RESEARCH PAPER

The opioid methadone induces a local anaesthetic-like inhibition of the cardiac Na⁺ channel, Na_v1.5

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BACKGROUND AND PURPOSE

Treatment with methadone is associated with severe cardiac arrhythmias, a side effect that seems to result from an inhibition of cardiac hERG K⁺ channels. However, several other opioids are inhibitors of voltage-gated Na⁺ channels. Considering the common assumption that an inhibition of the cardiac Na⁺ channel Na_v1.5, is the primary mechanism for local anaesthetic (LA)-induced cardiotoxicity, we hypothesized that methadone has LA-like properties leading to a modulation of Na_v1.5 channels.

EXPERIMENTAL APPROACH

The whole-cell patch clamp technique was applied to investigate the effects of methadone on wild-type and mutant human Na_v1.5 channels expressed in HEK293 cells. A homology model of human Na_v1.5 channels was used to perform automated ligand-docking studies.

KEY RESULTS

Methadone inhibited Na_v1.5 channels in a state-dependent manner, that is, tonic block was stronger with inactivated channels than with resting channels and a use-dependent block at 10 Hz. Methadone induced a concentration-dependent shift of the voltage dependency of both fast and slow inactivation towards more hyperpolarized potentials, and impaired recovery from fast and slow inactivation. The LA-insensitive mutants N406K and F1760A exhibited reduced tonic and use-dependent block by methadone, and docking predictions positioned methadone in a cavity that was delimited by the residue F1760. Dextromethadone and levomethadone induced discrete stereo-selective effects on Na_v1.5 channels.

CONCLUSIONS AND IMPLICATIONS

Methadone interacted with the LA-binding site to inhibit Na_v1.5 channels. Our data suggest that these channels are a hitherto unrecognized molecular component contributing to cardiac arrhythmias induced by methadone.

Abbreviations

LA, local anaesthetic

Introduction

Methadone is a potent synthetic opioid commonly used not only for treatment of chronic and neuropathic pain, but also

for maintenance therapy after opioid and heroin abuse. Unlike all other clinically used opioids, therapeutic dosages of methadone can trigger ventricular arrhythmias such as torsade de pointes tachycardias, which appear to be

responsible for cases of sudden cardiac death in patients receiving methadone (Walker *et al.*, 2003; Chugh *et al.*, 2008). These proarrhythmic properties of methadone are believed to be due to a prolonged time for repolarization in the myocardium (Kornick *et al.*, 2003; Krantz *et al.*, 2003; Andrews *et al.*, 2009). In a clinical setting, these effects present as a prolongation of the QT interval and ultimately to the typical pattern of torsade de pointes tachycardias. Like several other drugs known to induce a long QT syndrome, methadone was demonstrated to inhibit cardiac hERG K⁺ channels (Katchman *et al.*, 2002; Eap *et al.*, 2007; Grilo *et al.*, 2010; channel nomenclature follows Alexander *et al.*, 2013). Methadone is a chiral substance commonly applied as a racemic mixture, and the stereoisomers induce distinct analgesic and proarrhythmic effects. Although levomethadone predominantly induces analgesia via the μ -opioid receptor, dextromethadone has very little activity on opioid receptors but seems to be mainly responsible for the inhibition of hERG K⁺ channels and thus for cardiac toxicity (Kristensen *et al.*, 1995; Eap *et al.*, 2007).

Because multiple ion channels are required for a proper electrogenesis in the heart, it seems likely that multiple mechanisms underlie the cardiac toxicity of methadone. Although the local anaesthetic (LA) bupivacaine inhibits hERG K⁺ channels as well (Gonzalez *et al.*, 2002), its high cardiotoxic potential rather seems to be due to an inhibition of the cardiac Na⁺ channel, Na_v1.5 (Clarkson and Hondeghem, 1985; Butterworth, 2010). Like other LAs, bupivacaine inhibits Na⁺ channels by interacting with a LA-binding site located in the pore cavity (Nau *et al.*, 2000b; Nau and Wang, 2004). Similar to the action of methadone on hERG channels, this inhibitory effect of bupivacaine on Na_v1.5 channels is stereoisomer-dependent, with R(+) bupivacaine being more potent than S(−) bupivacaine (Valenzuela *et al.*, 1995).

We and other laboratories have shown that opioids such as buprenorphine, sufentanil and fentanyl are inhibitors of voltage-gated Na⁺ channels, and that they can specifically interact with the LA-binding site (Haeseler *et al.*, 2006; Leffler *et al.*, 2012). However, the plasma levels of these opioids are lower than the concentrations required for a substantial block of Na⁺ channels. In this study, we investigated if methadone interacts with Na_v1.5 channels in a way that could contribute to its high cardiotoxic potential. To address this question, we performed standard whole-cell patch clamp recordings on human Na_v1.5 channels expressed in HEK293 cells. We found that methadone inhibits Na_v1.5 channels at clinically relevant concentrations, but in a rather stereo-nonselective manner. Experiments on LA-insensitive mutant constructs of Na_v1.5 channels as well as computational docking predictions suggest that methadone may interact with the canonical LA receptor site to inhibit Na_v1.5 channels.

Methods

Cell culture and heterologous expression

The cDNA of human Na_v1.5 channels in pTracer was a generous gift from Dr Dirk Isbrandt (Hamburg, Germany). Using HEK293 cells, we constructed stably expressing hNa_v1.5-HEK293 cells. Cells were cultured in DMEM (Gibco-

Invitrogen, Darmstadt, Germany), supplemented with 10% FBS (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Gibco-Invitrogen) and 0.4% Zeocin (Gibco-Invitrogen). Cells were held in cell culture flasks at 37°C in air with 5% CO₂. Mutagenesis of N406K and F1760A was performed according to the instructions of the manufacturer (QuikChange XL Kit; Qiagen GmbH, Hilden, Germany). HEK293t cells were transfected with F1760A (2 μ g) or N406K (5 μ g) mutant channels by using a NanoFectin transfection kit (PAA Laboratories GmbH, Pasching, Austria). To visualize expression for patch clamp experiments, cells were co-transfected with pEGFP-N1 (0.5 μ g; Clontech, Palo Alto, CA, USA). After transfection, cells were replated into Petri dishes and used within 12–24 h for patch clamp recordings.

Electrophysiology and data analysis

Membrane sodium currents were recorded by performing whole-cell patch clamp experiments using an EPC9 patch clamp amplifier (HEKA Electronics, Lambrecht, Germany). For recordings and analysis, the Pulse and Pulse Fit software programs were used (HEKA Electronics). Data analysis, curve fitting as well as the creation of figures were performed with the Microcal Origin 6.1 software (OriginLab Corp., Northampton, MA, USA). Currents were filtered at 5 kHz, acquired 20 kHz and stored for off-line analysis on a personal computer. In order to minimize voltage errors, the series resistance was compensated by 70% and the capacitance artefact was reduced using the amplifier circuitry. Linear leak subtraction was performed for all experiments and is based on the resistance estimates from four hyperpolarizing pulses applied before the test pulse.

Patch pipettes were pulled from borosilicate glass capillaries (GB150EFT-10; Science Products, Hofheim, Germany) to a resistance of 1–2 M Ω after heat polishing. Control and test solutions were applied by a gravity-driven application system with an outlet positioned ~100 μ m from the cell. All experiments were carried out at room temperatures of 21–23°C.

IC₅₀ values were calculated by normalizing peak current amplitudes at different drug concentrations to the value obtained in control solution. The data were fitted with the Hill equation $y = y_{\max} \times \{IC_{50}^n / (IC_{50}^n + C^n)\}$, with $y_{\max}$ representing the maximal amplitude, IC₅₀ the concentration at which $y/y_{\max} = 0.5$, and n is the Hill coefficient. The Hill equation fits were not constrained to a maximum of 1.0. To obtain the inactivation curves, peak currents evoked by a test pulse were measured, normalized and plotted against the conditioning pre-pulse potential. The data were fitted by the Boltzmann equation $y = 1 / [1 + \exp((E_{pp} - h0.5)/kh)]$, where E_{pp} is the membrane potential, $h0.5$ is the voltage at which $y = 0.5$, and kh is the slope factor.

Automated ligand docking

A homology model of the closed-state Na_v1.5 pore was generated as described previously (O'Reilly *et al.*, 2012). The crystal structures of L-methadone (reference code METHAD) and D-methadone (reference code MEADOB) were downloaded from the Cambridge Structural Database (<http://www.ccdc.cam.ac.uk/>). The structure of bupivacaine was generated using Marvin (version 5.7) software (ChemAxon, Budapest, Hungary). Ligands were processed using AutoDock-

Tools (version 1.5.4) (Molecular Graphics Laboratory, Scripps Research Institute, La Jolla, CA, USA) to add define rotatable bonds and polar hydrogens and merge non-polar hydrogens. Automated docking predictions were carried out using AutoDock Vina (version 1.1.2) software (Trott and Olson, 2009) using default docking parameters and a grid of 60 × 60 × 60 points, with 1 Å spacing centred on the pore. A docking run for each ligand was performed, and docking poses ranked by predicted binding affinity were visually compared to identify a common binding site. Figures were produced using PyMOL (DeLano Scientific, San Carlos, CA, USA).

Data analysis

Data are displayed as mean ± SEM or fitted value ± SE of the fit. Statistical significance was calculated with the Student's *t*-test and determined at *P* < 0.05.

Materials

Racemic methadone, dextromethadone and bupivacaine were purchased from Sigma-Aldrich (Schnellendorf, Germany) and prepared as stock solutions in DMSO. Veratridine was purchased from Tocris, Wiesbaden-Nordenstadt, Germany. The final concentration of DMSO was never higher than 0.3% (at 300 μM), a concentration that does not affect Na_v1.5 channels when applied alone (data not shown). Levomethadone was obtained from Sanofi-Aventis (L-Polamidon®, Frankfurt, Germany). Stock solutions were stored at 4°C, and test solutions for experiments were prepared directly prior to patch clamp recordings.

The bath solution contained (in mM): 70 NaCl, 70 choline chloride, 3 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES and 15 glucose for all experiments with cells stably expressing Na_v1.5 channels. The reduced concentration of Na⁺ was chosen because of the very high expression of Na_v1.5 channels in these stable cells, that is, to prevent voltage errors when performing whole-cell patch clamp recordings. For HEK293t cells transfected with Na_v1.5-N406K or Na_v1.5-F1760A channels, 140 mM NaCl was used instead of 70 mM NaCl and 70 mM choline chloride. The pipette solution contained (in mM): 140 CsF, 10 NaCl, 1 EGTA and 10 HEPES. Pipette and bath solutions were adjusted to pH 7.4 by adding tetramethylammonium hydroxide (bath solution) or CsOH (pipette solution). The osmolality of all solutions was adjusted to 290–300 mOsm. All solutions were stored at 4°C.

Results

Methadone inhibits human Na_v1.5 channels

We first examined if racemic methadone inhibits or modifies wild-type human Na_v1.5 channels. Cells were held at –120 mV and 20 ms depolarizing pulses ranging from –90 to 40 mV evoked fast-activating and inactivating sodium currents (Figure 1A). Application of 100 μM methadone results in a strong reduction of the peak current amplitudes (54 ± 1%, *n* = 13), but only a small hyperpolarizing shift of the current–voltage relationship (Figure 1B). As shown in Figure 1C, methadone indeed induced a concentration-dependent tonic block of resting Na_v1.5 channels examined in cells held at –120 mV. The corresponding IC₅₀ value of

90 ± 7 μM (Hill coefficient –1.15 ± 0.09, *n* = 12) was calculated with the Hill equation (Figure 1D). Apart from this tonic block, methadone also induces a use-dependent block of Na_v1.5 channels, when these were activated at 10 Hz with 20 ms pulses in cells held at –120 mV (Figure 1E). Use-dependent block was concentration-dependent, as revealed by a stronger block by 100 μM (26 ± 2%, *n* = 10), compared with 10 μM (6 ± 1%, *n* = 10) methadone (Figure 1F; *P* < 0.01, unpaired *t*-test). We also examined use-dependent block in a more physiological context by applying 400 ms test pulses at 1 Hz, that is, mimicking the cardiac action potential. When using this protocol, use-dependent block was more pronounced with a total block of 54 ± 5% (*n* = 11). Thus, methadone induces both tonic and use-dependent block of Na_v1.5 channels, that is, properties typical for many LAs (Nau and Wang, 2004). Another typical property of LAs is their high-affinity block of inactivated and open Na⁺ channels. In order to explore the action of methadone on open Na_v1.5 channels, we examined tonic block of persistent currents induced by addition of veratridine (50 μM). As demonstrated in Figure 2A, veratridine induced a prominent persistent current activated by 50 ms pulses in cells held at –120 mV. The calculated IC₅₀ value of 62 ± 4 μM (Hill coefficient –1.4 ± 0.1, *n* = 13; Figure 2B) suggests that methadone is a blocker of human Na_v1.5 channels, thus again mimicking the inhibitory properties of classical LAs.

We now proceeded with a thorough characterization of the effects of methadone on inactivated Na_v1.5 channels. Fast inactivation was induced by 100 ms pre-pulses ranging from –150 to –25 mV in steps of 5 mV before a test pulse to 0 mV was applied. As shown in Figure 3A, methadone induced a concentration-dependent shift of the steady-state fast inactivation (control: h0.5 –52.0 ± 0.3 mV; 10 μM methadone: h0.5 –58.0 ± 0.3 mV; 100 μM methadone: h0.5 –66.0 ± 0.3 mV, *n* = 18). In order to examine tonic block of fast inactivated channels, a 100 ms pre-pulse at –70 mV was applied prior to the test pulse to 0 mV. As indicated by the low IC₅₀ value of 10 ± 6 μM (Hill coefficient –0.6 ± 0.11) (Figure 3B; *n* = 12), fast inactivated channels displayed a strongly enhanced tonic inhibition by methadone, compared with resting channels. The effect of methadone on the recovery from fast inactivation was examined by a protocol consisting of two test pulses to 0 mV applied with increasing time intervals between 0 and 84.7 ms. Methadone impaired recovery from fast inactivation in a concentration-dependent manner (control: τ 2.9 ± 0.1 ms, *n* = 10; 3 μM methadone: τ 5.1 ± 0.2 ms, *n* = 10; 30 μM methadone: τ 8.6 ± 0.4 ms, *n* = 10; Figure 3C).

We next investigated the effects of methadone on slowly inactivated channels. Slow inactivation was induced by 10 s pre-pulses ranging from –120 to –10 mV in steps of 10 mV, followed by a 100 ms pulse at –120 mV allowing recovery from fast inactivation and finally a test pulse to –10 mV. Methadone induced a prominent shift of the voltage dependency of slow inactivation of Na_v1.5 channels (control: V_{1/2} –57 ± 4.3 mV; 3 μM methadone: V_{1/2} –67 ± 2.5 mV, *n* = 15; Figure 3D). For experiments exploring tonic block of slow inactivated channels, the test pulse to –10 mV was preceded by a 10 s pulse to –70 mV and a 100 ms pulse at –120 mV. With this protocol, the calculated IC₅₀ value was 42 ± 6.4 μM (Hill coefficient –1.1 ± 0.15; Figure 3E, *n* = 12). The recovery of slow and fast inactivated channels was explored using a

Figure 1

Methadone inhibits human $\text{Na}_v1.5$ channels. (A) Representative current traces generated by human $\text{Na}_v1.5$ channels in the presence of control solution or 100 μM methadone. Cells were held at -120 mV, and currents were elicited by 20 ms test pulses ranging from -90 to 40 mV in steps of 10 mV. (B) Normalized current-voltage curves of currents obtained in (A). Currents were normalized to the peak current amplitude and plotted against the corresponding membrane potential. (C) Typical current traces of Na^+ currents generated by human $\text{Na}_v1.5$ channels, displaying inhibition by increasing concentrations of methadone. Cells were activated by 20 ms test pulses from -120 to -10 mV in intervals of 10 s. (D) Concentration-dependent block of resting $\text{Na}_v1.5$ channels studied at a holding potential of -120 mV. Peak amplitudes of Na^+ currents at different drug concentrations were normalized with respect to the peak amplitude in control solution and plotted against the concentration of methadone. Data were fitted with the Hill equation as indicated by the solid line. (E) Representative current traces of $\text{Na}_v1.5$ channels in HEK293 cells activated at 10 Hz in the presence of 100 μM methadone. Currents were elicited by 60 test pulses lasting 25 ms, and cells were held at -120 mV. (F) Development of use-dependent block of $\text{Na}_v1.5$ channels by 10 and 100 μM methadone. Peak currents were normalized to the amplitude of the first pulse and plotted against the pulse number. All data are presented as mean $\pm$ SEM.

two-pulse paradigm, that is, a 10 s inactivating pulse at -70 mV was followed by a test pulse to 0 mV. The interval between these pulses varied between 0 and 7.3 s. With this protocol, we obtained a time course with a biphasic course, that is, it was best fitted with a double exponential fit giving

each phase its own time constants (τ) (Figure 3F). In the absence of drug, the fast time constant represents recovery from fast inactivation, and the slower time constant is due to recovery from slow inactivation. In control solution, $\sim 70\%$ of the current recovered with a fast time constant, $\tau_1 7 \pm 1$ ms,

Figure 2

Methadone inhibits persistent Na_v1.5 currents. (A) Representative current traces of Na_v1.5 channels in the presence of 50 μM veratridine. Cells were held at -120 mV, and currents were elicited by 50 ms pulses to 0 mV in the presence of increasing concentrations of methadone. (B) Concentration-dependent block of the persistent Na_v1.5 current. Peak amplitudes of the persistent Na⁺ currents at different drug concentrations were normalized with respect to the peak amplitude in control solution and plotted against the concentration of methadone. Data were fitted with the Hill equation as indicated by the solid line.

and ~30% with a slower time constant, τ_2 215 ± 34 ms (n = 14). In the presence of a drug, τ_1 represents recovery of unblocked channels from fast inactivation, and τ_2 represents recovery of inactivated channels that were blocked during the 10 s pre-pulse, including rebinding and dissociation from resting channels and recovery from slow inactivation (Leffler *et al.*, 2007). In the presence of 10 μM methadone, the fractions of the current recovering with a fast (~70%) and a slow (~30%) time constants were not changed; however, both time constants were prolonged to 22 ± 3 and 774 ± 146 ms respectively (n = 14; Figure 3F). This result suggests that methadone impaired recovery from both fast and slow inactivation, but that the presence of methadone did not lead to a slow recovery from inactivated drug-bound channels. This would have resulted in a strongly prolonged slow time constant and a higher proportion of channels recovering with a slow time constant, reflecting a slow dissociation of the drug from inactivated channels (Leffler *et al.*, 2007). In order to substantiate this interpretation we next performed comparative experiments with bupivacaine, known to dissociate slowly from inactivated Na_v1.5 channels (Clarkson and Hondeghem, 1985; Nau *et al.*, 2000b). As shown in Figure 4A, tonic block of resting Na_v1.5 channels revealed an even lower blocking potency for bupivacaine (IC_{50} 198 ± 14 μM, n = 14) than that found for methadone. Use-dependent block at 10 Hz with 10 μM bupivacaine, however, revealed that bupivacaine was a more potent use-dependent blocker than methadone (10 μM bupivacaine 30 ± 1%, n = 10; Figure 4B, P < 0.001, unpaired t -test). Finally, recovery of drug-bound inactivated channels was investigated with the same protocol as described earlier for Figure 2F. As shown in Figure 4C, application of 10 μM bupivacaine resulted in a strong prolongation of both time constants as compared with the control situation; τ_1 22 ± 4 ms and τ_2 2619 ± 445 ms (n = 7). Furthermore, the fraction of current recovering with a fast time constant was reduced to ~40%, resulting in ~60% of the current recovering with a very

slow time constant. These data very well agree with previous reports showing that bupivacaine dissociates slowly from inactivated channels, and also implies that methadone dissociates as rapidly. Possible implications of these pharmacological differences are discussed in detail below.

Stereo-specific effects of methadone on Na_v1.5 channels

We next explored if methadone inhibited Na_v1.5 channels in a stereo-specific manner as a correlate for the clinically relevant high cardiotoxic potential of dextromethadone. Tonic block of resting Na_v1.5 channels was examined as described earlier at -120 mV. Surprisingly, dextromethadone (IC_{50} 131 ± 14 μM, n = 10) blocked Na_v1.5 channels with a significantly lower potency, compared with that of levomethadone (IC_{50} 81 ± 10 μM, n = 8; Figure 5A,B, P < 0.01, unpaired t -test). For use-dependent block by 100 μM at 10 Hz, however, dextromethadone (46 ± 3%, n = 8) induced a significantly stronger block than levomethadone (32 ± 3%, n = 8; Figure 5C, P < 0.01, unpaired t -test). The recovery of drug-bound inactivated channels was investigated with the same protocol as described earlier. As illustrated in Figure 5D, application of 10 μM dextromethadone (τ_1 35 ± 4 ms and τ_2 662 ± 128 ms, n = 6) and levomethadone (τ_1 36 ± 5 ms and τ_2 519 ± 124 ms, n = 6) did not reveal any relevant stereo-specific differences (P > 0.05, unpaired t -test).

Methadone interacts with the LA-binding site on Na_v1.5 channels

As methadone produces a state-dependent inhibition of Na_v1.5 channels that closely resembled the effects of LAs, we next asked if methadone interacted with the proposed binding site for LAs. As shown in Figure 6A,B, the LA-insensitive mutant channels, Na_v1.5-F1760A (A) and Na_v1.5-N406K (B) (Nau *et al.*, 2000b) still exhibited a concentration-dependent tonic block in cells held at -120 mV (i.e. resting channels). However, the calculated IC_{50} values of 650 ± 28 μM for Na_v1.5-F1760A (Hill coefficient -1.3 ± 0.1, n = 10) and 188 ± 21 μM for Na_v1.5-N406K (Hill coefficient -0.9 ± 0.1; n = 14) reveal a significant reduction of the tonic-blocking potency of methadone on both mutants as compared with wild-type Na_v1.5 channels (Figure 6C; P < 0.001 for both mutants, unpaired t -tests). Moreover, neither of the mutants display an enhanced use-dependent block when 100 μM methadone is applied as compared with recordings in control solution: N406K: control 3 ± 1%, 100 μM methadone 8 ± 1% (n = 10, P = 0.30, paired t -test) and F1760A: control 8 ± 0.2%, 100 μM methadone 9 ± 0.4% (n = 10, P = 0.56, paired t -test; Figure 6D). Notably, the ability of methadone to shift the voltage dependency of both fast and slow inactivation was conserved with both N406K and F1760A mutant channels (data not shown).

The determination of the first Na⁺ channel crystal structure – Na_vAb from *Arcobacter butzleri* (Payandeh *et al.*, 2011) – facilitated homology modelling of the closed-state Na_v1.5 pore domain and the examination of binding interactions of tonic-blocking compounds (O'Reilly *et al.*, 2012). Tonic block of Na_v1.5 channels by dextro- and levomethadone was comparable, which suggests that they may share an overlapping binding site in the pore. Furthermore, the blocking efficacy of

Figure 3

Methadone modifies inactivation properties of $\text{Na}_v1.5$ channels. (A) Steady-state fast inactivation of $\text{Na}_v1.5$ channels in the presence of control solution, 10 and 100 μM methadone. Fast inactivation was induced by 100ms pre-pulses ranging from -150 to -30 mV in steps of 10 mV. Solid lines represent fits obtained with the Boltzmann equation. (B) Dose-response curve for tonic block of fast inactivated channels. Channels were inactivated by 100ms pre-pulses at -70 mV, and methadone was applied in increasing concentrations. Peak amplitudes were normalized to the peak amplitude obtained in control solution. The solid line represents a fit with the Hill equation. (C) Time course of recovery from fast inactivation of $\text{Na}_v1.5$ channels with control solution, 3 and 30 μM methadone. Fast inactivation was induced by 100ms pre-pulses to -10 mV, followed by increasing time intervals at -120 mV and by a test pulse to -10 mV. Current amplitudes were normalized to the maximal values obtained at the end of the protocol and plotted against the time interval. Data were fitted with a single exponential function. (D) Voltage dependency of slow inactivation of $\text{Na}_v1.5$ channels in control solution and with 3 μM methadone. Slow inactivation was induced by 10s pre-pulses ranging from -120 to -10 mV, followed by a 100ms interval at -120 mV allowing recovery from fast inactivation, and finally by a test pulse to -10 mV. Peak currents were normalized to the amplitude obtained at -120 mV and plotted against the membrane potential. The solid line represents fits calculated with the Boltzmann equation. (E) Dose-response curve for tonic block of slow inactivated channels. Channels were inactivated by 10s pre-pulses at -70 mV, followed by a 100ms interval at -120 mV allowing recovery from fast inactivation, and finally by a test pulse to -10 mV. Peak amplitudes were normalized to the peak amplitude obtained in control solution. The solid line represents a fit with the Hill equation. (F) Time course for the recovery of drug-bound inactivated channels. Inactivation was induced by 10-s-long pre-pulses to -70 mV, and the test pulse to -10 mV was applied after a variable time interval at -120 mV. Peak current amplitudes were normalized to the maximal value obtained at the end of the protocol and plotted against the length of the interval at -120 mV. The data were best fitted with a double exponential function to reveal two time constants.

methadone was most strongly attenuated by the F1760A mutation, which identifies this residue as a major binding determinant. Therefore, the $\text{Na}_v1.5$ channel closed-state model was employed with automated ligand docking software to identify binding sites for levo- and dextromethadone, and the top docking predictions in terms of calculated affinity were compared for each stereoisomer to identify similarities in their binding interactions.

Figure 7 shows that methadone can be accommodated in a binding site located at the interface of DIII-S6, DIV-S6 and the DIII-Ploop. This receptor adjoins the DIII-DIV fenestration, which was characterized previously as a feasible ingress route for small molecule tonic blockers of $\text{Na}_v1.5$ channels (O'Reilly *et al.*, 2012). Both stereoisomers of methadone share an overlapping docking pose. Deviation from this structural overlap is localized to the chiral centre, which produces a difference in orientation of the methadone amine group. As this tertiary amine can function as a hydrogen bond acceptor,

it is possible that the positional difference of this moiety and therefore modified hydrogen bond interactions may contribute to the difference in tonic block that we observe with dextro- and levomethadone. The side chain hydroxyl groups of S1458 and T1461 are <4 Å from the amine of both docked stereoisomers, which identifies these residues as potential hydrogen bond donors.

The overlapping portions of the docked stereoisomers form van der Waals interactions with the hydrophobic side chains of L1413, A1416, I1757 and I1756. In addition, a key binding contact is a face-to-face aromatic interaction with the side chain of F1760. Substitution of the 1760 side chain with a non-aromatic small-chain residue would eliminate the ring stacking between methadone and receptor that putatively stabilizes the bound ligand conformation. We therefore propose that this may be the structural basis for the decreased blocking potency displayed by methadone with the F1760A mutant of the $\text{Na}_v1.5$ channel. In contrast to F1760, the side

Figure 4

Inhibition of Na_v1.5 channels by bupivacaine. (A) Concentration-dependent block of resting Na_v1.5 channels studied at a holding potential of −120 mV. Peak amplitudes at different drug concentrations were normalized with respect to the peak amplitude in control solution and plotted against the concentration of bupivacaine. Data were fitted with the Hill equation represented by the solid line. (B) Development of use-dependent block of Na_v1.5 channels by 10 μM bupivacaine compared with control solution. Peak currents were normalized to the amplitude of the first pulse and plotted against the pulse number. (C) Time course for the recovery of drug-bound inactivated channels. Data were obtained as described under Figure 2E, but with 10 μM bupivacaine instead of methadone.

chain of N406 is not orientated towards the lumen in the closed-state pore (Figure 7). Therefore, the effect of the N406K mutation may not be the elimination of a ligand-binding determinant but instead allosteric modulation of the ligand-binding site (McNulty *et al.*, 2006).

Discussion and conclusions

In this *in vitro* study, we demonstrate that methadone was a state-dependent inhibitor of the cardiac sodium channel, Na_v1.5. Our data suggest that methadone inhibited Na⁺ channels by interacting with the local LA site, and that the tonic-blocking potency of methadone was comparable with that of the most potent and cardiotoxic LA bupivacaine. Our data reinforce our hypothesis that a modulation of Na_v1.5 channels is likely to contribute to the clinically relevant proarrhythmic potential of methadone.

Methadone is a chiral synthetic opioid that is mostly administered as the racemic mixture consisting of dextro- and levomethadone. They are in clinical use for opiate replacement therapy as well as treatment of chronic and neuropathic pain. In contrast to many other opioids, methadone is available at low cost and has a long *t*_{1/2} of up to 60 h. However, it has been suggested that the duration of analgesia is shorter than the time of metabolism, and consequently there is an increased risk of accumulation. Furthermore, the metabolism of methadone is subject to great interindividual variability due to a hepatic elimination by CYP3A4 and CYP2B6 (Eap *et al.*, 2002). The activity of these enzymes is determined by genetic and environmental factors, including co-administration of drugs that are also metabolized by CYP complexes (Eap *et al.*, 2002). Consequently, the plasma concentrations of methadone can vary significantly. A study performed by de Vos *et al.* (1995) found that plasma levels of methadone in drug addicts can reach concentrations of up to 3.6 μM. However, 'slow metabolizers' of methadone might develop higher even plasma levels within the range of the concentrations shown to inhibit Na_v1.5 channels in this paper. Such patients are more susceptible to developing cardiac arrhythmias when receiving methadone. In fact, several case reports have described torsade de pointes tachycardias as well as sudden cardiac death in patients treated with methadone (Walker *et al.*, 2003; Chugh *et al.*, 2008). These fatal cardiac side effects are unique for an opioid, and hitherto they were considered to be mediated by an inhibition of cardiac hERG K⁺ channels that are crucial for a proper repolarization. A study from Katchman *et al.* (2002) demonstrates that methadone blocks hERG channels in a state-dependent manner with a high affinity for open and inactivated channels. Furthermore, Eap *et al.* (2002) demonstrated that methadone inhibits hERG channels in a stereospecific manner, that is, dextromethadone (IC₅₀ ~12 μM) is more potent than levomethadone (IC₅₀ ~29 μM).

With our present study, we identify the cardiac Na⁺ channel, Na_v1.5, as another putatively relevant target of methadone in terms of cardiac toxicity. Methadone displays typical pharmacological properties known for LAs, and to date the Na_v1.5 channel is considered the most important target molecule for LAs in terms of cardiac toxicity (Butterworth, 2010). Block of human Na_v1.5 channels by methadone is state dependent, and our experiments with the LA-insensitive mutant Na_v1.5-F1760A suggest that methadone interacts with the LA-binding site. Similar effects on human Na_v1.5 channels have previously been demonstrated for other cardiotoxic substances, including amitriptyline and bisphenol A (Nau *et al.*, 2000a; O'Reilly *et al.*, 2012). When considering the IC₅₀ value for tonic block of resting Na_v1.5

Figure 5

Stereo-specific effects of methadone on $\text{Na}_v1.5$ channels. (A) Typical current traces of $\text{Na}_v1.5$ channels inhibited by dextromethadone and levomethadone. Currents were activated every 10 s, and cells were held at -120 mV. (B) Dose-response curves for tonic block of resting $\text{Na}_v1.5$ channels by dextromethadone and levomethadone. Peak currents were normalized to the current obtained in control solution, and data were fitted with the Hill equation. (C) Development of use-dependent block of $\text{Na}_v1.5$ channels by 100 μM dextromethadone and levomethadone. Peak currents were normalized to the amplitude of the first pulse and plotted against the pulse number. (D) Time course for the recovery of drug-bound inactivated channels. Data were obtained as described under Figure 2E, but with 10 μM dextromethadone and levomethadone.

channels, it is clear that methadone is at least as potent as bupivacaine, the most potent and cardiotoxic LA. Although this parameter might give a good idea about the potency of methadone as a tonic Na^+ channel blocker, it does not allow us to draw the conclusion that inhibition of $\text{Na}_v1.5$ channels must be a clinically relevant action of methadone. Considering published data on plasma concentrations of methadone up to 4 μM , one could argue that clinically relevant concentrations would hardly induce a substantial tonic block *in vivo* (IC_{50} 90 μM). However, it is important to note that the recording of resting channels at strongly hyperpolarized potentials like -120 mV is artificial and not representative for the membrane properties of cardiomyocytes *in vivo*. With a physiological resting membrane potential around -80 mV and with an ongoing cardiac activity, the clinical relevance of a Na^+ channel blocker is probably better estimated from recordings on inactivated channels (IC_{50} ~ 10 μM in our study). We also found that methadone stabilizes both fast and slow inactivation of $\text{Na}_v1.5$ channels. Methadone prevents a proper repolarization in the myocardium primarily by blocking hERG channels. This effect is also likely to prevent recovery from inactivated Na^+ channels, and thus to further increase the blocking efficacy of methadone on $\text{Na}_v1.5$ channels. However, it is not just the tonic block of $\text{Na}_v1.5$ channels that

may be crucial for a high cardiac toxicity. Bupivacaine is known to induce a strong use-dependent block likely to be of importance for cardiotoxicity (Nau *et al.*, 2000b), and our comparative experiments with methadone and bupivacaine suggested that methadone is a rather poor use-dependent blocker. The previously described slow dissociation of bupivacaine and amitriptyline from inactivated channels has been suggested to favour cardiotoxicity, leading to the commonly used description for these substances as “fast-in, but slow-out” blockers (Clarkson and Hondeghem, 1985; Nau *et al.*, 2000a,b). With these blockers, cardiac Na^+ channels may not fully recover between two action potentials, leading to an accumulation of drug-bound inactivated channels and eventually to cardiac arrhythmias. As is clear from our experiments, methadone dissociates considerably faster from inactivated channels as compared with bupivacaine. Methadone has a protonable tertiary amine (pK_a 9.65; Kaufman *et al.*, 1975) that is predominantly charged at physiological pH, and the drug can therefore form the cation- π interaction with the F1760 side chain that is the hallmark of use-dependent block by LAs (Ahern *et al.*, 2008). However, whereas most LAs including bupivacaine are structurally analogous in that the tertiary amine group is linked by an amide or ester bond to a single aromatic ring, the tertiary amine of methadone is

Figure 6

Methadone interacts with the LA-binding site of Na_v1.5 channels. (A,B) Typical current traces of Na_v1.5-F1760A (A) and Na_v1.5-N406K inhibited by methadone. Currents were activated every 10 s, and cells were held at -120 mV. (C) Dose-response curves for tonic block of resting Na_v1.5-F1760A and Na_v1.5-N406K channels by methadone. Peak currents were normalized to the current obtained in control solution and data were fitted with the Hill equation. (D) Development of use-dependent block of Na_v1.5-F1760A and Na_v1.5-N406K by 100 μ M methadone. Peak currents were normalized to the amplitude of the first pulse and plotted against the pulse number.

connected by an alkane linker to a carbon substituted with two aromatic rings and a butanone group. The considerable molecular size of this linked group may prevent, through steric hindrance, the optimal binding of methadone in the open or inactivated channel, which could account for its relatively poor use-dependent block and fast off-rate when

Figure 7

Stereograms of docking predictions for methadone (top panels) and bupivacaine (bottom panels) in the closed-state Na_v1.5 pore. The channel is represented as ribbons (S6 helices: green; P-loops: brown) with residues F1760 and N406 shown as sticks. Calculated binding affinity is -7.4 kcal·mol⁻¹, L-methadone (purple); -6.9 kcal·mol⁻¹, D-methadone (orange); -6.7 kcal·mol⁻¹, bupivacaine (cyan).

compared with bupivacaine. As such, methadone may therefore not be the prototypical Na⁺ channel blocker for which a high cardiotoxic potential would be predicted. However, the simultaneous block of hERG channels causes prolonged depolarizations and may thus replace a slow dissociation of methadone from Na_v1.5 channels. This in turn would produce an accumulation of inactivated Na⁺ channels that are potentially blocked by methadone.

A stereo-selective effect of methadone is not only restricted to the inhibition of hERG channels. The agonistic effect on the μ -opioid receptor is almost exclusively induced by levomethadone (Kristensen *et al.*, 1995). Furthermore, block of NMDA receptors by methadone was reported to be mainly mediated by dextromethadone (Holtman and Wala,

2007; Shimoyama *et al.*, 1997). In regard to the action of methadone on Na_v1.5 channels, we observed that levomethadone was a more potent tonic blocker as compared with dextromethadone. *Vice versa*, dextromethadone was the more potent use-dependent blocker. To date, we cannot say if any of these discrete stereo-specific differences are of clinical relevance. With regard to cardiac toxicity, it seems likely that the inhibition of Na_v1.5 channels derives from a rather stereo-nonspecific action of methadone.

Limitations and conclusion

It is important to acknowledge that our study has some relevant limitations. We performed *in vitro* patch clamp experiments that cannot perfectly reproduce *in vivo* conditions, and we must assume that methadone targets other membrane molecules, apart from hERG and Na_v1.5 channels, in the myocardium. Therefore, our study should not be interpreted as a claim of an essential role of Na_v1.5 channels for methadone-induced cardiotoxicity, but rather as a thorough characterization of a yet unrecognized pharmacological property of methadone as a Na⁺ channel blocker with typical LA properties. Nevertheless, our findings do offer an additional mechanism for cardiac arrhythmias and sudden cardiac death in conjunction with methadone treatment.

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Conflict of interest

None.

References

Ahern CA, Eastwood AL, Dougherty DA, Horn R (2008). Electrostatic contributions of aromatic residues in the local anesthetic receptor of voltage-gated sodium channels. *Circ Res* 102: 86–94.

Alexander SPH *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Overview. *Br J Pharmacol* 170: 1449–1867.

Andrews CM, Krantz MJ, Wedam EF, Marcuson MJ, Capacchione JF, Haigney MC (2009). Methadone-induced mortality in the treatment of chronic pain: role of QT prolongation. *Cardiol J* 16: 210–217.

Butterworth JFT (2010). Models and mechanisms of local anesthetic cardiac toxicity: a review. *Reg Anesth Pain Med* 35: 167–176.

Chugh SS, Socoteanu C, Reinier K, Waltz J, Jui J, Gunson K (2008). A community-based evaluation of sudden death associated with therapeutic levels of methadone. *Am J Med* 121: 66–71.

Clarkson CW, Hondeghem LM (1985). Mechanism for bupivacaine depression of cardiac conduction: fast block of sodium channels during the action potential with slow recovery from block during diastole. *Anesthesiology* 62: 396–405.

Eap CB, Buclin T, Baumann P (2002). Interindividual variability of the clinical pharmacokinetics of methadone: implications for the treatment of opioid dependence. *Clin Pharmacokinet* 41: 1153–1193.

Eap CB, Crettol S, Rougier JS, Schlapfer J, Sintra Grilo L, Deglon JJ *et al.* (2007). Stereoselective block of hERG channel by (S)-methadone and QT interval prolongation in CYP2B6 slow metabolizers. *Clin Pharmacol Ther* 81: 719–728.

Gonzalez T, Arias C, Caballero R, Moreno I, Delpon E, Tamargo J *et al.* (2002). Effects of levobupivacaine, ropivacaine and bupivacaine on HERG channels: stereoselective bupivacaine block. *Br J Pharmacol* 137: 1269–1279.

Grilo LS, Carrupt PA, Abriel H (2010). Stereoselective inhibition of the hERG1 potassium channel. *Front Pharmacol* 1: 1–11.

Haeseler G, Foadi N, Ahrens J, Dengler R, Hecker H, Leuwer M (2006). Tramadol, fentanyl and sufentanil but not morphine block voltage-operated sodium channels. *Pain* 126: 234–244.

Holtman JR, Jr, Wala EP (2007). Characterization of the antinociceptive and pronociceptive effects of methadone in rats. *Anesthesiology* 106: 563–571.

Katchman AN, McGroary KA, Kilborn MJ, Kornick CA, Manfredi PL, Woosley RL *et al.* (2002). Influence of opioid agonists on cardiac human ether-a-go-go-related gene K(+) currents. *J Pharmacol Exp Ther* 303: 688–694.

Kaufman JJ, Semo NM, Koski WS (1975). Microelectrometric measurement of the pK's and partition and drug distribution coefficients of narcotics and narcotic antagonists and their pH and temperature dependence. *J Med Chem* 18: 647–655.

Kornick CA, Kilborn MJ, Santiago-Palma J, Schulman G, Thaler HT, Keefe DL *et al.* (2003). QTc interval prolongation associated with intravenous methadone. *Pain* 105: 499–506.

Krantz MJ, Kutinsky IB, Robertson AD, Mehler PS (2003). Dose-related effects of methadone on QT prolongation in a series of patients with torsade de pointes. *Pharmacotherapy* 23: 802–805.

Kristensen K, Christensen CB, Christrup LL (1995). The mu₁, mu₂, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. *Life Sci* 56: PL45–PL50.

Leffler A, Reiprich A, Mohapatra DP, Nau C (2007). Use-dependent block by lidocaine but not amitriptyline is more pronounced in tetrodotoxin (TTX)-Resistant Nav1.8 than in TTX-sensitive Na⁺ channels. *J Pharmacol Exp Ther* 320: 354–364.

Leffler A, Frank G, Kistner K, Niedermirtl F, Koppert W, Reeh PW *et al.* (2012). Local anesthetic-like inhibition of voltage-gated Na(+) channels by the partial mu-opioid receptor agonist buprenorphine. *Anesthesiology* 116: 1335–1346.

McNulty MM, Kyle JW, Lipkind GM, Hanck DA (2006). An inner pore residue (Asn406) in the Nav1.5 channel controls slow inactivation and enhances mibefradil block to T-type Ca²⁺ channel levels. *Mol Pharmacol* 70: 1514–1523.

Nau C, Wang GK (2004). Interactions of local anesthetics with voltage-gated Na⁺ channels. *J Membr Biol* 201: 1–8.

- Nau C, Seaver M, Wang SY, Wang GK (2000a). Block of human heart hH1 sodium channels by amitriptyline. *J Pharmacol Exp Ther* 292: 1015–1023.
- Nau C, Wang SY, Strichartz GR, Wang GK (2000b). Block of human heart hH1 sodium channels by the enantiomers of bupivacaine. *Anesthesiology* 93: 1022–1033.
- O'Reilly AO, Eberhardt E, Weidner C, Alzheimer C, Wallace BA, Lampert A (2012). Bisphenol A binds to the local anesthetic receptor site to block the human cardiac sodium channel. *Plos ONE* 7: e41667.
- Payandeh J, Scheuer T, Zheng N, Catterall WA (2011). The crystal structure of a voltage-gated sodium channel. *Nature* 475: 353–358.
- Shimoyama N, Shimoyama M, Elliott KJ, Inturrisi CE (1997). d-Methadone is antinociceptive in the rat formalin test. *J Pharmacol Exp Ther* 283: 648–652.
- Trott O, Olson AJ (2009). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 31: 455–461.
- Valenzuela C, Snyders DJ, Bennett PB, Tamargo J, Hondeghem LM (1995). Stereoselective block of cardiac sodium channels by bupivacaine in guinea pig ventricular myocytes. *Circulation* 92: 3014–3024.
- de Vos JW, Geerlings PJ, van den Brink W, Ufkes JG, van Wilgenburg H (1995). Pharmacokinetics of methadone and its primary metabolite in 20 opiate addicts. *Eur J Clin Pharmacol* 48: 361–366.
- Walker PW, Klein D, Kasza L (2003). High dose methadone and ventricular arrhythmias: a report of three cases. *Pain* 103: 321–324.