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Hypokalaemia induces Ca^{2+} overload and Ca^{2+} waves in ventricular myocytes by reducing Na^+,K^+-ATPase α_2 activity

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Key points

- Hypokalaemia is a risk factor for development of ventricular arrhythmias.
- In rat ventricular myocytes, low extracellular K^+ (corresponding to clinical moderate hypokalaemia) increased Ca^{2+} wave probability, Ca^{2+} transient amplitude, sarcoplasmic reticulum (SR) Ca^{2+} load and induced SR Ca^{2+} leak.
- Low extracellular K^+ reduced Na^+,K^+-ATPase (NKA) activity and hyperpolarized the resting membrane potential in ventricular myocytes. Both experimental data and modelling indicate that reduced NKA activity and subsequent Na^+ accumulation sensed by the Na^+, Ca^{2+} exchanger (NCX) lead to increased Ca^{2+} transient amplitude despite concomitant hyperpolarization of the resting membrane potential.
- Low extracellular K^+ induced Ca^{2+} overload by lowering NKA α_2 activity. Triggered ventricular arrhythmias in patients with hypokalaemia may therefore be attributed to reduced NCX forward mode activity linked to an effect on the NKA α_2 isoform.

Abstract Hypokalaemia is a risk factor for development of ventricular arrhythmias. The aim of this study was to determine the cellular mechanisms leading to triggering of arrhythmias in ventricular myocytes exposed to low K_o. Low K_o, corresponding to moderate hypokalaemia, increased Ca^{2+} transient amplitude, sarcoplasmic reticulum (SR) Ca^{2+} load, SR Ca^{2+} leak and Ca^{2+} wave probability in field stimulated rat ventricular myocytes. The mechanisms leading to Ca^{2+} overload were examined. Low K_o reduced Na^+,K^+-ATPase (NKA) currents, increased cytosolic Na^+ concentration and increased the Na^+ level sensed by the Na^+, Ca^{2+} exchanger (NCX). Low K_o also hyperpolarized the resting membrane potential (RMP) without significant alterations in action potential duration. Experiments in voltage clamped and field stimulated ventricular myocytes, along with mathematical modelling, suggested that low K_o increases the Ca^{2+} transient amplitude by reducing NKA activity despite hyperpolarization of the RMP. Selective inhibition of the NKA α_2 isoform by low dose ouabain abolished the ability of low K_o to reduce NKA currents, to increase Na^+ levels sensed by NCX and to increase the Ca^{2+} transient amplitude. We conclude that low K_o, within the range of moderate hypokalaemia, increases Ca^{2+} levels in

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ventricular myocytes by reducing the pumping rate of the NKA α_2 isoform with subsequent Na^+ accumulation sensed by the NCX. These data highlight reduced NKA α_2-mediated control of NCX activity as a possible mechanism underlying triggered ventricular arrhythmias in patients with hypokalaemia.

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Abbreviations AP, action potential; APD, action potential duration; K_o, extracellular potassium concentration; NCX, Na^+, Ca^{2+} exchanger; NKA, Na^+,K^+-ATPase; RMP, resting membrane potential; SR, sarcoplasmic reticulum.

Introduction

Hypokalaemia is a common electrolyte disturbance present in over 20% of hospitalized patients (Paice *et al.* 1986), and increases the risk of ventricular tachycardia and fibrillation more than four-fold (Goyal *et al.* 2012). Hypokalaemia is suggested to increase the propensity for both early and delayed afterdepolarizations, in addition to promoting re-entry circuits in the myocardium (Osadchii, 2010).

Low extracellular K^+ has been reported to result in aftercontractions after a positive inotropic response to K^+-free superfusion in guinea pig papillary muscles (Eisner & Lederer, 1979*a*,*b*). The inotropic and pro-arrhythmic effect of low K_o was suggested to be caused by reduced Na^+,K^+-ATPase (NKA) activity, leading to increased intracellular Na^+ concentration and subsequent Ca^{2+} overload through modulation of Na^+, Ca^{2+} exchanger (NCX) activity. Later studies have linked aftercontractions in myocardial samples to induction of Ca^{2+} waves at the cellular level (Orchard *et al.* 1983). In contrast to these findings, hyperpolarization of the resting membrane potential (RMP) has been reported to induce lower cellular Ca^{2+} levels in experiments in isolated ventricular myocytes superfused with 1 mM K^+ (Bouchard *et al.* 1995). Therefore, previous investigations suggest that low K_o may exert two opposite effects on NCX activity, namely increasing cytosolic Na^+ levels (increasing cellular Ca^{2+} levels) and inducing hyperpolarization of the RMP (reducing cellular Ca^{2+} levels).

The net effect of low K_o on NCX activity depends on the balance between the reduced NKA activity, RMP hyperpolarization and influence of other K_o-sensitive currents. None of the aforementioned studies are directly applicable to hypokalaemic patients, as these studies were performed with either no extracellular K^+ or K_o levels lower than observed in hypokalaemic patients. Moderate hypokalaemia is defined as serum K^+ levels of 2.5–3.0 mM, at which NKA will pump at reduced rates, in contrast to experiments conducted with 0 K_o, which abolishes NKA activity. Thus, whether low K_o within the clinically relevant range induces Ca^{2+} overload and subsequent Ca^{2+} waves in ventricular myocytes is not known.

The first aim of the present study was to determine whether low K_o (corresponding to clinically moderate hypokalaemia) induces Ca^{2+} overload and Ca^{2+} waves, which can be a substrate for delayed afterdepolarization in ventricular myocytes. Further, we wanted to determine the relative role of NKA inhibition, hyperpolarization of the RMP and altered action potential duration (APD) in controlling the cellular Ca^{2+} levels during low K_o. Our second aim was to determine the relative role of NKA α_1 and α_2 isoforms in mediating the cellular response to low K_o. While NKA α_1 is uniformly distributed throughout the sarcolemma, NKA α_2 is clustered in the t-tubules and is a potent regulator of NCX activity in ventricular myocytes by a yet undetermined mechanism (Swift *et al.* 2007; Despa *et al.* 2012). NKA α_1 and NKA α_2 also differ in terms of K_o sensitivity; NKA α_2 having a lower K^+ affinity than NKA α_1, with a reported K_o sensitivity in a range corresponding to levels found in hypokalaemic patients (Han *et al.* 2009). Here we show that reduced NKA α_2 activity is the main mechanism leading to Ca^{2+} overload in response to low K_o in ventricular myocytes, providing a cellular mechanism for triggered arrhythmias in hypokalaemic patients.

Methods

Animals and cell isolation

Male Wistar-Hannover rats (Møllegaard, Denmark; ~300 g) were housed in a temperature-regulated room with 12 h day/12 h night cycling, with *ad libitum* access to food and water. Rats were sedated with a mixture of 4% isoflurane, 64% N_2O and 31% O_2, before endotracheal intubation and ventilation (Zoovent, Triumph Technical Services, Milton Keynes, UK) with a mixture of 68% N_2O, 29% O_2 and 2.5% isoflurane. Then, 200 IU of heparin was injected I.V. prior to excision of the heart in surgical anaesthesia. The heart was rapidly cannulated and retrogradely perfused using the following solution (mM): NaCl 130, Hepes 25, $MgCl_2$ 0.5, KCl 5.4, NaH_2PO_4 0.4, D-glucose 22, pH adjusted to 7.4 with NaOH. Collagenase was added to the perfusion as previously described (Bokenes *et al.* 2008).

Ca^{2+} transients

Ca^{2+} transients were obtained from isolated ventricular myocytes, loaded with 20 μM fluo-4 AM (Molecular Probes, Eugene, OR, USA) and subsequently superfused at 37°C with the following solution (mM): NaCl 140, Hepes 5, KCl 5.0 or 2.7, $CaCl_2$ 1.0, $MgCl_2$ 0.5, D-glucose 5.5, NaH_2PO_4 0.4, pH adjusted to 7.4 with NaOH. Cells were excited at 488 nm and emitted light was registered with a photomultiplier (Photon Technology International, Monmouth Junction, NJ, USA) mounted on a Nikon inverted microscope as previously described (Louch *et al.* 2010). To correct for background fluorescence, cell-free fluorescence was obtained after each experiment and subtracted from the primary recording. The resulting Ca^{2+} transients were analysed with Clampfit 9.0 (Axon Instruments, Foster City, CA, USA). The following sets of experiments were performed using this setup: (1) Ca^{2+} transient amplitude, measured as the ratio between maximal (F) and basal (F_0) fluorescence during one twitch; (2) Ca^{2+} wave probability, measured as the probability of evoking a Ca^{2+} wave within 30 s following field stimulation at 1, 2 and 4 Hz. Ca^{2+} wave probability was measured at K_o = 5.0 mM, before switching to K_o = 2.7 mM, and back to K_o = 5.0 mM to test reversibility; (3) sarcoplasmic reticulum (SR) Ca^{2+} load, measured as the ratio between maximal and basal fluorescence after rapid application of 10 mM caffeine; (4) rate constant of Ca^{2+} extrusion mediated by SERCA2, NCX and other Ca^{2+} transport mechanisms. The SERCA2 rate constant was calculated as the difference between the rate constant for regular Ca^{2+} transients and the caffeine-evoked Ca^{2+} transient. Rate constants were calculated as 1/tau, where tau was obtained by a monoexponential fit of the extrusion phase of the Ca^{2+} transient (Diaz *et al.* 1997). The NCX rate constant was measured as the difference between the rate constant after rapid application of caffeine ± 10 mM Ni^+ in the superfusate (Bokenes *et al.* 2008). (5) SR Ca^{2+} leak, measured as the difference in resting fluorescence ± 1 mM tetracaine (Shannon *et al.* 2002). Ventricular myocytes were field stimulated in normal Tyrode's solution containing (mM): NaCl 140, KCl 5.0 or 2.7, $CaCl_2$ 1.0, $MgCl_2$ 1.0, D-glucose 10, Hepes 5, pH adjusted to 7.4 with NaOH. Field stimulation was stopped and leak was measured in a 0 Na^+/0 Ca^{2+} solution (mM): LiCl 140, KCl 5.0 or 2.7, $MgCl_2$ 1, D-glucose 10, Hepes 5, EGTA 10, pH adjusted to 7.4 with LiOH, ± 1 mM tetracaine.

NKA and NCX activity

NKA currents and NCX currents were measured using whole-cell voltage clamp as previously described (Swift *et al.* 2008), using an Axopatch 200B amplifier (Axon Instruments). The pipette solution contained (mM): Hepes 10, tetraethylammonium chloride 20, L-aspartate 42, EGTA 42, $CaCl_2$ 29.7, Na_2phosphocreatine 5, MgATP 10, NaOH 40, pH adjusted to 7.2 with CsOH. Free Ca^{2+} concentration was calculated to 300 nM, using WinMAX C 2.01 software (C. Patton, Stanford University, CA, USA). Average pipette resistance was ∼1 MΩ. After gaining whole-cell access, ventricular myocytes were superfused (37°C) with (mM): NaCl 147, $MgCl_2$ 2, EGTA 0.1, D-glucose 5.5, Hepes 5, $BaCl_2$ 2, nicardipine 0.001, pH adjusted to 7.40 with NaOH. The holding potential was –50 mV, and NKA currents were interpreted as the K_o-sensitive current induced by superfusion with either 2.7 or 5.0 mM KCl. I_{NCX} was elicited with 2 mM $CaCl_2$, and was elicited both in the presence and in the absence of K_o to study the effect of NKA-mediated regulation on NCX activity. A low dose of ouabain (0.3 mM) was used to selectively block the NKA α_2 isoform (Swift *et al.* 2007).

NKA I–V relationship was measured as described previously (Swift *et al.* 2007). Briefly, ventricular myocytes were depolarized (50 ms) to +70 mV from the holding potential of −50 mV, then hyperpolarized to −120 mV ($dV/dt = 380\ mV\ s^{-1}$), and back to −50 mV. The difference between the recorded current at baseline and during activation of NKA represented the I–V relationship of NKA.

Cytosolic Na^+ measurements

To measure the cytosolic Na^+ concentration, isolated ventricular myocytes were loaded at room temperature in sodium-binding benzofurzan isophthalate (SBFI) for 120 min, in the presence of 0.15% Pluronic F-127. Isolated ventricular myocytes were superfused with the same solution used for fluo-4 experiments. SBFI ratios were recorded with 5.0 mM KCl in the superfusate during field stimulation (1 Hz), before switching to 2.7 mM KCl. Each cell was calibrated by superfusing the cell with a solution containing gramicidin 2 $\mu g\ ml^{-1}$, ouabain 1 mM, Hepes 5 mM, glucose 5.5 mM, EGTA 2 mM, NaCl 0 or 14 mM, and KCl 140 or 0 mM, pH adjusted to 7.2 with NaOH or KOH. Cytosolic Na^+ concentration was calculated as previously described (Swift *et al.* 2007).

Voltage clamp protocols

For Ca^{2+} transient measurements, ventricular myocytes were voltage clamped using discontinuous mode (switching rate 9 kHz) on an Axoclamp 2B amplifier and pCLAMP software (Axon Instruments). Patch pipettes (1.5-2.5 MΩ) were filled with (in mM): CsCl 120, TEACl 20, Hepes 10, Na_2ATP 5, CsEGTA 0.02, and fluo-5F pentapotassium salt 0.1, pH adjusted to 7.2 with CsOH. Cells were patched in a solution containing (in mM): NaCl 140, Hepes 5, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 0.5, D-glucose 5.5, NaH_2PO_4 0.4, pH adjusted to 7.4 with NaOH. After

gaining whole cell access, cells were superfused with the following solution (in mM): NaCl 134, glucose 10, Hepes 10, $MgCl_2$ 1, KCl 5, 4-aminopyridine 5, $BaCl_2$ 0.1 and probenecid 2, pH adjusted to 7.4 with NaOH. The holding potential was −45 mV, and Ca^{2+} transients were triggered at 1 Hz by a 100 ms square voltage step from −45 to 0 mV. After stable Ca^{2+} transients were obtained, the superfusate was switched to a comparable solution, except KCl was reduced to 2.7 mM. Ca^{2+} transients were obtained with Cairn Research Optoscan Monochromator (excitation 485 nm, emission 515 nm long pass) (Cairn Research Ltd., Faverham, UK).

Background K^+ current was measured in ventricular myocytes by continuous mode voltage clamp using patch pipettes (2.0–2.5 MΩ) filled with the following solution (in mM): KCl 130, NaCl 10, Hepes 10, MgATP 5, $MgCl_2$ 1, EGTA 0.5, pH adjusted to 7.2 with KOH. The myocytes were superfused with a solution containing (in mM): NaCl 135, Glucose 10, Hepes 10, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, $CdCl_2$ 0.2, pH adjusted to 7.4 with NaOH. $CdCl_2$ was used to inhibit Ca^{2+} currents. The voltage clamped myocytes were held at -80 mV, and currents were elicited by 500 ms voltage steps to various test potentials in the range −170 mV to 50 mV. In order to study the effect of Ba^{2+} on background K^+ currents, 2 mM $BaCl_2$ (same concentration as used to study NKA and NCX currents) was added to the superfusate. The stable K^+ currents were measured in the same myocytes both with and without Ba^{2+} present.

Current clamp protocols

RMP and action potentials (APs) were recorded as previously described (Mork *et al.* 2009). APs were triggered by a 3 ms suprathreshold current injection. The pipette solution contained (in mM) potassium aspartate 120, $MgCl_2$ 0.5, NaCl 6, EGTA 0.06, Hepes 10, glucose 10, KCl 25 and K_2-ATP 4, pH adjusted to 7.2 with KOH.

Mathematical modelling

A computational simulation of the rat ventricular myocytes was performed using a whole-cell model (Niederer & Smith, 2007). This model integrates the electrophysiological components of the endocardial model of Pandit *et al.* (2009), the Ca^{2+} handling model of Hinch *et al.* (2004) and the contraction model of Niederer *et al.* (2006). The model features a single cytoplasmic pool of Ca^{2+} and Na^+, i.e. without involving a specific subspace whose specific concentration is sensed by NKA. The mathematical code was implemented in the CellML language (www.cellml.org; Terkildsen *et al.* 2008) and the differential equations integrated with the COR software (Cellular Open Resource, cor.physiol.ox.ac.uk), using the CVODE solver. Further analysis was performed in Matlab (MathWorks, Natick, MA, USA). The hypokalaemia experiments were simulated by instantaneously switching the value of the K_o exposed to each K^+-transporting channel separately, to allow the evaluation of the contribution of each channel in isolation.

Statistics

All data are presented as means ± SEM. Numbers of observations are presented as *n*. Statistical significance was calculated by Student's *t* test using either paired or unpaired tests. $P < 0.05$ was considered significant.

Results

Low K_o increased propensity for Ca^{2+} waves in isolated ventricular myocytes

Superfusion with K_o = 2.7 mM increased the Ca^{2+} wave probability at all frequencies tested (Fig. 1*A–C*, $P < 0.05$, $n = 7$). None of the included ventricular myocytes showed Ca^{2+} waves at 1 Hz with K_o = 5.0 mM, while 8 and 17% of the same cells showed Ca^{2+} waves at 2 and 4 Hz, respectively. Ca^{2+} wave probability was 63%, 92% and 92% in ventricular myocytes superfused with $K_o = 2.7$ mM and field stimulated at 1, 2 and 4 Hz, respectively. After again superfusing the cells with K_o = 5.0 mM, the Ca^{2+} wave probability was largely reversed, as 25%, 0% and 20% of the ventricular myocytes showed Ca^{2+} waves at 1, 2 and 4 Hz, respectively.

Low K_o increased Ca^{2+} transient amplitude, SR Ca^{2+} load and SR Ca^{2+} leak

Switching from K_o = 5.0 to 2.7 mM induced a biphasic response in Ca^{2+} transient amplitude in ventricular myocytes field stimulated at 1 Hz (Fig. 1*D* and *E*). Initially, within 30–60 s after the switch, the Ca^{2+} transient amplitude decreased in amplitude by 13 ± 2% ($P < 0.05$, $n = 9$)(1st phase in Fig. 1*D*). Subsequently, the Ca^{2+} transient amplitude gradually increased and reached a steady state 25 ± 7% above baseline within 4–6 min (2nd phase in Fig. 1*D*).

The increase in Ca^{2+} transient amplitude by low K_o was associated with a 36% increase in SR Ca^{2+} load compared to K_o = 5.0 mM (Fig. 1*F*, $P < 0.05$, n = 6–9). No SR Ca^{2+} leak was detected in cells superfused with K_o = 2.7 mM, while there was significant SR Ca^{2+} leak at K_o = 2.7 mM (Fig. 1*G*, $P < 0.05$, $n = 9$). Hence, increased frequency of Ca^{2+} waves in intact cells was associated with Ca^{2+} overload after exposure to low K_o.

Low K_o hyperpolarized RMP without altering AP duration

To determine the underlying cellular mechanism leading to Ca^{2+} overload by low K_o in field-stimulated ventricular myocytes, we investigated the relative contribution of RMP hyperpolarization and APD. In voltage-clamped ventricular myocytes stimulated at 1 Hz, switching K_o from 5.0 to 2.7 mM induced a significant hyperpolarization of the RMP (Fig. 2*A* and *B*, $P < 0.05$, $n = 6$). None of the analysed APD parameters were significantly altered by low K_o (Fig. 2*C*).

Voltage-clamped ventricular myocytes did not exhibit the 1st negative phase induced by low K_o

To test whether the increase in Ca^{2+} transient amplitude was dependent upon RMP hyperpolarization, we next recorded Ca^{2+} transients in cells with clamped RMP to prevent the hyperpolarization by low K_o at 1 Hz stimulation. As shown in Fig. 2*D*, the Ca^{2+} transient amplitude gradually increased after switching K_o from 5.0 to 2.7 mM, reaching a stable state in ~60 s. Notably, in contrast to the intact field-stimulated ventricular myocytes, Ca^{2+} transient amplitude increased in all cells compared to baseline at all analysed time points after switching K_o from 5.0 to 2.7 mM (Fig. 2*D*–*G*). In the same ventricular myocytes, both peak and the integral of L-type Ca^{2+} currents were decreased by switching K_o from 5.0 to 2.7 mM (Fig. 2*H* and *I*). Decreased L-type Ca^{2+} currents concomitant with increased Ca^{2+} transient amplitude indicate low K_o that to increase Ca^{2+} levels by enhancing SR fluxes relative to trans-sarcolemmal Ca^{2+} cycling, possibly by reducing forward mode NCX activity.

Low K_o decreased NKA activity and increased Na^+ concentration in ventricular myocytes

To test whether reduced NKA activity mediated the increased Ca^{2+} levels observed by low K_o, we next examined NKA and NCX currents in a series of experiments shown in Fig. 3. The NKA current was 25±1% lower by adding 2.7 mM K^+ to the superfusate compared

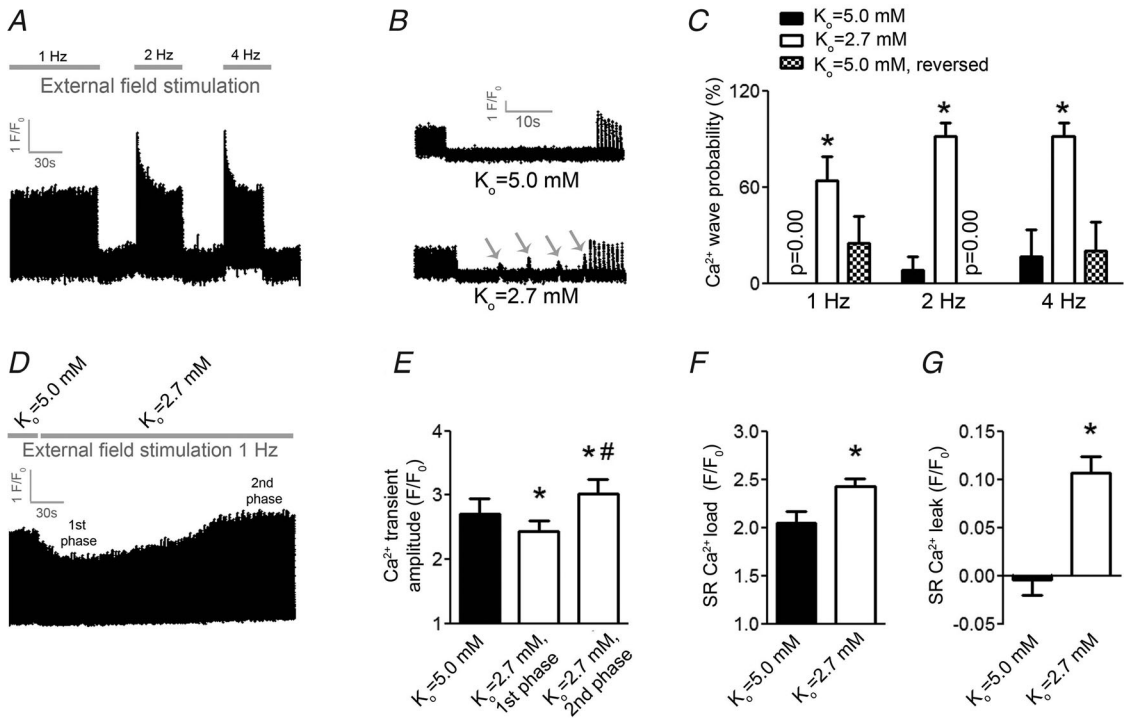

Figure 1. Low extracellular K^+ increases probability for Ca^{2+} waves, Ca^{2+} transient amplitude, SR Ca^{2+} load and SR Ca^{2+} leak in ventricular myocytes
A, protocol for evaluation of Ca^{2+} wave frequency in field-stimulated ventricular myocytes. Stimulation frequencies were as indicated, with 30 s pause after each stimulation frequency. *B*, representative tracings of cellular fluorescence after superfusion with $K_o = 5.0$ mM (top) and $K_o = 2.7$ mM (bottom) at 1 Hz stimulation frequency. Grey arrows indicate Ca^{2+} waves. *C*, number of cells exerting Ca^{2+} waves at various stimulation frequencies. *D*, representative tracing of Ca^{2+} transient amplitude in 1 Hz field-stimulated ventricular myocytes after switch from $K_o = 5.0$ to 2.7 mM. *E*, Ca^{2+} transient amplitude at baseline, and 1st and 2nd phase as indicated in *D*. *F*, SR Ca^{2+} load evoked by rapid caffeine application after reaching stable (2nd) phase at 1 Hz field stimulation. *G*, SR Ca^{2+} leak. $^*P < 0.05$ *vs.* $K_o = 5.0$ mM, $^{\#}P < 0.05$ *vs.* $K_o = 2.7$ mM.

to 5.0 mM K^+ (Fig. 3*H*, $P < 0.05$, $n = 7$), and NKA currents were lower by switching K_o from 5.0 to 2.7 mM at all membrane potentials (Fig. 3*A* and *B*). Based on the NKA *I-V* plot shown in Fig. 3*B* and the detected hyperpolarization of the RMP by $K_o = 2.7$ mM (Fig. 2*B*), switching K_o from 5.0 mM to 2.7 mM was calculated to reduce NKA currents by ~48%. Corresponding to the reduced NKA activity, we detected an increase in cytosolic Na^+ in intact cells field stimulated at 1 Hz (Fig. 3*C* and *D*; $P < 0.05$, $n = 8$).

Low K_o increased Na^+ levels sensed by NCX in voltage-clamped ventricular myocytes

Whether low K_o increases Na^+ concentration sensed by NCX was next tested in a voltage clamp experiment designed to measure NKA-dependent control of NCX activity (Fig. 3*G*). To control for background K^+ currents, we first obtained *I–V* plots for the background K^+ current and tested the effect of adding Ba^{2+} to the superfusate. As shown in Fig. 3*E* and *F*, Ba^{2+} blocked the background K^+ currents at −60 to −30 mV.

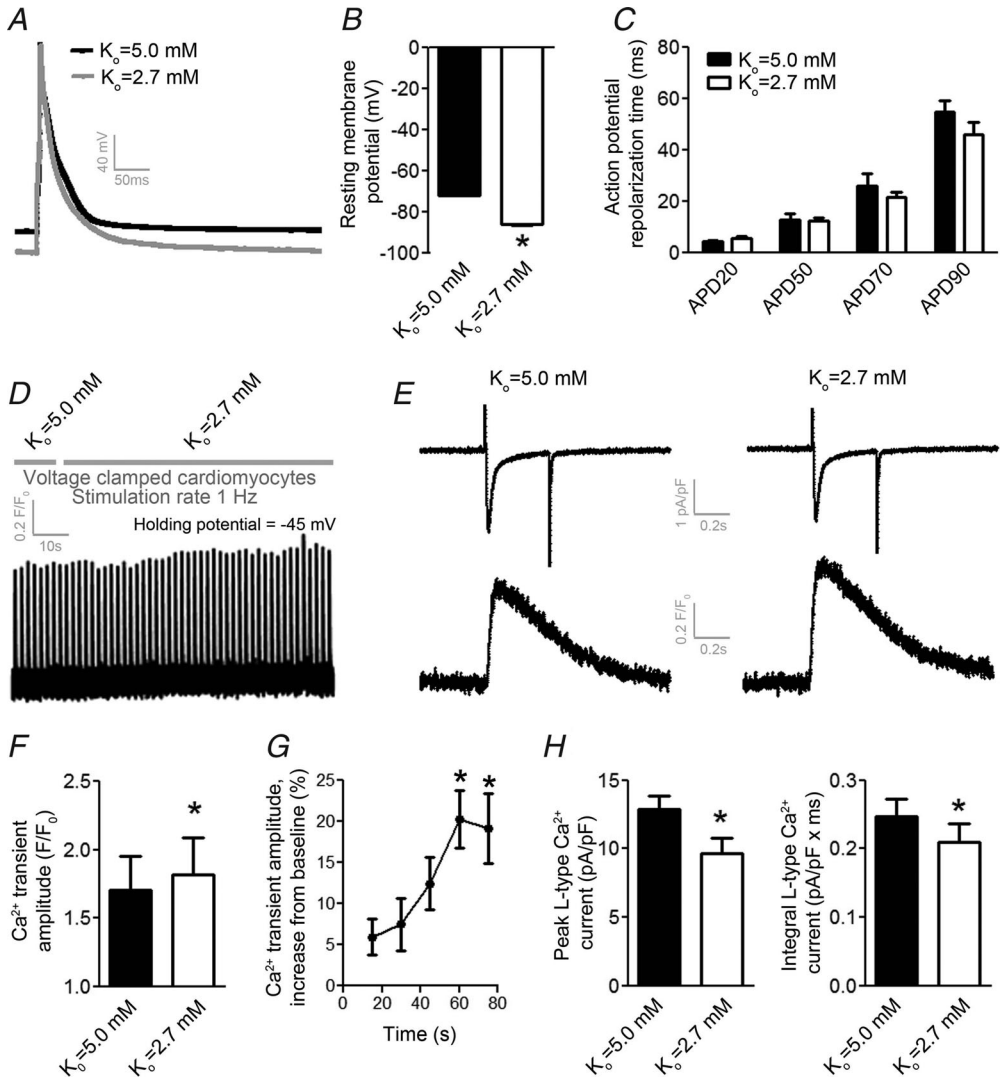

Figure 2. Low extracellular K^+ increases Ca^{2+} transient amplitude in ventricular myocytes with clamped membrane potential
A, representative tracings of RMPs and APs at 1 Hz stimulation. *B*, mean RMPs. *C*, mean AP repolarization time. *D*, representative tracing of Ca^{2+} transients obtained from voltage-clamped cells and stimulated at 1 Hz (holding potential –45 mV). *E*, representative Ca^{2+} transients and L-type Ca^{2+} currents during a single twitch in the same cells at $K_o = 5.0$ mM and after switch to $K_o = 2.7$ mM. *F*, mean Ca^{2+} transient amplitude in voltage-clamped ventricular myocytes. *G*, time development of Ca^{2+} transient amplitude after switch from $K_o = 5.0$ to 2.7 mM. *H*, peak and integral L-type Ca^{2+} current before and after switch from $K_o = 5.0$ to 2.7 mM. $^*P < 0.05$ *vs.* baseline.

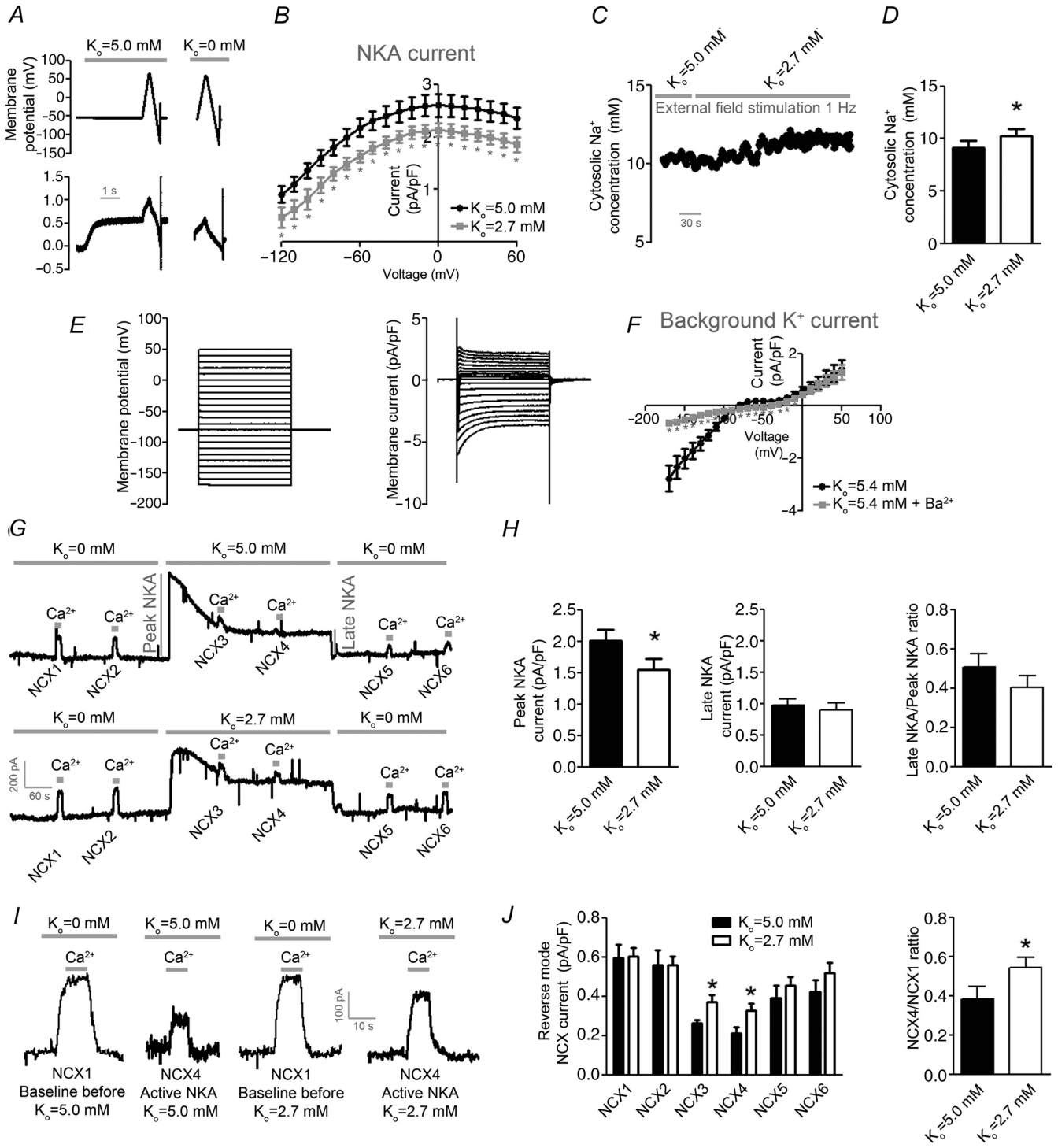

Figure 3. Low extracellular K^+ reduces NKA activity and increases Na^+ level sensed by NCX
A, protocol and representative tracing used to obtain *I–V* plots for NKA currents. *B*, *I–V* plots for NKA currents. *C*, representative tracing of cytosolic Na^+ level converted from SBFI fluorescence in 1 Hz field-stimulated ventricular myocytes. *D*, cytosolic Na^+ concentration. *E*, protocol and representative tracing used to obtain *I–V* plots of background K^+ current. *F*, *I–V* plot for background K^+ currents with and without Ba^{2+} present in the superfusate. *G*, representative tracing of protocol used to obtain NKA-dependent regulation of NCX currents. *H*, NKA currents in whole cell voltage-clamped ventricular myocytes. *I*, representative tracings of reverse mode NCX currents evoked by activation of Ca^{2+} before and after activation of NKA with either K_o = 5.0 mM (left) or K_o = 2.7 mM (right). *J*, mean NCX currents (left) and NCX4/NCX1 ratio (right). $^*P < 0.05$.

Based on the obtained *I–V* plots for NKA and background K^+ currents, we examined NKA-dependent regulation of NCX at a holding potential of −45 mV and in the presence of Ba^{2+}. Stepping K_o from 0 to 5.0 mM induced a larger peak NKA current than stepping K_o from 0 to 2.7 mM (Fig. 3*H*, left panel). The NKA current gradually declined in all cells examined, and stabilized at a lower level than the initial peak current (late NKA current in Fig. 3*G*, *H*). The NCX current during activation of NKA was ∼30% higher in cells superfused with K_o = 2.7 mM compared to K_o = 5.0 mM (NCX3–4) (Fig. 3*I*, *J*, $P < 0.05$, $n = 6$–8). Because we measured reverse mode NCX current in this protocol, these data suggest that NKA activation by 2.7 mM K^+ increases the Na^+ level sensed by the NCX compared to 5.0 mM K^+.

Low K_o reduced NCX-mediated Ca^{2+} extrusion

The results described above suggest low K_o both reduces NKA activity and leads to RMP hyperpolarization, which would have opposite effects on NCX driving forces in intact cells. As shown in Fig. 4, the rate constant for NCX-mediated Ca^{2+} extrusion (forward mode) was lower in cells superfused with 2.7 mM K^+ compared to 5.0 mM K^+ ($P < 0.05$, $n = 6$–9) at 1 Hz field stimulation. The removal of cytosolic Ca^{2+} by SERCA2 and additional, slow Ca^{2+} extrusion mechanisms were not significantly different between cells superfused with 2.7 and 5.0 mM K^+. These results support a model where reduced NKA activity exerts a more pronounced effect on NCX activity than hyperpolarization of RMP.

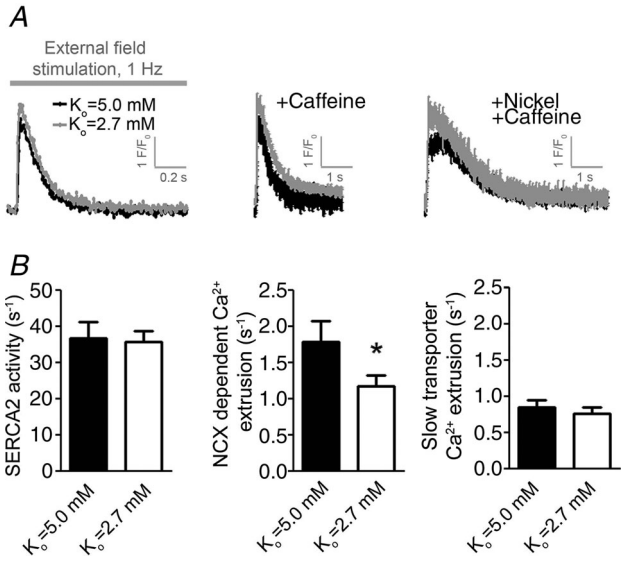

Figure 4. Low extracellular K^+ decreases NCX-mediated Ca^{2+} extrusion in ventricular myocytes
A, representative tracings of Ca^{2+} transients in field-stimulated (1 Hz) ventricular myocytes (left), after rapid application of caffeine (middle) and after rapid application of caffeine in the presence of nickel (right). *B*, rate constant of SERCA2 activity (left), NCX-mediated Ca^{2+} extrusion (middle) and Ca^{2+} extrusion by other Ca^{2+} transporters (right). *$P < 0.05$.

Modelling supported a role for NKA in mediating increased Ca^{2+} levels during low K_o

To further investigate the role of NKA activity in the Ca^{2+} response induced by low K_o, we utilized a previously published mathematical model of rat ventricular myocyte electro-mechanical function (Niederer & Smith, 2007). In line with the experimental results, the model predicted that switching K_o from 5.0 to 2.7 mM would induce a comparable biphasic response in Ca^{2+} transient amplitude (Fig. 5*A*, *B*) at 1 Hz contraction frequency. The 2nd phase with increased Ca^{2+} transient amplitude was further characterized by increased SR Ca^{2+} load, reduced NKA currents, increased cytosolic Na^+ concentration and reduced NCX currents (Fig. 5*C*).

A factorial analysis was performed (Fig. 5*D*) to test the relative role of NKA versus other ion transporters sensitive to K_o in mediating the increased Ca^{2+} levels by low K_o. In this analysis, the 2nd positive phase observed experimentally was not present when holding NKA pump activity at the rate obtained with K_o at 5.0 mM while all other ion transporters were exposed to K_o at 2.7 mM. Thus, the factorial analysis suggests that reduced NKA activity is the main mechanism leading to the increase in Ca^{2+} transient amplitude by low K_o.

Inhibition of NKA α_2 abolished the increase in Ca^{2+} transient amplitude by low K_o

We next investigated the hypothesis that reduction of NKA α_2 currents mediates the increase in Ca^{2+} transient amplitude by low K_o. NKA *I–V* relationships at K_o at 5.0 and 2.7 mM in voltage clamp cardiomyocytes exposed to a low dose of ouabain that selectively inhibits NKA α_2 (Swift *et al.* 2007) are shown in Fig. 6*A* and *B*.

To test whether NKA α_2 mediates the increase in cellular Ca^{2+} levels by low K_o, we obtained Ca^{2+} transients in ventricular myocytes field stimulated at 1 Hz in the presence of 0.3 μM ouabain. At baseline, pretreatment with ouabain increased Ca^{2+} transient amplitude (data not shown). Switching K_o from 5.0 to 2.7 mM induced the 1st negative phase (Fig. 6*C*, *D*, $P < 0.05$, $n = 9$) within a comparable time frame as in cells without ouabain present. Importantly, the 2nd positive phase was abolished in cells pretreated with ouabain (Fig. 6*C*, *D*), indicating that reduced NKA α_2 current is a key mediator of the increase in Ca^{2+} transient amplitude seen with low K_o.

Selective NKA α_2 inhibition blunted the ability of low K_o to increase Na^+ levels sensed by NCX

As low K_o was not able to increase the amplitude of Ca^{2+} transients in ventricular myocytes pretreated with ouabain, we investigated whether Na^+ levels sensed by NCX was increased by low K_o in the presence of ouabain (Fig. 6*H–K*). Both peak and late-stage NKA currents were comparable at K_o at 5.0 and 2.7 mM in ventricular myocytes exposed to ouabain. Importantly, neither of the measured NCX currents (NCX3–4) or the NCX4/NCX1 ratio were different between cells perfused with 5.0 and 2.7 mM K^+ in the presence of ouabain. These results are consistent with a model where low K_o increases Na^+ levels sensed by NCX by preferentially lowering NKA α_2 activity in ventricular myocytes.

Selective NKA α_2 inhibition did not alter RMP or AP response to low K_o

To exclude that 0.3 μM ouabain altered the RMP or AP response to low K_o, we obtained RMP and AP in ventricular myocytes superfused with ouabain before and after switching K_o from 5.0 to 2.7 mM. As for experiments without ouabain, switching K_o from 5.0 to 2.7 mM hyperpolarized the RMP in the presence of ouabain (Fig. 6*E*, *F*, $P < 0.05$, $n = 6$). None of the analysed APD times were significantly altered by switching K_o from 5.0 to 2.7 mM in the presence of ouabain (Fig. 6*G*).

Discussion

In this study, we show that low K_o, corresponding to the serum K^+ levels in patients with moderate hypokalaemia, leads to increased Ca^{2+} transient amplitude, SR Ca^{2+} load and Ca^{2+} wave probability in ventricular myocytes. As we found that reduced NKA activity exerts a more pronounced effect on NCX activity than the concomitant RMP hyperpolarization, we suggest that reduced NKA activity is the main modulator of Ca^{2+} levels in ventricular myocytes in response to low K_o. Ca^{2+} overload induced by low K_o was not exacerbated by the presence of an NKA α_2 selective dose of ouabain, suggesting that triggered arrhythmias in hypokalaemic patients are linked to reduced NKA α_2-mediated control of NCX activity.

Previous studies have reported both negative (Bouchard *et al.* 2004) and positive inotropic effects (Eisner & Lederer, 1979*a*,*b*; Christe, 1983; White & Terrar, 1991) in response to low K_o. Hyperpolarization of the RMP, altered APD and NKA inhibition have all been suggested to contribute to the inotropic response to low K_o in various cardiac preparations. In the present study, we detected a biphasic response eventually leading to an increase in Ca^{2+} transient amplitude by switching K_o from 5.0 to 2.7 mM

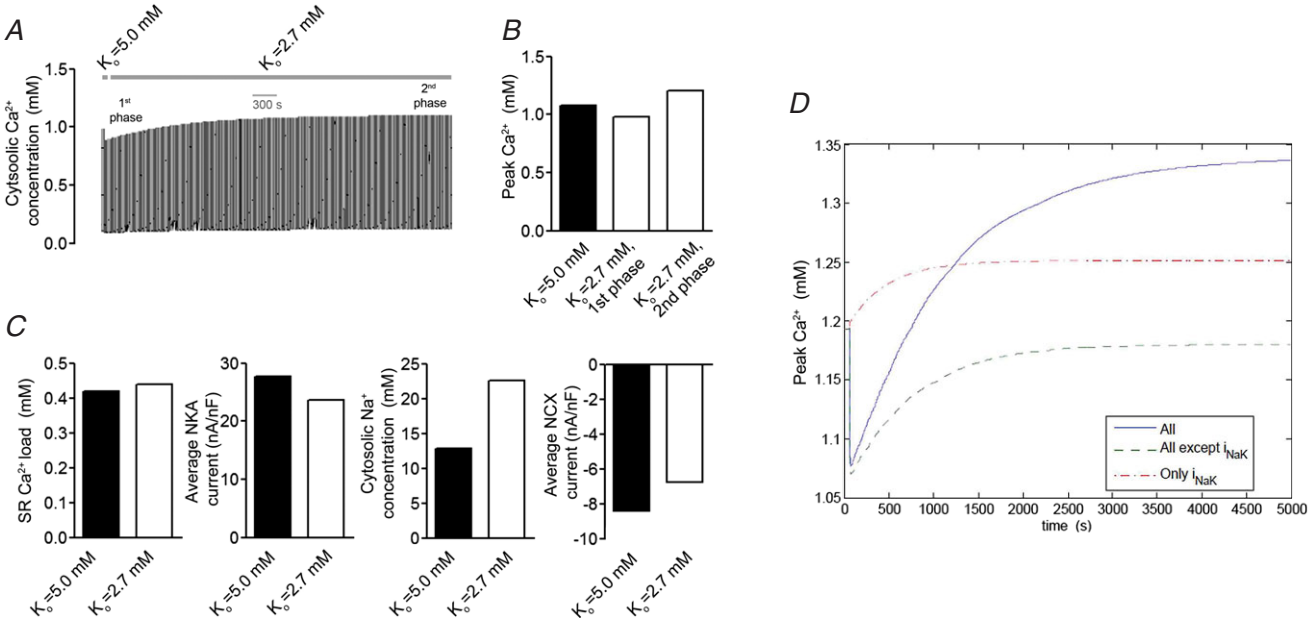

Figure 5. Modelling predicts NKA inhibition as a mechanism for increased Ca^{2+} transients by low extracellular K^+
A, cytosolic Ca^{2+} concentration at 1 Hz contraction rate in whole cell model after switch from K_o = 5.0 to 2.7 mM. *B*, peak Ca^{2+} levels in 1st and 2nd phase after switch from K_o = 5.0 to 2.7 mM. *C*, SR Ca^{2+} load, mean NKA current, cytosolic Na^+ concentration and mean NCX current after switch from K_o = 5.0 to 2.7 mM. *D*, factorial analysis of peak Ca^{2+} levels after switch from K_o = 5.0 to 2.7 mM without K_o dependency of NKA or K_o dependency of all K_o-sensitive ion transporters other than NKA.

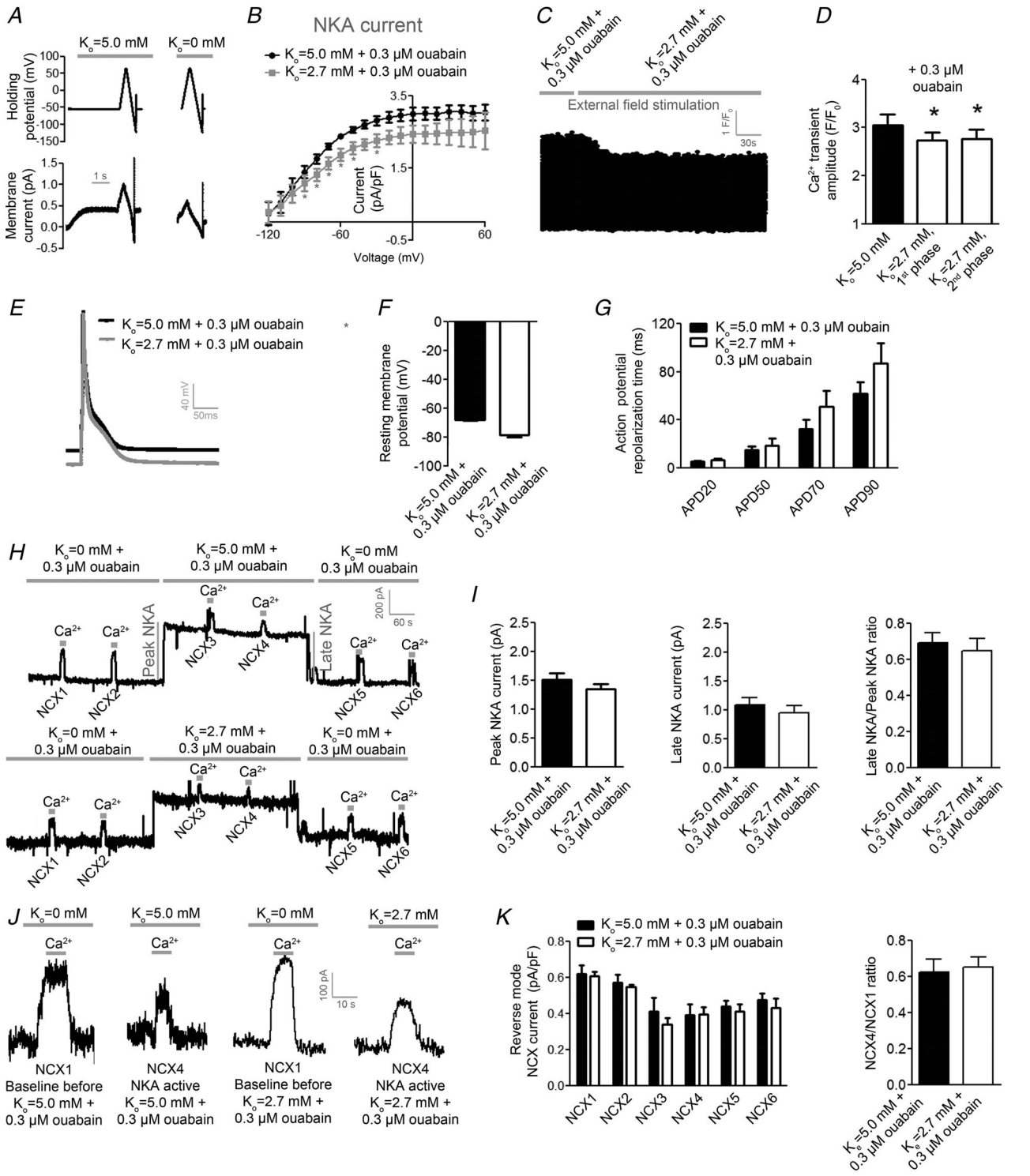

Figure 6. NKA α_2 inhibition abolishes the increase in Ca^{2+} transient by low extracellular K^+
A, protocol and representative tracing used to obtain *I–V* plots for NKA currents. *B*, *I–V* plots for NKA currents in the presence of 0.3 μM ouabain. *C*, representative tracing of Ca^{2+} transients in ventricular myocytes after switch from K_o = 5.0 to 2.7 mM in the presence of 0.3 μM ouabain and field stimulated at 1 Hz. *D*, mean Ca^{2+} transient amplitude. *E*, representative tracings of RMPs and APs. *F*, mean RMP. *G*, mean AP repolarization time. *H*, representative tracings of experiments used to obtain NKA-dependent regulation of NCX currents in the presence of 0.3 μM ouabain. *I*, NKA currents in whole cell voltage-clamped ventricular myocytes in the presence of 0.3 μM ouabain. *J*, reverse mode NCX currents evoked by Ca^{2+} in whole cell voltage-clamped ventricular myocytes. *K*, mean NCX currents (left) and NCX4/NCX1 ratio. $^*P < 0.05$.

in field-stimulated ventricular myocytes, corresponding to the 'early' and 'late' effects previously reported in guinea-pig papillary muscles (Eisner & Lederer, 1979*a*). Several investigators have linked the positive inotropic response to low K_o to reduced NKA activity (Eisner & Lederer, 1979*b*; Godfraind & Ghysel-Burton, 1980), although it has been reported that alterations in NKA activity could depress contractility through excessive Ca^{2+} loading (Schouten *et al.* 1990).

We found that NKA currents were sensitive to switching K_o from 5.0 to 2.7 mM (Fig. 2*B*), in accordance with earlier reports (Nakao & Gadsby, 1986; Han *et al.* 2009). The decline of NKA currents observed over minutes in the protocol used to study NKA-dependent control of NCX activity (Fig. 3*G*) may be caused by a gradual cellular Na^+ depletion as a result of NKA activation. However, it might also involve post-translational modifications of NKA (Aronsen *et al.* 2013). In the present study, $K_o = 5.0$ mM induced a larger peak NKA current than $K_o = 2.7$ mM, while the late phase NKA currents were similar. To explain these findings, we suggest that low K_o initially decreases NKA currents due to the K_o dependency of NKA, and subsequently leads to increased cellular Na^+ levels. In line with this idea, reverse mode NCX currents during stable state NKA currents were higher at $K_o = 2.7$ mM than at 5.0 mM (Fig. 3*I*, *J*). As NCX currents in this protocol are expected to be mostly regulated by the intracellular Na^+ levels, these data suggest that low K_o leads to cytosolic Na^+ accumulation sensed by the NCX.

Low K_o has previously been reported to decrease cellular Ca^{2+} levels by increasing forward mode NCX due to hyperpolarization of the RMP (Bouchard *et al.* 2004). In line with several earlier studies (Eisner & Lederer, 1979*a*; Christe 1983; Bouchard *et al.* 2004), we observed a marked hyperpolarization of the RMP by low K_o. In a protocol designed to study Ca^{2+} transients with a fixed membrane potential, low K_o was still able to increase Ca^{2+} levels in ventricular myocytes without the initial decline in Ca^{2+} transient amplitude (1st phase). Based on this observation, we suggest that the 1st and negative phase observed in field-stimulated ventricular myocytes is due to hyperpolarization of the RMP by low K_o, while the stable 2nd phase is due to reduced NKA activity.

Mathematical modelling showed that the K_o dependency of NKA is necessary for low K_o to increase Ca^{2+} transient amplitude, and thus supports the experimental data. Two notable discrepancies between the model and the experiments were observed. First, the time from switching to low K_o until the Ca^{2+} transient amplitude was increased compared to baseline was longer in the model simulation compared to the experiments (~15 *vs.* ~5 min). The model is parameterized at room temperature, which may significantly prolong the time until equilibrium. Furthermore, the model predicted an increase in cytosolic Na^+ that was ~10 times larger than what was observed experimentally. The model does not take into account any of the suggested subcellular Na^+ gradients that could regulate Ca^{2+} levels in ventricular myocytes (Aronsen *et al.* 2013). Absence of such microdomains in the model could explain the slow development of the cellular response to low K_o and the higher predicted Na^+ concentration. Future experimental and mathematical studies will be needed to shed more light on the NKA-dependent control of NCX activity within possible cellular microdomains.

We and others have previously reported that the NKA α_2 isoform is a key determinant of cardiac contractility and excitation–contraction coupling (James *et al.* 1999; Swift *et al.* 2007, 2008; Despa *et al.* 2012). Selective reduction of NKA α_2 currents have been shown to increase Ca^{2+} transient amplitude independent of the global cytosolic Na^+ level (Swift *et al.* 2007, Despa *et al.* 2012). Based on these reports, we proposed that low K_o induces Ca^{2+} overload by reducing NKA α_2 activity and thereby increases Na^+ concentrations sensed by NCX. In line with this idea, in contrast to experiments without ouabain, low K_o was not able to increase NCX currents in the presence of ouabain. Furthermore, we did not observe any increase in Ca^{2+} transient amplitude by switching K_o from 5.0 to 2.7 mM in cells pretreated with ouabain. Based on the NKA currents reported in Figs 3 and 6, NKA α_2 was calculated to contribute by 24% and 12% to the total NKA current at

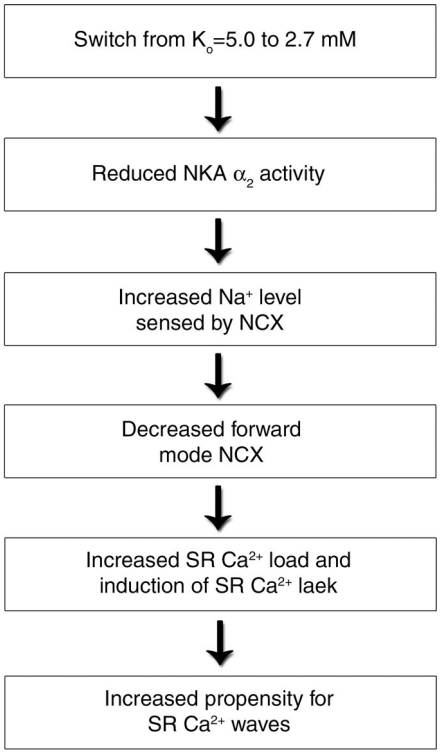

Figure 7. Proposed mechanism for increased propensity for Ca^{2+} waves in ventricular myocytes by low extracellular K^+

$K_o = 5.0$ and 2.7 mM, respectively, which is in line with previous reports that NKA α_2 is more sensitive to low K_o than NKA α_1 in the range of clinical hypokalaemia (Han *et al.* 2009). We did not detect any significant alterations in AP duration by low K_o, indicating that increased intracellular Ca^{2+} levels by low K_o is not caused by AP alterations as previously reported (White & Terrar, 1991). Altogether, we suggest a model where low K_o induces Ca^{2+} overload by lowering NKA α_2 activity in ventricular myocytes, thereby increasing Na^+ levels sensed by NCX (Fig. 7).

In conclusion, we demonstrate that low K_o, within the range of moderate hypokalaemia, leads to increased Ca^{2+} wave probability in isolated ventricular myocytes. This effect was linked to a reduction in NKA α_2 activity and a subsequent increase of Na^+ levels that is sensed by the NCX. This provides a possible mechanism underlying triggered ventricular arrhythmias in patients with hypokalaemia.

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Additional information

Competing interests

None of the authors has any conflict of interest.

Author contributions

J.M.A., J.S., W.E.L., K.H., M.K.S., F.S., O.M.S., I.S.: conception and design of the cellular experiments, and collection, analysis and interpretation of data from the cell experiments. A.L., S.A.N., N.P.S.: conception and design of the mathematical models, computational simulations and analysis of simulation results. All authors approved the final version of the manuscript.

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