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TRANSLATIONAL RESEARCH

The opioid tramadol blocks the cardiac sodium channel $Na_v1.5$ in HEK293 cells

Lixia Jia [1], Marieke W. Veldkamp [1], Arie O. Verkerk [1,2], and Hanno L. Tan [1,3]*

[1]Department of Clinical and Experimental Cardiology, Heart Center, Amsterdam UMC, University of Amsterdam, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands; [2]Department of Medical Biology, Amsterdam Cardiovascular Sciences, Amsterdam UMC, University of Amsterdam, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands; and [3]Netherlands Heart Institute, Moreelsepark 1, Utrecht 3511 EP, The Netherlands

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Aims Opioids are associated with increased risk of sudden cardiac death. This may be due to their effects on the cardiac sodium channel ($Na_v1.5$) current. In the present study, we aim to establish whether tramadol, fentanyl, or codeine affects $Na_v1.5$ current.

Methods and results Using whole-cell patch-clamp methodology, we studied the effects of tramadol, fentanyl, and codeine on currents of human $Na_v1.5$ channels stably expressed in HEK293 cells and on action potential (AP) properties of freshly isolated rabbit ventricular cardiomyocytes. In fully available $Na_v1.5$ channels (holding potential −120 mV), tramadol exhibited inhibitory effects on $Na_v1.5$ current in a concentration-dependent manner with an IC_{50} of 378.5 ± 33.2 μM. In addition, tramadol caused a hyperpolarizing shift of voltage-gated (in)activation and a delay in recovery from inactivation. These blocking effects occurred at lower concentrations in partially inactivated $Na_v1.5$ channels: during partial fast inactivation (close-to-physiological holding potential −90 mV), IC_{50} of $Na_v1.5$ block was 4.5 ± 1.1 μM, while it was 16 ± 4.8 μM during partial slow inactivation. The tramadol-induced changes on $Na_v1.5$ properties were reflected by a reduction in AP upstroke velocity in a frequency-dependent manner. Fentanyl and codeine had no effect on $Na_v1.5$ current, even when tested at lethal concentrations.

Conclusion Tramadol reduces $Na_v1.5$ currents, in particular, at close-to-physiological membrane potentials. Fentanyl and codeine have no effects on $Na_v1.5$ current.

* Corresponding author. Tel: +31 20 566 3264. *E-mail address*: h.l.tan@amsterdamumc.nl

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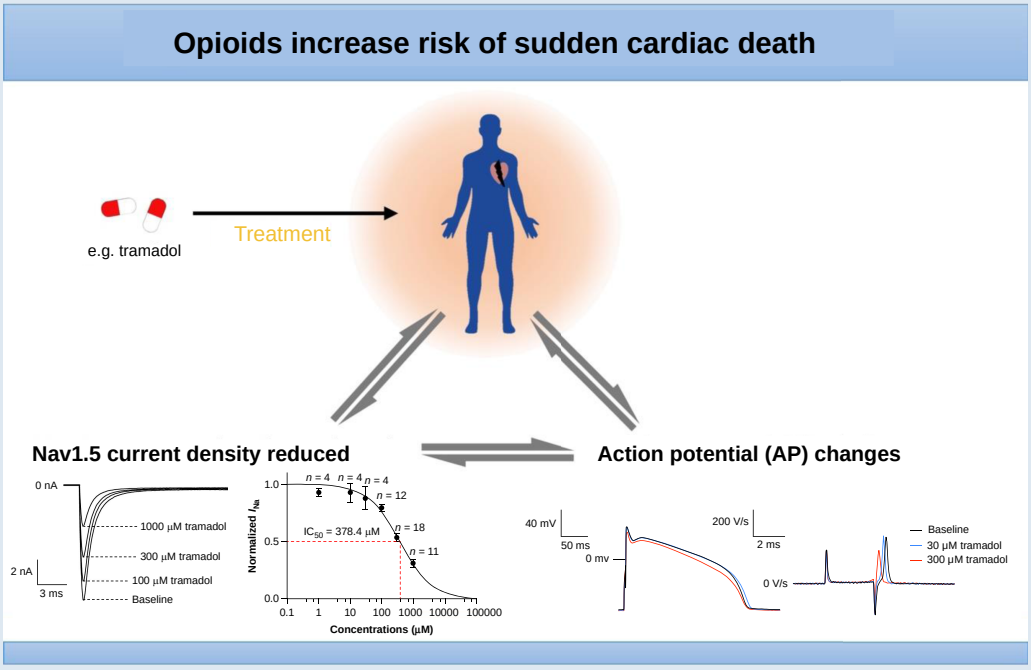
Graphical Abstract



Keywords Sudden cardiac death • Tramadol • Action potential • $Na_v1.5$ current • Overdose • Risk association

What's new?

Opioids associated with an increased risk of sudden cardiac death due to life-threatening cardiac arrhythmias:

- Tramadol reduced current of $Na_v1.5$ channels in a fully available state in a dose-dependent manner and caused gating changes at lethal concentrations.
- These effects were mirrored by reductions in the action potential upstroke velocity and reduction in action potential amplitude.
- Fentanyl and codeine had no effects on $Na_v1.5$ channels in a fully available state at lethal concentrations.

Translational Perspective

Opioid use is increasing, along with death rates associated with it. This may be due to cardiovascular effects of these drugs. Use of various opioids is associated with an increased risk of sudden cardiac death (SCD) in the general population. This may be due to the ability of opioids to induce life-threatening cardiac arrhythmias by impacting on the cardiac ion channels that underlie cardiac electrophysiology. We sought to establish the cellular electrophysiological effects of the commonly used opioids tramadol, fentanyl, and codeine on currents of the human cardiac sodium channel $Na_v1.5$ and repolarizing ion channels. We found that tramadol, but not fentanyl or codeine, reduced $Na_v1.5$ currents and action potential upstroke velocity in a dose-dependent and rate-dependent manner at clinically relevant concentrations, but exhibited no significant effects on action potential duration. These findings are of potential clinical relevance, because they may help define individuals who are at elevated risk of tramadol-induced SCD such as individuals whose $Na_v1.5$ current reserve is reduced because of inherited factors (genetic $Na_v1.5$ variants) and/or acquired conditions (comorbidities such as ischemic heart disease). Identification of elevated risk may be the basis for personalized treatment of these individuals (lowering of tramadol dosages or avoidance of its use altogether) that aims at preventing drug-induced SCD.

Introduction

Opioids are increasingly prescribed for the treatment of severe and chronic pain.[1] The incidence of opioid use disorders due to long-term exposure to opioids is increasing, along with death rates associated with unintentional opioid overdoses.[2–4] This may be due to cardiovascular effects of these drugs.[2,5] For instance, we demonstrated that use of opioids—in particular tramadol, oxycodone, codeine, fentanyl, morphine, and methadone (the six most widely prescribed opioids in The Netherlands)[6]—is associated with an increased risk of sudden cardiac death (SCD) in the general population.[7,8] This may be due to the ability of these drugs to induce life-threatening cardiac arrhythmias[5] by impacting on the cardiac ion channels whose concerted activity underlies the cardiac action potential (AP).[9]

Two potentially relevant ion channel targets for opioids are the cardiac sodium channel, $Na_v1.5$ (which is important for AP initiation and propagation),[10] and the hERG potassium channel (crucial for sarcolemma repolarization and AP termination).[11] Increased SCD risk of opioids (in particular oxycodone, codeine, fentanyl, and morphine) is mostly ascribed to hERG block[12] and is reflected by QT prolongation of the electrocardiogram (ECG).[13,14] The possibility that opioids block $Na_v1.5$ channels is less recognized and studied.[7,15,16] Yet, $Na_v1.5$ dysfunction may also increase SCD risk.[17,18] Of note, drug-induced

$Na_v1.5$ current block and increased SCD risk were not only reported for drugs whose therapeutic effect is based on this effect (Vaughan-Williams class I antiarrhythmic drugs)[16] but also for drugs used for noncardiac disease for which $Na_v1.5$ block is an undesired off-target effect. This is increasingly recognized for a growing number of noncardiac drugs.[7] At present, this group contains tricyclic antidepressants[19,20] and antiepileptic drugs.[21] We hypothesize that this group may also contain opioids and opioid agonists. Indeed, various opioids, including morphine, U-50, 488H, oxycodone, methadone, loperamide, and buprenorphine, inhibit $Na_v1.5$ channels and/or cardiac AP upstroke velocities.[22–27] Currently, the effects of tramadol, codeine, and fentanyl on $Na_v1.5$ current are unknown. However, tramadol and fentanyl block neuronal ($Na_v1.2$ and $Na_v1.7$) voltage-gated sodium channels[26,28] and because the $Na_v1.5$ isoform is highly homologous with neuronal sodium channels,[29] it is likely that these opioids also reduce $Na_v1.5$ current. The aim of the present study was to establish whether tramadol, codeine, or fentanyl reduces $Na_v1.5$ current and affects the morphology of APs. To this end, we carried out patch-clamp experiments on human embryonic kidney 293 (HEK293) cells stably expressing human $Na_v1.5$ channels and determined the effects of the drugs on the APs of freshly isolated rabbit left ventricular cardiomyocytes.

Methods

HEK293 cell culture

We used a HEK293 cell line with stable human $Na_v1.5$ channel expression.[30] The HEK293 cells were cultured in DMEM with Glutamax (Gibco) supplemented with 10% FBS (Biowest), L-glutamine (Gibco), penicillin–streptomycin (Gibco), and Zeocin (of 200 μg/mL, Invitrogen) in a 5% CO_2 incubator (Shel Lab) at 37°C. The cells were passaged every 3–4 days at 70% confluency in 25 mL flasks by using 0.25% trypsin (Gibco) treatments of around 2 min. On the day of the patch-clamp measurements, cells were trypsinized, stored at room temperature, and used within 3 h.

Rabbit ventricular cardiomyocyte preparation

Male New Zealand white rabbits (3.0–3.5 kg) were anesthetized by a combination of ketamine (intramuscular 100 mg) and xylazine (intramuscular 20 mg), heparinized (Heparin LEO 5000 IU), and killed by an injection of pentobarbital (240 mg). The hearts were excised and transported to the laboratory in cold (4°C) Tyrode's solution containing (in mM) NaCl 128, KCl 4.7, $CaCl_2$ 1.5, $MgCl_2$ 0.6, $NaHCO_3$ 27, Na_2HPO_4 0.4, and glucose 11 (pH 7.4) by equilibration with 95% O_2 and 5% CO_2. Subsequently, the hearts were mounted on a Langendorff perfusion apparatus and left ventricular midmyocardial cardiomyocytes were isolated by enzymatic dissociation from the most apical part of the left midmyocardial ventricular free wall as described previously.[31] Animal procedures were performed in accordance with governmental and institutional guidelines for animal use in research and were approved by the Animal Experimental Committee of Amsterdam UMC, The Netherlands.

Patch-clamp recording

We applied the whole-cell configuration of the patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices, San Jose, CA, USA). Borosilicate glass patch pipettes (GC100F-10; Harvard Apparatus, UK) were pulled using a custom microelectrode puller and had a resistance of 2–3 MΩ after filling with the pipette solutions (for compositions, see below). All signals were low-pass filtered (5 kHz) and digitized at 33 kHz. Series resistance was compensated by ≥80%. Custom software (Scope, kindly provided by J.G. Zegers, and MacDAQ, kindly provided by A.C.G. van Ginneken) was used to record and analyze $Na_v1.5$ currents and APs.

Sodium current measurements

$Na_v1.5$ current was measured in single HEK293 cells using the ruptured patch-clamp technique at room temperature. The pipette solution contained (in mM) NaF 10, CsCl 10, CsF 110, EGTA 11, $CaCl_2$ 1.0, $MgCl_2$ 1.0, Na_2ATP 2.0, and HEPES 10 (pH adjusted to 7.2 with CsOH). The bath solution contained (in mM) NaCl 20, CsCl 120, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.5, and HEPES 5.0 (pH adjusted to 7.4 with CsOH).[30] $Na_v1.5$ current was measured in response to depolarizing voltage steps from a holding potential of −120 mV (cycle length of 5 s). At −120 mV, all $Na_v1.5$ channels are fully available for activation, and we named this the 'fully available' state of the channel. $Na_v1.5$ current was defined as the difference between peak and steady-state current. The dose–response curves were fitted by the Hill equation: $Y = 1/[(1 + (IC_{50}/X)^n)]$, where Y is the current normalized to baseline condition, IC_{50} is the dose required for 50% current block, and n is the Hill coefficient.

$Na_v1.5$ (in)activation current was measured with a double-pulse protocol (see inserted protocols in figures). During the first depolarizing pulses (P1), $Na_v1.5$ current activates and the currents analyzed here are used to determine current–voltage (I–V) relationships and the voltage dependency of activation. The second pulse (P2) is used to determine the voltage dependency of inactivation. The I–V relationships were corrected for driving force, normalized to maximum peak current, and fitted to a Boltzmann distribution curve. Voltage dependence of activation and inactivation curves was fitted with Boltzmann function: $I/I_{max} = A/\{1.0 + \exp[(V_{1/2} - V)/k]\}$, where $V_{1/2}$ is the midpoint of channel (in)activation and k is the slope factor of the (in)activation curve. The rate of recovery from inactivation was measured with a double-pulse protocol with two depolarizing steps (P1 and P2) from −120 to −20 mV and a variable interpulse interval (see inserted protocols in figures). Currents measured during P2 were normalized to currents measured during P1. Recovery from inactivation curves were fitted by a double exponential function: $y = y0 + A_f[1 - \exp(-t/\tau_f)] + A_s[1 - \exp(-t/\tau_s)]$, where τ_f and τ_s are the fast and slow time constants of recovery from inactivation, and A_f and A_s are the fractions of fast and slow recovery from inactivation. Use-dependent block was determined by application of 30 activating pulses with a duration of 200 ms from −120 to −20 mV at a frequency of 4 Hz (see inserted protocols in figures). $Na_v1.5$ currents were normalized to the current of the first pulse. In addition to testing the drug effects on fully available $Na_v1.5$ channels, we also tested drug effects on $Na_v1.5$ channels that were partially inactivated. Effects on $Na_v1.5$ current in partially fast-inactivated state were tested by applying a 1000 ms prepulse at −90 mV, followed by a 50 ms test pulse at −40 mV (see inserted protocols in figures). Effects on $Na_v1.5$ current in partially slow-inactivated state were tested by applying a 1000 ms prepulse at −40 mV, followed by a 20 ms pulse at −120 mV allowing recovery from fast inactivation, and finally a test pulse to −40 mV (see inserted protocols in figures).

Action potential measurement

Action potentials were measured in single rabbit ventricular cardiomyocytes at 36°C using the amphotericin-perforated patch-clamp technique. Cells were superfused with modified Tyrode's solution containing (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.5, and HEPES 5.0 (pH adjusted to 7.4 with NaOH). Pipette solution contained (in mM) K-gluconate 125, KCl 20, NaCl 5.0, Amphotericin-B 0.44, and HEPES 10 (pH adjusted to 7.2 with KOH). Action potentials were evoked at 1–3 Hz using square 3-ms current pulses through the patch pipette, and potentials were corrected for the calculated liquid junction potential.[32] We analyzed resting membrane potential (RMP), AP amplitude (APA), maximal AP upstroke velocity (dV/dt_{max}), and AP duration at 20, 50, or 90% repolarization (APD_{20}, APD_{50}, and APD_{90}, respectively). Action potential parameters from 10 consecutive APs were averaged.

Preparation of opioids

Tramadol, fentanyl, and codeine (purity ≥98%), purchased from Sigma-Aldrich, were freshly dissolved in the bath solution to the desired concentration just before use. $Na_v1.5$ current was measured at baseline conditions (no drug) and after 5–8 min wash-in of these drugs at various concentrations that are close to the lethal concentrations of these drugs.[33–35] Tramadol was measured at 1, 10, 30, 100, 300, or 1000 μM. Fentanyl was measured at 1 μM. Codeine was measured at 100 μM.

Statistical analysis

Values are presented as mean ± SEM. Curve fitting and statistics was performed using Prism8 GraphPad (GraphPad Software, LLC, USA). One-way ANOVA or two-way ANOVA was used to assess the statistical

significance of the differences among multiple groups. One-way repeated measures (RMs) ANOVA followed by pairwise comparison using the Holm–Sidak multiple comparisons test or one-way RM ANOVA on ranks (Friedman test) followed by Dunn's multiple comparison test for *post hoc* analyses was used when data were not normally distributed. Differences between two groups were tested using paired Student's *t*-tests or two-way RMs ANOVA followed by pairwise comparison using the Holm–Sidak multiple comparisons test. Details on normalization are given in Methods or in the figure legends. $P < 0.05$ was considered to be statistically significant.

Results

Effects on $Na_v1.5$ current amplitude in HEK293 cells

We first studied whether high (lethal) concentrations of tramadol, fentanyl, or codeine impact $Na_v1.5$ current amplitude in HEK293 cells by applying single 100 ms depolarizing pulses from −120 to −40 mV (*Figure 1A–C*). We found that 1 μM fentanyl and 100 μM codeine had no effect on fully available $Na_v1.5$ current amplitude (*Figure 1B and C*). In contrast, 1000 μM tramadol reduced $Na_v1.5$ current amplitude by 69 ± 3.7% ($n = 11$) (*Figure 1A*). We therefore conducted additional studies with tramadol to assess its effects on $Na_v1.5$ biophysical properties in more detail.

Effects of tramadol on current amplitude and gating properties of $Na_v1.5$ channels in HEK293 cells

Using a similar depolarizing step as used in *Figure 1*, we studied the concentration dependency of tramadol-induced block (*Figure 2A*) of fully available $Na_v1.5$ current and found that the IC_{50} of current reduction was 378.5 ± 33.2 μM. Subsequently, we tested if 300 μM tramadol, a concentration close to the IC_{50}, caused changes in gating properties of $Na_v1.5$. *Figure 2B* shows typical $Na_v1.5$ currents under baseline condition and in the presence of 300 μM tramadol measured over a wide range of depolarizing voltages (for protocol, see *Figure 2B*, inset). *Figure 2C* shows the average *I–V* relationships and indicates that tramadol significantly decreased $Na_v1.5$ current in the voltage range from −60 to 30 mV, e.g. by 56.4 ± 4.5% at −40 mV ($n = 6$, $P < 0.05$). Tramadol induced a hyperpolarizing shift in voltage dependency of both activation and inactivation (*Figure 2D*). The average $V_{1/2}$ of channel activation was at −54.6 ± 1.2 mV in the absence and −61.3 ± 1.6 mV in the presence of tramadol ($n = 6$, $P < 0.05$). The average $V_{1/2}$ of channel inactivation were −96.0 ± 2.2 mV (baseline) and −105.2 ± 2.7 mV (tramadol), respectively ($n = 6$, $P < 0.05$, *Table 1*). The slope of the activation curve, *k*, was significantly changed after the application of tramadol from 5.9 ± 0.4 to 5.2 ± 0.3 mV ($n = 6$, $P < 0.05$, *Table 1*). To study the rate-dependent effects of tramadol, we applied double-pulse protocols as shown in *Figure 2E and F*. We found that tramadol delayed recovery from steady-state inactivation (*Figure 2E* and *Table 1*) with τ_f and τ_s significantly changed from 10.9 ± 2.3 to 34.5 ± 8.0 ms, and from 293.6 ± 70.4 to 800.1 ± 139.3 ms, respectively ($n = 5$, $P < 0.05$, *Table 1*). Consistent with this observation, the reduction in $Na_v1.5$ current density at rising pulse numbers increased more in the presence of tramadol, e.g. by 81.7 ± 3.6% at the 30th pulse ($n = 9$, $P < 0.05$) (*Figure 2F*). Time constants of current inactivation at −20 mV were fitted with biexponential fits and were not significantly affected by 300 μM tramadol (data not shown).

Effects of tramadol on density of partially inactivated $Na_v1.5$ current in HEK293 cells

A previous study in neuronal sodium channels showed that tramadol induced more pronounced effects when these channels were in a partially inactivated state than when they were in a fully available state.[28] To study whether this also occurs in $Na_v1.5$ channels, we studied the concentration dependency of tramadol on $Na_v1.5$ current of channels in a partially fast-inactivated or partially slow-inactivated state in HEK293 cells (*Figure 3*). The used voltage clamp protocols are shown in the left panels; the middle panels depict typical current traces. *Figure 3A* shows that tramadol blocked partially fast-inactivated $Na_v1.5$ current in a concentration-dependent manner, with an IC_{50} of 4.5 ± 1.1 μM. *Figure 3B* shows that tramadol blocked partially slow-inactivated $Na_v1.5$ current in a concentration-dependent manner, with an IC_{50} of 16 ± 4.8 μM.

Effects of tramadol on action potentials in rabbit ventricular cardiomyocytes

To verify the functional implication of the tramadol-induced effects on $Na_v1.5$ current, we tested the effects of tramadol (10, 30, 100, and 300 μM) on APs of isolated rabbit left ventricular cardiomyocytes. *Figure 4A* shows typical APs elicited at 1 Hz under baseline conditions and in the presence of 30 and 300 μM tramadol; average AP parameters after application of 10–300 μM tramadol are summarized in *Figure 4B–G*. Tramadol reduced dV/dt_{max} in a concentration-dependent manner (*Figure 4D*), while other AP parameters (RMP, APA, APD_{20}, APD_{50}, and APD_{90}) were not significantly changed (*Figure 4B, C*, and *E–G*). Subsequently, we studied the effect of 300 μM tramadol on APs at faster stimulation frequencies. *Figure 5A* shows typical APs elicited at 1, 2, and 3 Hz under baseline conditions and in the presence of 300 μM tramadol; average AP parameters at 1–3 Hz are summarized in *Figure 5B–G*. Tramadol caused statistically significant decreases in APA and dV/dt_{max} in a frequency-dependent manner (*Figure 5C and D*). For example, APA decreased by 4.1 ± 1.5% (from 119.2 ± 1.2 to 114.2 ± 1.0 mV, $n = 6$, $P < 0.05$) at 1 Hz and by 9.0 ± 1.3% (from 119 ± 2.3 to 108.3 ± 2.3 mV, $n = 6$, $P < 0.05$) at 3 Hz (*Figure 5C*). Similarly, dV/dt_{max} was significantly decreased at all rates, e.g. by 41.7 ± 9.0% (from 327.7 ± 44.3 to 192.2 ± 37.8 V/s, $n = 6$, $P < 0.05$) at 1 Hz and by 58.2 ± 6.7% (from 320.2 ± 51.7 to 136 ± 31.4 V/s, $n = 6$, $P < 0.05$) at 3 Hz (*Figure 5D*). Thus, the reductions in APA and dV/dt_{max} were larger at higher stimulation frequencies ($n = 6$, $P < 0.05$), consistent with the reduced rate of recovery from inactivation of $Na_v1.5$ (*Figure 2E and F*). Resting membrane potential, APD_{20}, APD_{50}, and APD_{90} at 1–3 Hz were not significantly affected by tramadol (*Figure 5B and E–G*).

Discussion

Our main findings were as follows: (i) tramadol reduced current of $Na_v1.5$ channels in a fully available state in a dose-dependent manner, while fentanyl and codeine had no effect even when tested at lethal concentrations; (ii) tramadol shifted the voltage dependency of (in)activation and slowed the recovery from inactivation of $Na_v1.5$; (iii) tramadol caused block of $Na_v1.5$ channels at lower concentrations when these channels were in a partially inactivated state than when they were in a fully available state; (iv) tramadol reduced dV/dt_{max} and APA in rabbit cardiomyocytes with a larger amount of reduction at fast pacing rates; and (v) tramadol did not affect AP repolarization.

Our results on $Na_v1.5$ are consistent with previous findings on the effects of tramadol on neuronal sodium channels. Of note, the concentration of tramadol required for 50% inhibition of $Na_v1.5$ currents when measured at a holding potential of −120 mV (IC_{50} 378.5 ± 33.2 μM) was only mildly greater than the concentrations needed to obtain 50% block of TTX-sensitive neuronal sodium currents in rodent neuroblastoma ND7/23 cells (194 ± 9 μM)[24] and of rat $Na_v1.2$ in HEK293 cells (103 ± 8 μM).[28] Very recently, Bok *et al.*[36] also tested the effect of tramadol on $Na_v1.7$ and $Na_v1.5$ currents in HEK293 cells. They found that tramadol reduced $Na_v1.5$ current, consistent with our findings, with an IC_{50}

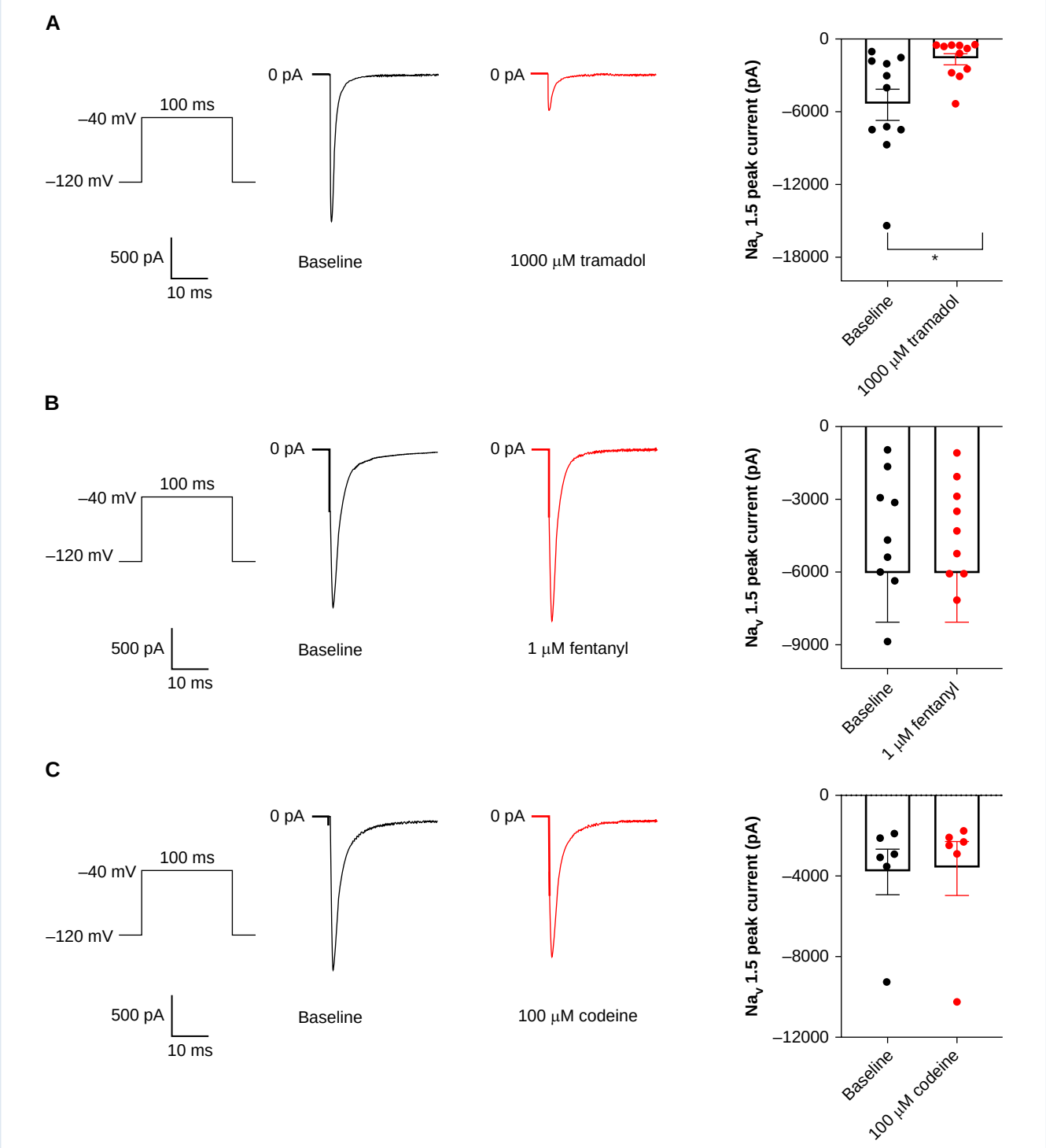


Figure 1 Effects of opioids on current densities of $Na_v1.5$ channels expressed in HEK293 cells. (*A*–*C*) Typical $Na_v1.5$ current elicited by application of 100 ms depolarizing pulses from −120 to −40 mV (left panels) and average current amplitudes (right panels) in the absence or presence of 1000 μM tramadol (*A*), 1 μM fentanyl (*B*), and 100 μM codeine (*C*). Data are expressed as the mean ± SEM. $*P < 0.05$, tramadol vs. baseline (paired Student's *t*-tests).

of 0.85 ± 0.04 mM at a −120 mV holding potential. We also used a holding potential of −120 mV, but we found an IC_{50} of 0.38 ± 0.03 mM, which is thus 2.2 times smaller than in the study of Bok *et al.*[36] In both studies, the IC_{50} decreases upon more depolarized holding potentials indicating that the blocking effect of tramadol is increased in the presence of more inactivated $Na_v1.5$ channels. This principle is also observed for $Na_v1.5$ and $Na_v1.2$ currents in HEK293 cells.[28,36] In our study, the $V_{1/2}$ of $Na_v1.5$ inactivation is slightly more negative under control conditions

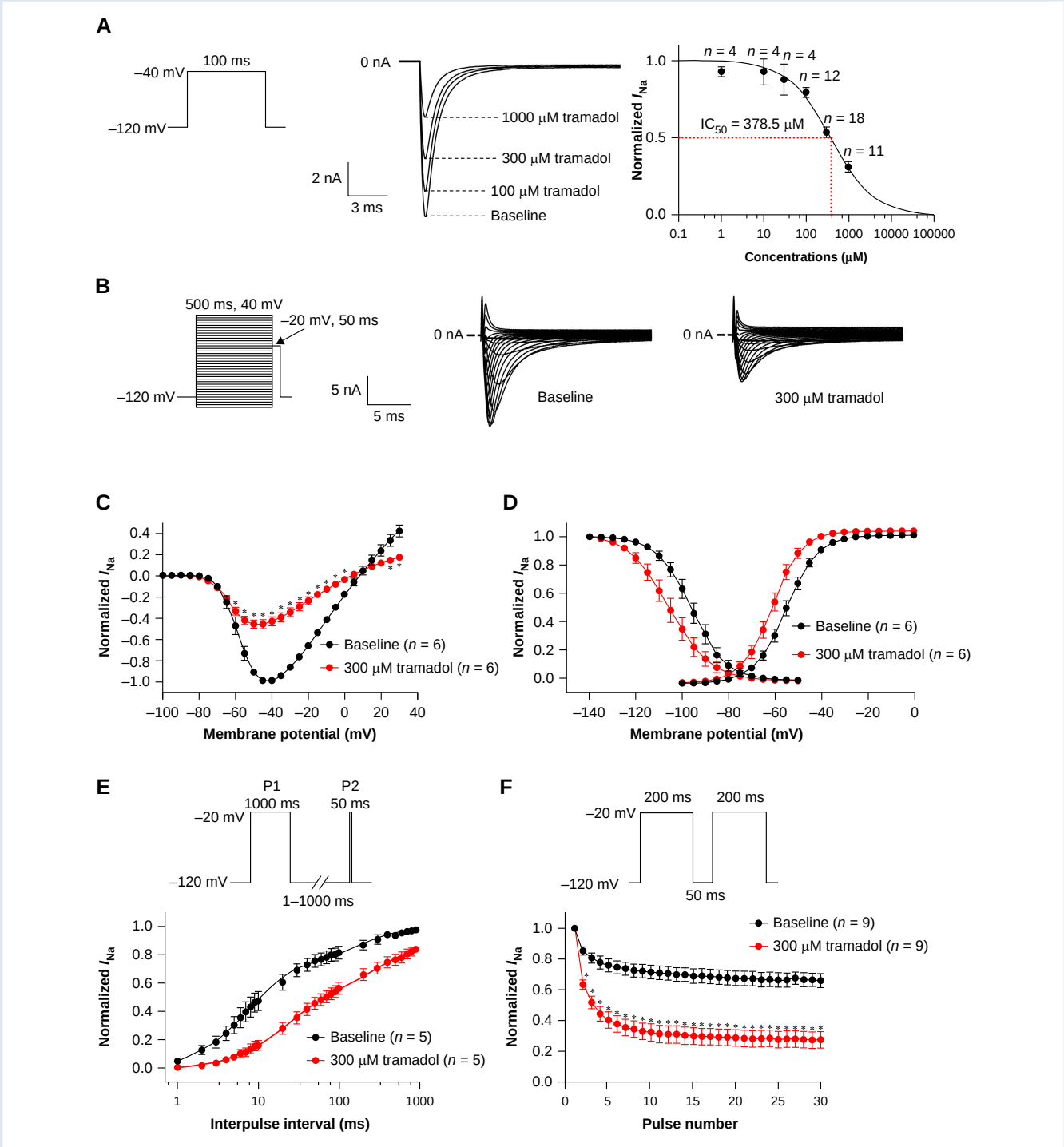


Figure 2 Effects of tramadol on amplitude and gating kinetics of $Na_v1.5$ currents expressed in HEK293 cells. (*A*) Typical $Na_v1.5$ currents under baseline conditions and in the presence of 100–1000 μM tramadol; examples (left panel) and concentration dependency (right panel) of $Na_v1.5$ current inhibition. The solid line is a Hill fit to the average data. Values are normalized to the values measured under baseline conditions. Numbers near symbols indicated the number of cells (*n*) measured at the given concentrations. Inset: voltage clamp protocol used. (*B*) Typical $Na_v1.5$ currents under baseline conditions and in the presence of 300 μM tramadol. Inset: voltage clamp protocol used to measure current–voltage (*I–V*) relationships and the voltage dependency of (in)activation. Cycle length was 5 s. (*C*) The $Na_v1.5$ *I–V* relationships before and after the application of 300 μM tramadol. $Na_v1.5$ current was normalized to the maximal peak amplitude under baseline conditions, but peak current was set to −1 to retain the well-known inward direction of the sodium current. (*D*) Effects of tramadol on the voltage dependency of $Na_v1.5$ current (in)activation. Solid lines are Boltzmann fits to the average data. (*E*) Recovery from inactivation of $Na_v1.5$ current in the absence or presence of 300 μM tramadol measured with a double-pulse protocol with variable interpulse intervals (inset). (*F*) Use dependency under baseline conditions and in the presence of tramadol measured during a train of 30 depolarizing pulses with an interpulse interval of 50 ms (inset). *$P < 0.05$, tramadol vs. baseline (two-way RM ANOVA).

Table 1 Cardiac sodium current properties in the absence or presence of 300 μM tramadol

		Baseline	Tramadol
Activation	$V_{1/2}$ (mV)	−54.6 ± 1.2 (n = 6)	−61.3 ± 1.6 (n = 6)*
	k (mV)	5.9 ± 0.4 (n = 6)	5.2 ± 0.3 (n = 6)*
Inactivation	$V_{1/2}$ (mV)	−96.0 ± 2.2 (n = 6)	−105.2 ± 2.7 (n = 6)*
	k (mV)	6.6 ± 0.4 (n = 6)	7.5 ± 0.6 (n = 6)
Recovery from inactivation	τ_f (ms)	10.91 ± 2.28 (n = 5)	34.52 ± 7.93 (n = 5)*
	τ_s (ms)	293.6 ± 70.35 (n = 5)	800.1 ± 139.3 (n = 5)*
	$A_s/(A_s + A_f)$ (ms)	0.29 ± 0.03 (n = 5)	0.47 ± 0.02 (n = 5)*

Data are expressed as the mean ± SEM, and n indicates the number of cells.
*$P < 0.05$, tramadol vs. baseline (paired Student's t-tests).

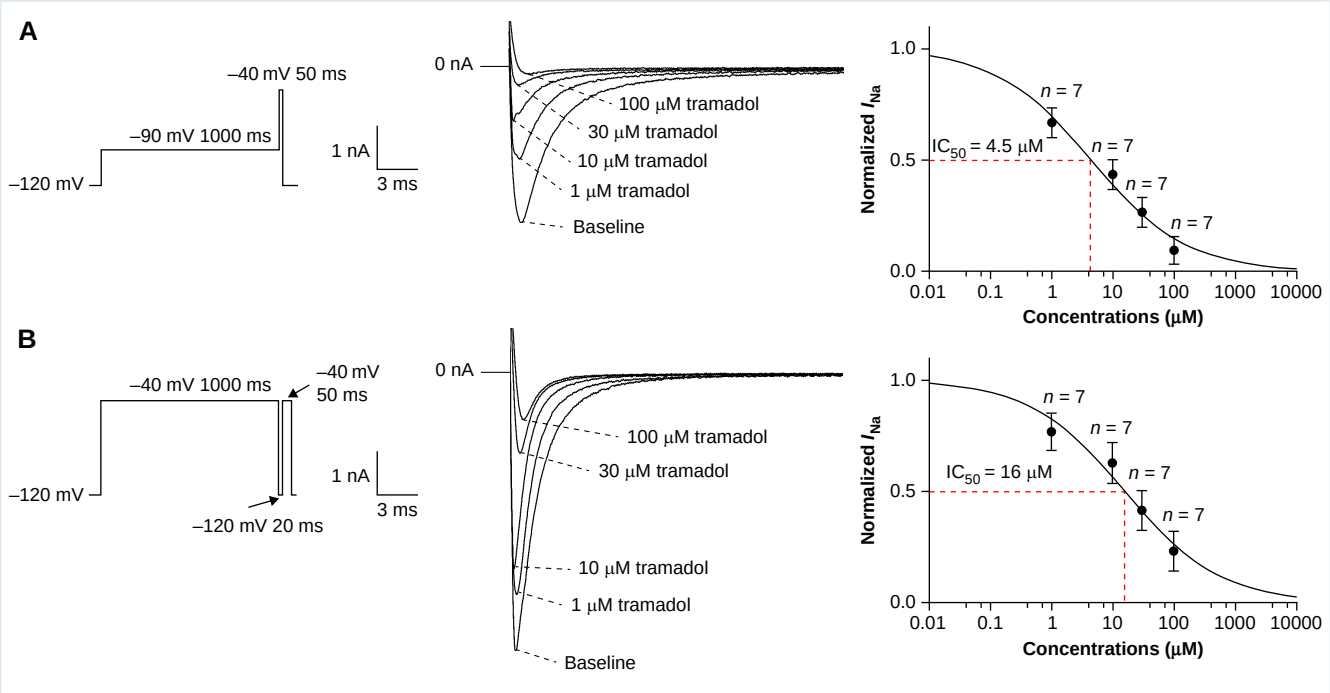


Figure 3 Effects of tramadol on amplitude of partially inactivated $Na_v1.5$ current in HEK293 cells. (*A* and *B*) Block of fast-inactivated (*A*) and slow-inactivated (*B*) $Na_v1.5$ current by tramadol in a concentration-dependent manner. Inset: voltage clamp protocol used to measure (left panel) and typical $Na_v1.5$ currents under baseline conditions and in the presence of 1–100 μM tramadol (middle panel). We achieved partial fast inactivation (*A*) by applying a −90 mV prepulse and studied the effect of partial fast inactivation by applying the test pulse immediately following this prepulse. We achieved partial slow inactivation (*B*) by applying a −40 mV prepulse, during which $Na_v1.5$ channels enter into both a fast-inactivated and a slow-inactivated state. This was followed by a brief (20 ms long) repolarizing pulse to −120 mV, which allows almost full recovery from fast inactivation, but not slow inactivation. Thus, the ensuing test pulse activates only $Na_v1.5$ channels that have recovered from fast inactivation, but not $Na_v1.5$ channels that are still in a slow inactivation state. Cycle length was 5 s. Solid lines are Hill fits to the average data. Values are normalized to the values measured under baseline conditions. Numbers near symbols indicated the number of cells (n) measured at the given concentrations.

compared to the study of Bok *et al.* As a result, we might have more inactivated $Na_v1.5$ channels at −120 mV resulting in a more pronounced inhibition and a shift in IC_{50} to lower concentrations compared to the study of Bok *et al.*[36] Moreover, the tramadol-induced negative shift of voltage-gated inactivation and the use-dependent block of $Na_v1.5$ that we found agree with the findings of Bok *et al.*,[36] and this was also observed for $Na_v1.2$ current[28] and $Na_v1.7$.[36] We found also a negative shift in the $V_{1/2}$ of activation, and this was also found by Bok *et al.* for $Na_v1.7$, but not for $Na_v1.5$.[36] While our findings are thus largely consistent with the study of Bok *et al.*, our study offers significant advances beyond that study by also including other opioids and AP studies in cardiomyocytes. Action potential studies are of particular relevance because previous studies have suggested that tramadol may impact not only on cardiac depolarization and $Na_v1.5$ current but also on cardiac repolarization and AP duration. Tramadol reduced dV/dt_{max} and APA in rabbit cardiomyocytes in a concentration-dependent and frequency-dependent manner,

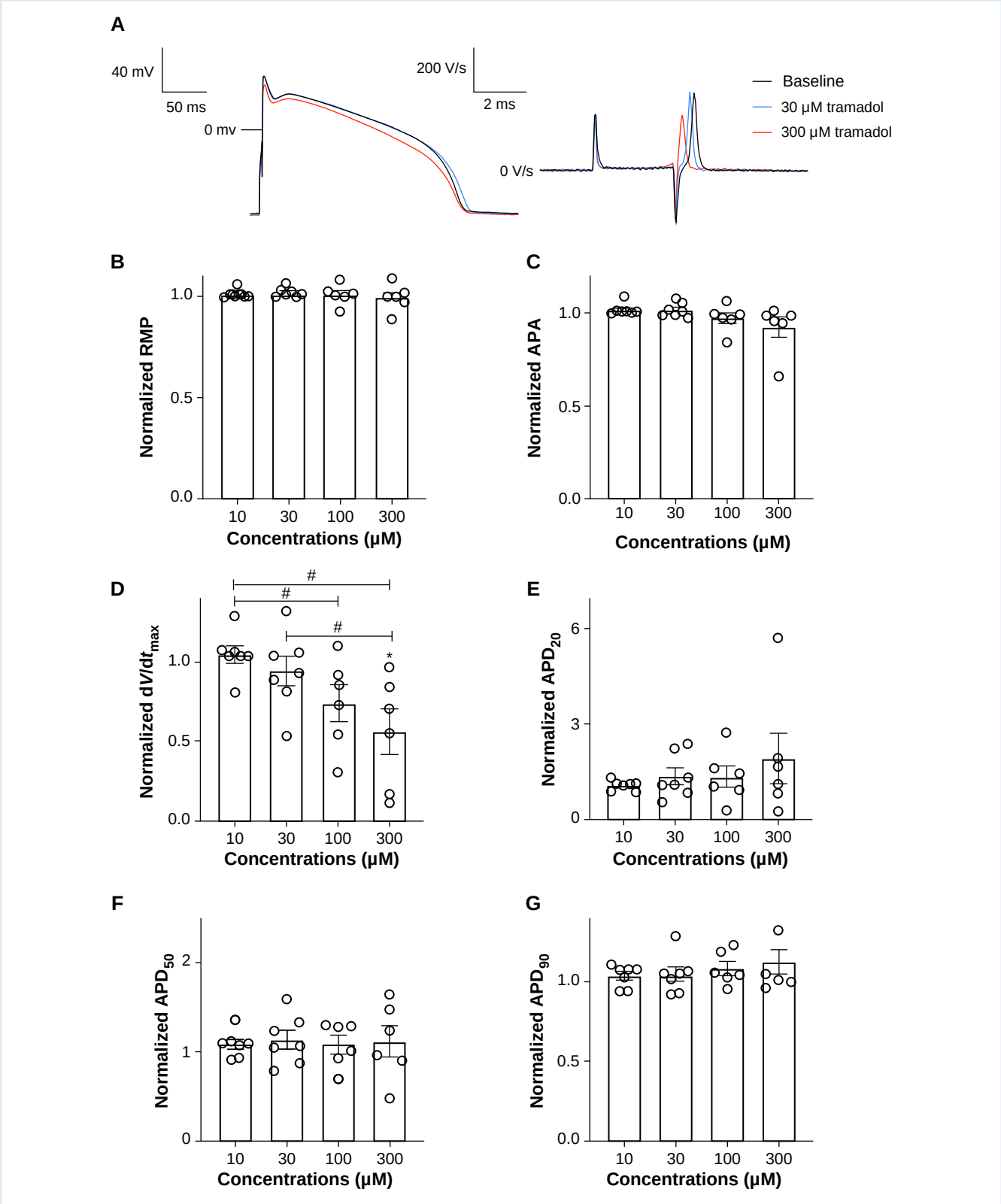


Figure 4 Concentration dependency of effects of tramadol on action potentials (APs) of single rabbit ventricular cardiomyocytes. (*A*) Superimposed representative APs at 1 Hz under baseline conditions and in the presence of 30 and 300 μM tramadol. Inset: time derivatives of the AP upstrokes. (*B*–*G*) Average AP characteristics at 1 Hz of resting membrane potential (*B*), AP amplitude (*C*), maximal AP upstroke velocity (*D*), AP duration at 20% of repolarization (*E*), AP duration at 50% of repolarization (*F*), and AP duration at 90% of repolarization (*G*) in the absence or presence of 10, 30, 100, or 300 μM tramadol. Data are mean ± SEM. $^{*}P < 0.05$, tramadol vs. baseline (paired *t*-test); $^{\#}P < 0.05$, drug effects observed with different concentrations (one-way RM ANOVA). APA, AP amplitude; APD_{20}, AP duration at 20% of repolarization; APD_{50}, AP duration at 50% of repolarization; APD_{90}, AP duration at 90% of repolarization; dV/dt_{max}, maximal AP upstroke velocity; RMP, resting membrane potential.

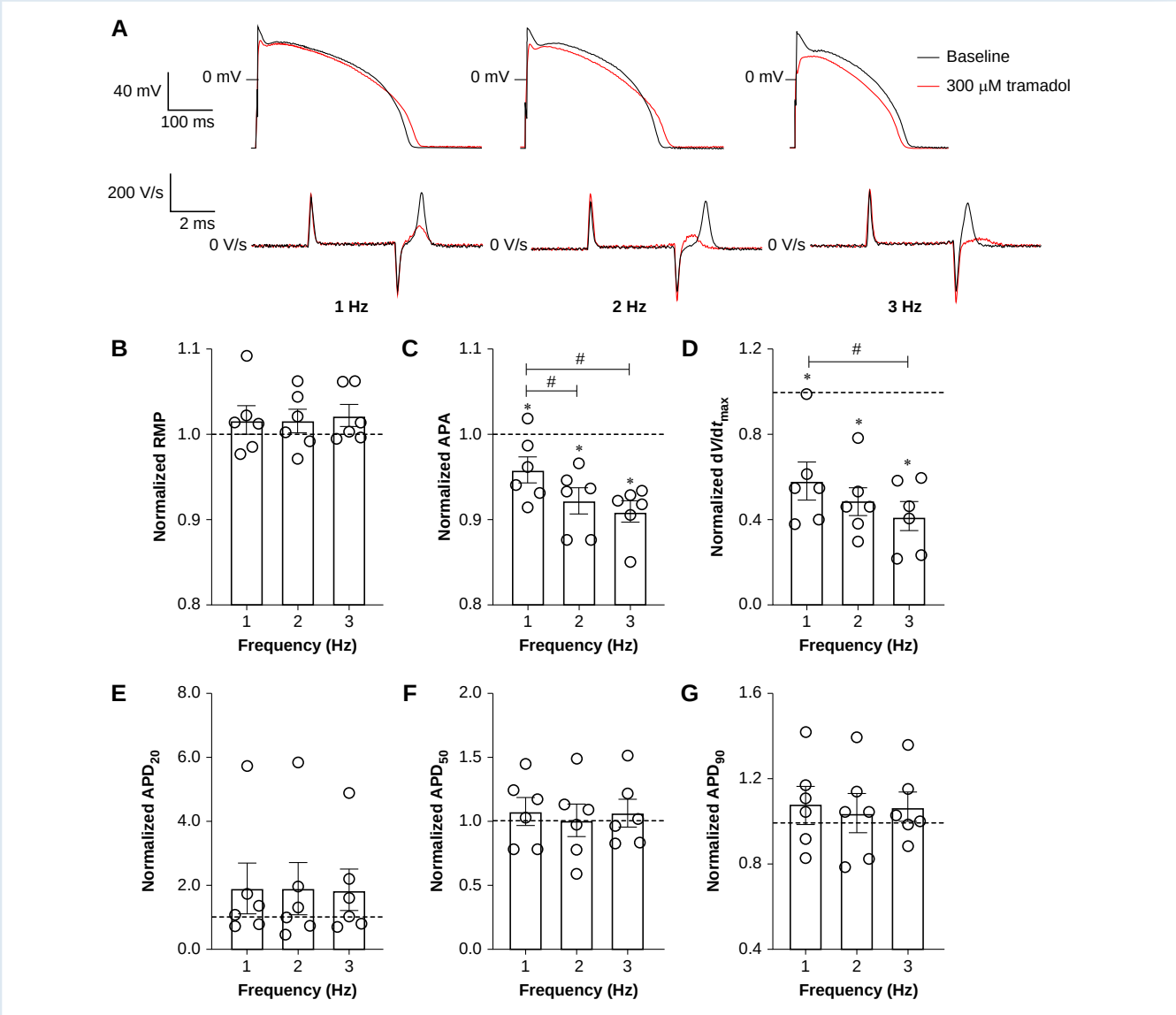


Figure 5 Frequency dependency of effect of 300 μM tramadol on APs of rabbit ventricular cardiomyocytes. (*A*) Superimposed representative APs at 1, 2, and 3 Hz under baseline conditions and in the presence of 300 μM tramadol. Inset: time derivatives of the AP upstrokes. (*B*–*G*) Average AP characteristics at 1, 2, and 3 Hz of RMP (*B*), APA (*C*), dV/dt_{max} (*D*), APD_{20} (*E*), APD_{50} (*F*), and APD_{90} (*G*). Action potential parameters are normalized to the AP parameters measured under baseline conditions in the same cell. Data are mean ± SEM. $^{*}P < 0.05$, tramadol vs. baseline (paired *t*-test); $^{\#}P < 0.05$, drug effects observed with different frequencies (one-way RM ANOVA).

consistent with our measurement of $Na_v1.5$ current in HEK293 cells. It has been reported that tramadol poisoning could induce tachycardia,[37] and the use dependency of tramadol's effects that we observed would exaggerate tramadol's inhibitory action on $Na_v1.5$.

This inhibitory action is in line with ECG changes such as QRS widening and Brugada syndrome (BrS) pattern, of patients with tramadol poisoning[37–39] which are importantly due to reduced $Na_v1.5$ channel function.[40,41] We report that the IC_{50} of tramadol to block fully available $Na_v1.5$ channels is 378.5 ± 33.2 μM. This is far above the therapeutic range (0.38–1.14 μM).[42] When we analyzed tramadol's effects on fully available $Na_v1.5$ currents, we used a strongly hyperpolarized holding potential of −120 mV. While this method is routinely used for electrophysiological studies, it may hamper extrapolation to physiological conditions, where the RMP of cardiac myocytes is around −80 mV. In fact, at −80 mV, many $Na_v1.5$ channels are in an inactivated state. Therefore, the effects of drugs, including tramadol, on partially inactivated $Na_v1.5$ channels are clinically more relevant. When we analyzed tramadol's effects on $Na_v1.5$ channels that were partially in a fast-inactivated state (by utilizing a RMP of −90 mV), we found that the inhibition of $Na_v1.5$ current was strongly enhanced. In fact, the IC_{50} on $Na_v1.5$ channels that were in a partially fast-inactivated state was now 4.5 ± 1.1 μM, which is close to therapeutic concentrations (up to 1.14 μM). This effect was also documented by Bok *et al.*[36] In the meantime, we also notice that this change is not so obvious in the AP measurements in which the cardiomyocytes have a RMP of around −80 mV. Our result showed that the tramadol-induced dV/dt_{max} reduction is concentration dependent, and tramadol-induced reduction of dV/dt_{max} was only present at 300 μM in comparison to the baseline condition. However, compared

to 10 μM, tramadol could also reduce the dV/dt_{max} at 100 μM (*Figure 4D*) in rabbit ventricular cardiomyocytes. This may be due to the different experiment conditions, such as measurement temperature, the sodium and fluoride concentrations of the used solutions, or the different cell models.[20,43–45] Even so, at clinically relevant high concentrations, tramadol-induced overdose toxicity still may be achieved in clinical cases. For instance, the tramadol concentrations in peripheral blood were reported from 1.6 mg/L (6.1 μM) to 15.1 mg/L (57.3 μM) in an overview report, which is close to our measured IC_{50} of $Na_v1.5$ channels in a partially fast-inactivated or slow-inactivated state.[46] In another report, tramadol fatal concentrations ranged from 0.03 to 134 mg/L (0.1–508.7 μM).[34]

While the effects of tramadol on $Na_v1.5$ channels occurred at concentrations that are somewhat higher than therapeutic concentrations, a relevant consideration is that certain patient groups may have increased sensitivity to the $Na_v1.5$ blocking effects of tramadol.[37] This may stem from acquired comorbidities and/or from inherited susceptibility. A highly prevalent acquired condition is myocardial ischemia and infarction.[47] The concept that these comorbidities may permit or facilitate the occurrence of fatal cardiac arrhythmia and SCD upon the use of $Na_v1.5$ current blocking drugs was discovered in the Cardiac Arrhythmia Suppression Trial. In this trial, patients randomized to the class 1c antiarrhythmic drugs flecainide and encainide—potent $Na_v1.5$ current blockers—suffered excess SCD rates compared to placebo-treated patients.[16] Excess SCD rates occurred in patients with myocardial ischemia.[48] Inherited susceptibility may stem from carrying variants in genes that encode subunits of the $Na_v1.5$ channel, in particular variants in *SCN5A*, the gene that encodes its α-subunit.[49,50] Loss-of-function mutations in this gene underlie the BrS[51] and cardiac conduction disease,[52] inherited cardiac arrhythmia syndromes associated with elevated SCD risk.

Tramadol was reported to block hERG current in NG108-15 neuronal cells (IC_{50} 25 μM),[53] and in neuronal APs modified by 4-aminopyridine (4-AP) in rat sciatic nerves (measured at 4 mM),[54] and to prolong the duration of the QTc interval of the ECG,[55] although the latter is not a consistent finding.[56] We found that tramadol had no significant effect on APD_{20}, APD_{50}, or APD_{90}. This suggests that tramadol may not reduce hERG current in cardiomyocytes or that our used concentrations of tramadol may have been insufficient to influence AP repolarization. An alternative explanation is that a reduction in outward hERG current is counterbalanced by reduced inward I_{Na} (this study and Bok *et al.*[36]) and L-type Ca^{2+} current,[57] culminating in no net change in AP duration. We also found no significant effect of tramadol on RMP, suggesting that I_{K1} is largely unaltered. Because RMP was unchanged, we had no evidence that reduction in $Na_v1.5$ channel availability contributed to the observed tramadol-induced reduction in dV/dt_{max}.[58]

We found no evidence that fentanyl or codeine blocked fully available $Na_v1.5$ current, even when tested at fatal concentrations (1 and 100 μM, respectively), although fentanyl was shown to block neuronal sodium current with IC_{50} of 141 ± 6 μM in rat $Na_v1.2$ current and 153.2 μM in rat cultured thalamic neurons.[28,59] Our findings are consistent with the study of Tschirhart *et al.*[60] who also found no effects of fentanyl (10 μM) on the sodium current of neonatal rat ventricular myocytes. Taken together, we found evidence that tramadol blocks $Na_v1.5$ current at clinically relevant concentrations. It is possible that this effect contributes to the increased SCD incidence observed among users of these drugs. Conversely, we found no evidence that fentanyl or codeine blocks $Na_v1.5$ current at clinically relevant concentrations.

Conclusion

In conclusion, we found that tramadol reduces $Na_v1.5$ current by reducing its current amplitude and changing its gating properties; these effects are reflected in changes in AP properties. Fentanyl and codeine have no effects on fully available $Na_v1.5$ current, even when tested at fatal concentrations.

Authors' contributions

H.L.T. conceived and designed the study. L.J. and A.O.V. structured and designed the patch-clamp studies. L.J. carried out the patch-clamp experiments and statistical analysis of the patch-clamp data and drafted the first version of the manuscript. M.W.V. contributed to sample preparation and edited the manuscript. All authors contributed to manuscript revision and approved the final version.

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Conflict of interest: None declared.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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