Na^+. Similarly to the studies mentioned above (i.e., under Na^+-free conditions), the most important cardiac isoforms of T-type Ca^{2+} current ($Ca_v3.1$ and $Ca_v3.2$) were shown to be resistant to TTX. On the other hand, little is known about the effect of TTX on the L-type Ca^{2+} channels ($Ca_v1.2$), except for one study performed in mice where no effect of TTX on L-type Ca^{2+} channel could be observed. Therefore, we aimed to reexamine the effect of TTX on L-type calcium current ($I_{Ca,L}$) in isolated canine ventricular myocytes. This preparation was chosen because its electrophysiological parameters are believed to be most similar to those of human regarding the distribution and kinetic properties of transmembrane ion currents.

In order to understand in detail how TTX blocks voltage gated $Ca_v1.2$ channels, we had to develop a theoretical model to explain TTX binding to $Ca_v1.2$ channels. This model is based on the selectivity filter region of the $Na_v1.4$ channel in complex with the TTX molecule and on the known homology between the $Na_v1.4$, $Na_v1.5$ and $Ca_v1.2$ channels.

Furthermore, the limited selectivity of TTX could reveal that the cross actions between these channels seem to be inescapable in case of using cardiac voltage-gated ion channels blockers in medical therapy and experimental research.

LITERARY REVIEW

Voltage-gated Ca^{2+} channels

The voltage-gated Ca^{2+} channels, situated in the cell surface membrane, act as main transducers of membrane depolarization into local intracellular Ca^{2+} transients that serve as a base of several physiological phenomena. In response to membrane depolarization, opening of voltage-gated calcium channels results calcium influx, which regulates intracellular processes such as muscle contraction, secretion, neurotransmission, gene expression and several enzyme activities in many different cell types.

Ca^{2+} channels are complex proteins consisting of 4 or 5 subunits, encoded by relatively many genes. The α_1 subunit of 190 to 250 kDa is the largest among the subunits, which involves the conduction pore of the channel, the voltage sensor, the gating apparatus, and most of those sites playing role in channel regulation to which second messengers, drugs, and toxins can bind. Similar to the α subunit of Na^+ channels, the α_1 subunit of Ca^{2+} channels is composed of four homologous domains (I-IV) and each of them consists of six transmembrane segments (S1-S6). The S4 segment acts as the voltage sensor and the pore forming loop between transmembrane segments S5 and S6

determines ion selectivity and conductance in both cases. Selectivity filter of the voltage-gated Ca^{2+} channels is formed by the glutamate-glutamate-X-X (so-called as EEXX ring) amino acid sequence. The entirely intracellular β subunits ($\beta_1 - \beta_4$) increase the expression of the channel up to ten folds and accelerate the activation and inactivation kinetics. Transmembrane, disulfide-linked α_2/δ subunit is also present in most Ca^{2+} channels. The γ subunit can be found typically in skeletal muscle Ca^{2+} channels, while similar subunits are also expressed in brain and heart. Although these auxiliary subunits affect the properties of the channel, the pharmacological and the electrophysiological diversity of Ca^{2+}channels derive particularly from the presence of different α_1 subunits. $Ca_v1.2$ channels are present primarily in myocardium, in there they mediate the L-type calcium current.

L-type Ca^{2+} channels are located mainly in the sarcolemma in the ventricular myocytes, principally in the T-tubule surface facing to the sarcoplasmic reticulum (SR). Ca^{2+} entry through the opening of $Ca_v1.2$ channels is the most important inducing factor of the Ca^{2+}-induced Ca^{2+}-release from the SR under physiological conditions. $I_{Ca,L}$ plays an important role in excitation-contraction coupling, has an influence on shaping the action potential and its pathological function can lead to cardiac arrhythmias.

L-type Ca^{2+} channels can be activated by potentials positive to -40 mV, the maximal amplitude of the current is reached between 0 and +10 mV, thus its current-voltage relationship can be characterized mostly by a U shaped curve. Regarding its kinetics, the activation is fast, the inactivation is relatively slow. Decay of the current can be best described as a sum of a fast and a slow component. The time constant of activation is approximately 2-3 ms, while the time constants of inactivation are in the magnitude of 10 and 100 ms. Time-dependent inactivation of $I_{Ca,L}$ is both Ca^{2+}-, and voltage-dependent. The relative contribution of Ca^{2+}-dependent inactivation to the total is higher at negative potentials, where the extent of voltage-dependent inactivation is low.

Accordingly, during β-adrenergic stimulation also the Ca^{2+}-dependent inactivation becomes the most prominent mechanism, first due to the increasing systolic ic. Ca^{2+} concentration, and secondly, as a result of slowing the voltage-dependent inactivation. Ca^{2+}-dependent inactivation serves as a negative feedback process in the regulation of Ca^{2+} entry and hereby it could be a protective mechanism against the harmful Ca^{2+}-overload of cells.

Effect of tetrodotoxin on voltage-gated Na^+ channels

Tetrodotoxin is widely applied to block fast Na^+ channels in experimental studies. Almost all of the voltage-gated Na^+ channel types were demonstrated to be present in different regions of the mammalian heart; however, ventricular myocardium contains dominantly $Na_v1.5$ channels. In contrast to skeletal muscle and neural tissues, where the main voltage-gated Na^+ channel subtypes are TTX-sensitive (IC_{50} in the nanomolar range), the $Na_v1.5$ channel is relatively resistant to TTX (IC_{50} in the micromolar range). As a consequence of the above mentioned differences in TTX-sensitivity, approximately hundred or thousand times higher TTX concentration is necessary to block fast Na^+ in the heart than in the case of skeletal muscles or neural preparations. However, several studies reported a TTX-suppressible fraction of Ca^{2+} current (($I_{Ca(TTX)}$), when the toxin was applied in such high concentration.

Overview about the previously described tetrodotoxin-sensitive Ca^{2+} currents

TTX-sensitive Ca^{2+} current components have been reported in several species, including the rainbow trout, mouse, rat, guinea pig, dog and human cardiac cells. Furthermore, the presence of a TTX-sensitive Ca^{2+} current has been demonstrated also in heterologously transfected cells and neural preparations. Until the last years, tetrodotoxin has been regarded as one of the

most selective inhibitors of voltage-gated Na^+ channels. Although TTX-suppressible Ca^{2+} currents have been recognized many years ago, they were probably mediated by Na^+ channels. The truth about the origin of this kind of current is still unclear; however, two alternative theories have been developed to explain that. (1) Modification of gating properties of the classical fast Na^+ channel under peculiar conditions may result in promiscuous ion selectivity. (2) A distinct population of Na^+ channels was claimed to be present in some species even under physiological circumstances. The fact that $I_{Ca(TTX)}$ has been detected in a physiological ionic milieu is in line with the second explanation.

Considering the first possibility one may conclude that $I_{Ca(TTX)}$ should appear only under specific conditions, like the absence of external Na^+, activation the β-adrenergic pathway by isoproterenol or cAMP, or following application of cardiotonic steroids such as ouabain and digoxin. According to this first hypothesis Ca^{2+} flows through classical cardiac Na^+ channels ($Na_v1.5$) in case of Na^+-free external solution by reversibly altering the ion selectivity. In the explanation of Santana *et al.* the classical Na^+ channels become „promiscuous" by phosphorylation (as a consequence of β-adrenergic activation) or on the effect of nanomolar concentrations of cardiotonic steroids, allowing Ca^{2+} to permeate the modulated channel. This property of the channel state was named as slip-mode conductance.

According to the second hypothesis, Aggarwal *et al.* and Chen-Izu *et al.* suggested that $I_{Ca(TTX)}$ was generated by a novel Na^+ channel protein, distinct from classical Na^+ channels ($Na_v1.5$). This hypothesis is supported by several facts. (a) Different kinetic properties are observed: $I_{Ca(TTX)}$ activates and inactivates slower than the classical cardiac Na^+ current. (b) Voltage dependencies obtained for activation and inactivation of $I_{Ca(TTX)}$ are shifted to more negative potentials. (c) Different permeability features: $Na_v1.5$ channels shows very little permeability to Ca^{2+} and Cs^+, while channels responsible for $I_{Ca(TTX)}$ have substantially higher permeability to those cations. (d) TTX-

sensitivity is also somewhat different, having higher IC_{50} value for $I_{Ca(TTX)}$. (e) The amplitude of current (and consequently the density of channels) is much less in the case of $I_{Ca(TTX)}$. However, channels which mediate $I_{Ca(TTX)}$ are still Na^+ channels, because they display higher Na^+ permeability, even at high extracellular Ca^{2+} concentrations, and their pharmacological profile is also congruent with that of Na^+ channels, since the current was unaffected by nifedipine, La^{3+} or Ni^{2+}, while showed high sensitivity to scorpion toxins and veratridine.

Su *et al.* as well as Sun *et al.* previously demonstrated that high concentrations of STX block T-type and L-type Ca^{2+} channels, and this finding slightly derogated the prominent channel selectivity of heterocyclic guanidinium marine toxins. However, direct inhibitory action of TTX on Ca^{2+} channel has not been described yet in cardiomyocytes. Moreover, until recently only a few experiments with $I_{Ca(TTX)}$ have been reported in the heart of larger mammals, such as humans and dogs. Among these one was performed in failing human heart and two in atrial myocytes. Conversely, myocardial cells in heart failure show quite different expression patterns of ion channels compared to healthy ones. $I_{Ca(TTX)}$ generated by Na^+ channels have not been described in the heart of larger mammals under physiological conditions yet, these channels seem to be a specialty of rodents, such as rat, mouse and guinea pig, and also of pathological hearts observed after myocardial remodelling.

Limited selectivity of drugs acting on cardiac voltage-gated Na^+ and Ca^{2+} channels

Inhibitors of voltage-gated Ca^{2+} and Na^+ channels are generally used in the daily routine of almost every medical specialty and biomedical research. These drugs are among so common pharmacological groups such as local anaesthetics, antiarrhythmics, antiepileptics, antidepressants and certain antihypertensive agents. Drugs acting on cardiac voltage-gated Na^+ and Ca^{2+}

channels are frequently reported to cross react with the structural homologous, but not primarily targeted channel type (e.g. Ca^{2+} channel blockers on Na^{+} channels and *vice versa*). This interaction is generally not favourable, especially during experimental conditions aiming to measure a specific ion movement across the cell membrane, but can also produce side effects in the clinical practice. It is more likely to happen than it could be an unexpected event. On the other hand, the cross action might be useful in a few special cases, including antiarrhythmic agents such as lidocaine and verapamil, however, their proarrhythmic property could also be enhanced. Regarding the extensive clinical use of Na^{+} and Ca^{2+} channel blockers, it seems likely that the limited selectivity does not indicate necessarily a disadvantage, because the treatment with these drugs is frequently very effective. Based on the molecular similarities, the voltage-gated Na^{+} and Ca^{2+} channels are supposed to have a common ancestor, thus the phenomenon would trace back in the development of four domain channels. Voltage-gated Ca^{2+} channels together with voltage-gated Na^{+} channels and certain voltage-gated K^{+} channels are members of an ion channel superfamily.

AIM

TTX-sensitive Ca^{2+} current was examined previously by many working groups in different excitable tissues, including cardiac muscle. Published data are controversial and species-dependent therefore our aims were the followings:

1. To examine the concentration-dependent effect of TTX on L-type Ca^{2+} current amplitude, voltage-dependence and inactivation kinetics in canine ventricular cardiomyocytes.
2. To study the use-dependent blockade of TTX on L-type Ca^{2+} channels.
3. To study the influence of phosphorylation, pH and redox potential changes in the binding of TTX to L-type calcium channels and explain the impact of these modifications using a theoretical model.

4. To develop a computational model to understand how occurs the binding of TTX to cardiac voltage-sensitive Na^+ ($Na_v1.5$) and L-type Ca^{2+} ($Ca_v1.2$) channels in detail.
5. To study the effect of TTX on $Ca_v1.2$ channels expressed in HEK tsA-201 cells.

MATERIALS AND METHODS

Isolation of canine left ventricular myocytes

Experiments were carried out on enzymatically isolated canine left ventricular cardiomyocytes. Single myocytes were obtained from adult dogs bred for experimental purposes using the anterograde segment perfusion technique.

After the isolation procedure 30-60 % of the cells were rod shaped, showed intact striation, sharp edges and clear cytoplasm when the external calcium was restored to 2.5 mM. The experiments were started 2-3 hours after the isolation. The cells were stored in Minimum Essential Medium Eagle solution (pH=7.3) at 14 °C until use.

Preparation of HEK tsA-201 cells expressing $Ca_v1.2$ channels

CACNA1C plasmid, encoding for $Ca_v1.2$ channels, (a kind gift from Prof. Charles Antzelevitch) were co-transfected into HEK tsA-201 cells along with plasmids encoding for EGFP. EGFP positive transfectants were identified with a Nikon TE2000U fluorescence microscope. More than 70 % of the EGFP positive cells expressed the $Ca_v1.2$ ion channels. Currents were recorded 24 hours after transfection.

Electrophysiological measurements

When starting the experiments, myocardial cells were transferred to a thermoregulated plexiglass chamber (1 ml volume) allowing continuous superfusion with oxygenized Tyrode's solution (pH=7.4) at 37 °C. Axoclamp 2B (Axon Instruments) and MultiClamp 700B (Molecular Devices) amplifiers were used for the electrophysiological measurements. Output signals from the clamp amplifier were digitized using an A/D converter (Digidata 1440A, Molecular Devices) and recorded at a sampling rate of 100 kHz under software control (pClamp 6.0 and 10.0).

Borosilicate microelectrodes, having tip resistance of 2-3 MΩ, were used for the measurements in whole-cell configuration of patch-clamp technique. Ionic currents were normalized to cell capacitance, determined in each cell using short hyperpolarizing pulses from −10 mV to −20 mV. The series resistance was typically 4–8 MΩ before compensation (usually 50–80 %) prior to voltage clamp. Ca^{2+} current was separated during the experiments by using appropriate voltage commands and ion channel blockers.

Ionic current measurements with action potential voltage-clamp technique

Action potentials of cardiomyocytes were recorded in current clamp mode through the patch pipette, while the cells were continuously paced at steady-state stimulation frequency of 1 Hz using 1 ms wide rectangular current pulse with suprathreshold amplitude. The action potential obtained this way was delivered to the same cell at the identical frequency as command voltage after switching the amplifier to voltage clamp mode. The current trace obtained under these conditions was a horizontal line positioned at the zero level except for the capacitive transient. The profile of the TTX-sensitive current was determined by subtracting the postdrug curve from the predrug one. Therefore, the TTX-sensitive current appeared as an inwardly directed current.

Simulation of tetrodotoxin binding to $Ca_v1.2$ and $Na_v1.5$ channel proteins

The theoretical structure of the selectivity filter region of the voltage-gated $Na_v1.4$ channel in complex with tetrodotoxin has been previously published by Tikhonov and Zhorov, which was used as starting geometry to construct our models for the human $Na_v1.5$ and $Ca_v1.2$ in complexes with the tetrodotoxin molecule. The amino acid sequence homology for $Na_v1.4$ and $Na_v1.5$, as well as between $Na_v1.4$ and $Ca_v1.2$ is already known in details based on the publications of Sun et al. as well as Tikhonov and Zhorov, which were used without modification. In these models the highly conserved DEKA rings of Na^+ channels correspond to the conserved EEXX rings in Ca^{2+} channels. The residue mutations corresponding to $Na_v1.4$ to $Na_v1.5$ and $Ca_v1.2$ transformations were then carried out by using the YASARA software package. The energy of TTX-$Na_v1.5$ and TTX-$Ca_v1.2$ complex were minimized using the same software. The graphical representations of the complex geometries were prepared by the Chimera suites of software. The simulations were carried out using the Amber 12 software package, while the visual molecular dynamics (VMD) package was used for visualization.

Statistics

All values presented are arithmetic means±SEM. Statistical significance of differences was evaluated by using one way analysis of variance (ANOVA) followed by Student's t-test for paired or unpaired data, as appropriate. Differences were considered significant when the P value was less than 0.05.

The investigation was conducted in accordance with the „Guide for the Care and Use of Laboratory Animals" (US NIH publication no. 85-23., revised 1996) and the principles outlined in the Declaration of Helsinki. The study protocol was also approved by the local Ethical Committee of the University of Debrecen.

RESULTS

Inhibition of L-type Ca^{2+} channels by tetrodotoxin

First, the effect of TTX was studied at a relatively high concentration (30 μM), although that concentration of toxin is often used in order to achieve full suppression of cardiac sodium current in different studies. I_{Na} fell to zero, while $I_{Ca,L}$ was only partially, but significantly blocked by 30 μM TTX. The amplitude of $I_{Ca,L}$ decreased from 8.61±1.09 pA/pF in control to 6.08±1.05 pA/pF after perfusion of 30 μM TTX (n=4, $p<0.01$). Importantly, the onset of TTX-induced inhibition of $I_{Ca,L}$ was fast and fully reversible upon washout in every case.

$I_{Ca,L}$ was progressively suppressed by increasing concentrations of TTX. When data obtained in four cells were fitted to the Hill equation, an IC_{50} value of 55±2 μM was obtained. The Hill coefficient of unity (1.0±0.04) is congruent with a single binding site of TTX on the Ca^{2+} channel.

Since several inhibitors exert their effects by shifting the voltage-dependence of a current to more positive membrane potentials, the action of TTX on voltage-dependence of $I_{Ca,L}$ was also tested. TTX effectively and reversibly suppressed $I_{Ca,L}$ in the full voltage range studied, however, the current-voltage relationship was unaltered, so the maximal current amplitude could be measured at +5 mV, similar to control conditions.

Inactivation of $I_{Ca,L}$ followed biexponential kinetics in accordance with literature data, thus the drug effect on the time-dependence of $I_{Ca,L}$ inactivation was studied by biexponential fitting of the current decay. No significant changes in the decay time constants were observed; however, the amplitudes of both the fast and slow components were significantly decreased by 30 μM TTX.

It is important to know that the current recorded under our experimental conditions was, in fact, L-type Ca^{2+} current. To demonstrate this, the effect of 30 μM TTX was tested after pretreatment with nisoldipine, the potent and highly selective blocker of $I_{Ca,L}$. Nisoldipine blocked 93 % of $I_{Ca,L}$, and TTX failed to

modify the current significantly in the presence of nisoldipine. In another series of experiments, the current remaining after partial (30 μM) TTX blockade was successfully eliminated by nisoldipine. These results confirm that the measured current was indeed L-type calcium current.

The profile of an ion current may be markedly different when comparing under conventional voltage-clamp and action potential-clamp conditions. An advantage of the action potential clamp technique is that it enables us to record true current profiles flowing during an actual cardiac action potential. 30 μM TTX dissected an inward current with two peaks under action potential voltage-clamp conditions. The first inward peak consisted of two components, one of them as a result of the fast sodium channel inhibition, while the other one had a typical fingerprint of the L-type calcium channel block. However, the role of L-type calcium channels is definitively assumed in the formation of the second inward peak during the plateau phase of the action potential. It was formerly determined by our working group that the double-peaked calcium current is a characteristic feature of a subepicardial ventricular myocytes. Both inward current peaks disappeared after the washout of TTX, which implies that this effect of TTX was fully reversible. After that the experiment was repeated in the presence of nisoldipine. Where 30 μM TTX failed to dissect current, except for a narrow, early inward peak corresponding to sodium channel blockade. The effect of TTX was again fully reversible upon washout. These results strongly support the view that the TTX-sensitive current found in canine ventricular cells is really $I_{Ca,L}$.

Use-dependent properties of TTX-induced $I_{Ca,L}$ blockade

In the experiments above, $I_{Ca,L}$ was activated by 200 ms-long depolarisations delivered at a frequency of 0.2 Hz. In other words, the repetition time of stimulation was 5 s. This was long enough to maintain a reasonably

normal availability of $I_{Ca,L}$; therefore, the effect of TTX observed under these conditions was considered as the magnitude of tonic block. However, a significant rate-dependent component of block was built up in the presence of 10 μM TTX, when the cycle length of stimulation was decreased to 1 s. The magnitude of this rate-dependent block was 12.7±1.0 % *vs.* the 4.3±1.1 % decrease of $I_{Ca,L}$ observed without TTX. The 200 ms duration of these pulses did not allow to study of the effect of TTX at higher frequencies; therefore, short depolarisations, having durations of only 10 ms, were applied in control and in the presence of TTX. Surprisingly, these short pulses caused a rate-dependent enhancement of $I_{Ca,L}$ in the presence of 10 μM TTX, but not in control. Increases in $I_{Ca,L}$ amplitudes were larger at higher stimulation rates: increases of 2.1±1.1 %, 3.9±1.2 % and 6.3±1.2 % were obtained at cycle lengths of 5 s, 1 s and 0.3 s, respectively. These results indicate that in addition to the tonic block, an additional rate-dependent block is also evoked by TTX; however, the magnitude of this block was relatively moderate compared to the tonic component. More importantly, the TTX-induced inhibition of $I_{Ca,L}$ seems to be restricted to resting and inactivated channel states, since frequent opening of the channel (by using short depolarisations at high frequencies) results in a relief from the block, suggesting that the affinity of TTX to the open channel state is markedly reduced.

Effect of $I_{Ca,L}$ modulation on TTX-mediated inhibition

Effect of channel phosphorylation

Since phosphorylation of the channel protein is one of the most effective ways to control $I_{Ca,L}$ in many excitable and non-excitable tissues, the blocking effect of TTX was compared on native and PKA-phosphorylated Ca^{2+} channels. Forskolin is known to induce a stable activation of adenylate cyclase, resulting in high cAMP levels and, consequently, in full activation of protein kinase A, an

enzyme responsible for phosphorylation of the pore forming α_1 and the auxiliary β subunit of the Ca^{2+} channel. This phosphorylation – through allosteric interactions – effectively increases the open probability of the pore-forming α_1 channel subunit, while it has little effect on the selectivity filter. 3 μM forskolin increased the peak amplitude of $I_{Ca,L}$ (from 5.5 ± 0.4 pA/pF to 16.3 ± 0.8 pA/pF, n=5). TTX caused a concentration-dependent and reversible suppressive effect on $I_{Ca,L}$ in forskolin-treated cardiomyocytes. Furthermore, fitting the data to the Hill equation yielded an IC_{50} value of 50±5 μM (n=5), which is very similar to that obtained in control. The Hill coefficient was also close to unity (1.02±0.09) in the presence of forskolin.

Effect of extracellular pH

Our model, constructed to explain the TTX-sensitivity of $I_{Ca,L}$, predicts that TTX binds to the selectivity filter of the channel. More specifically, this connection is based on the negatively charged (at pH values close to the physiological range) selectivity filter region of the channel and the positively charged guanidine group of the toxin. Consequently, changing the strengths of this electrostatic interaction by increasing or decreasing the protonation of either the selectivity filter region of the channel or of the TTX molecule itself is expected to modulate the magnitude of the TTX-induced blockade. Our assumption was confirmed, alkalization significantly decreased (to 26±2 % at pH=8.4, n=6), while acidification increased (to 60±2 % at pH=6.4, n=6) the inhibition of $I_{Ca,L}$ caused by 55 μM TTX (which was estimated to be exactly 50 % at the physiological pH value of 7.4).

Effect of redox potential changes

The effects of redox potential changes were very similar to those initiated by changes in pH. Accordingly, in a strongly oxidant milieu, induced by

application of 100 μM H_2O_2, 55 μM TTX suppressed only 31±3 % of $I_{Ca,L}$ (n=5), in contrast to the 62±6 % $I_{Ca,L}$ blockade observed in a reductant environment (n=8, 1 mM reduced glutathione + 1 mM ascorbic acid + 1 mM dithiothreitol).

Simulation of TTX binding to $Na_v1.5$ és $Ca_v1.2$ channel proteins

In order to understand how TTX blocks voltage-gated $Ca_v1.2$ and $Na_v1.5$ channels in detail, structural information on their selectivity filter would be desirable. Based on the theoretical model elaborated originally for the $Na_v1.4$ Na^+ channel, a model describing the binding of TTX to cardiac $Na_v1.5$ and $Ca_v1.2$ channels has been proposed. In our model, similar to $Na_v1.4$ channels, the guanidinium group binds to D (aspartate) and E (glutamate) in repeats I and II, respectively. Other acidic residues at the selectivity filter region may also contribute to ligand binding either directly or indirectly. This interaction is carried out by a formation of a negative electrostatic potential in the ligand binding cavity. A hydrophobic site of TTX interacts with the side chain of tryptophan (W), which residues (W904, W1421) assumed to play a role in ion permeation, are likely to contribute to binding of TTX.

Despite the even more acidic highly conserved EEEE ring of the selectivity filter region of $Ca_v1.2$, only one E can form a salt bridge with TTX (E736), when it is bound to $Ca_v1.2$. In addition to that, possibly a subsequent aspartate side chain (both from repeat II) can contribute to binding of the TTX. A few other residues can participate in TTX binding; however, their contribution seems to have less importance. It should also be mentioned that the conserved W residues (W1145, W1146) may have non-negligible roles in forming the ligand binding cavity and/or in the lipophilic interaction of ligand binding. According to our model, it is also evident that the number of residues making direct contact with the TTX molecule is less in $Ca_v1.2$ than in $Na_v1.5$. These results predict a weaker interaction between the $Ca_v1.2$ and TTX than in the complex of the

$Na_v1.5$ and TTX, which ultimately results in a diminished blocking potency of TTX against $Ca_v1.2$ channels.

Since the pK_a value (8.7) of the most acidic OH in the TTX molecule is relatively close to the pH range of 6.4–8.4 applied in our experiments, both the protonated form (i.e., positively charged due to the guanidinium group and the C10-OH) and the neutral form (the guanidinium group of TTX is still protonated, but the C10–OH is deprotonated to C10-O^-) of TTX were considered in our calculations. Docking experiments revealed that TTX binds to the selectivity filter in both of its states; however, the binding is stronger (8.22 kcal/mol *vs.* 7.55 kcal/mol) when TTX is positively charged (i.e., when the proton is not dissociated from the C10-OH position). This can be the consequence of the dominantly negative formal charge of the selectivity filter.

TTX fails to inhibit $Ca_v1.2$ channels expressed in HEK cells

Surprisingly, putting a new face on the matter, TTX failed to induce any significant inhibitory effect on $Ca_v1.2$ channels expressed in HEK tsA-201 cells by transfection of CACNA1C gene. $Ca_v1.2$ currents, measured after perfusion of 30 and 100 μM TTX, were 97.81±1.75 % and 94.06±3.87 % of control, respectively (not significant, n=5).

When comparing the kinetic properties of $Ca_v1.2$ current and $I_{Ca,L}$ by applying normalization from peak to pedestal values of the current, a reasonably good overlap could be achieved. This indicates that many properties of the transfected $Ca_v1.2$ current are identical or very similar to those of the native $I_{Ca,L}$ - in spite of the absence of several auxiliary subunits (α_2, β and δ) from the former. Indeed, the current mediated by the single pore forming subunit (α_1) showed properties similar to those of the native current - except for sensitivity to TTX.

DISCUSSION

Importance of cardiac L-type Ca^{2+} channel inhibition by tetrodotoxin

Although TTX and STX are generally believed to be selective inhibitors of fast Na^+ current, data in literature are accumulating to indicate that this is not exactly the case. For instance, both toxins have been demonstrated to interact with native and cloned T-type Ca^{2+} channels, while STX was shown by Su *et al.* to inhibit L-type Ca^{2+} channels. However, this is the first report to demonstrate the inhibitory action of TTX on cardiac $I_{Ca,L}$.

Cardiac I_{Na} (mediated by $Na_v1.5$ channels) is relatively insensitive to TTX compared to that of skeletal muscle and neural Na^+ channels. Indeed, a concentration of 30 μM was required to fully suppress I_{Na} in ventricular cardiomyocytes. Since this high concentration of TTX suppressed 35 % of $I_{Ca,L}$ in our canine ventricular cardiomyocytes, serious errors can thus be introduced, depending on experimental approach. The problem is especially accentuated when trying to study late Na^+ current using TTX under action potential voltage-clamp conditions. Chorvatova *et al.* demonstrated that the amplitude of this current is in the range of some tens of picoamperes in healthy myocytes which was shown to increase up to 200 pA in hypertrophied hearts. It is easy to imagine the magnitude of error introduced by blocking only a small fraction of $I_{Ca,L}$ by TTX. Similarly, the reported hypertrophy-induced changes in the late Na^+ current might seriously be contaminated by the TTX-induced suppression of $I_{Ca,L}$.

Based on our results, native $I_{Ca,L}$ was shown to be TTX-sensitive in canine ventricular myocytes. Since these toxins still appear to be relatively selective to I_{Na} over $I_{Ca,L}$, they are widely applied in biomedical research. However, depending on the experimental conditions, extra care must be taken when using these agents to block I_{Na} selectively.

Molecular determinants of interaction between tetrodotoxin and L-type Ca^{2+} channels, explanation the effects of pH- and redox potential changes

The 55 μM value of IC_{50} obtained with TTX for $I_{Ca,L}$ is relatively high, even if considering that the cardiac Na^+ channel isoform ($Na_v1.5$) shows much lower affinity to TTX than the neural ($Na_v1.1$–1.3) or skeletal ($Na_v1.4$) ones. The aromatic side chain of the residue following the DEKA ring builder Asp residue was proposed to play a critical role in the specific TTX-ion channel interaction, and its replacement (e.g., to Cys residue in $Na_v1.5$) may be the main reason for the reduced affinity. In the sequence alignment proposed by Tikhonov and Zhorov, which was used when building the model in the present study, a Gly residue can be found at this key position. This fact can plausibly explain why $Ca_v1.2$ channels bind TTX with considerably lower affinity than neural or skeletal Na^+ channels.

Regarding the effect of pH on the amplitude of $I_{Ca,L}$, the following interpretation can be given. Considering the approximately 6.5 pK_a value of histidine, its imidazole side chain can be protonated, even in a mild acidic milieu. Accordingly, the histidine residues located in the conduction pathway of the channel (including the selectivity filter, as well) are at least partially protonated at the acidic pH of 6.4, which resulted in a less favourable environment for a positively charged ion. In addition, one (or even more than one) of the glutamate side chains at the highly negatively charged EEEE ring of the channel can be protonated, too, which can further reduce cationic current. Indeed, similarly to our results, $I_{Ca,L}$ and I_{Na} have been reported to decrease in acidic milieu. On the other hand, TTX exists almost exclusively in its positively charged form at pH 6.4, since the pK_a value of the most acidic C10 hydroxyl group is close to 8.7. Therefore, the positively charged TTX molecule is expected to increase TTX binding (compared to the neutral zwitterionic form, which is dominant at the higher, alkaline pH range, but also existing partially at

pH=8.4 and in a small fraction even at pH=7.4) to the negatively charged selectivity filter region resulting in a stronger inhibition of $I_{Ca,L}$. Furthermore, due to the reduced strength of electrostatic interactions (which is valid for both the double charged Ca^{2+} and – although to a lesser extent – for the protonated TTX molecule, because of its single positive charge), the relative contribution of the non-ionic hydrophilic and hydrophobic terms in the interaction between TTX and the selectivity filter of the channel is likely to increase. All these effects resulted in a reduction of $I_{Ca,L}$ in acidic environment, which could be reduced by TTX in a higher degree. In contrast, at the alkaline pH of 8.4, most of the histidine side chains are neutral and largely half of the free cysteine SH groups can be deprotonated, since the corresponding pK_a is 8.5. This is certainly a more favourable environment (even compared to the neutral situation, *i.e.*, to the case of pH=7.4) for attracting and binding of the positively charged Ca^{2+} ions to the channel; consequently, the ion flux through the channel may increase. TTX may exist partially in its neutral form at mild alkaline pH values, due to deprotonation of the C-10 hydroxyl group, allowing for development of a weaker electrostatic attraction between TTX and the selectivity filter. The stronger Ca^{2+} channel interaction combined with a weaker interaction between TTX and the channel protein necessarily yields a smaller inhibition of $I_{Ca,L}$ by TTX under alkaline conditions. The increased amplitude of $I_{Ca,L}$ and the decreased TTX blockade, observed at alkaline milieu, can easily be interpreted based on the simplified electrostatic model and the protonation equilibrium of the zwitterionic TTX outlined above in good agreement with the pH dependence of the TTX blockade observed on Na channels by Ulbricht and Wagner. The increased inhibitory effect of TTX on $I_{Ca,L}$ at acidic pH seems to contradict the earlier results in the literature regarding I_{Na} inhibition, suggesting a reduced TTX block at low pH values. It is possible that at pH=6.4, the conformation of the selectivity filter region of the Ca^{2+} channel may change in a way, becoming more suitable for TTX-binding. Moreover, the 3 %–5 % non-effective

(zwitterionic) fraction of TTX, estimated at pH=7.4, is converted into cationic form at pH=6.4, which may also contribute to the increase of the TTX-induced block.

The role of the redox potential in modulating $I_{Ca,L}$ and its suppressibility by TTX needs some more effort to be interpreted. In line with our observations, hydrogen peroxide has been shown by Guo *et al.* to increase the amplitude of $I_{Ca,L}$ in various mammalian cardiac tissues, while Zhang *et al.* described that the current was decreased by a reductant environment. Since H_2O_2 is a strong oxidant, it oxidizes the free sulfhydryl groups to disulfide bonds. In contrast, a reducing environment may prevent the formation of disulfide bridges or break them once already preformed. These disulfide bridges seem to stabilize a structure relatively resistant to TTX binding; thus, the inhibitory action of TTX on $I_{Ca,L}$ increases in a reductant environment, while it decreases in the presence of H_2O_2. An additional explanation arises when considering that the density of $I_{Ca,L}$ correlates well with oligomerization of the $Ca_v\beta$ subunits of Ca^{2+} channels (demonstrated by Lao *et al.*). Assuming that H_2O_2 can stabilize these oligomeric states by the formation of disulfide bridges, both the higher $I_{Ca,L}$ amplitudes, as well as the weaker inhibition by TTX can be explained.

Finally, it must be noted that the effects of a reductant milieu, both on the amplitude of $I_{Ca,L}$ and on its suppressibility by TTX, were very similar to those of acidification. This strongly suggests that application of a reducing milieu may facilitate the protonation of some sensitive groups – in spite of the large buffering capacity of the system. This might explain the good coincidence observed in the TTX-induced inhibition of $I_{Ca,L}$, comparing it in an acidic *vs.* alkaline and in a reductant *vs.* oxidant milieu.

The results obtained with the application of forskolin suggest that the protein-kinase A-mediated phosphorylation-induced allosteric changes in the structure of the α_1 subunit, although having serious consequences on channel gating, have little influence on TTX binding. The value of unity obtained for the

Hill coefficients is congruent with the presence of one single TTX-binding site on the α_1 subunit.

Possible explanations for the lack of TTX inhibition of $Ca_v1.2$ channels expressed in HEK tsA-201 cells

This is exactly the opposite of that one could expect from the results obtained in native canine myocytes. To explain these paradoxical findings there are numerous other factors, having an influence on the structure and function of the Ca^{2+} channel, to be taken into account. The most plausible explanation for these findings might be to postulate that TTX is bound to some of the auxiliary subunits. However, previous results, based on detailed investigations of TTX-sensitivity as a function of pH, redox potential and channel phosphorylation, completed with 3D modelling of the protein structure of the channel, clearly showed that the binding site for TTX is located in a similar position in both Ca^{2+} and Na^{+} channels, which is an integral part of the selectivity filter of the conducting pore. Of course, the possibility of TTX-binding to any of the auxiliary subunits can not be fully excluded (from this point of view, α_2 might be the most relevant candidate due to its extracellular position), but in this case the chemical environment of this hypothetical binding site should be very similar to that of the selectivity filter, which option, however, seems to be quite unlikely. It is more reasonable to assume that TTX is bound to the pore-forming α_{1C} subunit (as it has been proposed earlier), but – as a consequence of allosteric interactions – only in the presence of one or more auxiliary subunit. Since there are further auxiliary subunits in the native cells, these may allosterically alter the final assembly of channel structure. Furthermore, several splice variations, specific for the given cell types, exist for the α_1 subunit and each individual splice variant might carry a different sensitivity for antagonists.

Alternatively, it is also possible that the expression system itself is responsible for the observed TTX-insensibility, since it is not exceptional that a well documented effect on Ca^{2+} current can not be reproduced in an expression system. There is a wide range of theoretical options in this case, including the differences in the lipid environment, variations in internal electrolyte composition, as well as the presence or absence of important second messengers, like cAMP, cGMP, or $[Ca^{2+}]_i$, which factors may also modify the channel function resulting in differences in TTX-sensitivity as well. Until a satisfactory explanation for the TTX-insensitivity of the $Ca_v1.2$ channels expressed in HEK cells is not found, extra cautiousness is required when interpreting results of electrophysiological and pharmacological studies performed using expression systems. In any case, TTX increases the number of agents which exert Ca^{2+} channel blocking activity in addition to their well-known specific actions.

Limited selectivity of drugs action on voltage-gated Na^+ and Ca^{2+} channels

It is not exceptional that a drug believed to selectively interact with I_{Na} binds to and thus modifies other transmembrane ion channels such as L-type Ca^{2+} channels. These cross-reactions can be well explained with the known similarities in the structures of Na^+ and Ca^{2+} channels.

According to the results obtained with TTX, one may conclude that it has a some tens of nanomolar concentration of IC_{50} values in neuronal and skeletal muscle Na_v channels, about hundreds to thousand times higher IC_{50} in cardiac $Na_v1.5$ channel, and similar in T-type Ca^{2+} channels, whereas compared to cardiac Na^+ channels ($Na_v1.5$) again about 10 to 50 times higher IC_{50} on L-type Ca^{2+} channels. These studies on the effects of tetrodotoxin provided congruent results with the evolution of voltage-gated Na^+ and Ca^{2+} channels consisting of

four homologous domains from a common ancestor. The previously mentioned data support the fact that closer similarity can be detected in the pore region of classical cardiac Na^+ channels to T-type Ca^{2+} channels than to L-type Ca^{2+} channels.

Therefore, it seems to be a general property that almost every agent exerting an effect on Na^+ or Ca^{2+} channels also influences the other structurally homologous channel subtypes. The explanation of this phenomenon can be found in the similarities observed in the structures and amino acid sequences of the channels generating $I_{Ca,L}$, $I_{Ca,T}$ and I_{Na}. In summary, cross actions between these four domain channels seem to be inescapable, and therefore it is crucial to be considered in future drug design, medical therapy and experimental investigations. Understanding of this feature may be useful not only when applying cardioactive agents, but also in the action of other drugs, such as antiepileptics, antidepressants, and medications of neurodegenerative diseases or chronic pain.

SUMMARY

Background: Tetrodotoxin (TTX) is believed to be the most selective inhibitor of voltage-gated fast Na^+ channels in excitable tissues, including nerve, skeletal muscle, and heart, although TTX-sensitivity of the latter is lower than that of the formers by at least three orders of magnitude.

Experimental approach: In the present study the TTX sensitivity of L-type Ca^{2+} current ($I_{Ca,L}$) was studied in isolated canine ventricular cells using conventional voltage-clamp and action potential-clamp techniques, at 37 °C.

Results: TTX was found to block $I_{Ca,L}$ in a dose-dependent and reversible manner without altering its inactivation kinetics. Fitting results to the Hill equation an IC_{50} value of 55±2 μM was obtained with a Hill coefficient of 1.0±0.04. $I_{Ca,L}$ changed from 8.61±1.09 pA/pF to 6.08±1.05 pA/pF in the presence of 30 μM TTX (n=4; p<0.01). The current was fully abolished by 1 μM nisoldipine indicating that it was indeed $I_{Ca,L}$. Under action potential voltage clamp conditions the TTX-sensitive current displayed a fingerprint of $I_{Ca,L}$, which was absent in the presence of nisoldipine. Phosphorylation of the channel protein (induced by forskolin) failed to modify the inhibitory potency of TTX, an IC_{50} value of 50±4 μM was found in the presence of 3 μM forskolin. TTX in 55 μM concentration (IC_{50} value obtained under physiological conditions) caused 60±2 % inhibition of $I_{Ca,L}$ in acidic (pH=6.4), while only a 26±2 % block in alkaline (pH=8.4) milieu. Similarly, the same concentration of TTX induced 62±6 % suppression of $I_{Ca,L}$ in a reductant milieu (containing glutathione + ascorbic acid + dithiothreitol, 1 mM each), in contrast to the 31±3 % blockade obtained in the presence of a strong oxidant (100 μM H_2O_2). These results are in a good accordance with the predictions of our model, indicating that TTX binds, in fact, to the selectivity filter of cardiac L-type Ca^{2+} channels.

Conclusions: This is the first report demonstrating that TTX inhibits L-type calcium current in the heart. Since this toxin still appears to be more selective to Na^+ current (I_{Na}) than $I_{Ca,L}$, it is widely applied in the biomedical research. However, extra care must be taken when using this agent to block I_{Na} selectively, depending on the experimental conditions. Since voltage-gated Ca^{2+} and Na^+ channels derive from an evolutionary common ancestor, the poor selectivity of agents acting on these channels indicates an intrinsic property of drug receptors, which has to be taken into account when designing new cardioactive compounds for either medical therapy or experimental research in the future.

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List of publications related to the dissertation

1. Szentandrássy, N., Papp, F., **Hegyi, B.**, Bartók, Á., Krasznai, Z., Nánási, P.P.: Tetrodotoxin blocks native cardiac L-type calcium channels but not CaV1.2 channels expressed in HEK cells.
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