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Steady-state Coupling of Plasma Membrane Calcium Entry to Extrusion Revealed by Novel L-type Calcium Channel Block

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Abstract

The L-type Ca^{2+} channel ($Ca_V1.2$) is the main pathway for trans-sarcolemmal (SL) Ca^{2+} influx in cardiac myocytes. To maintain Ca^{2+} homeostasis, chronic SL Ca^{2+}-influx must be matched by chronic SL efflux. In this study we tested the hypothesis that chronic down-regulation of SL Ca^{2+}-entry regulates SL extrusion. We studied mRNA and Ca^{2+} handling responses to chronic down-regulation of Ca^{2+} channel current induced by over-expression of the small GTPase Rem. Rem lowered net SL diastolic Ca^{2+}-entry, and reduced the twitch Ca^{2+} amplitude. Rem also significantly slowed Ca^{2+} transient decay kinetics ($p<10^{-3}$). Rem reduced NCX1.1 protein level and function. To measure Na-Ca^{2+}-exchange (NCX) function and sarcoplasmic reticulum (SR) store load we perfused Ca^{2+}-free bath for 25s followed by rapid application of 50mM caffeine. In control, caffeine transient relaxations were described by a bi-exponential decay with a fast phase that was 10mM Ni^{2+}-senstive. Rem significantly slowed caffeine-induced relaxation time course (Rem vs control, $p<10^{-6}$). To test whether extrusion slowing was mediated by insufficient basal Ca^{2+} for allosteric NCX activation we measured the effect of increasing bath Ca^{2+} from 1.8 to 6mM on caffeine-induced relaxation kinetics. 6mM Ca^{2+} did not alter kinetics of control cells, but in Rem-over-expressed cells 6mM Ca^{2+} sped kinetics. We conclude that chronic block of $Ca_V1.2$ channel-mediated SL entry alters NCX expression, and coincidentally controls SR Ca loading and SL Ca^{2+} efflux.

Keywords

Ca-channel; Na / Ca exchanger; G-proteins; calcium (cellular); sarcolemma

1. Introduction

Sarcolemmal (SL) Ca^{2+} flux is crucial for beat to beat excitation-contraction coupling (ECC) in cardiac myocytes (CM). In mature CM, SL Ca^{2+} flux is dominated by L-type Ca^{2+} channel

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($Ca_V1.2$) entry, this L-type Ca^{2+} current ($I_{Ca,L}$) provides the trigger for Ca^{2+}-induced Ca^{2+} release (CICR) of sarcoplasmic reticular (SR) Ca^{2+}. During the relaxation phase Ca^{2+} is extruded from the cytosol by activation of forward mode Na^+-Ca^{2+} exchange current, and SR Ca^{2+} is returned via the SERCa-ATPase. A SL Ca-ATPase also extrudes cytosolic Ca^{2+}, but it is slower and a relatively minor contributor in most preparations[1,2]. To maintain Ca^{2+} homeostasis, CMs must balance SL Ca^{2+}-entry to SL Ca^{2+}-efflux. Although beat to beat imbalances may occur, a sustained mismatch of SL Ca^{2+} influx/efflux would result in a chronic change of internal Ca^{2+}.

For any given beat the fraction of Ca^{2+} from SL/SR varies depending on species and age. In mature rodents only ~8% of Ca^{2+} flux originates from the extracellular space, whereas in humans and other large mammals ~30% of Ca^{2+} flux is trans-SL[3]. Similarly, an important distinction between mature and developing systems is the relative importance of trans-SL Ca^{2+} compared to SR as a source of cytosolic Ca^{2+}. In the developing heart there is a greater reliance on SL Ca^{2+}; although the SR is functional [4,5]. In early development NCX protein levels[6] and NCX activity is elevated compared to mature heart[5].

Embryonic cardiac myocytes express L- and T-type Ca^{2+} channel currents $I_{Ca,L}$, $I_{Ca,T}$ encoded by $Ca_V1.2/1.3$, and $Ca_V3.1$, respectively[7]. $I_{Ca,L}$ is preferentially blocked by dihydropyridine and arylalkylamine drugs. More recently, small G-proteins of the RGK class are a novel means to selectively block $I_{Ca,L}$ to the exclusion of effects on $I_{Ca,T}$ [8-10].

Many studies have focused on compensatory Ca^{2+} handling changes induced by over-expression, knock-out, or conditional knock-out of NCX. In embryonic cardiac myocytes SL Ca^{2+} efflux is accomplished by NCX1.1 and to a lesser extent PM Ca^{2+}-ATPase[5]. NCX1.1 knock-out is embryonic lethal at about embryonic day 11 (ED11;[11]-[12],) but prior to lethality transients exist due to the function of the slower efflux pathway provided by PM Ca^{2+}-ATPase[12]. Most recently it was shown that conditional NCX knock-down resulted in compensatory reduction of $I_{Ca,L}$[13]. This suggests that the reciprocal may also be true. That is, we posit that PM Ca entry, primarily via Ca_V1 channels, ultimately determines Ca^{2+} regulation. This postulate takes on additional importance considering that $I_{Ca,L}$ can be down-regulated either in disease[14]-[15], or as a consequence of therapeutic doses of Ca channel block in hypertensive patients. We now show that chronic regulation of SL Ca^{2+} entry is coupled with SL Ca^{2+} efflux via two coincident compensatory mechanisms: NCX1.1 protein level is reduced, and homeostatic regulation of diastolic cytosolic Ca^{2+} down regulates NCX function.

2. Materials & Methods

2.1 Cell culture & Cytosolic Calcium Imaging

E10 embryonic ventricular myocytes (EVMs) for Ca-imaging were isolated as in references [7,8](details in expanded materials and methods). Cells were transfected with cDNA plasmids encoding either GFP-Rem or GFP (control), and cells were tested ~48 hours post-transfection. Cardiac myocytes were loaded with 2 μM fura-2-AM for 10 minutes in a 5% CO_2 incubator and then de-esterified in Tyrodes (140 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 Hepes (free acid), 5.4 KCl, 10 Glucose, pH = 7.4, NaOH) solution for ~20 minutes. All recordings were performed at 37°C. The measurement of net SL diastolic calcium-entry was made by subtracting the baseline of recording (baseline defined as the mean end-diastolic Ca level for 3 successive beats) in 1.8 mM bath Ca^{2+} solution from lowest point between 20 and 25 seconds after 0 bath Ca^{2+} (140 NaCl, 1 $MgCl_2$, 10 Hepes (free acid), 5.4 KCl, 10 Glucose, 5 EGTA, pH = 7.4, NaOH was introduced (basal calcium level). The time for 90% (or 50%) decay of Ca transients (t_{90} or t_{50}) was measured by first obtaining the difference amplitude preceding the onset to the

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peak, and then the time for the Ca transient from the peak to reach 90% (or 50%) decay was reported.

2.2 Real-time RT-PCR and Western blots

Details and primer sequences are provided in expanded materials and methods (supplement). NCX1.1 densitometry was normalized to GAPDH. Equal protein (20 μg) was run in all lanes.

2.3 Computer simulations

To simulate calcium transients in a myocyte, we used the Shannon-Bers Model [16] which can be downloaded from: (http://www.luhs.org/depts/physio/personal_pages/bers_d/index.html). The Shannon-Bers model is a set of ordinary differential equations which simulate excitation-contraction coupling in a mature rabbit ventricular myocyte. To reproduce some features of the experimental results, we removed the reverse mode of the SERCA pump, and we adjusted the following parameters: GCaB (0.00025 -> 0.0015 mS/μF); INaCaX_Vmf(9.0 -> 18.0 A/F); and ISRCaP_Vmf(286 -> 200 μmol/l cytosol/s). To model acute LCC block, we decreased the permeability of ions through LCC by a factor of 0.01.

3. Results

To maintain the native intracellular environment we measured Ca^{2+} kinetics from intact, spontaneously active embryonic day 10 ventricular myocytes (E10 EVMs). Figure 1 shows representative spontaneous, rhythmic Ca^{2+} transients exhibited by control cells and by cells over-expressing Rem. Three features are obvious: Rem slows the spontaneous rate (control, 0.41+/-0.07 Hz, n=14; Rem, 0.22+/-0.04 Hz, n =20; p=0.01), reduces individual twitch amplitude (control, 0.18+/-0.02 $\Delta F_{340}/F_{380}$, n =14; Rem 0.05+/-0.01 $\Delta F_{340}/F_{380}$, n=20; $p<10^{-7}$), and significantly slows the twitch decay kinetics, t50, control 0.36 ± 0.04 (n=14) versus Rem, 0.56±0.04 (n=20; $p=10^{-3}$); and t90 for control 1.0 ± 0.10 (n=14), versus Rem, 1.8 ± 0.2 (n=20; $p<10^{-3}$).

3.1 Rem partially inhibits NCX1.1 expression

Rem and NCX1.1 mRNAs are expressed by E10 VM (Figure 2A). Rem over-expression may slow Ca^{2+} extrusion by down-regulating NCX. However, Rem over-expression does not significantly change NCX mRNA levels (Figure 2A). To evaluate NCX1.1 protein level a typical Western blot is shown in Figure 2B. Rem-over-expressing E10 VMs compared to control VMs show a 47% decrease of NCX1.1 protein. Early embryonic stages have more reliance on SL, but early stages pose a practical limitation - the low tissue weight creates a challenge to obtain sufficient quantity of protein for Western blot analysis. As a compromise, and secondarily to test for embryonic age-independent effects, we repeated Western blot analysis of Rem-induced changes of NCX. Rem over-expression significantly decreases NCX1 protein in E16 VMs (Figure 2B), and this decrease is similar to that in E10 EVM.

3.2 Rem inhibits Ca^{2+} efflux

We previously established that Rem blocks cardiac $I_{Ca,L}$ in E10 VM [8]. The prolonged Ca^{2+} extrusion kinetics shown in Figure 1, and reduced protein level (Figure 2) suggest a Rem effect on NCX as well. To assess SL Ca^{2+} efflux, we switched the bath solution to 0 Ca^{2+} bath (=0-added Ca^{2+}, 5mM EGTA) for 25s, and then abruptly added 50 mM caffeine. Removal of bath Ca^{2+} causes an instantaneous cessation of spontaneous activity and a drop of cytosolic Ca^{2+} (Figure 3A, dashed line). Subsequent addition of caffeine induces a large transient (CaffTr) with relaxation kinetics (Figure 3B) that is well-defined by bi-exponential function in control cells, but not adequately fitted by a single exponential function (Figure 3C-D). In the presence of Ni^{2+} relaxation kinetics required only a single, slow exponential function

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(supplemental data). 10 mM Ni^{2+} eliminated the fast component of relaxation, but had no effect on the slow component. 20 mM Ni^{2+} slowed relaxation further (Figure 3B).

Rem over-expression significantly reduces net SL diastolic Ca^{2+}-entry (control, 0.049+/-0.005 $\Delta F_{340}/F_{380}$, n=14; Rem, 0.030+/-0.003 $\Delta F_{340}/F_{380}$, n=20; p=0.003) (Figure 4A-**dashed line**), and significantly slows the caffeine relaxation time (Figure 4A; Table 1). This Reminduced slowing is progressively more pronounced for t50 than t75 or t90 (Table 1). In 10 of 20 cells no fast component of relaxation was observed (Figure 4C), and in the remaining 10 cells the fast component was significantly slower than that in control cells. 10 mM and 20 mM Ni^{2+} progressively slowed relaxation rates (Figure 4B; Supplemental Table). In control ionic conditions, chronic Rem over-expression significantly reduced the fast relaxation of the caffeine transient (47.3+/-2.0%, n=14, versus 22.0+/-5.5%, n=20; control versus Rem, $p<10^{-3}$); Figure 4D). These results suggest that Rem over-expression inhibits NCX function.

3.3 Cytosolic Ca^{2+} Homeostasis as a Mechanism of Rem-induced reduction of trans-PM Ca^{2+} efflux

In this study, the beating frequency ranged from 0 (quiescent) to ~0.9 Hz. As shown above (Figure 1), Rem slows the natural frequency. Thus an important caveat is that frequency may regulate SR Ca^{2+} load secondary to Rem effects. To address this issue we plotted the amplitude of caffeine-releasable Ca^{2+} transient (CaffTr) as a function of beat frequency. Figure 5 shows that there is no dependence of SR Ca^{2+} load (defined as CaffTr amplitude) on frequency within the Rem or control groups. However, both SR Ca^{2+} load (control, 0.522+/-0.038 ΔF340/F380, n=14; Rem, 0.411+/-0.030 ΔF340/F380, n=20; p=0.01), and frequency (Figure 1) are reduced by Rem. Thus, we conclude that $I_{Ca,L}$-mediated Ca^{2+} entry regulates SR Ca^{2+} load and beat rate independently.

We next tested the hypothesis that the kinetics of the CaffTr relaxation was slower in Rem because low diastolic Ca^{2+} caused a memory-dependent inactivation of NCX[17]. To address this we tested the effect of raising bath Ca from 1.8 to 6mM on the decay kinetics of Ca^{2+} transients induced by caffeine in the absence of bath Ca^{2+}. Figure 6A shows that raising bath Ca^{2+} had no effect on the fast phase of CaffTr in control (1.8 mM Ca^{2+}, 44.8+/-2.1%, n=6; 6 mM Ca^{2+}, 43.0+/-3.7%, n=6). In contrast, elevated bath Ca^{2+} significantly increased the fast component in cells over-expressing Rem (1.8 mM Ca^{2+}, 20.9+/-8.6%, n=5; 6 mM Ca^{2+}, 35.8 +/-2.4%, n=5; p=0.04). This fast component of relaxation is inhibited by 10mM Ni^{2+} as expected for NCX. Similarly, elevated bath Ca^{2+} speeded kinetics of the caffeine transient relaxation in Rem over-expressing cells (Figure 6C), but kinetics was unchanged in control cells (Figure 6B).

Given that NCX is deactivated by low internal Ca^{2+}, and that Rem blocks Ca^{2+} entry we posited that an explanation for Rem inhibition of NCX function is attributable to lowered cytosolic Ca^{2+}. Raising bath Ca^{2+} to 6 mM significantly increased NCX function in Rem-expressed cells (Figure 6). Elevated bath Ca^{2+} also caused an increase of twitch amplitude (1.8 mM Ca^{2+}, 0.066+/-0.008 $\Delta F_{340}/F_{380}$ (n=5); 6 mM Ca^{2+}, 0.120+/-0.023 $\Delta F_{340}/F_{380}$ (n=5; p=0.02; Figure 7A), and diastolic amplitude (1.8 mM Ca^{2+}, 0.280+/-0.043 F_{340}/F_{380} (n=5); 6 mM Ca^{2+}, 0.315 +/-0.024 F_{340}/F_{380}; n=5; p=0.002; Figure 7B). Similar trends occurred in control cells consistent with bath Ca^{2+} increasing Ca^{2+} entry. SR Ca^{2+} load was not altered by elevated bath Ca^{2+} (1.8 mM Ca^{2+}, 0.51+/-0.06 $\Delta F_{340}/F_{380}$, (n=6); 6 mM Ca^{2+}, 0.48+/-0.5 $\Delta F_{340}/F_{380}$; n=6; Figure 7C), but in Rem-over-expressed cells SR Ca^{2+} load was significantly increased (1.8 mM Ca^{2+}, 0.34+/-0.02 $\Delta F_{340}/F_{380}$, n=5; 6 mM Ca^{2+}, 0.50+/-0.05 $\Delta F_{340}/F_{380}$, n=5; p=0.01; Figure 7C). We conclude that partial SL Ca^{2+}-entry inhibition by Rem can be overcome by elevated bath Ca^{2+} leading to normalized SR load and increased diastolic Ca^{2+}. As a result of increased cytosolic Ca^{2+}, NCX function is normalized despite chronic $I_{Ca,L}$ partial blockade.

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3.4 Effect of short term block of $I_{Ca,L}$ block on trans-SL Ca^{2+} efflux

We next determined whether acute Ca^{2+} channel block (CCB) yields similar effects as chronic $I_{Ca,L}$ inhibition. Acute CCB by 10 μM n ifedipine ceased all spontaneous activity. After 1 minute of 10 μM nifedipine in physiological salt solution, the cells were bathed in 0 Ca^{2+} bath solution with 10 μM nifedipine for 25 seconds and then 50 mM caffeine (also containing10 μM nifedipine) was applied abruptly (Figure 8). We found no significant difference in net SL diastolic Ca^{2+}-entry in controls in response to acute CCB for 1 minute (Figure 8, dashed line). CaffTr amplitude (SR load) was significantly reduced, (Figure 8B; control, 0.56+/-0.06 $\Delta F_{340}/F_{380}$, n=6; 10 μM Nifedipine, 0.34+/-0.05 $\Delta F_{340}/F_{380}$, n=6; p=0.003). In contrast to chronic $I_{Ca,L}$ blockade, the acute blocked cells showed a slight slowing of CaffTr kinetics, but the Rem-effect led to significantly slower CaffTr decay kinetics (Figure 8C). These results lead us to the conclusion that short term $I_{Ca,L}$ blockade alters loading of the SR, but chronic Rem-mediated $I_{Ca,L}$ blockade leads to further homeostatic regulation mechanisms governed by reduced diastolic calcium levels thereby inhibiting NCX forward mode.

To verify these conclusions we simulated Ca^{2+} transients with an established model for mature cardiac myocytes[16]. The model reproduced the effect of acute Ca^{2+} channel blockade by nifedipine, but could not reproduce the Rem-effect of slowing CaffTr relaxation. Thus, we conclude that long-term block of $I_{Ca,L}$ homeostatically regulates Ca^{2+} entry and commensurately regulates NCX-based Ca^{2+} efflux across the SL.

4. Discussion

The major finding of this study is that a chronic partial block of $I_{Ca,L}$ mediated by Rem over-expression results in a reduction of NCX-mediated extrusion. We show direct evidence for two non-competing mechanisms that may contribute to this result. First, Rem reduces NCX1.1 protein post-translationally as manifested by a decrease of protein, but without a change in mRNA. Second, Rem over-expression results in reduced cytosolic Ca^{2+}, but this reduction of Ca^{2+} can be restored by elevating bath Ca^{2+}, and this partially restores NCX function. The end-point of these findings - reduced $I_{Ca,L}$ leads to reduced NCX is reciprocal to finding in NCX knockout mice[13], but the compensatory mechanisms differ. Primary alteration of NCX leads to no change in $Ca_V1.2$ protein[18], whereas primary alteration of $Ca_V1.2$ leads to reduced NCX1 protein level (present study).

We suggest that a Ca^{2+} channel-centric perspective for control of Ca^{2+} handling is a critically important consideration. For example, in the $Ca_V1.2$ transgenic mouse model an $I_{Ca,L}$ increase precedes hypertrophy[19], supporting the notion that $I_{Ca,L}$ level is a cause rather than a consequence of Ca^{2+} dynamics remodeling in pathological conditions. In line with our findings, a modest increase of SL Ca^{2+} entry in this transgenic model induced a compensatory up-regulation of NCX activity[19]. Although $I_{Ca,L}$ up-regulation is the opposite experimental manipulation of our study, it invokes the same homeostatic balance mechanisms of SL Ca^{2+}. Importantly, pathological conditions naturally may reduce $I_{Ca,L}$ [20,21]. More work will be needed to determine whether reduction of $I_{Ca,L}$ is a potentially protective mechanism to prevent $Ca^{2+}{}_{Ca,L}$ overload by preventing Ca^{2+} entry, or exacerbates Ca^{2+} overload by triggering compensatory down-regulation of NCX based efflux.

NCX over-expression in mature rabbit ventricular myocytes yields a decrease of SR load[22, 23]. In the same vein, Rem over-expression in the present study decreases SR load. The commonality is that these manipulations decrease cytosolic Ca^{2+}, either by increasing exit or by decreasing entry, respectively. Rem selectively blocks L-type Ca^{2+} channels in muscle [10] reinforcing the notion that $I_{Ca,L}$ is a long-term regulator of SR Ca^{2+} loading, though increased Ca^{2+} entry may increase systolic Ca^{2+} to normalize SR load [24].

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We find that CaffTr decay is well described by a biexponential function. A biexponential decay suggests a sequential process, yet under conditions of sustained caffeine exposure the relevant major cytosolic extrusion pathways - NCX and sarcolemmal Ca-ATPase are of course arranged in parallel. A plausible explanation is that NCX is allosterically deactivated by declining cytosolic Ca. As the caffeine-induced Ca transient decays -that is as cytosolic Ca lowers, NCX deactivates[25],[26]. This well established feature of NCX [25,27-31]is captured in the Shannon-Bers model[16], and here we find that a simulation of a caffeine-induced transient in 0 bath Ca results in a Ca decay that is biexponential (not shown). The time course of allosteric activation of NCX upon increasing cytosolic Ca^{2+} is controversial. In some studies activation occurs in <200 ms[25],[27], though in others a time constant of ~0.6s was noted[32], and a lag was detected[17]. Regardless these times are well within the seconds-long CaffTr decay. With NCX deactivated, the remaining extrusion is dominated by the relatively slow sarcolemmal Ca-ATPase. Thus, two molecular mechanisms arranged in parallel may lead to an apparent biexponential process as long as NCX deactivation is accounted for.

In our work, the fast component of CaffTr decay is completely eliminated by 10mM Ni^{2+}. Several past works, including that by Trafford, Diaz, & Eisner document that the 10 mM Ni^{2+}-sensitive component of the caffeine transient is largely ascribable to NCX mediated Ca-extrusion (reviewed by [2]). Diaz et al. [33]) found a biexponential decay of the caffeine transient - the fast component was attributed to Ca buffers. Diaz et al. (2001) was, in part, built on earlier work that showed a slower decay as a function of peak Ca released[34]. Thus, it follows that one possible explanation could be that our observation of a slower decay in Rem-treated cells is secondary to the decrease of caffeine-releasable Ca in these cells. However, two experiments argue against a buffering-only explanation for the slower decay in Rem-treated cells. First, when we applied acute nifedipine we measured decreased CaffTr amplitude without any effect on the time-course of CaffTr decay. Fortuitously, CaffTr amplitude following acute nifedipine in control cells was similar to CaffTr amplitude for Rem-treated cells without acute addition of drug. Second, Ni^{2+} blocked the fast component completely, but did not alter the amplitude of the caffeine-releasable Ca transient. This does not negate buffering as a consideration for interpreting Ca transient kinetics in intact cells. However, the reproducible attenuation of Ni^{2+}-sensitivity is consistent with our new hypothesis that Rem decreases NCX function.

Given the complex nature of Ca^{2+} dynamics, computational models contain many unconstrained parameters. Nevertheless, the application of such models can be informative. We used the Shannon-Bers model[16] which was developed for a mature myocyte. Although the time scale of the calcium dynamics is an order of magnitude faster than the experimental results, features captured by the modeling are revealing. First, the decay of the CaffTr depends on the operation of NCX and the SL calcium pump. Second, acute CCB effects on Ca^{2+} dynamics are reproduced: a switch to 0 bath Ca^{2+} decreases diastolic Ca^{2+} and CaffTr kinetics is unaffected. Features not captured by modeling are also revealing. Following the removal of bath calcium, the experimental results showed a sudden drop in cytosol calcium, which was not reproduced by the computational model [16]. We found that increasing the conductance of the background calcium current helped the model mimic this experimental result. Assuming that Rem block is specific for $I_{Ca,L}$ [8,10], a possible implication is that leak $I_{Ca,L}$ is a contributor to steady-state, diastolic Ca^{2+} entry. Interestingly, the model simulation of the decay of the caffeine induced calcium transient does not depend on the length of time of the $I_{Ca,L}$ block. Yet, the experimental results indicate that the sudden drop in $[Ca]_i$ disappears with chronic $I_{Ca,L}$ block. Therefore, some component of the background calcium current through the SL is associated with the long term operation of the L-type Ca^{2+} channel.

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In conclusion, we suggest that long-term regulation of trans-SL Ca^{2+}-pathways is an essential regulator of the Ca^{2+} handling. This study uncovers the importance of long-term homeostatic regulation of Ca^{2+} dynamics initiated by long-term partial blockade of $I_{Ca,L}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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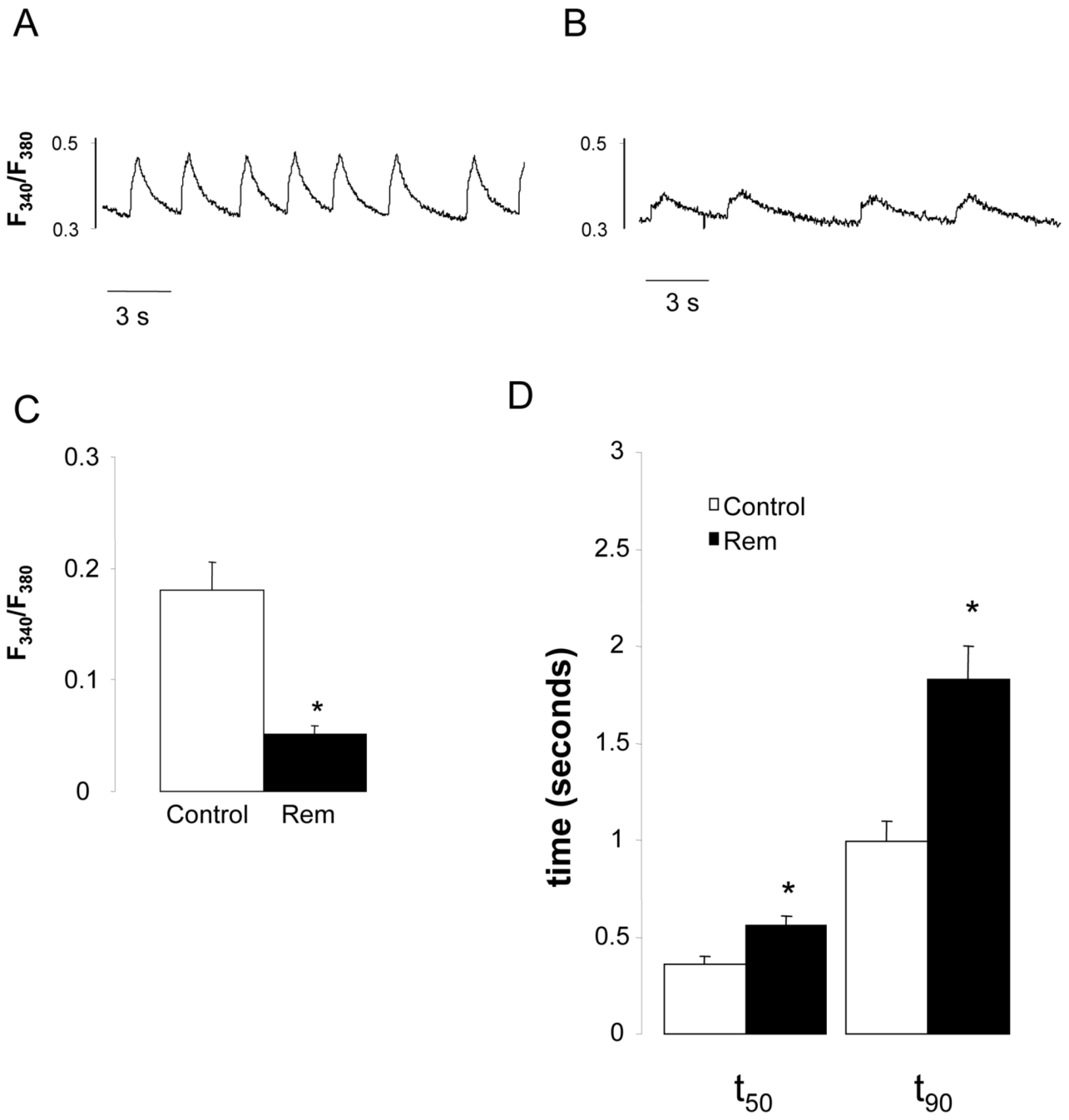

Figure 1.

Rem slows spontaneous beat rate, reduces Ca^{2+} transient amplitude and slows Ca^{2+} transient kinetics. A,B) Representative Ca^{2+} transients from a control cell (A), and Rem-transfected cell (B). C) Rem significantly reduces twitch amplitude ($p<10^{-7}$). D) Rem slows twitch Ca^{2+} 90% and 50% decay times (t90 and t50, $p<10^{-3}$; n=14 control, n=20 Rem transfected).

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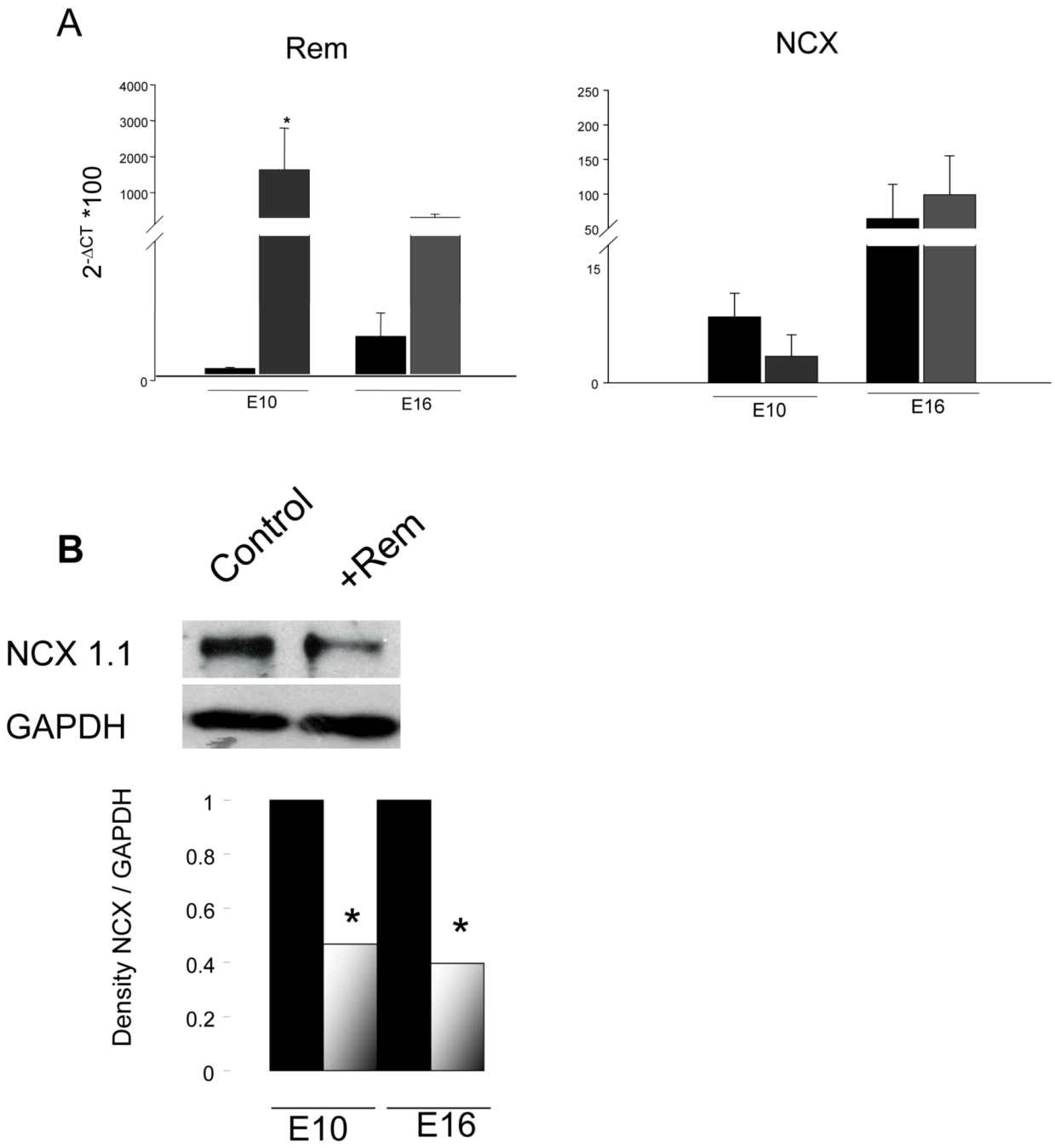

Figure 2.

Rem has no effect on NCX1.1 mRNA level, but decreases NCX1.1 protein similarly in E10 and E16 embryonic ventricular myocytes. A) qRT-PCR for Rem (left) and NCX1.1 (right) in control versus Rem over-expressing cells. (E10, n=3; E16 n=4; triplicates for each n=1). B) Western blot of NCX1.1 and GAPDH in control and Rem over-expressing cells. *p<0.001. (E10, n=3; E16, n=6).

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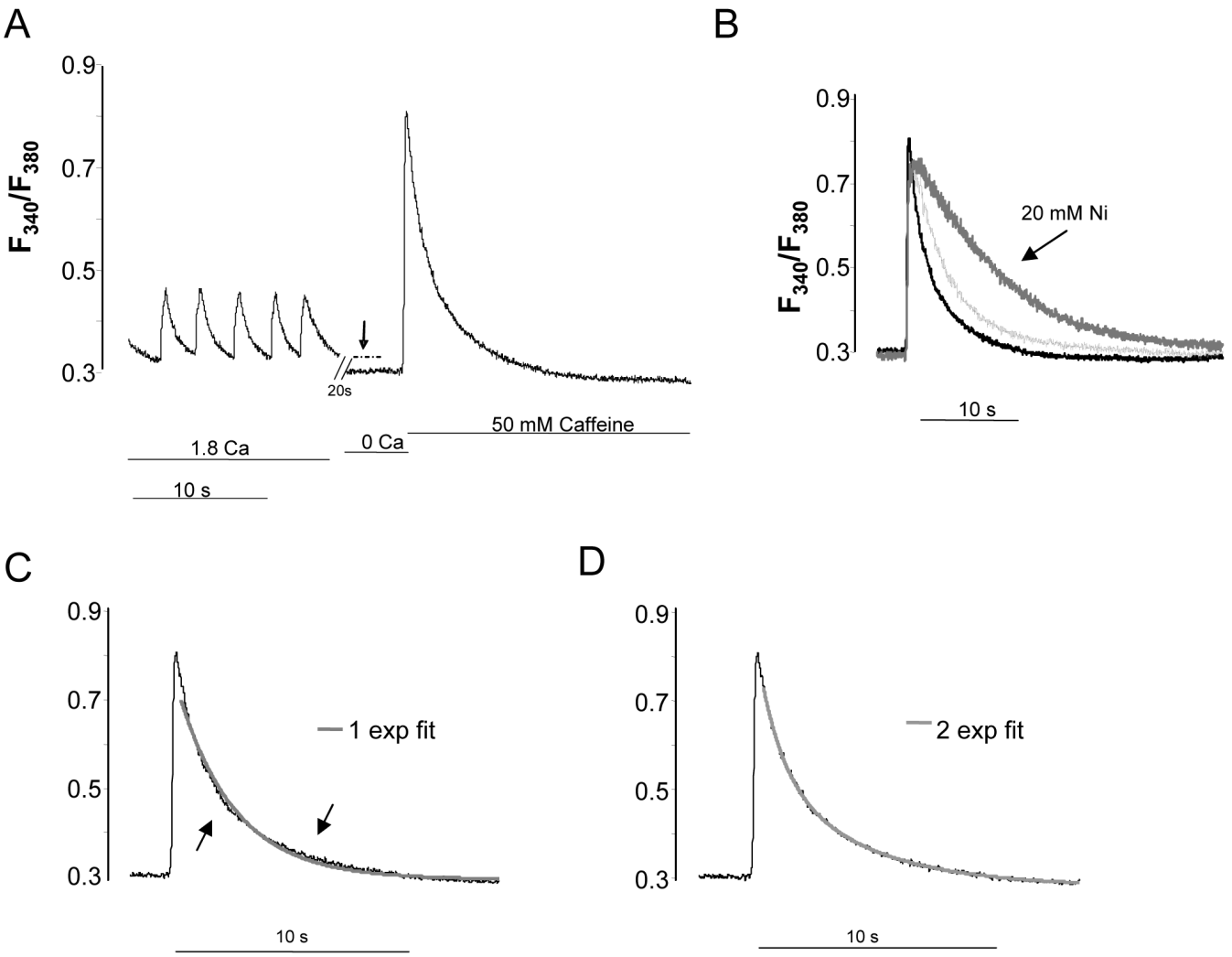

Figure 3.

Representative control cell demonstrating SL contribution, SR load, and SL efflux. A) Ca^{2+} dynamics in response to sequential change of solutions from normal tyrodes, to Ca^{2+}-free, to Ca^{2+}-free+50 mM caffeine. The vertical arrow and dashed line highlights the difference between diastolic Ca of spontaneously beating cells, and Ca^{2+} in the absence of SL Ca^{2+} influx. For all experiments, 25 s lapsed between onset of Ca^{2+}-free and addition of caffeine to Ca^{2+}-free bath solution. B) Caffeine-induced Ca^{2+} transient in Ca^{2+}-free bath solution superimposed over that elicited with the addition of 10 (light grey) or 20 mM Ni^{2+} (dark grey). Note that increasing Ni^{2+} progressively slows decay kinetics. C,D) The caffeine-induced Ca^{2+} transient decay (CaffTr) is not well described by a single exponential function (C), but is well-described by a bi-exponential function (D). CaffTr + Ni^{2+} exhibited only the slow exponential component (not shown).

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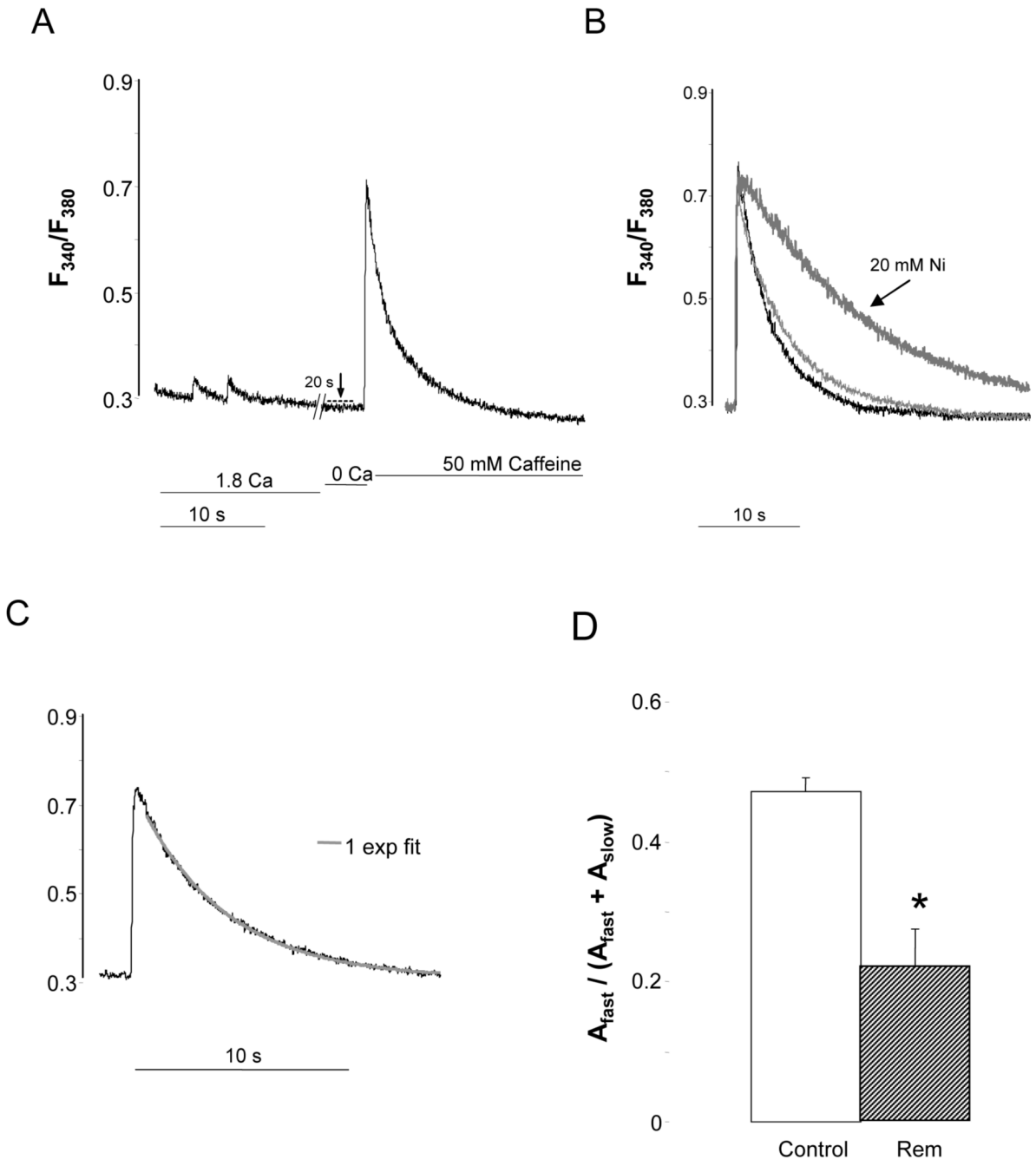

Figure 4.

Rem decreases SL Ca^{2+}-entry and slows CaffTr kinetics. A) Representative Ca^{2+} dynamics in Rem-transfected CM using same protocol as Figure 2A. B) CaffTr in Ca^{2+}-free bath (black trace) and in response to 10 mM Ni^{2+} (light grey) and 20 mM Ni^{2+}. C) Single-exponential fit describes CaffTr in representative Rem-transfected cells. D) Pooled data of the fraction of fast amplitude of CaffTr. Control amp_{fast} was significantly greater than control cells with 10 mM Ni^{2+}, or control cells versus Rem-transfected cells ($p<10^{-3}$; n=20 Rem, n=14 control).

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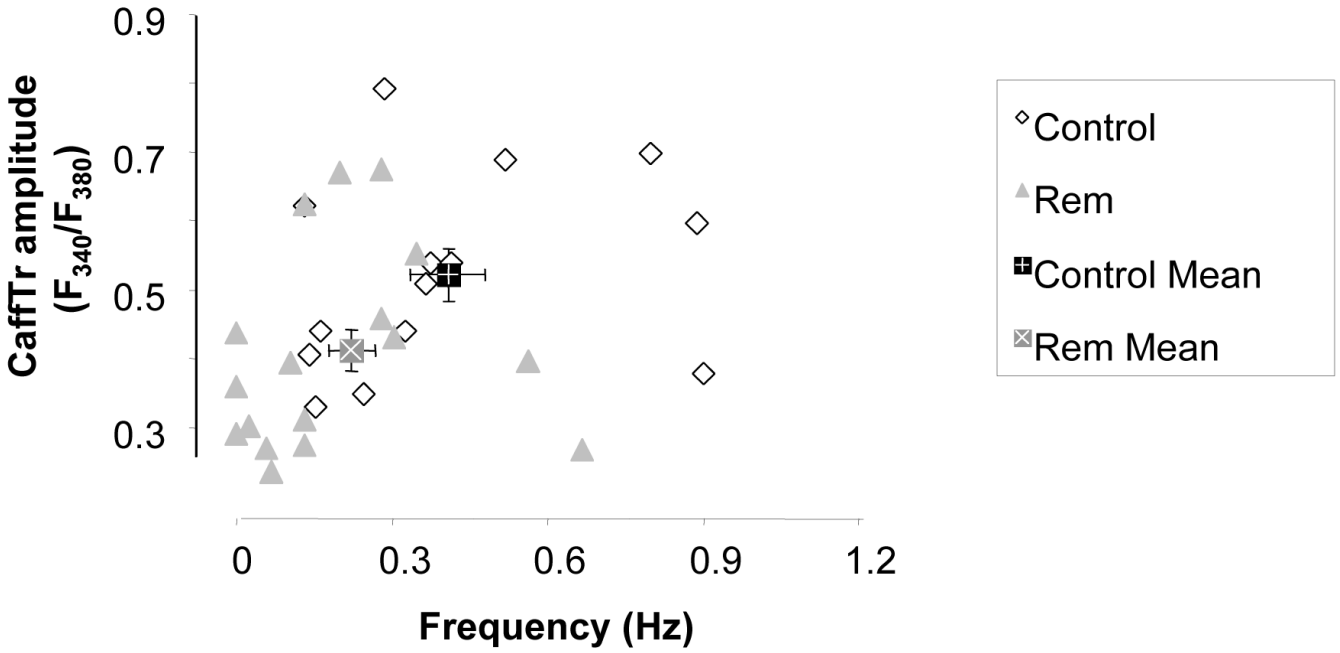

Figure 5.

SR Ca^{2+} load does not significantly vary as a function of natural beating frequency. Caffeine-induced Ca^{2+} transient amplitude plotted versus prior spontaneous beat frequency for control cells (open diamonds) and Rem-transfected cells (closed triangles). However, mean frequency and mean SR load are significantly different between control (closed square), and Rem-transfected cells (grey).

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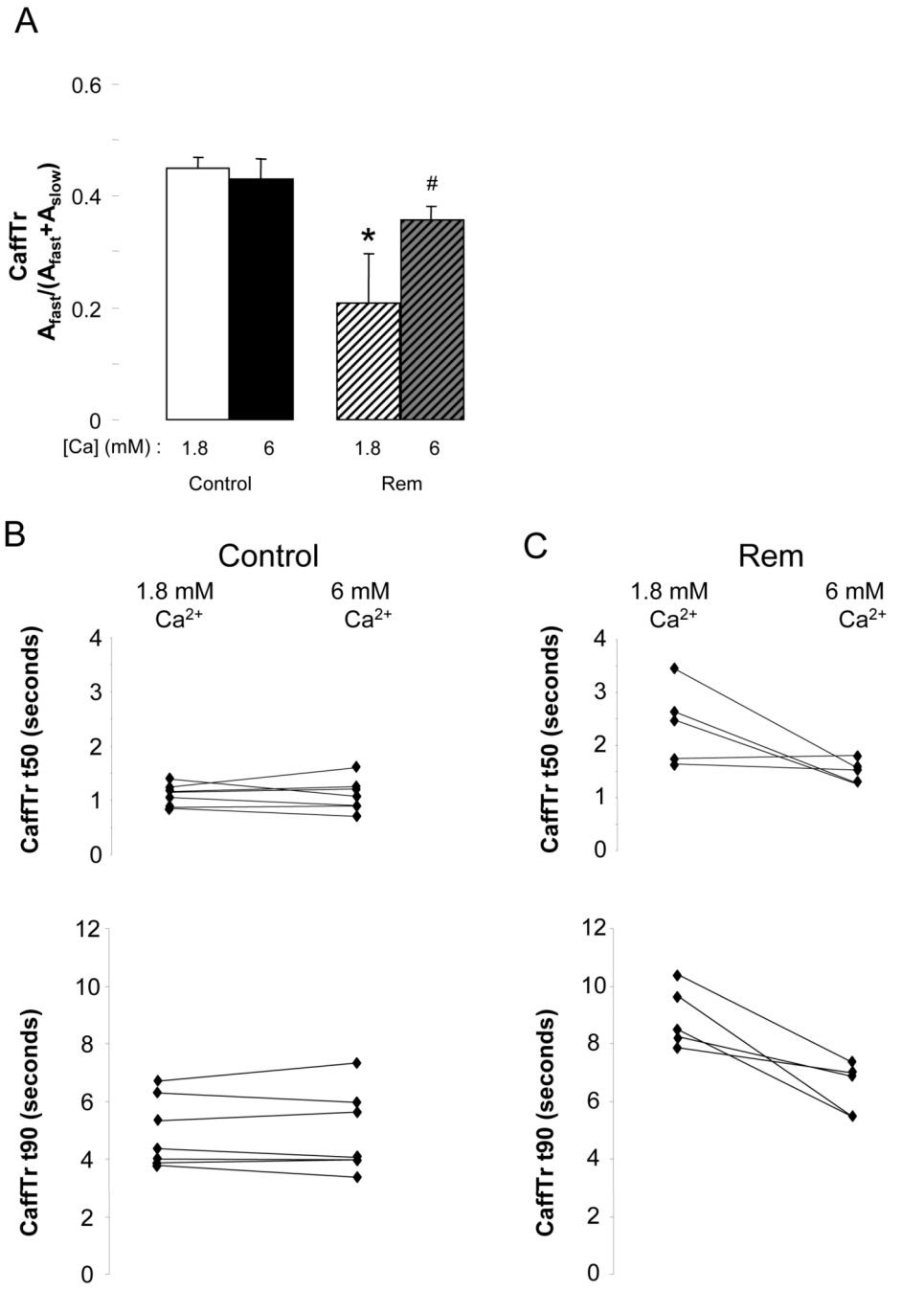

Figure 6.

Increasing bath Ca^{2+} from 1.8 to 6 mM restores fast component of CaffTr relaxation. A) Pooled fractional amplitudes of bi-exponential decay fit of CaffTr relaxation. In control cells elevated Ca^{2+} has no effect; in Rem transfected cells elevated Ca^{2+} significantly increases fast component. * indicates $p<0.005$, compared to 1.8 mM control cell; # indicates $p<0.05$ compared to 1.8 mM Rem cell. B, C) Scatter plot of t50 and t90 of CaffTr in (B) control and (C) Rem transfected cells in response to elevation of bath Ca^{2+} from 1.8 to 6 mM. Note that bath Ca^{2+} elevation had no effect in control cells, but decreased CaffTr total time in all Rem-transfected cells ($p<0.02$).

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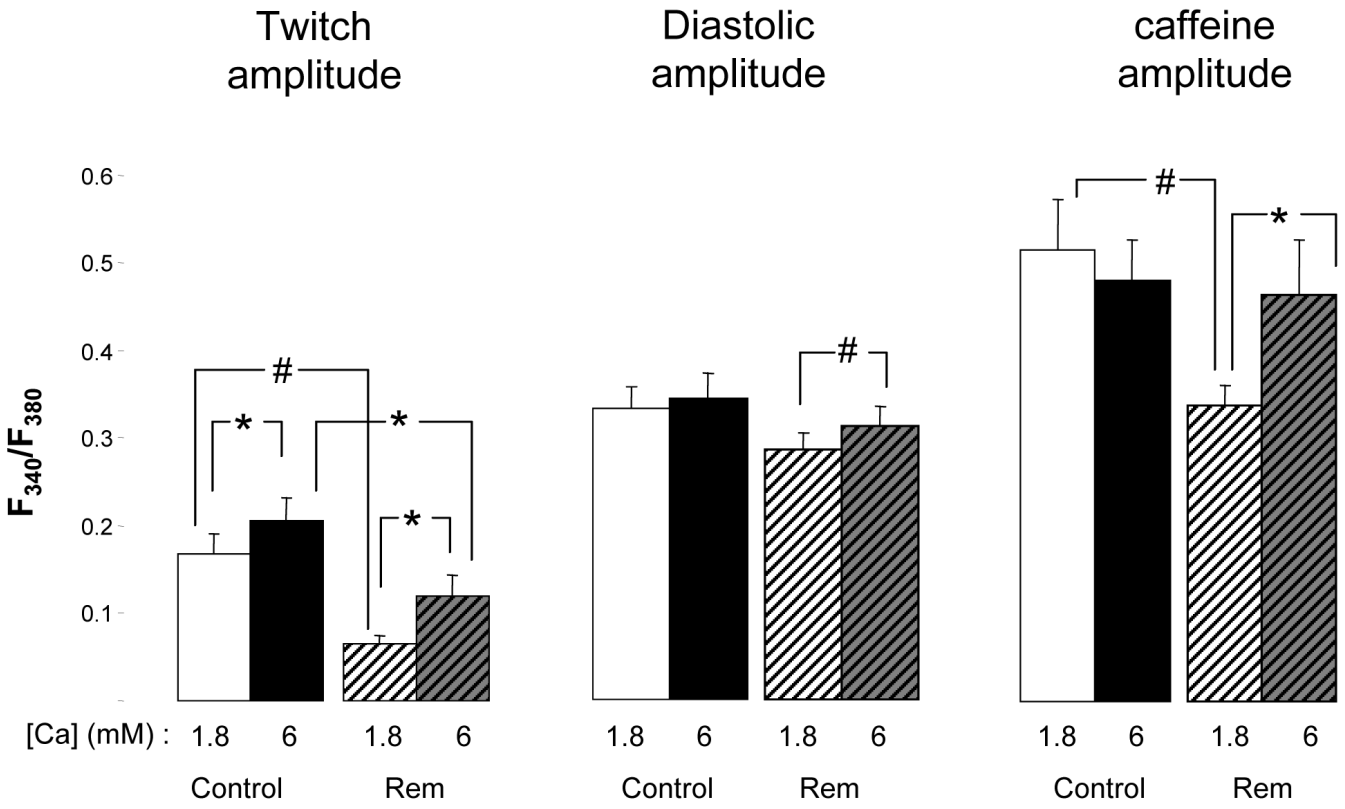

Figure 7.

Ca responses to elevation of bath Ca from 1 to 6 mM in control and Rem over-expressing cells. Left) Twitch amplitude is significantly increased regardless of Rem expression. Also, Rem cells have significantly lower amplitude than that of control controls. Middle) Net SL diastolic Ca^{2+}-entry is only increased in Rem over-expressing cells. Right) CaffTr amplitude, a measure of SR Ca load, is only increased in Rem over-expressing cells. Note that in 1.8mM bath Ca, SR Ca is significantly less in Rem over-expressing cells than control, but elevation of bath Ca normalizes SR Ca load. *$p<0.02$; #$p<0.002$; n=14 and 20 for control and Rem-transfected, respectively.

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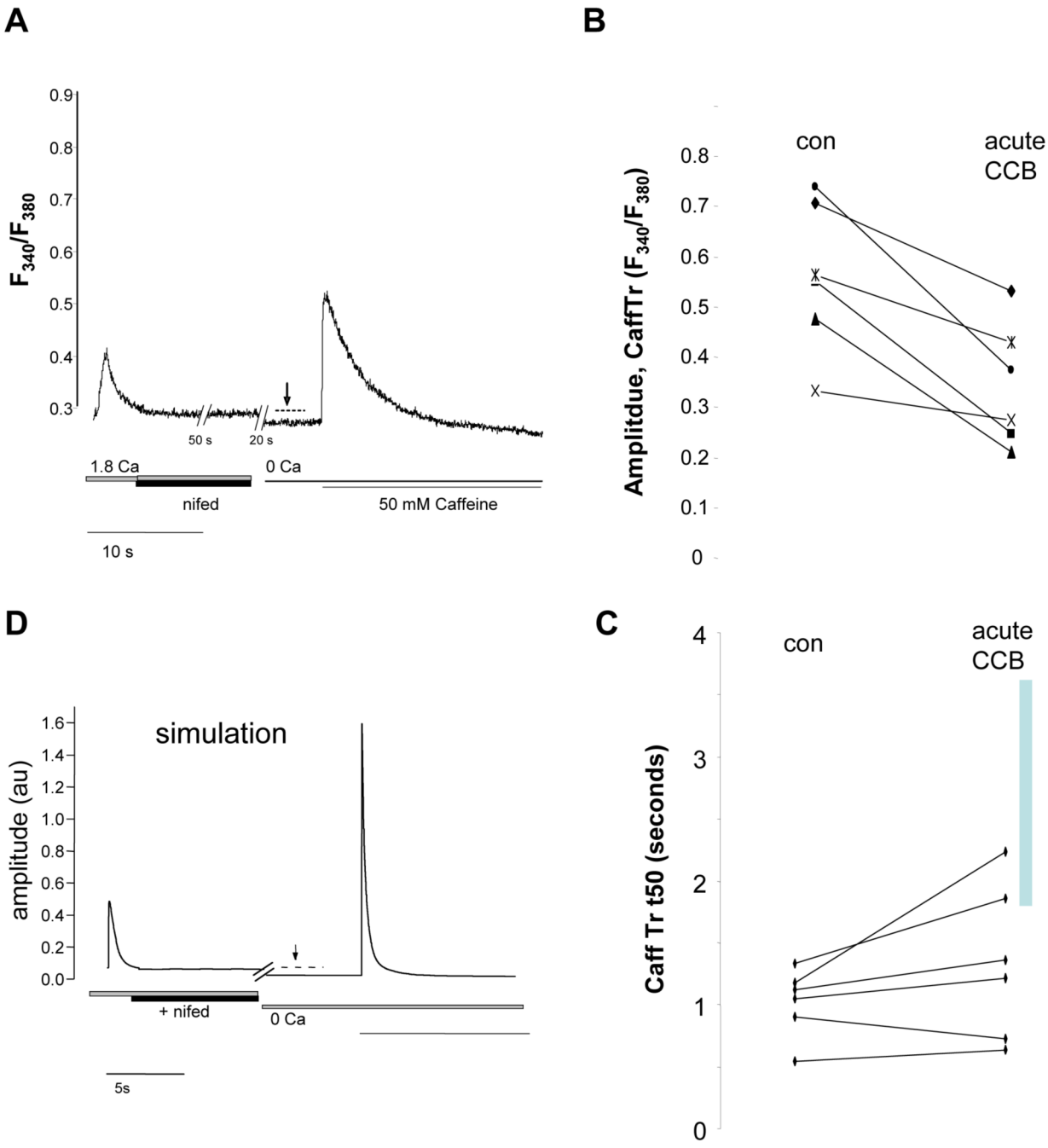

Figure 8.

Acute blockade of $I_{Ca,L}$ by 10 μM nifedipine does not block CaffTr relaxation time. A) Representative Ca^{2+} dynamics; sequential bath solutions were: normal tyrodes, normal tyrodes +10 μM nifedipine for 60s, 0 Ca/ EGTA bath solution for 25s, 0Ca / EGTA + 50 mM caffeine. Note that nifedipine rapidly blocks spontaneous activity, but in distinction to Rem-transfected cells the diastolic Ca^{2+} level is not lower than control cells in normal tyrodes. B) Acute nifedipine reduces CaffTr amplitude. C) Acute nifedipine has no significant effect on CaffTr kinetics. The vertical gray bar shows the range of t50 for Rem treated cells. Acute block is CaffTr decay is significantly faster than Rem over-expressing cells ($p<10^{-3}$). D) Computer

simulation of the effect of $I_{Ca,L}$ blockade. Nifedipine had no effect on CaffTr. Nifedipine and control traces are superimposed from 0 Ca onward and are indistinguishable.

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Table 1

Caffeine transient decay times

Condition	Caffeine Decays (n)	t50 (s)	t75 (s)	t90 (s)
Control	14	1.15 ± 0.05	2.59 ± 0.13	4.82 ± 0.25
Rem	20	3.11 ± 0.28*	6.37 ± 0.50*	10.41 ± 0.63*
Fold-slowing		2.7	2.5	2.2

* $p<10^{-7}$