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Short Communication

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TETRODOTOXIN BLOCKS NATIVE CARDIAC L-TYPE CALCIUM CHANNELS BUT NOT $CA_V1.2$ CHANNELS EXPRESSED IN HEK CELLS

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Tetrodotoxin (TTX) has been believed for a long time to be a selective inhibitor of voltage-gated fast Na^+ channels in excitable tissues, including mammalian myocardium. Recently TTX has been shown to block cardiac L-type Ca^{2+} current ($I_{Ca,L}$). Furthermore, this inhibition was ascribed to binding of TTX to the outer pore of the Ca^{2+} channel, contributing to the selectivity filter region. In this study the TTX-sensitivity of $Ca_v1.2$ channels, expressed in HEK cells, was tested using the whole cell version of the patch clamp technique and compared to the TTX-sensitivity of native canine $I_{Ca,L}$. $Ca_v1.2$ channels mediate Ca^{2+} current in ventricular myocardium of various mammalian species. Surprisingly, TTX failed to inhibit $Ca_v1.2$ current up to the concentration of 100 μM - in contrast to $I_{Ca,L}$ - in spite of the fact that the kinetic properties of the $I_{Ca,L}$ and $Ca_v1.2$ currents were similar. The possible reasons for this discrepancy are discussed. Present results may question the suitability of a single pore-forming channel subunit, expressed in a transfection system, for electrophysiological or pharmacological studies.

Key words: *calcium channels, dog heart, tetrodotoxin, voltage clamp, ventricular myocytes, calcium, cell transfection, sodium channels*

INTRODUCTION

It is generally believed that the marine guanidine toxin tetrodotoxin (TTX), similarly to saxitoxin, is a highly selective inhibitor of voltage-gated Na^+ channels in mammalian excitable tissues including nerve, skeletal muscle, and heart, although the TTX-sensitivity of the latter is lower than the formers by at least three orders of magnitude (1). Therefore very high TTX concentrations (sometimes tens of micromoles) have to be applied in order to achieve a full suppression of Na^+ current in voltage clamped cardiac cells (2, 3), except for the Na^+ channels of the fish heart which displays TTX-sensitivity in the nanomolar range (4). Even this relative selectivity to Na^+ channels was questioned by a recent study demonstrating that TTX blocks L-type Ca^{2+} current ($I_{Ca,L}$) in ventricular cardiomyocytes isolated from healthy dogs (5), *i.e.* in a preparation having electrophysiological properties most similar to those of human ventricular myocardium regarding the distribution and kinetic properties of transmembrane ion currents (6, 7). The blocking effect of TTX on $I_{Ca,L}$ had an EC_{50} value of 55 μM, it was readily reversible upon washout and could be fully prevented by nisoldipine-pretreatment indicating that it was indeed $I_{Ca,L}$ being inhibited by TTX (5). In addition, in a more recent study, the TTX-induced inhibition of $I_{Ca,L}$ was shown to be a consequence of TTX binding to the outer channel pore, contributing to the selectivity filter region (8). If so, *i.e.* if the TTX-binding site is carried by the pore-forming channel subunit, it is expected also to be suppressible by TTX when expressed in a transfected cell. This hypothesis was tested in the present study by examining the TTX-sensitivity of the current flowing through $Ca_v1.2$ channels ($I_{Cav1.2}$) expressed in HEK cells. These channels mediate Ca^{2+} current in ventricular myocardium of various mammalian species including dogs and humans (9-11). Quite surprisingly, TTX failed to inhibit $Ca_v1.2$ channels up to the concentration of 100 μM in spite of the fact that the kinetic properties of the $I_{Ca,L}$ and $Ca_v1.2$ currents were similar. These results reveal the limitations of using a single pore-forming channel subunit for electrophysiological and pharmacological studies.

MATERIALS AND METHODS

Measurement of $Ca_v1.2$ current in transfected HEK cells

HEK tsA-201 cells, characterized by Graham *at al*. (12), as human embryonic kidney (HEK) cells, were grown in Dulbecco's minimum essential medium - high glucose supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin-G, and 100 μg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2 and 95% air-humidified atmosphere. Cells were passaged twice per week after 7-min incubation in a PBS solution containing 0.2 g/L EDTA. 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. Transfections were done at an EGFP: channel DNA molar ratio of 1:10 using lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA), and cultured under standard conditions. Currents were recorded 24 hours after transfection. 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.

Whole-cell currents were measured in voltage-clamped cells using Multiclamp 700B and Axopatch 200A amplifiers connected to a personal computer using Axon Instruments Digidata 1440 or 1320 data acquisition boards (Molecular Devices, Sunnyvale, CA, USA). Series resistance compensation up to 70% was used to minimize voltage errors and achieve good voltage-clamp conditions. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries in five stages and fire-polished, resulting in electrodes with 4–6 M of resistance in the bath. The normal bath solution contained (in mM) NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2.5; glucose, 5.5; HEPES, 10 (pH=7.35, 305 mOsm). In the case of calcium-free external solution the concentration of $MgCl_2$ was elevated to 3.5 mM and the bath was supplemented with 1 mM EGTA. The pipette solution consisted of (in mM) KF, 140; $MgCl_2$, 2; $CaCl_2$, 1; HEPES, 10; EGTA, 11; at pH 7.22. The KF content of the pipette solution helped to maintain stable gigaseals. Perfusion was achieved using a continuous perfusion system driven by gravity. Current through $Ca_v1.2$ channels were recorded at 0 mV using test pulses of 100 ms duration arising from the holding potential of –100 mV. These test pulses were delivered every 15 s. In some experiments the I-V relation of peak $Ca_v1.2$ curren was studied at various test potentials. For data acquisition and analysis, pClamp 9 and 10 software packages (Molecular Devices, Sunnyvale, CA, USA) were applied. Current signals were low-pass filtered using the analog four-pole Bessel filters of the amplifiers and sampled at 10 kHz.

Measurement of L-type Ca^{2+} current in canine ventricular myocytes

Adult beagle dogs of either sex were anaesthetized with intravenous injections of 10 mg/kg ketamine hydrochloride (Calypsol, Richter Gedeon, Budapest, Hungary) + 1 mg/kg xylazine hydrochloride (Sedaxylan, Eurovet Animal Health BV, Bladel, The Netherlands) according to a protocol approved by the local ethical committee and conforming to the Declaration of Helsinki. The hearts were quickly removed and placed in Tyrode solution. Single myocytes were obtained by enzymatic dispersion using the segment perfusion technique as previously described (13, 14).

The rod-shaped viable cells showing clear striation were sedimented in a plexiglass chamber allowing continuous superfusion with oxygenized Tyrode solution containing (in

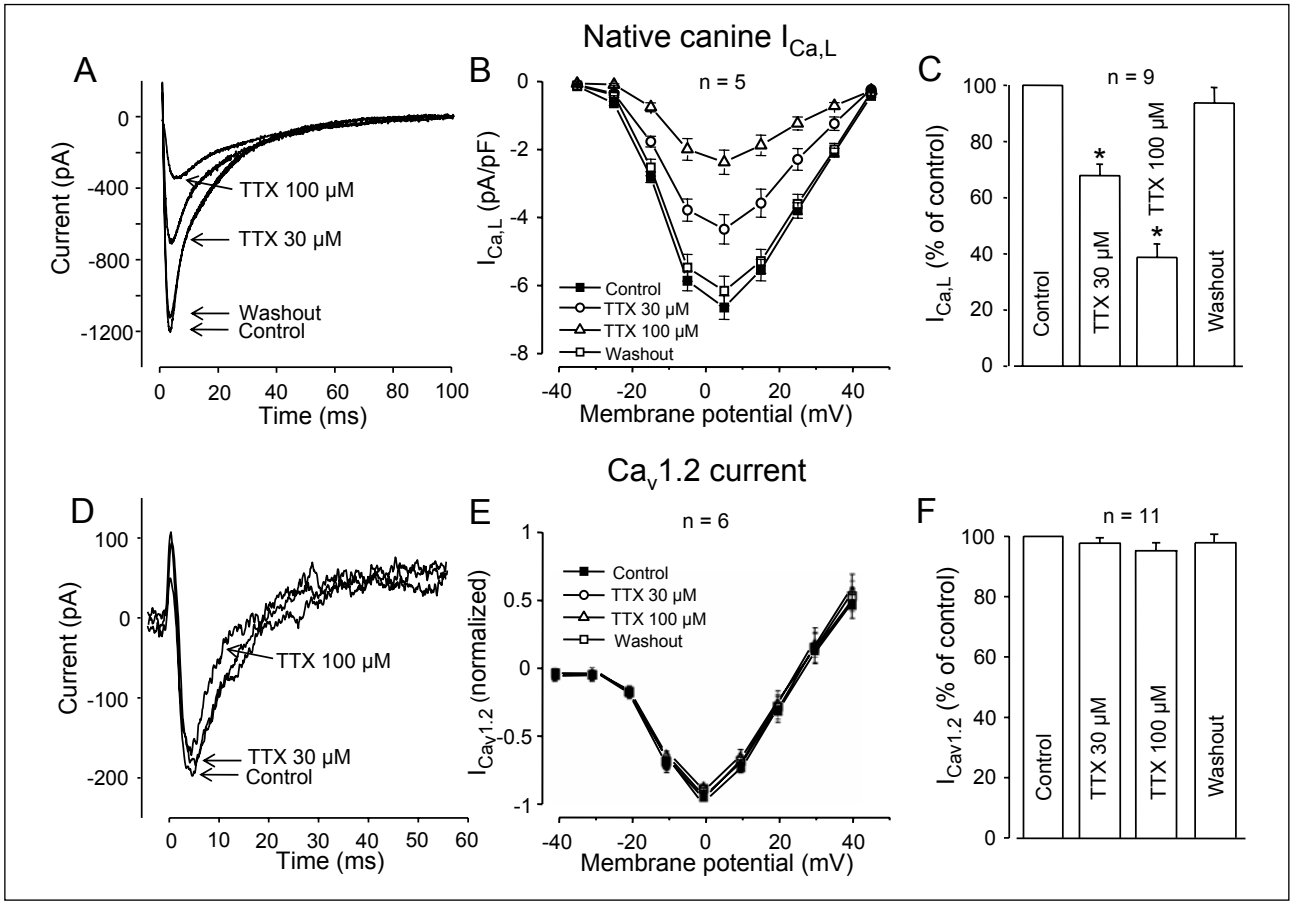

Fig. 1. Effects of TTX on calcium current in native canine ventricular myocytes (*A-C*) and in HEK cells transfected with $Ca_v1.2$ channels (*D-F*). *A*, *D*: Superimposed representative $I_{Ca,L}$ and $Ca_v1.2$ current records, respectively, obtained before and in the presence of 30 µM and 100 µM TTX (washout was also performed in canine myocytes). Only a shorter initial segment of the full records are shown. *B*, *E*: Current-voltage relationships obtained for $I_{Ca,L}$ and $Ca_v1.2$, respectively. Peak currents, normalized to cell capacitance, were plotted against the respective test potential in myocytes, while the currents were normalized to the corresponding control values in transfected HEK cells. *C*, *F*: Average results showing the TTX-induced blockade of $I_{Ca,L}$ and $Ca_v1.2$, measured at +5 and 0 mV, respectively. In both cases peak currents, recorded in the presence of TTX, were normalized to the pre-TTX control values. Columns and bars are means ± S.E.M., asterisks denote significant changes from control, n denotes the number of experiments.

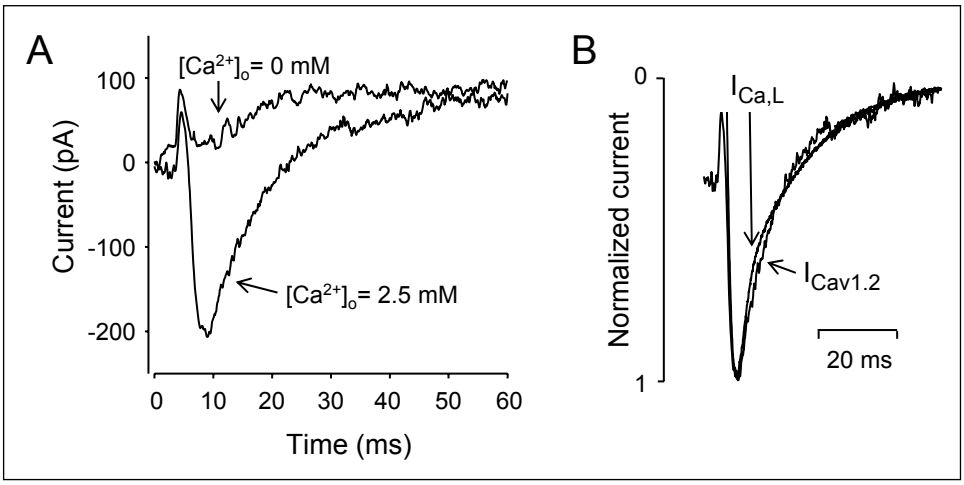

Fig. 2. A: Superimposed $Ca_v1.2$ current records obtained in the presence of normal (2.5 mM) external Ca^{2+} concentration and after removal of Ca^{2+} from the bathing medium.
B: Superimposed representative $I_{Ca,L}$ and $Ca_v1.2$ current records. For the better comparability currents were normalized to unity by matching their peak and pedestal values while keeping original temporal resolution.

mM): NaCl, 140; KCL, 5.4; $CaCl_2$, 2.5; $MgCl_2$, 1.2; Na_2HPO_4, 0.35; HEPES, 5; glucose, 10; pH=7.4 at 37°C. Suction pipettes, fabricated from borosilicate glass, had tip resistance of 2 M Ω after filling with pipette solution containing in (mM): KCl, 110; KOH, 40; HEPES, 10; EGTA, 10; TEACl, 20; K-ATP, 3; pH=7.2. $I_{Ca,L}$ was recorded in Tyrode solution, completed with 3 mM 4-aminopyridine to suppress transient outward current, with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) using the whole cell configuration of the patch clamp technique (15). The current was activated at +5 mV using 400 ms long depolarizations arising from the holding potential of –40 mV. In some experiments the I-V relation of peak $I_{Ca,L}$ was studied at test potentials ranging between –35 and +45 mV. After establishing high (1–10 GΩ) resistance seal by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by further suction or by applying 1.5 V electrical pulses for 1 ms. The series resistance was typically 4–8 MΩ before compensation (usually 50–80%). Experiments were discarded when the series resistance was high or substantially increasing during the measurement. Outputs from the clamp amplifier were digitized at 100 kHz under software control (pClamp version 9, Molecular Devices, Sunnyvale, CA, USA). Ion currents were normalized to cell capacitance, which was determined in each cell using short hyperpolarizing pulses from –10 mV to –20 mV. The effects of 30 μM and 100 μM TTX were tested in a cumulative manner being each concentration superfused for 3 min, washout lasted for 10 min. These incubation and washout periods were sufficient to develop steady-state drug effects and practically full reversion.

Results are expressed as mean ± S.E.M. values. Statistical significance of differences was evaluated using Student's t-test for paired data. Differences were considered significant when p was less than 0.05. Drugs not specified otherwise were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

RESULTS

For the sake of comparability, first the effect of 30 and 100 μM TTX is demonstrated on the amplitude of $I_{Ca,L}$ recorded from isolated canine ventricular cells. As shown in *Figs. 1A-1C*, these concentrations of TTX effectively and reversibly suppressed $I_{Ca,L}$ in the full voltage range studied (between –35 and +45 mV (*Fig. 1B*). Present results are fully congruent with previous data on the TTX-suppressibilty of native $I_{Ca,L}$ (5, 8).

In contrast to $I_{Ca,L}$, similar concentrations of TTX failed to cause any significant change in the amplitude of $Ca_v1.2$ current, expressed in HEK cells (*Figs. 1D-1F*). This current was really mediated by Ca^{2+}, as indicated by its I-V relationship (*Fig. 1E*) and because it was almost fully abolished after removal of Ca^{2+} from the external solution (*Fig. 2A*). 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 (*Fig. 2B*). 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

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 modeling 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 (8), which is an integral part of the selectivity filter of the conducting pore (16, 17). 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_1 subunit (as it has been proposed earlier), but - as a consequence of allosteric interactions - only in the presence of one or more auxiliary subunit.

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 (18). 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 $[Ca^{2+}]_i$. Until finding satisfactory explanation for the TTX-insensitivity of the $Ca_v1.2$ channels expressed in HEK cells, 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 (19, 20).

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Conflict of interests: None declared.

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