The effect of Ca^{2+}–calmodulin-dependent protein kinase II on cardiac excitation–contraction coupling in ferret ventricular myocytes

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1. The effect of Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) on excitation–contraction coupling (E–C coupling) was studied in intact ferret cardiac myocytes using the selective inhibitor KN-93. KN-93 decreased steady-state (SS) twitch $[Ca^{2+}]_i$ (by 51%), resting Ca^{2+} spark frequency (by 88%) and SS sarcoplasmic reticulum (SR) Ca^{2+} content evaluated by caffeine application (by 37·5%).

2. Increasing extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) to 5 mM in KN-93 restored SR Ca^{2+} load and Ca^{2+} spark frequency towards that in control (2 mM Ca_o^{2+}), but SS twitch $[Ca^{2+}]_i$ was still significantly depressed by KN-93.

3. KN-93 decreased Ca^{2+} transient amplitude of SS twitches much more strongly than the amplitude of post-rest (PR) twitches. In the control, the time constant (τ) of $[Ca^{2+}]_i$ decline of SS twitches was faster than that for PR twitches. This stimulation-dependent acceleration of $[Ca^{2+}]_i$ decline was abolished by KN-93.

4. Voltage-clamp experiments demonstrated that KN-93 significantly inhibited sarcolemmal L-type Ca^{2+} current (I_{Ca}) during repetitive pulses by slowing recovery from inactivation. This may explain the preferential action of KN-93 to suppress SS *vs.* PR twitches.

5. In KN-93, even when both I_{Ca} and SR Ca^{2+} load were matched to the control levels by manipulation of conditioning voltage-clamp pulses, contraction and twitch Ca^{2+} transients were still both significantly depressed (to 39 and 49% of control, respectively).

6. Since KN-93 reduced SR Ca^{2+} release channel (RyR) activity during E–C coupling, even for matched SR Ca^{2+} load and trigger I_{Ca}, we infer that endogenous CaMKII is an important modulator of E–C coupling in intact cardiac myocytes. Effects of KN-93 on I_{Ca} and SS twitch $[Ca^{2+}]_i$ decline also indicate that endogenous CaMKII may have stimulatory effects on I_{Ca} and SR Ca^{2+} uptake.

Ca^{2+} entering mammalian cardiac cells via sarcolemmal L-type Ca^{2+} channels controls the release of Ca^{2+} from the sarcoplasmic reticulum (SR), leading to cell contraction in the process known as excitation–contraction coupling (E–C coupling; Wier, 1990; Bers, 1991; Stern, 1992). The SR Ca^{2+} release channel or ryanodine receptor (RyR) plays an essential role in this process. Several potential regulatory systems have been reported to alter the properties of the SR RyR in studies using SR vesicles or with RyR incorporated into lipid bilayers for single channel measurements (Bers, 1991; Coronado, Morisette, Sukhareva & Vaughan, 1994).

Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) is highly enriched in the nervous system and mediates many actions of Ca^{2+} (Schulman, Hanson & Meyer, 1992). CaMKII also exists in cardiac cells (Kennedy & Greengard, 1981; Edman & Schulman, 1994) where it phosphorylates several proteins including the RyR and phospholamban (PLB) in response to Ca^{2+} signals. Phosphorylation of the cardiac RyR by CaMKII can alter single channel gating properties (Witcher, Kovacs, Schulman, Cefali & Jones, 1991; Hain, Onoue, Mayrleitner, Fleischer & Schindler, 1995). At rest the auto-inhibitory segment of the kinase (amino acids 281–309) sterically blocks the access of substrates (Braun & Schulman, 1995). Several amino acid residues within this segment have been identified as being important for inhibitory potency (Brickey, Bann, Fong, Perrino, Brennan & Soderling, 1994). The calmodulin (CaM) binding site which activates CaMKII is also within this segment (amino acids 296–311; Smith, Colbran, Brickey & Soderling, 1992).

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Rapid direct activation of CaMKII by Ca^{2+}–calmodulin can also be greatly prolonged by autophosphorylation, whereby the enzyme can remain active for many seconds after the fall of intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$). This aspect may be important in the integrating action of CaMKII upon repetitive $[Ca^{2+}]_i$ increases (Schulman *et al.* 1992; Hanson, Meyer, Stryer & Schulman, 1994).

Recent evidence has shown that *in vitro* CaMKII phosphorylates a distinct site in the RyR and either increases or decreases release channel openings in bilayers (Takasago, Imagawa, Furukawa, Ogurusu & Shigekawa, 1991; Witcher *et al.* 1991; Wang & Best, 1992; Lokuta, Fuentes, Lederer, Rogers & Valdivia, 1995; Hain *et al.* 1995). Valdivia, Kaplan, Ellis-Davies & Lederer (1995) showed that phosphorylation of the RyR by cAMP-dependent protein kinase (PKA) increased the responsiveness of the channel to Ca^{2+}, but accelerated the kinetics of adaptation. That is, upon abrupt increase of $[Ca^{2+}]$ the PKA phosphorylated channel showed a greater open probability initially and then quickly decreased to a level below control.

While the effect of CaMKII has been extensively studied in cell-free systems, relatively little is known about how CaMKII might alter E–C coupling in intact cardiac myocytes. The aim of this study was to understand how CaMKII regulates E–C coupling and Ca^{2+} flux, focusing on the regulation of RyR in intact ventricular myocytes. Several experimental techniques were used, including measurements of cell contraction, $[Ca^{2+}]_i$, Ca^{2+} current (I_{Ca}) and Na^+–Ca^{2+} exchange current (I_{Na-Ca}), as well as the frequency of resting Ca^{2+} sparks (using laser scanning confocal microscopy). We used a new synthetic CaMKII inhibitor (an isoquinoline sulphonamide derivative, KN-93), which selectively inhibits CaMKII, without appreciable effect on other protein kinases at the 1 μM concentration used here (Sumi *et al.* 1991). Our results suggests that CaMKII increases the responsiveness of Ca^{2+}-induced Ca^{2+} release during cardiac E–C coupling. Some of this work has been presented in abstract form (Li & Bers, 1996).

METHODS

Cardiac myocyte preparation

Ferret ventricular myocytes were isolated as previously described (Hryshko, Stiffel & Bers, 1989). Briefly, adult male ferrets (1–1·3 kg) were killed by injection of sodium pentobarbitone (70 mg kg^{-1}, I.P.) and rapid excision of the heart. The cell isolation used Langendorff perfusion and collagenase (1 mg ml^{-1}; Type B) and 0·16 mg ml^{-1} pronase. Myocytes were plated onto superfusion chambers, with glass bottoms pretreated with laminin (Gibco) to increase cell adhesion. Myocyte shortening was measured as previously described (Bassani, Bassani & Bers, 1992). The cells were superfused with normal Tyrode solution at room temperature (22–23 °C). Cells were transilluminated by a red light source, to avoid interference with indo-1 fluorescence measurement (below), and shortening was measured using a video-edge detection system (Crescent Electronics, Sandy, UT, USA).

Measurement of intracellular $[Ca^{2+}]$

Ratiometric measurement. Intracellular calcium concentration was measured under voltage-clamp conditions essentially as described by Bassani *et al.* (1992). Cells were loaded with indo-1 by incubation with the acetoxymethyl ester form of the dye (indo-1 AM, 10 μM; Molecular Probes, Inc.) for 20 min at room temperature and washed for at least 30 min. Fluorescence excitation was at 355 ± 5 nm (Chroma Technology, Brattleboro, VT, USA) via a 380 nm dichroic mirror with emission measured at 405 and 485 nm (40 nm bandwidth, Chroma Technology) with the emission field restricted to a single cell. Mean background fluorescence at each wavelength from cells not loaded with indo-1 was subtracted before the fluorescence ratio ($R = F_{405}/F_{485}$) was calculated and converted to free $[Ca^{2+}]_i$ using the equation:

$$[Ca^{2+}]_i = K_d \beta [(R - R_{min})/(R_{max} - R)]$$

(Grynkiewicz, Poenie & Tsien, 1985), assuming a dissociation constant (K_d) of 800 nM (Bassani, Bassani & Bers, 1995*a*). The β value (the ratio of the free to bound indo-1 fluorescence at 485 nm) was 3·3. The minimum and maximum values of R (R_{min} and R_{max}) were determined *in vivo* in indo-1 AM-loaded cells superfused with solutions containing either 5 mM EGTA (and nominally Ca^{2+} free) or 20 mM Ca^{2+}, respectively, in the presence of the non-fluorescent Ca^{2+} ionophore Br-A23187 (10 μM; Calbiochem, La Jolla, CA, USA). During R_{min} and R_{max} measurement, 50 mM 2,3-butanedione monoxime (BDM) was present to inhibit cell contraction.

Analysis of Ca^{2+} sparks with laser scanning confocal microscopy. Ca^{2+} sparks were measured as previously described (McCall, Li, Satoh, Blatter & Bers, 1996; Satoh, Blatter & Bers, 1997) on a laser scanning confocal microscope (LSM-410, Carl Zeiss) equipped with an argon ion laser (model 2014 series, 25 mW; Uniphase, San Jose, CA, USA) coupled to an inverted microscope (Axiovert 100, Carl Zeiss) with a Zeiss ×40 oil-immersion Plan-Neofluor objective (numerical aperture, 1·3; excitation at 488 nm; emission > 515 nm). Briefly, myocytes were loaded with fluo-3 AM (10 μM; Molecular Probes) for 30 min at room temperature. Line-scan mode was used, where a single myocyte was scanned repeatedly (250 Hz) along a line parallel to the longitudinal axis, avoiding nuclei. The pixel size was ~0·05 μm^2 and with a z resolution of ~1 μm, the volume of each pixel (voxel) was ~0·05 μm^3. Data were analysed on a Macintosh computer with IDL image-processing software (Research Systems, Boulder, CO, USA).

Fluo-3 fluorescence was transformed to $[Ca^{2+}]_i$ by a 'pseudo-ratio' method (Cheng, Lederer & Cannell, 1993). That is,

$$[Ca^{2+}]_i = K_d(F/F_0)/(K_d/[Ca^{2+}]_{i,rest} + 1 - F/F_0),$$

where K_d for fluo-3 was taken as 1·1 μM (Harkins, Kurebayashi & Baylor, 1993), F is the fluorescence intensity and F_0 is the intensity at rest (mean of lowest 50 pixels at each point) and was assumed to be 150 nM (Bassani *et al.* 1995*a*).

After myocytes were field stimulated at 1 Hz to SS, the spark frequency was analysed during a 20 s period of rest (10 line-scan images). The criteria for inclusion as a Ca^{2+} spark were that the peak amplitude of the Ca^{2+} transient exceeded 60 nM (mean of five adjacent pixels) and the duration of the half-amplitude exceeded 8 ms. The number of sparks counted along each line-scan image was normalized spatially and temporally as the spark frequency per unit volume ($pl^{-1}\,s^{-1}$), where one spark per line-scan image corresponds to ~20 $pl^{-1}\,s^{-1}$. We have not analysed kinetic aspects of individual Ca^{2+} sparks here.

Assessment of SR Ca^{2+} load: caffeine contractures

The rapid application of a caffeine-containing solution induces release of the entire SR Ca^{2+} load, causing a Ca^{2+} transient and contracture in cardiac myocytes. The amplitude of the caffeine-induced contracture and Ca^{2+} transient as well as integrated Ca^{2+} flux via Na^+–Ca^{2+} exchange current can be used as indices of SR Ca^{2+} content (Kitazawa, 1988; Smith, Valdeolmillos, Eisner & Allen, 1989; Bassani *et al.* 1992). Caffeine (10 mM) was added to a normal Tyrode solution containing Na^+, such that Ca^{2+} extrusion and $[Ca^{2+}]_i$ decline were attributable primarily to Na^+–Ca^{2+} exchange. This solution was introduced into the chamber via a quick-switching device, similar to that described by Bassani *et al.* (1992). Caffeine application was continued for 8 s, by which time the myocytes had relaxed completely.

I_{Ca} and I_{Na-Ca} measurement

Whole cell L-type Ca^{2+}-channel current was measured using the perforated-patch technique (Horn & Marty, 1988), allowing the cell to retain its usual intracellular environment and limiting I_{Ca} run down. An Axopatch 200A amplifier (Axon Instruments) was used with patch electrodes of 1–2 MΩ resistance. Electrodes were backfilled with 240 μg ml^{-1} amphotericin-B in a pipette solution (see below). Once a gigaseal was established, the patch was allowed to perforate for 15–30 min, after which time stable whole-cell recordings with a series resistance of 5–10 MΩ could be made.

Myocytes were initially superfused with modified Tyrode solution. To achieve steady-state SR Ca^{2+}, loaded cells were held at −70 mV and then depolarized to 0 mV for 200 ms at 0·5 Hz for five pulses. After caffeine-induced SR Ca^{2+} depletion more pulses were required to return the SR Ca^{2+} load to the steady state. The test pulse was initiated 2 s after the start of the last conditioning pulse. The test pulses included an initial depolarizing ramp to −45 mV over 500 ms to inactivate Na^+ current, holding at −45 mV for 20 ms and then further depolarization for 200 ms to a test potential between −30 and +40 mV. K^+ currents were blocked by replacement of K^+ with Cs^+ in bath and pipette solutions. I_{Ca} magnitude was measured as the difference between peak current and the residual current at the end of the pulse. The purpose of the conditioning trains was to load the SR and also to activate endogenous CaMKII by repeated elevations of $[Ca^{2+}]_i$. This protocol was repeated after 5 min equilibration in modified Tyrode solution containing 1 μM CaMKII inhibitor KN-93 or the inactive analogue KN-92, except that the conditioning pulses were adjusted to achieve matching SR Ca^{2+} load in both control and in the presence of KN-93. This was accomplished by increasing either the depolarizing duration or voltage during the conditioning pulses. The constancy of the SR Ca^{2+} load was assessed by the amplitude of $[Ca^{2+}]_i$ change ($\Delta[Ca^{2+}]_i$) and integration of I_{Na-Ca} induced by rapid caffeine application. To study the voltage- and time-dependent Ca^{2+} channel recovery (Figs 5 and 6), the ruptured patch configuration was used and both pipette and bath solutions were Na^+ free (see below).

Reagents and solutions

Unless otherwise stated experimental reagents used were of analytical grade and supplied by Sigma. 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulphonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methyl benzylamine (KN-93) and 2-[*N*-(4-methoxybenzenesulphonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN-92) were from Seikagaku America, Inc. (Rockville, MD, USA). A 10 mM stock solution was made up in water (KN-93) or DMSO (KN-92) and stored at 4 °C (final DMSO concentration 0·01 %). Aliquots of these solutions were added to the perfusate immediately prior to use. The normal Tyrode (NT) solution contained (mM): 140 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 5 Hepes, with the pH adjusted to 7·4 with NaOH at 22 °C. The modified normal Tyrode solution used for voltage clamp recordings contained the same components except that KCl was replaced with CsCl. In a 0 Na^+, 0 Ca^{2+} solution, NaCl in NT solution was replaced with LiCl and $CaCl_2$ was replaced

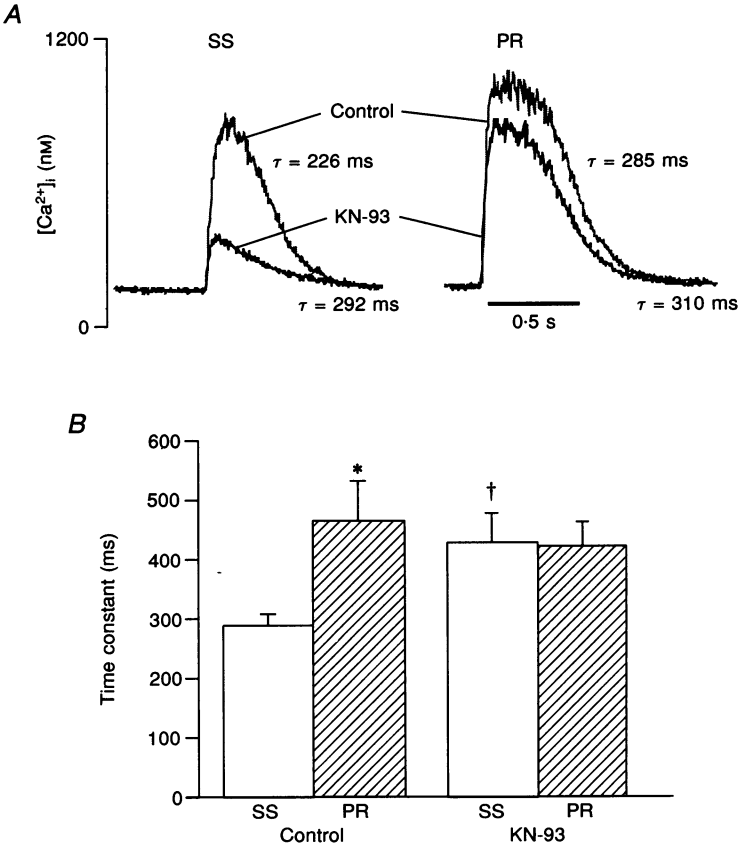


Figure 1. Effects of KN-93 on steady-state (SS) and post-rest (PR) twitch $[Ca^{2+}]_i$

A, original records of Ca^{2+} transients from SS twitch (left) and after 60 s rest (PR, right) obtained in a representative fluo-3-loaded ferret ventricular myocyte. Time constants (τ) of $[Ca^{2+}]_i$ decline during individual Ca^{2+} transients are indicated. *B*, pooled mean τ values of SS and PR $[Ca^{2+}]_i$ decline obtained under control conditions (left) and with 1 μM KN-93 (right). Data shown are from 8 cells and are expressed as means ± S.E.M. * and † indicate significant difference from control SS; $P < 0·05$. In a subset of 4 paired measurements under control conditions the mean SS τ (284 ± 31 ms) was significantly faster than the PR τ (407 ± 131 ms), but in the presence of KN-93 the SS τ (381 ± 72 ms) was about the same as in PR (347 ± 35 ms) and both were similar to control PR.

with 1 mM EGTA. The intracellular solution for the current measurement in perforated patches contained (mM): 55 CsCl, 70 caesium methanesulphonic acid (CsMes), 8 NaCl, 1 $MgCl_2$, 10 Hepes, 0·1 EGTA, with pH adjusted to 7·3 with CsOH at 22 °C. For the ruptured patch configuration, the bath solution contained (mM): 140 TEACl, 6 CsCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 Hepes, 10 glucose, with pH adjusted to 7·4 using TEAOH. The pipette solution contained (mM): 125 CsCl, 10 MgATP, 20 Hepes, 10 EGTA, with pH adjusted to 7·3 using CsOH. The solution for *in vivo* R_{min} measurement contained (mM): 60 NaCl, 50 KCl, 1 $MgCl_2$, 5 Hepes, 10 glucose, 50 BDM, 5 EGTA (pH 7·35). The R_{max} solution was the same except that instead of EGTA, 20 mM $CaCl_2$ was added.

RESULTS

Steady-state (SS) and post-rest (PR) twitches

Figure 1*A* shows original records of Ca^{2+} transients during SS stimulation of twitches at 1 Hz and the first twitch after a 60 s rest. It is clear that 1 μM KN-93 decreased the amplitude of Ca^{2+} twitch transients in the SS (from 931 ± 41 to 455 ± 112 nM, $n = 4$), but the difference was not significant for the PR Ca^{2+} transient (from 1033 ± 87 to 762 ± 96, $n = 4$). The ratio of PR:SS peak $[Ca^{2+}]_i$ was significantly increased after KN-93 (179 ± 27% in KN-93, 122 ± 13% in control, $n = 8$, $P < 0·01$), mainly due to stronger depression of peak $[Ca^{2+}]_i$ during the SS twitch (see Fig. 1*A*).

The time constant (τ) of SS $[Ca^{2+}]_i$ decline was also lengthened by KN-93 treatment (Fig. 1*B*; τ was 284 ± 31 ms in control and 381 ± 72 ms in KN-93; $n = 4$, $P < 0·05$). On the other hand, the τ of $[Ca^{2+}]_i$ decline for PR contractions was not altered significantly by KN-93 (407 ± 131 ms in control and 347 ± 35 ms in KN-93). Furthermore, the τ values of the control PR and both SS and PR in KN-93 were all similar (347–407 ms). This result is consistent with the faster $[Ca^{2+}]_i$ decline in control SS twitches being attributable to the stimulus-dependent activation of endogenous CaMKII, as reported for rat myocytes (Bassani, Mattiazzi & Bers, 1995*c*).

KN-93 and diastolic SR Ca^{2+} release

Ca^{2+} sparks are believed to represent elementary events of local SR Ca^{2+} release during both rest and E–C coupling in heart (Cheng *et al.* 1993; Cannell, Cheng & Lederer, 1994; Lopez-Lopez, Shacklock, Balke & Wier, 1995). If RyR

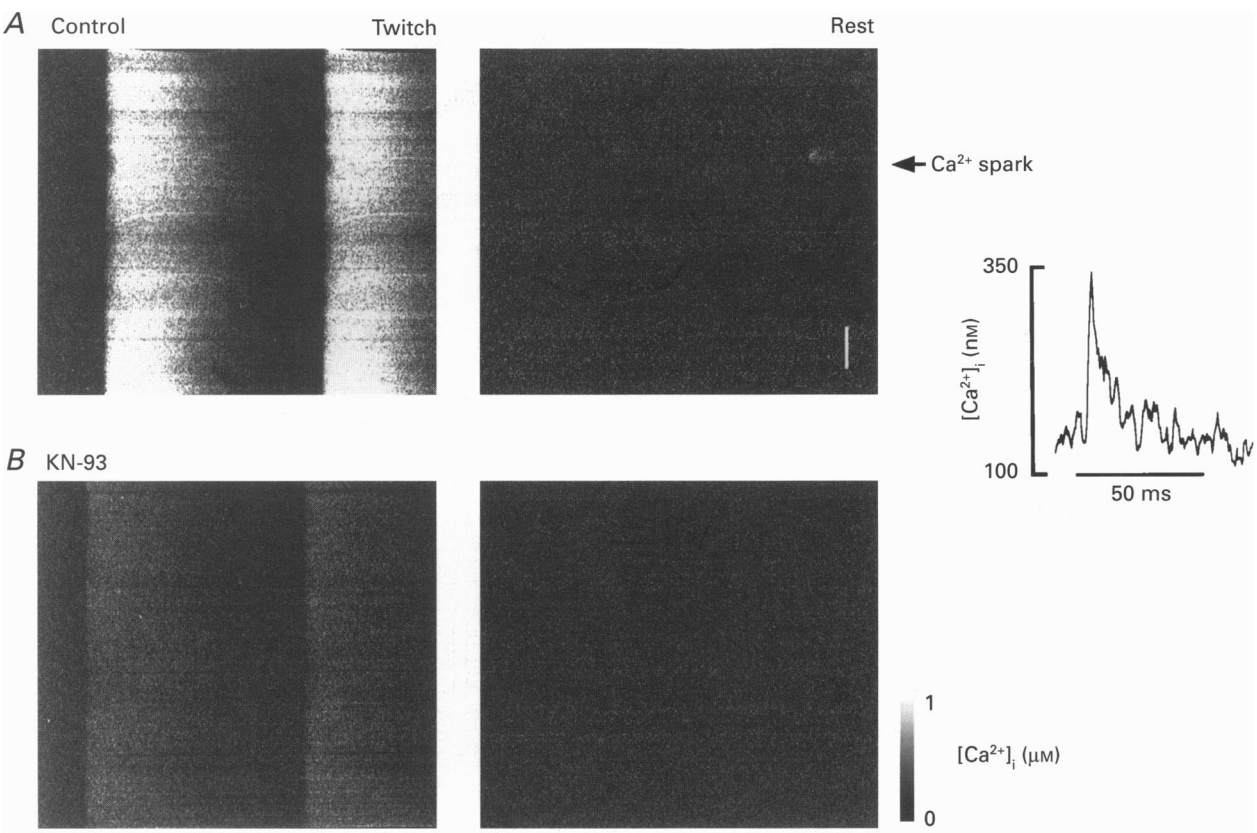


Figure 2. Effect of KN-93 on resting Ca^{2+} sparks

Line-scan images of Ca^{2+} sparks during rest in the absence (*A*), and presence (*B*) of 1 μM KN-93 in a ferret myocyte at 2 mM Ca_o^{2+}. The line-scan images indicate the twitch $[Ca^{2+}]_i$ (1 Hz, left) and the resting images (right) 6 s after the last steady-state twitch. Each 2 s image includes 512 lines at 250 Hz. The line recording of a Ca^{2+} spark shown on the right was taken from the spot indicated by the arrow. The $[Ca^{2+}]_i$ reached a peak value of 349 nM at ~20 ms and declined with a time constant of 67 ms. The white calibration bar indicates 10 μm.

phosphorylation by CaMKII increases the SR Ca^{2+} release channel activity during a period of stimulation to SS, Ca^{2+} spark frequency should be higher directly after SS stimulation. Figure 2 shows line-scan images of sparks in a ferret myocyte in the absence (*A*) and presence (*B*) of 1 μM KN-93. The spark frequency during 20 s of rest was decreased about eightfold by KN-93 (mean values were $23{\cdot}4 \pm 3{\cdot}6$ pl^{-1} s^{-1} in ten control cells and $2{\cdot}7 \pm 1{\cdot}3$ pl^{-1} s^{-1} in seven KN-93-treated cells, $P < 0{\cdot}01$, see Fig. 3*C*). However, since the SS twitch $[Ca^{2+}]_i$ transients were smaller in KN-93, the SR Ca^{2+} content might be reduced and this could be the cause of the reduced Ca^{2+} spark frequency (Cheng *et al.* 1993; Satoh *et al.* 1997).

SR Ca^{2+} content and twitch $[Ca^{2+}]_i$

SR Ca^{2+} load is an important factor regulating both resting Ca^{2+} sparks and Ca^{2+} release during E–C coupling (Isenberg & Han, 1994; Bassani, Yuan & Bers, 1995*b*; Satoh *et al.* 1997). Thus, SR Ca^{2+} content was measured using caffeine-induced contractures (CafC, in 0 Na^+, 0 Ca^{2+} solution) in experiments like those in Figs 1 and 2. Figure 3 shows that KN-93 decreases not only the SS twitch $[Ca^{2+}]_i$ but also the SR Ca^{2+} content. On average, peak $[Ca^{2+}]_i$ during CafC was 1246 ± 71 nM in control ($n = 13$, Fig. 3*B*) and 779 ± 113 nM in KN-93 ($n = 11$, $P < 0{\cdot}01$). We also tried to raise the SR Ca^{2+} content in the presence of KN-93 by increasing $[Ca^{2+}]_o$ to 5 mM. The resulting caffeine-induced Ca^{2+} transient

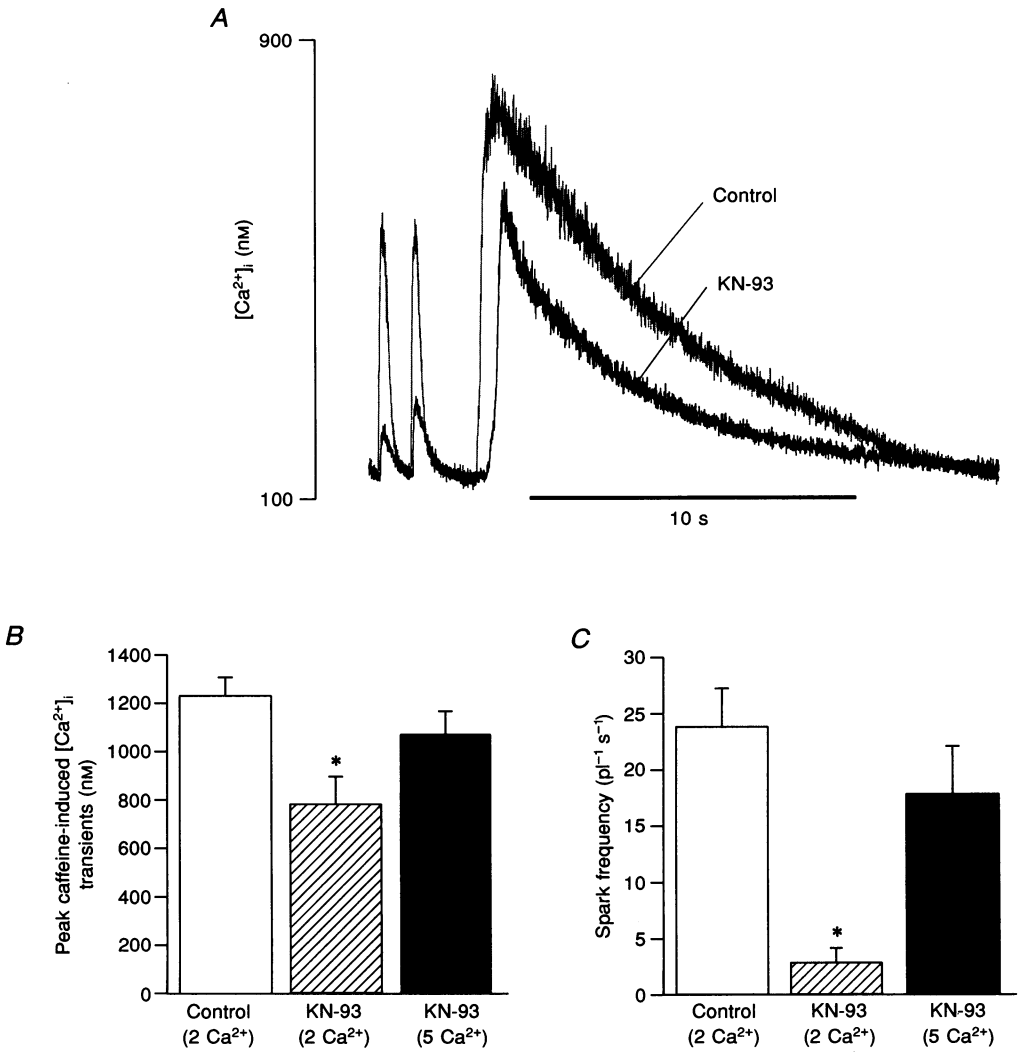


Figure 3. Effect of KN-93 on SR Ca^{2+} content and resting Ca^{2+} spark frequency

A, original record of SS twitch and caffeine-induced $[Ca^{2+}]_i$ transients (in 0 Na^+, 0 Ca^{2+} solution) in control and in the presence of KN-93. The smaller twitch Ca^{2+} transients are in KN-93. *B*, pooled data of peak $[Ca^{2+}]_i$ during caffeine-induced contractures in control (2 mM $[Ca^{2+}]_o$, 2 Ca^{2+}), KN-93 (2 Ca^{2+}) and KN-93 with 5 mM $[Ca^{2+}]_o$ (5 Ca^{2+}). The values are 1246 ± 71 nM ($n = 13$), 779 ± 113 nM ($n = 11$) and 1060 ± 102 nM ($n = 13$), respectively. $*P < 0{\cdot}01$ *vs.* control by one-way ANOVA. *C*, pooled data for mean spark frequency during 20 s of rest. The values are $23{\cdot}4 \pm 3{\cdot}6$ pl^{-1} s^{-1} in control ($n = 10$); $2{\cdot}7 \pm 1{\cdot}3$ pl^{-1} s^{-1} in KN-93 with 2 mM Ca_o^{2+} ($n = 7$) and $17{\cdot}5 \pm 4{\cdot}6$ pl^{-1} s^{-1} in KN-93 with 5 mM Ca_o^{2+} ($n = 10$). $*P < 0{\cdot}01$ *vs.* control by Student's paired *t* test.

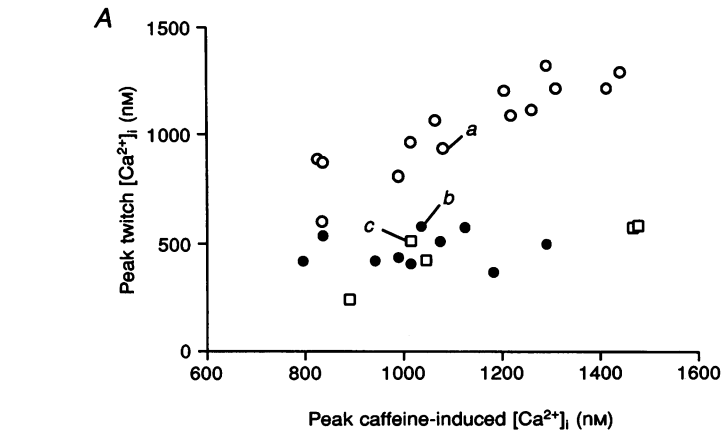
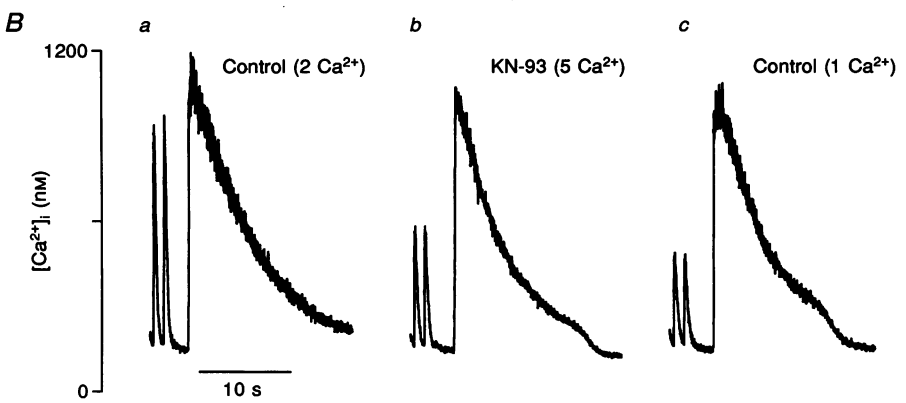


Figure 4. Relationship of SS twitch $[Ca^{2+}]_i$ *vs.* SR Ca^{2+} load
A, the relationship between peak caffeine-induced and SS twitch $[Ca^{2+}]_i$ transients in 2 mM and 1 mM Ca_o^{2+} Tyrode solution ($n = 14$ and 5; ○ and □, respectively) and in KN-93 with $[Ca^{2+}]_o$ elevated to 5 mM (●, $n = 10$). *B*, representative SS twitch and caffeine-induced $[Ca^{2+}]_i$ transients as marked in *A*. The values of peak $[Ca^{2+}]_i$ for twitch and CafC were 940 and 1087 nM, respectively, in control (*a*); 594 and 1087 nM in KN-93 with 5 mM Ca_o^{2+} (*b*); and 490 and 1063 nM in 1 mM Ca_o^{2+} (*c*).



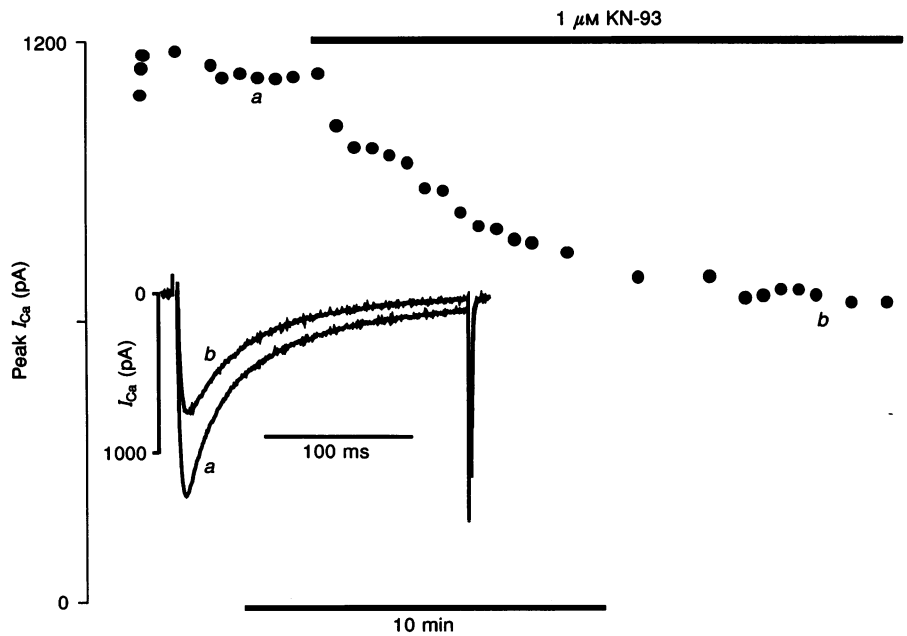


Figure 5. Effect of KN-93 on peak I_{Ca}
This representative cell was dialysed with 10 mM EGTA and exhibited no detectable contraction upon stimulation. Na^+ was omitted both in the pipette and extracellularly. Peak I_{Ca} is indicated for individual pulses of 200 ms duration to 0 mV from a holding potential of −70 mV with or without 1 μM KN-93. The inset shows superimposed original current traces in control (*a*) and the steady state in KN-93 (*b*).

reached a peak $[Ca^{2+}]_i$ of 1060 ± 102 nM in thirteen cells (Fig. 3*B*; not significantly different from control cells in 2 mM Ca_o^{2+}). As the SR Ca^{2+} content became similar to that in control, so did the resting spark frequency (Fig. 3*C*; 23·4 ± 3·6 $pl^{-1}\,s^{-1}$ in control, 2 mM Ca_o^{2+} and 17·5 ± 4·6 $pl^{-1}\,s^{-1}$ in KN-93, 5 mM Ca_o^{2+}, not significant). Figure 3*C* also shows the apparent steep relationship between SR Ca^{2+} content and spark frequency.

Figure 4*A* shows the relationship between SR Ca^{2+} content (CafC in 0 Na^+, 0 Ca^{2+}) and twitch $[Ca^{2+}]_i$ under three conditions: (a) control in 2 mM Ca_o^{2+}; (b) 1 μM KN-93 with increased $[Ca^{2+}]_o$ to 5 mM to restore SR Ca^{2+} load; (c) 1 mM Ca_o^{2+} to reduce I_{Ca} trigger in control. Figure 4*B* shows examples where the SR Ca^{2+} content was almost the same under the three conditions tested. Even with comparable SR Ca^{2+} load and elevated $[Ca^{2+}]_o$ (5 mM), KN-93 depressed twitch Ca^{2+} transient amplitude. Indeed, the decrease in the SS twitch Ca^{2+} transient with KN-93 even at 5 mM Ca_o^{2+} was comparable with that when $[Ca^{2+}]_o$ was reduced in control from 2 to 1 mM (Fig. 4*Bb vs. Bc*). These results raise the possibility that KN-93 depresses I_{Ca}, the E–C coupling process itself, or both.

Effect of KN-93 on I_{Ca}

The effects of KN-93 on sarcolemmal Ca^{2+} channels were studied first using the ruptured patch approach under controlled conditions where Ca^{2+} transients were prevented by EGTA in the dialysing pipette and Na^+-free, Cs^+-containing solutions prevented Na^+ and K^+ currents. Figure 5 shows that after peak I_{Ca} reached a steady state in normal Tyrode solution (Fig. 5*a*), 1 μM KN-93 decreased I_{Ca} gradually toward a new steady-state level (Fig. 5*b*). The inset shows superimposed I_{Ca} traces in *a* and *b*. In these ruptured patch experiments I_{Ca} was reduced to 78 ± 2% of control by KN-93 ($n = 3$, $P < 0{\cdot}01$). Similar depression was found in cells studied with perforated-patch clamp (I_{Ca} reduced to 72 ± 4% of control, $n = 9$, $P < 0{\cdot}001$). Since the holding potential (V_h) was −70 mV and pulses were only given every 30 s, this may indicate a decrease in overall channel availability. The degree of I_{Ca} depression can also be affected by the interpulse interval and V_h.

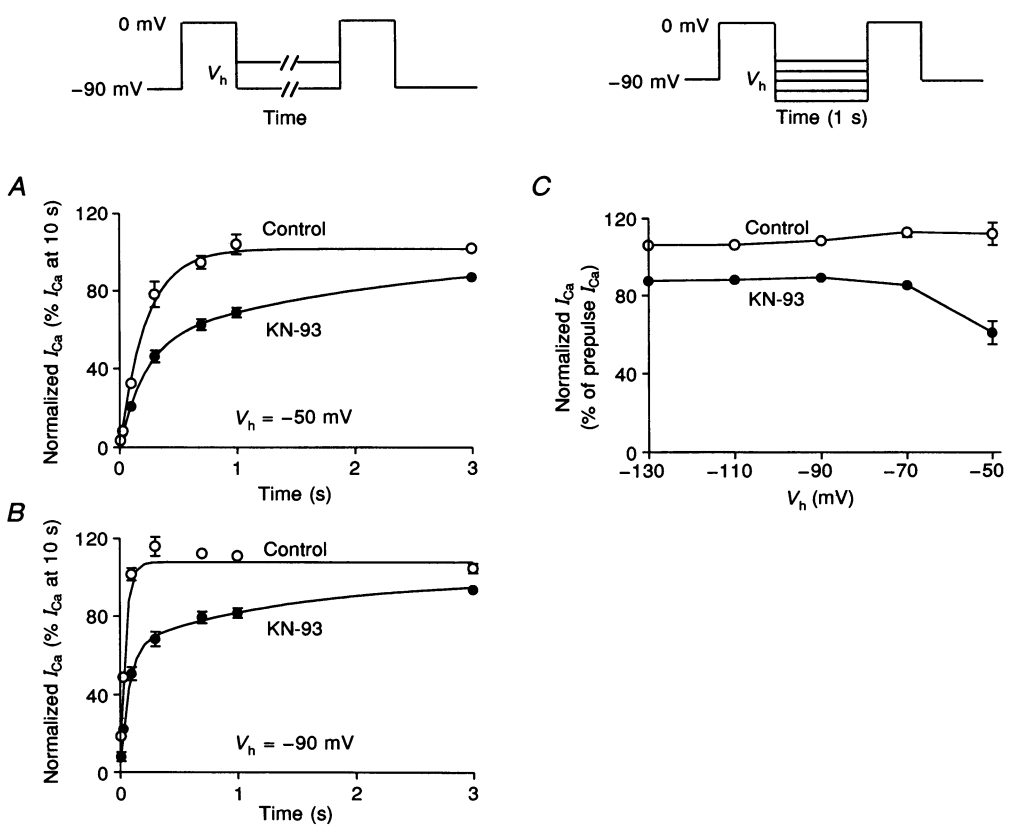


Figure 6. Recovery of I_{Ca} from inactivation in ferret ventricular myocytes

A, influence of KN-93 on time course of recovery at −50 mV. Cells were initially held at −90 mV. After a depolarizing pulse of 500 ms to 0 mV, cells were kept at a holding potential (V_h) of −50 mV for various times (10 ms to 10 s). Then a test pulse to 0 mV for 200 ms was given. I_{Ca} was normalized to the value measured after a 10 s interval. The same sequence was repeated in 1 μM KN-93. Mean data were best fitted by one and two exponential equations in control and KN-93, respectively. *B*, influence of KN-93 on time course of recovery at −90 mV. Same protocol as described in *A*, except that the V_h between the prepulse and test pulse was −90 mV. *C*, voltage-dependent recovery of I_{Ca}. After a 200 ms prepulse to 0 mV, cells were held at various voltages (−50 to −130 mV) for 1 s before a test pulse to 0 mV was given to assess Ca^{2+} channel recovery. I_{Ca} was normalized to I_{Ca} evoked by the prepulse (control: ○; KN-93: ●).

The kinetics and V_h dependence of channel recovery after inactivation are shown in Fig. 6. In panel *A*, cells were held at −50 mV for various times (10 ms to 10 s) after an initial 500 ms pulse from −90 to 0 mV (which fully inactivated I_{Ca}). Then test pulses to 0 mV for 200 ms were given to assess Ca^{2+} channel availability. The same sequence was repeated in the presence of KN-93. For Fig. 6*B* the protocol was the same except that the V_h between the prepulse and test pulse was −90 mV rather than −50 mV. Calcium current recovery in KN-93 was slower at both −50 and −90 mV V_h. In control, the recovery of I_{Ca} availability could be well described by a single exponential time constant ($\tau = 160$ and 31·6 ms for $V_h = -50$ and −90 mV, respectively). In the presence of KN-93, the fit for recovery required a second, much slower time constant ($\tau_{slow} = 1655$ and 972 ms for −50 and −90 mV, respectively) in addition to a comparable fast time constant ($\tau_{fast} = 146$ and 51 ms for −50 and −90 mV, respectively). Furthermore, ~50% of the overall recovery in the presence of KN-93 was in the new, very slow component.

We also tested the voltage dependence of recovery in a different way (Fig. 6*C*). After a 200 ms prepulse to 0 mV, cells were held at various voltages (−50 to −130 mV) for 1 s before a test pulse to 0 mV was delivered. For control the recovery of I_{Ca} was complete by 1 s at all voltages studied. However, in KN-93 some V_h dependence at −50 mV can be seen, as well as an ~15% reduction which appears to be independent of V_h and still observed after 1 s at −130 mV. Taken together, these results suggest that KN-93 decreased the I_{Ca} trigger due to an effect on Ca^{2+} channels. These results are similar to the reported effect of another CaMKII inhibitor, KN-62, on Ca^{2+} channels (Yuan & Bers, 1994). This slow I_{Ca} recovery may also explain the preferential depressant effect of KN-93 on SS twitch $[Ca^{2+}]_i$ transients (Fig. 1). Thus, to evaluate the effects of KN-93 on E–C coupling directly, it is clear that we must control I_{Ca} as well as the level of SR Ca^{2+} load.

SR Ca^{2+} load and I_{Na-Ca}

Since KN-93 has effects on both I_{Ca} and SR Ca^{2+} content, studying the effect of CaMKII on E–C coupling requires

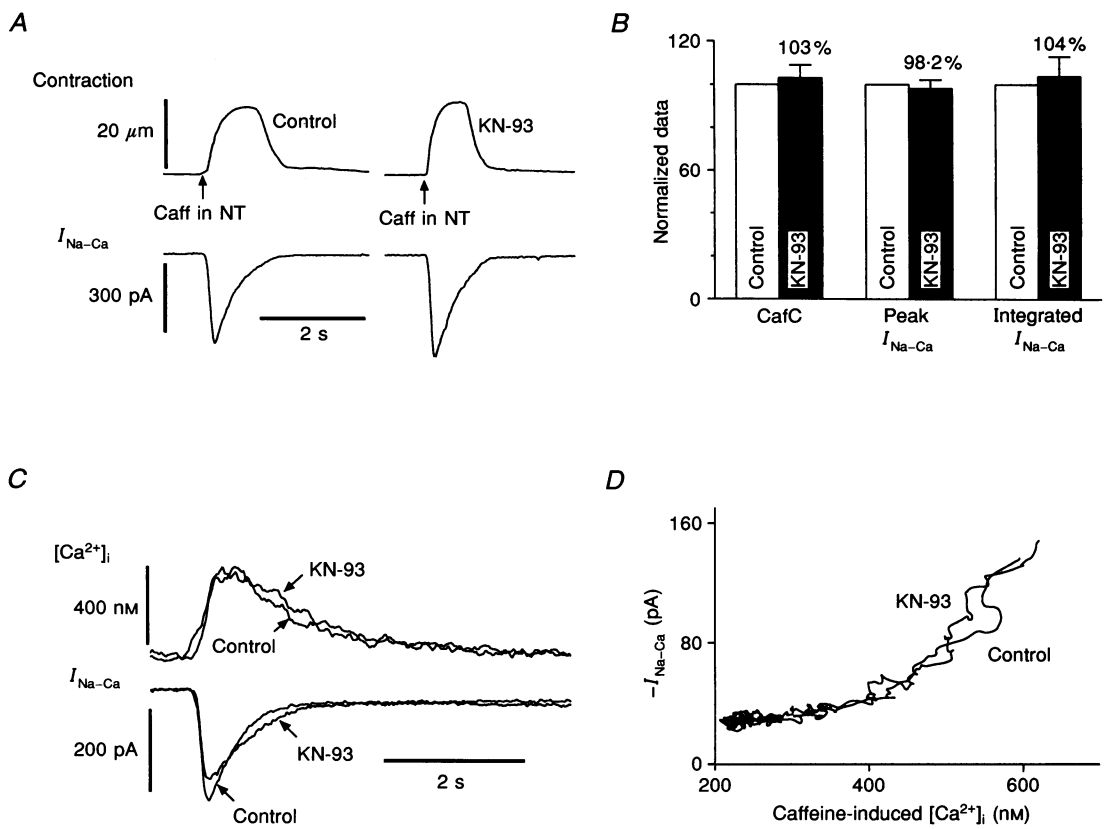


Figure 7. Measurement of SR Ca^{2+} content after conditioning pulses in voltage clamp

A, original record of caffeine-induced contracture (upper) and Na^+–Ca^{2+} exchange current (I_{Na-Ca}, lower) in control (left) and KN-93 (right). Five conditioning pulses to 0 mV for 200 ms were used in control. The pulses were prolonged to 400 ms in KN-93 to obtain the same SR Ca^{2+} load. *B*, pooled normalized mean caffeine-induced contracture amplitude (CafC), peak I_{Na-Ca} and integrated I_{Na-Ca} are shown ($n = 7$). The values of CafC, peak I_{Na-Ca} and integration of I_{Na-Ca} with KN-93 were not significantly different from control. *C*, caffeine-induced Ca^{2+} transient and I_{Na-Ca} in another myocyte before and after exposure to KN-93. In this cell the conditioning trains in control and KN-93 were as described for *A*. *D*, $[Ca^{2+}]_i$ dependence of I_{Na-Ca} for the cell shown in *C*. Data are shown only for the declining phase of the Ca^{2+} transient.

control of both SR Ca^{2+} load and trigger I_{Ca}. Thus, perforated voltage-clamp experiments were conducted where the SR Ca^{2+} load and I_{Ca} could be matched before and after KN-93 treatment (in a given cell). An essential first step was to be able to measure SR Ca^{2+} load before and after KN-93 and also to be able to manipulate the SR Ca^{2+} load by varying the conditioning voltage-clamp pulses.

Figure 7*A* shows an example where SR Ca^{2+} load was matched before and after KN-93. Typical records of cell contracture and I_{Na-Ca} induced by rapid caffeine application are shown. First, five conditioning pulses from −70 mV to 0 mV for 200 ms were applied to the control to set the SR Ca^{2+} content. Caffeine was applied 2 s after the last pulse and maintained for 8 s to release SR $Ca^{2+}$and activate I_{Na-Ca}. The same procedure was used after application of the CaMKII inhibitor KN-93 (1 μM), except that a more aggressive loading protocol was required to achieve the same SR Ca^{2+} load (compare Fig. 4, where $[Ca^{2+}]_o$ was increased in non-voltage-clamped cells in KN-93 for the same reason). In Fig. 7*A* the conditioning pulses in KN-93 were prolonged to 400 ms in order to match the SR content with control. In some other cells the conditioning depolarization was also increased (e.g. to +10 mV) to match the control SR Ca^{2+} load.

Figure 7*B* shows that SR Ca^{2+} load was effectively matched before and after KN-93 in cells which were used for further analysis (Figs 8 and 9 and Table 1). It is clear that both contracture amplitude (103 ± 6%, $n = 7$) and peak I_{Na-Ca} (98 ± 4%) were constant in control and in the presence of KN-93. The integrated I_{Na-Ca} induced by caffeine was also the same (104 ± 9%). Since, during this long caffeine pulse, Ca^{2+} is extruded mainly by sarcolemmal Na^+–Ca^{2+} exchange (Bassani, Bassani & Bers, 1994), the integral of I_{Na-Ca} is a good indicator of SR Ca^{2+} content (Varro, Negretti, Hester & Eisner, 1993). In some cells we also applied caffeine a second time, right after the first application. There was neither a $[Ca^{2+}]_i$ change nor measurable I_{Na-Ca}, indicating that all SR Ca^{2+} had been released by the first caffeine application and that no other current is activated directly by caffeine.

The increase in $[Ca^{2+}]_i$ upon caffeine application was also used in some experiments as an index of SR Ca^{2+} load in indo-1 AM-loaded myocytes (Figs 7*C* and 10). In this case we also have the advantage that $[Ca^{2+}]_i$ and I_{Na-Ca} are measured simultaneously. Thus we can plot I_{Na-Ca} as a function of $[Ca^{2+}]_i$ during the declining phase of the Ca^{2+} transient (Fig. 7*D*), where spatial gradients of $[Ca^{2+}]_i$ are not likely to complicate the results. Figure 7*D* shows that there was no apparent effect of KN-93 on the $[Ca^{2+}]_i$ dependence of I_{Na-Ca}.

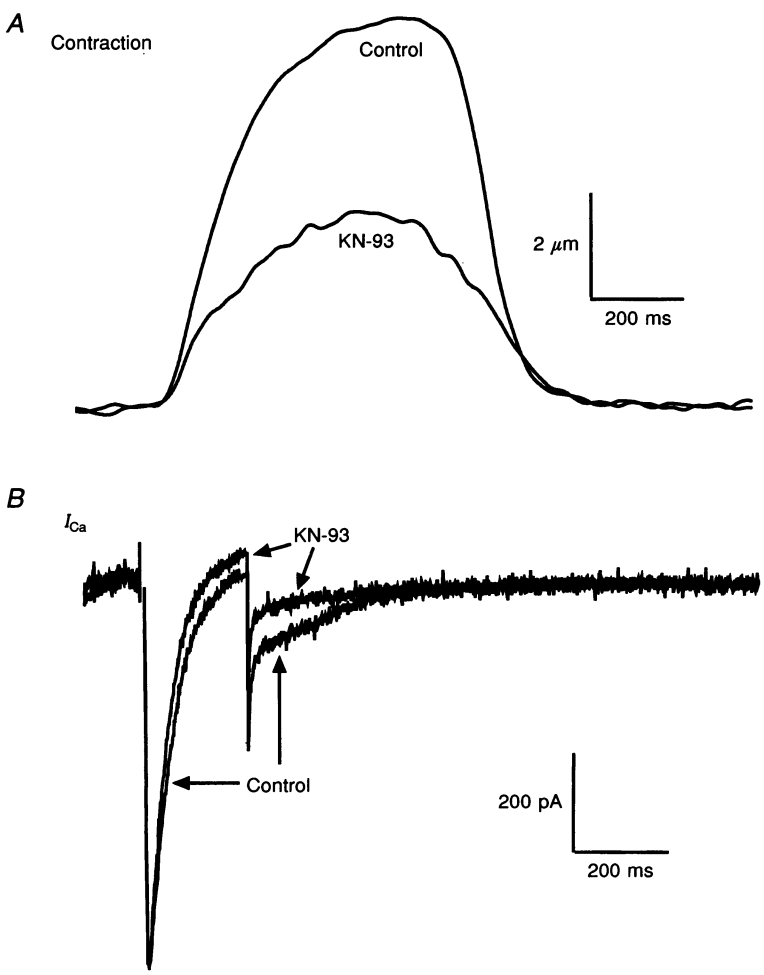


Figure 8. Contraction and I_{Ca} after conditioning pulses: effects of KN-93

I_{Ca} was generated by a test pulse to 0 mV for 200 ms after five conditioning pulses to 0 mV (*B*). To keep the SR Ca^{2+} load matched between control and KN-93, the conditioning pulse duration was increased from 200 ms in control to 400 ms in KN-93. Twitch contractions in response to the corresponding test pulses are shown in *A* (amplitude 7·4 μm in control, 3·7 μm in KN-93).

We should also emphasize that if we had used exactly the same conditioning protocol to load the SR in the presence of KN-93 then the SR Ca^{2+} load would have been smaller (e.g. Fig. 2*B*). However, by manipulating the conditioning pulses we could clearly achieve and document comparable SR Ca^{2+} loading (before and after KN-93) in all the cells used for further analysis of E–C coupling in Figs 7–10.

Effect of CaMKII on E–C coupling

Using the same cells and conditioning pulses which were documented to produce matching SR Ca^{2+} load above, the relationship between I_{Ca} and contraction or $[Ca^{2+}]_i$ was measured in the presence and absence of KN-93. Figure 8 shows cell contraction and I_{Ca} in a representative cell. It is apparent that the 0 mV test pulse generated a much smaller contraction in the presence of 1 μM KN-93 (panel *A*, 7·4 μm in control and 3·7 μm in KN-93), despite a constant SR Ca^{2+} content and comparable trigger I_{Ca} (728 pA in control and 772 pA in KN-93). In most cells a different test potential (V_m) was needed to match I_{Ca} (e.g. −10 mV in control and 0 mV in KN-93). The smaller contraction elicited in the presence of CaMKII inhibitor KN-93 for the same I_{Ca} and SR Ca^{2+} content implies that CaMKII has indeed modified E–C coupling. That is, CaMKII appears to increase the responsiveness of Ca^{2+}-induced Ca^{2+} release.

Figure 8*B* also shows that there is a larger inward tail current upon repolarization in the control series than with KN-93. This larger inward tail current is probably attributable to enhanced Na^+–Ca^{2+} exchange current, secondary to the larger Ca^{2+} transient and twitch contraction under control conditions.

Figure 9*A* shows complete current–voltage and contraction–voltage curves for the cell in Fig. 8 with conditioning pulses, where SR Ca^{2+} load should be the same at each test pulse. Both parameters exhibited bell-shaped voltage dependence in control and in the presence of KN-93. In this cell I_{Ca} was not appreciably depressed by KN-93 over the whole range of voltages tested. However, the contractions were much smaller in the presence of KN-93 throughout this range. The fact that we still see comparable depression of E–C coupling with KN-93 in the absence of altered I_{Ca} in this particular cell strengthens our conclusions with respect to E–C coupling, and indicates that the effects of KN-93 on I_{Ca} and E–C coupling are independent.

More typically I_{Ca} was somewhat depressed by KN-93 (to 72 ± 4% of control at 0 mV in this series). For these cells it is easier to compare contractions at a given trigger I_{Ca} using the relationship between I_{Ca} and contraction at the same SR Ca^{2+} load. This is shown for another cell in Fig. 9*B*. In this cell it can be seen that for a given I_{Ca} (and SR Ca^{2+} load) the contraction is always smaller with KN-93. The direction of the arrows indicates increasingly positive voltage steps. In this cell I_{Ca} was comparable for the control test pulse to +20 mV and KN-93 test pulse to 0 mV, but the contraction was much smaller in KN-93. The effect of KN-93 was reversible, but required a very long period of washout (e.g. more than 30 min, data not shown). This indicates that the

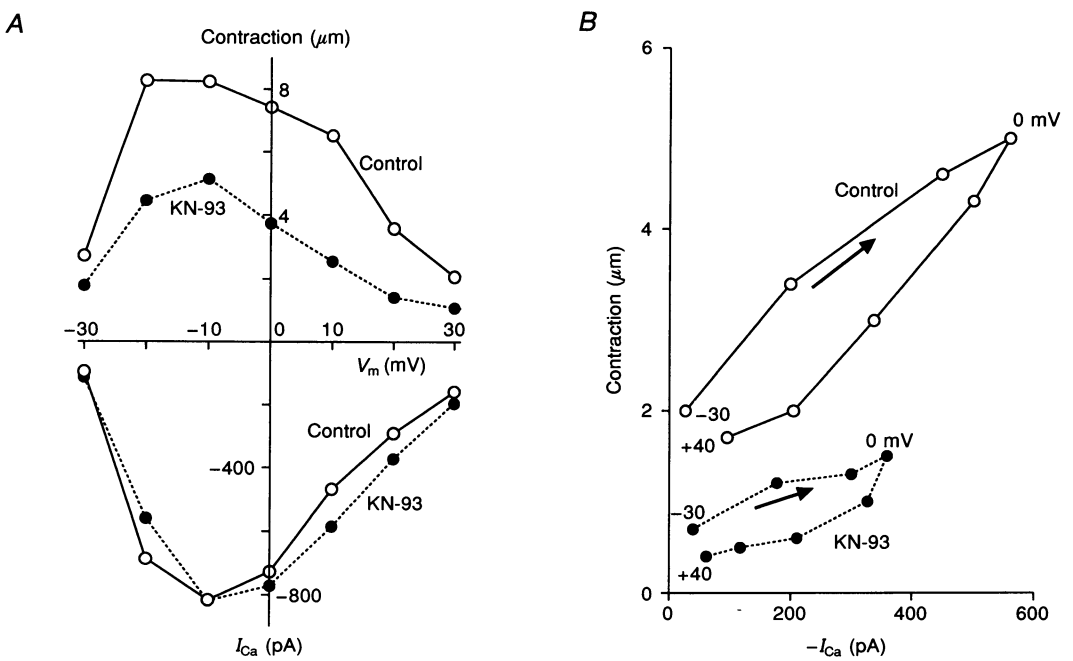


Figure 9. KN-93 depresses twitch contraction for a given I_{Ca} and SR Ca^{2+} load

A, full current–voltage and contraction–voltage relationships in the same cell as Fig. 8, under control conditions (○) and with 1 μM KN-93 (●). Prior to each test pulse, the cell was conditioned as described in the legend of Fig. 7. *B*, I_{Ca}–contraction relationships in the absence (○) and presence (●) of KN-93 in a different cell from that in *A*. The arrows indicate the direction of increasingly positive voltage-test steps.

Table 1. KN-93 depresses twitch contraction and $\Delta[Ca^{2+}]_i$ at constant SR Ca^{2+} load and I_{Ca}

	Set I Contraction only (% of control)	Set II Ca^{2+} transients (% of control)
SR Ca^{2+} load *	104 ± 9	96 ± 7
Calcium current †	104 ± 3	106 ± 4
Contraction or $\Delta[Ca^{2+}]_i$ ‡	39 ± 4§	49 ± 6§

KN-93 depresses E–C coupling even when SR Ca^{2+} load and I_{Ca} were matched. Data are shown as percentages of control (§ $P < 0{\cdot}0001$, $n = 7$ cells for each set). * In Set I SR Ca^{2+} load was based on $\int I_{Na-Ca}$ and in Set II it was based on the caffeine-induced increase of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$). † Peak I_{Ca} for the same test twitches, where test V_m was chosen to produce the same I_{Ca} in control and KN-93 for a given cell. Usually the KN-93 test was at the V_m which gave maximal I_{Ca}, while the chosen control gave a submaximal I_{Ca} matching that in KN-93 (typically 10–20 mV difference). ‡ Data are normalized to control for contraction amplitude (Set I) or $\Delta[Ca^{2+}]_i$ (Set II).

depressive effect of KN-93 on contraction is not due to run-down in these perforated patch experiments. This effect of KN-93 was seen in all seven cells studied with this strategy. Table 1 shows that in KN-93 the mean contraction was 39 ± 4% of control despite comparable SR Ca^{2+} load and trigger I_{Ca}. To assess whether KN-93 had any non-specific effects, its structural analogue KN-92 was also tested. KN-92 (1 μM) had no effects on either I_{Ca} or contraction amplitude (data not shown).

Additional experiments were performed with indo-1 AM-loaded cells where $[Ca^{2+}]_i$ was measured using the same experimental strategy. Quantitatively similar results were obtained as in Figs 8 and 9. That is, for comparable I_{Ca} and SR Ca^{2+} content, the twitch Ca^{2+} transient amplitude ($\Delta[Ca^{2+}]_i$) was reduced to 49 ± 6% of control (Table 1).

Figure 10 shows an example using Ca^{2+} transients of the converse experiment to that in Figs 8 and 9 and Table 1. In

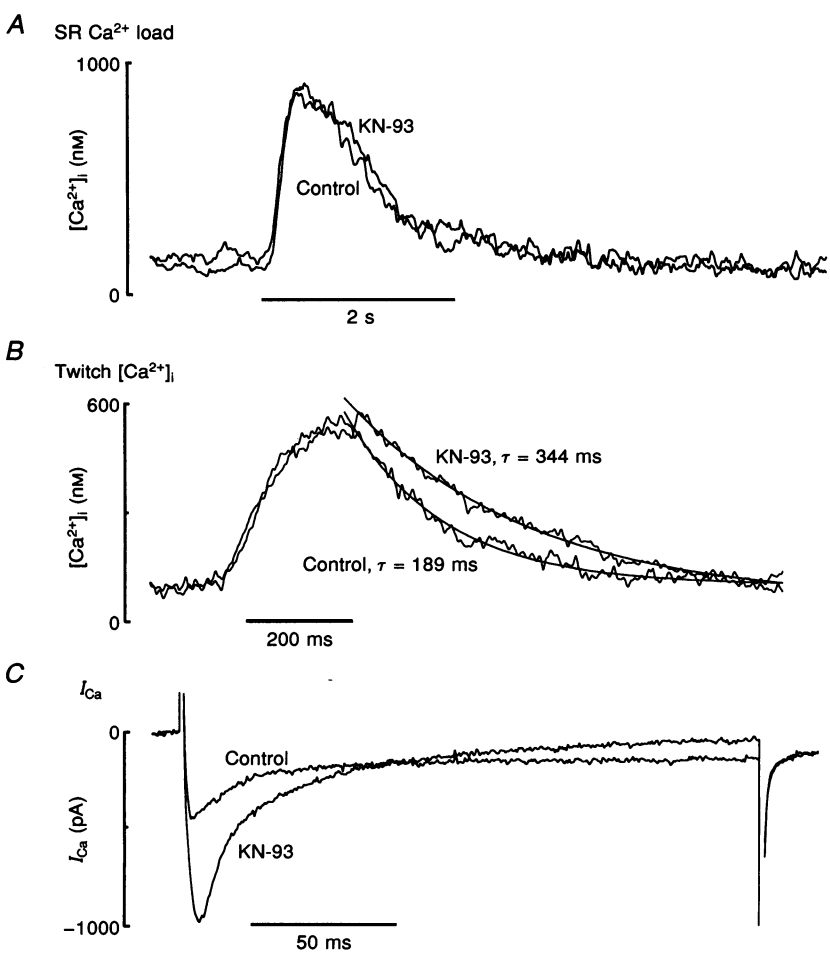


Figure 10. Comparable twitch $[Ca^{2+}]_i$ requires greater I_{Ca} trigger in KN-93

Caffeine-induced $[Ca^{2+}]_i$ transient, twitch $[Ca^{2+}]_i$ transient and corresponding I_{Ca} in a typical indo-1 AM-loaded cell. *A*, superimposed caffeine-induced $[Ca^{2+}]_i$ after conditioning pulses in control and after KN-93 treatment. *B*, twitch $[Ca^{2+}]_i$ in the absence and presence of KN-93 after the same series of conditioning pulses. Simple exponential curve fits to $[Ca^{2+}]_i = A\exp(-t/\tau)$ are superimposed and the time constants τ are indicated. *C*, I_{Ca} traces corresponding to *B*, where depolarization voltage was −30 mV in control and −10 mV in KN-93.

this case, the SR Ca^{2+} load was still matched to control in the presence of KN-93 (by increasing the conditioning pulses to 400 ms at +10 mV depolarization). In the comparison of traces in panels *A* and *B* of Fig. 10, the SR Ca^{2+} load and twitch $[Ca^{2+}]_i$ amplitude were the same. However, in this case a much larger I_{Ca} was required to achieve the same SR Ca^{2+} release with KN-93 (panels *B* and *C*). Ca^{2+} transient data from seven cells exhibited results similar to the contraction data shown in Fig. 9. We conclude that endogenous CaMKII increases the efficacy of E–C coupling. That is, SR Ca^{2+} release during SS pulses with CaMKII activated is greater for a given I_{Ca} and SR Ca^{2+} content.

In additional experiments, we also used a less negative holding voltage (−45 mV instead of −70 mV) in the control which produced weaker SS contractions and Ca^{2+} transients. In this case KN-93 did not depress contraction or twitch $[Ca^{2+}]_i$ for a given I_{Ca} and SR Ca^{2+} load (data not shown). This important negative result may suggest that Ca^{2+} transients need to reach a certain minimal level to trigger the activity of endogenous CaMKII. It also indicates that in the results we present here (Figs 7–10) the conditioning pulses were sufficient to activate endogenous CaMKII. The observation that KN-93 did not alter E–C coupling under these lower $[Ca^{2+}]_i$ conditions also suggests that the inhibitory effects of KN-93 on E–C coupling are not direct effects of the drug on E–C coupling, but rather depend on $[Ca^{2+}]_i$ and may indeed be CaMKII dependent.

KN-93 and $[Ca^{2+}]_i$ decline during relaxation

In Fig. 1 it was noted that the τ of $[Ca^{2+}]_i$ decline at the SS twitch was longer with KN-93. These results are consistent with KN-93 blocking a Ca^{2+} and stimulation-dependent acceleration of $[Ca^{2+}]_i$ decline (Bassani *et al.* 1995*c*). However, the smaller amplitude of the SS Ca^{2+} transient in KN-93 complicates this interpretation in Fig. 2, since a decrease in amplitude will intrinsically slow τ (Bers & Berlin, 1995). In Fig. 2 there could also have been changes in action potential duration which could complicate the interpretation. The example in Fig. 10 avoids both of these complications, since the amplitude of the Ca^{2+} transient and the duration of the test voltage-clamp pulse were unchanged. Even in this case KN-93 still slows Ca^{2+} decline for SS $[Ca^{2+}]_i$ transients (from 189 to 344 ms).

DISCUSSION

Specificity of the CaMKII inhibitor

We used a newly synthesized CaMKII inhibitor, KN-93, which is a methoxybenzenesulphonamide. An advantage of KN-93 is that the phosphate salt form dissolves readily in aqueous solution (Sumi *et al.* 1991). More importantly, KN-93 elicited potent inhibitory effects on CaMKII, competing with calmodulin (K_i, 0·37 μM; Sumi *et al.* 1991; Mamiya *et al.* 1993). The K_i values of KN-93 for other protein kinases such as protein kinase C, protein kinase A, casein kinase I and myosin light-chain kinase are all higher than 30 μM (Sumi *et al.* 1991). Thus KN-93 is more potent, selective and water soluble than the related CaMKII inhibitor KN-62.

However, these organic inhibitors may also affect other targets. Yuan & Bers (1994) found that KN-62 prevented Ca^{2+}-dependent I_{Ca} facilitation in cardiac myocytes as did dialysis with two different CaMKII inhibitory peptides. However, KN-62 also produced a slowing of I_{Ca} recovery from inactivation and shifted the SS inactivation curve to more negative V_m values and these effects on I_{Ca} were not mimicked by the specific peptides. It is therefore possible that some of these KN-62 effects were not due entirely to direct specific inhibition of CaMKII. Thus, while we are using KN-93 as a specific inhibitor of endogenous CaMKII, this constitutes an intrinsic assumption in our interpretations.

Effect of CaMKII on SR Ca^{2+} release channel

Biochemical experiments have demonstrated that the cardiac isoform of the RyR is a preferred substrate for CaMKII (Takasago *et al.* 1991; Witcher *et al.* 1991). Phosphorylation occurs at a single site (serine 2809). When cardiac junctional SR vesicles or purified RyR are fused with planar lipid bilayers, phosphorylation at this site can activate the Ca^{2+} channel (Witcher *et al.* 1991; Hain *et al.* 1995). On the other hand, Lokuta *et al.* (1995) reported that CaMKII decreased cardiac SR Ca^{2+} channel open probability when Ca^{2+} and calmodulin were present (i.e. when the channel was phosphorylated). This discrepancy might be partly explained in terms of the kinetics of RyR gating. Györke & Fill (1993) showed that cardiac SR Ca^{2+} release channel open probability is maximal immediately after the triggering rise in $[Ca^{2+}]$ and then declines with time, a process they called adaptation (and called inactivation by Schiefer, Meissner & Isenberg, 1995). Valdivia *et al.* (1995) showed that RyR adaptation happens within milliseconds when physiological concentrations of Mg^{2+} are present. They also showed that phosphorylation of the RyR by protein kinase A (PKA) increased the responsiveness of the channel to Ca^{2+}, but also accelerated the kinetics of adaptation. Upon channel phosphorylation by PKA, the initial channel open probability increased in response to a given $[Ca^{2+}]_i$ change but quickly dropped to a new steady-state level even lower than the steady state of control. Thus, their results showed that phosphorylation enhanced Ca^{2+} release channel activity in response to a rapid increase in local Ca^{2+} as may be expected near L-type Ca^{2+} channels, but decreased the SS activation by Ca^{2+}. If a similar phenomenon occurred with release channel phosphorylation by CaMKII, this could help explain the different CaMKII results obtained in bilayers.

In the present study we have provided an initial characterization of the effect of CaMKII on E–C coupling in intact ferret ventricular myocytes. Since CaMKII has dramatic effects on the RyR in biochemical and single channel studies as addressed above, it is important to understand its effects on RyR in the natural cellular environment.

KN-93 decreased resting Ca^{2+} spark frequency (Figs 2 and 3*C*). However, this effect seems to be due primarily to the decreased SS SR Ca^{2+} load, since restoring SR Ca^{2+} load also largely restores the control Ca^{2+} spark frequency towards the control level.

A central finding of the present study is that endogenous CaMKII increases the efficacy of Ca^{2+}-induced Ca^{2+} release. Since both SR Ca^{2+} content and trigger I_{Ca} are known to alter the fraction of SR Ca^{2+} release (Isenberg & Han, 1994; Bassani *et al.* 1995*b*) we were careful to match these parameters in the presence and absence of KN-93. Conditioning pulses were carefully adjusted such that SR Ca^{2+} content was constant before and after KN-93 treatment (Fig. 7, Table 1). We discarded any cells where we failed to make this match. Our comparison is also based on having established the same trigger I_{Ca} in control and KN-93. Although KN-93 inhibited I_{Ca} in most cells (discussed in next section), we have recorded I_{Ca} over the voltage range of −30 to 40 mV, such that we were able to find V_m values where I_{Ca} matched between control and KN-93. We, therefore, believe that KN-93 decreased the active SR Ca^{2+} release during E–C coupling, because of a decrease in the effectiveness of a given trigger I_{Ca} to release SR Ca^{2+} (resulting in smaller contractions and $[Ca^{2+}]_i$ transients in KN-93, Figs 8–10). It may be noted that this comparison did not depend on whether V_m was more positive or negative than control in KN-93. In general these V_m values were within 20 mV.

The mechanism of KN-93 inhibition of Ca^{2+} release channel function may be the inhibition of RyR phosphorylation by CaMKII. This conclusion fits well with recent observations of duBell, Lederer & Rogers (1996) that protein phosphatases can reduce SR Ca^{2+} release channel activity. Therefore, we conclude that phosphorylation of RyR by CaMKII increases Ca^{2+} release channel activity in intact cardiac myocytes during E–C coupling. This may be an important regulatory mechanism for direct control of RyR gating by Ca^{2+}-dependent phosphorylation. Once phosphorylation occurs in the cellular environment, the net effect is a change in the sensitivity of the RyR to activation by Ca^{2+}, such that greater SR Ca^{2+} efflux occurs for a given I_{Ca} trigger (Figs 8 and 9, Table 1). Similarly, a given twitch Ca^{2+} transient can be induced by a smaller I_{Ca} trigger (Fig. 10). Thus a progressive phosphorylation and sensitization of E–C coupling might contribute to the positive force–frequency relationship observed in cardiac muscle.

Stimulation of SR Ca^{2+} pump transport by CaMKII phosphorylation of PLB (or of the SR Ca^{2+}ATPase; Xu *et al.* 1993) in our experimental conditions could complicate our interpretations. However, this effect would be in the opposite direction required to explain the depressed twitches with KN-93. That is, a reduced rate of SR Ca^{2+} transport with KN-93 would be expected to increase the amplitude of the twitch Ca^{2+} transient if release were constant (Bassani *et al.* 1994). Indeed, if such a CaMKII-induced acceleration of SR Ca^{2+} transport occurs (see below), our results may underestimate the stimulatory effect of CaMKII on E–C coupling.

Effect of KN-93 on I_{Ca} and rest potentiation

Several groups have reported that Ca^{2+}-dependent I_{Ca} facilitation (positive staircase) is related to the activation of CaMKII (Yuan & Bers, 1994; Xiao, Cheng, Lederer, Suzuki & Lakatta, 1994; Anderson, Brau, Schulman & Premack, 1994). We also found that the positive I_{Ca} staircase was reversed by the CaMKII inhibitor KN-93 (data not shown), as it was by KN-62 (Yuan & Bers, 1994). KN-93 also shifted the voltage dependence of I_{Ca} gating towards a more negative voltage. This was associated with a slowing of Ca^{2+} channel recovery from inactivation at a given voltage (Fig. 6), such that fewer Ca^{2+} channels would be available during a test pulse at a given recovery time. This could partly explain the results shown in Figs 1 and 4. That is, KN-93 significantly depressed twitch $[Ca^{2+}]_i$, especially during SS twitches where Ca^{2+} channels have less time to recover. At 1 Hz SS simulation in KN-93, fewer sarcolemmal Ca^{2+} channels were available due to the slow recovery between pulses leading to a reduced SR Ca^{2+} release. Depressed SS I_{Ca} can also account for the lower SR Ca^{2+} content during SS field stimulation in Fig. 3*A* and *B*. This may explain the depression of the SS twitch Ca^{2+} transient in KN-93 in Figs 1–4. During long rest intervals (many seconds), nearly all sarcolemmal Ca^{2+} channels recover before the PR stimulation. This may be why the PR twitch Ca^{2+} transient was less inhibited by KN-93 (Fig. 1).

We cannot say precisely what fraction of the strong suppressive effect of KN-93 on the SS Ca^{2+} transient in Fig. 1 is due to depression of I_{Ca}, SR Ca^{2+} content or E–C coupling. Indeed, they all probably contribute. The effects of KN-93 on each of these have been independently assessed in the present study in specific controlled experiments. The reduction in SS SR Ca^{2+} content with KN-93 is surely secondary to the reduced Ca^{2+} entry via I_{Ca}. The data in Table 1 and Figs 8–10 also show that KN-93 clearly and strongly depresses E–C coupling to 39–49% of control, even when I_{Ca} and SR Ca^{2+} content are unchanged.

Study of twitch $[Ca^{2+}]_i$ decline

Bassani *et al.* (1995*c*) reported a frequency-dependent acceleration of SS twitch relaxation and $[Ca^{2+}]_i$ decline (*vs.* PR) in rat cardiac myocytes which could be inhibited by the CaMKII inhibitor KN-62. Phosphatase inhibitors also prevented the slowing of relaxation and $[Ca^{2+}]_i$ decline at post-rest twitches. This suggested that endogenous CaMKII was involved in the process. They attributed the faster $[Ca^{2+}]_i$ decline in SS *vs.* PR to CaMKII-dependent phosphorylation (possibly of PLB) with a consequent increased SR Ca^{2+} pump activity. In the present study in ferret myocytes, the $[Ca^{2+}]_i$ decline during SS twitches was also accelerated in control ferret cells (in comparison with PR, Fig. 1). KN-93 treatment abolished the effect of SS stimulation on the τ of SS and PR twitch $[Ca^{2+}]_i$ decline, consistent with the results of Bassani *et al.* (1995*c*) in rat.

We report here that CaMKII appears to increase both Ca^{2+}-induced Ca^{2+} release (for a given I_{Ca} and SR Ca^{2+} load) and the rate of $[Ca^{2+}]_i$ decline at SS twitches. These two effects may be co-ordinated physiologically so that at higher frequency SR Ca^{2+} release, release and re-uptake are both stimulated, allowing faster contraction and relaxation (and diastolic ventricular filling). This may complement the well-documented cAMP-mediated effects on the I_{Ca} trigger (causing increased SR Ca^{2+} release and loading) and SR Ca^{2+} uptake rate (speeding relaxation).

In summary, our results indicate that endogenous CaMKII activity increases the efficacy of Ca^{2+}-induced Ca^{2+} release during E–C coupling in intact cardiac myocytes. CaMKII may also be important in the rapid recovery of I_{Ca} and Ca^{2+} transport by the SR Ca^{2+} pump during repetitive pulses.

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