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RESEARCH PAPER

Ca^{2+}-calmodulin can activate and inactivate cardiac ryanodine receptors

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Background and purpose: Ca^{2+}-calmodulin (Ca^{2+}CaM) is widely accepted as an inhibitor of cardiac ryanodine receptors (RyR2); however, the effects of physiologically relevant CaM concentrations have not been fully investigated.
Experimental approach: We investigated the effects of low concentrations of Ca^{2+}CaM (50–100 nmol·L^{-1}) on the gating of native sheep RyR2, reconstituted into bilayers. Suramin displaces CaM from RyR2 and we have used a gel-shift assay to provide evidence of the mechanism underlying this effect. Finally, using suramin to displace endogenous CaM from RyR2 in permeabilized cardiac cells, we have investigated the effects of 50 nmol·L^{-1} CaM on sarcoplasmic reticulum (SR) Ca^{2+}-release.
Key results: Ca^{2+}CaM activated or inhibited single RyR2, but activation was much more likely at low (50–100 nmol·L^{-1}) concentrations. Also, suramin displaced CaM from a peptide of the CaM binding domain of RyR2, indicating that, like the skeletal isoform (RyR1), suramin directly competes with CaM for its binding site on the channel. Pre-treatment of rat permeabilized ventricular myocytes with suramin to displace CaM, followed by addition of 50 nmol·L^{-1} CaM to the mock cytoplasmic solution caused an increase in the frequency of spontaneous Ca^{2+}-release events. Application of caffeine demonstrated that 50 nmol·L^{-1} CaM reduced SR Ca^{2+} content.
Conclusions and implications: We describe for the first time how Ca^{2+}CaM is capable, not only of inactivating, but also of activating RyR2 channels in bilayers in a CaM kinase II-independent manner. Similarly, in cardiac cells, CaM stimulates SR Ca^{2+}-release and the use of caffeine suggests that this is a RyR2-mediated effect.
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Keywords: ryanodine receptor; calmodulin; cardiac; Ca^{2+}-release; Ca^{2+}-sparks; excitation–contraction coupling; sarcoplasmic reticulum

Abbreviations: AIP, autocamtide-2-related inhibitory peptide; CaM, calmodulin; CaMKII, CaM kinase II; EC-coupling, excitation–contraction coupling; RyR2, cardiac ryanodine receptor; RyRF, RyR2 peptide (RSKKAVWHKLLSKQRKRAVVACFRMAPLYNLP); SR, sarcoplasmic reticulum

Introduction

The cardiac ryanodine receptor (RyR2; nomenclature follows Alexander *et al.*, 2008) is the pathway for the release of intracellular Ca^{2+} during excitation–contraction (EC) coupling. It also acts as a scaffolding protein localizing numerous other proteins to the dyadic cleft regions. Calmodulin (CaM) binds very tightly to RyR2, but the physiological role of this direct association is unclear (Balshaw *et al.*, 2001; Ai *et al.*, 2005). CaM is a Ca^{2+}-sensing protein, which contains four Ca^{2+}-binding sites, two on the amino and two on the carboxyl lobe. CaM interacts with numerous proteins, usually in the Ca^{2+}-bound form (Ca^{2+}CaM), but can also modulate certain proteins in the non-Ca^{2+}-bound form (apoCaM). It has been well documented that Ca^{2+}CaM can cause partial inhibition of both cardiac and skeletal (RyR1) isoforms of RyR (Fruen *et al.*, 2000; Balshaw *et al.*, 2001). Additionally, apoCaM is known to activate RyR1, but has little effect on RyR2 (Smith *et al.*, 1989; Tripathy *et al.*, 1995; Fruen *et al.*, 2000).

The magnitude of the reported inhibition of RyR2 by Ca^{2+}CaM is often exceedingly small (Yamaguchi *et al.*, 2004; Xu and Meissner, 2004) and, interestingly, there are also rare reports suggesting that CaM may activate RyR2 (Fruen *et al.*, 2000; Chugun *et al.*, 2007). Experiments investigating the cellular actions of CaM also hint that CaM is not simply inhibiting RyR2. For example, in permeabilized cardiac myocytes, adding CaM to the perfusate can lead to a reduction in the frequency of Ca^{2+}-sparks (Guo *et al.*, 2006), but it has also

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been reported to enhance spark frequency (Lukyanenko and Gyorke, 1999). These divergent results led us to re-examine the effects of Ca^{2+}CaM on RyR2 single-channel behaviour and on sarcoplasmic reticulum (SR) Ca^{2+}-release in cardiac cells, paying particular attention to the effects of physiological levels of CaM. This is important because although the free [CaM] in cardiac cells has been recently estimated at only 50–75 nmol·L^{-1} (Wu and Bers, 2006), the majority of previous studies have examined the effects of high concentrations ((1 μmol·L^{-1}) of CaM on RyR2 channel function (Smith *et al.*, 1989; Xu and Meissner, 2004) and Ca^{2+}-spark generation in cardiac cells (Lukyanenko and Gyorke, 1999; Ai *et al.*, 2005; Guo *et al.*, 2006).

As CaM can be immunoprecipitated with RyR2 (Ai *et al.*, 2005), and as the $t_{1/2}$ for [^{35}S]CaM dissociation from cardiac SR is approximately 9 min for Ca^{2+}CaM and 40 s for apoCaM (Balshaw *et al.*, 2001), it is likely that some CaM is always bound tightly to RyR2 *in situ*, and may provide a certain level of 'constitutive' modulation of channel function. To experimentally investigate this modulation in cardiac cells, it is necessary first to remove the CaM attached to RyR2. We have therefore used suramin to displace RyR2-bound CaM from rat permeabilized cardiac myocytes. After incubation with suramin, we then add back a physiological concentration of CaM (50 nmol·L^{-1}) to the mock cytoplasmic solution.

Suramin has been shown to bind to the CaM binding site on RyR1 (Klinger *et al.*, 1999; Papineni *et al.*, 2002). We have previously demonstrated that suramin displaces CaM from cardiac SR (Hill *et al.*, 2004), but the mechanism underlying this displacement is not known. There are differences in the amino acid sequences comprising the RyR2 and RyR1 CaM binding sites and it is not known if suramin also binds to RyR2 at this site. By using suramin to dissociate preformed complexes of CaM and RyR2 CaM binding domain peptides, we now provide evidence suggesting that, as with RyR1, suramin does compete with CaM at this site on RyR2, hence displacing CaM.

Our study demonstrates for the first time that physiological levels of Ca^{2+}CaM (50 nmol·L^{-1}) can activate RyR2. We propose that CaM tightly bound to RyR2 in cardiac cells plays a facilitator role during excitation–contraction coupling (EC-coupling), enabling changes in cytosolic Ca^{2+} to be optimally effective.

Methods

Preparation of SR vesicles and planar lipid bilayers

Heavy SR membrane vesicles were prepared from sheep hearts (from a local abattoir) as described previously (Sitsapesan *et al.*, 1991), snap-frozen in liquid N_2 and stored at –80°C. Vesicles were fused with planar phosphatidylethanolamine lipid bilayers as described previously (Sitsapesan *et al.*, 1991). SR vesicles fused in a fixed orientation such that the *cis*-chamber corresponded to the cytosolic space and the *trans*-chamber to the SR lumen. The *trans*-chamber was held at ground and the *cis*-chamber held at potentials relative to ground. After fusion, the *cis*-chamber was perfused with 250 mmol·L^{-1} HEPES, 80 mmol·L^{-1} Tris, 15 μmol·L^{-1} free Ca^{2+}, pH 7.2, unless stated otherwise. The free [Ca^{2+}] was increased to 100 μmol·L^{-1} by addition of $CaCl_2$ solution. The *trans*-chamber was perfused with 250 mmol·L^{-1} glutamic acid and 10 mmol·L^{-1} HEPES, pH to 7.2 with $Ca(OH)_2$ (free [Ca^{2+}], approximately 50 mmol·L^{-1}). Experiments were carried out at room temperature (22 ± 2°C). The free [Ca^{2+}] and pH of the solutions were determined using a Ca^{2+} electrode (Orion 93-20) and a Ross-type pH electrode (Orion 81-55) as previously described (Sitsapesan *et al.*, 1991). Additions of CaM were made to the *cis*-chamber.

Data acquisition and analysis

Channel recordings were displayed on an oscilloscope and recorded on digital audiotape. Current recordings were filtered at 800 Hz (–3 dB) and digitized at 20 kHz using Pulse (HEKA Elektronik Lambrecht/Pfalz, Germany). Channel open probability (P_o) was determined over 3 min of continuous recording, unless otherwise stated, using the method of 50% threshold analysis (Colquhoun and Sigworth, 1983). Lifetime analysis was carried out only when a single channel incorporated into the bilayer. Events <1 ms in duration were not fully resolved and were excluded from lifetime analysis. Individual lifetimes were fitted to a probability density function by the method of maximum likelihood according to the equation:

$$g(x) = \sum_{i=1}^{N} a_i g_o(x - \ln \tau_i)$$

Where $\ln\tau_i$ is the logarithm of the ith time constant and a_i is the fraction of the total events represented by that component (Sigworth and Sine, 1987).

Complex formation between Ca^{2+}CaM and a fragment of RyR2 a.a. 3581–3612 (RyRF)

The synthetic peptide corresponding to the RyR2 CaM binding domain human sequence 3581–3612, RSKKAVWHKLLSKQRKRAVVACFRMAPLYNLP, was purchased from Peptide Protein Research Ltd. This 32-amino-acid peptide differs from the RyR1 CaM binding domain by three amino acids (RyR2 3581, 3596 and 3606). Rat brain CaM was expressed in *Escherichia coli* and purified with a phenyl Sepharose column as previously described (Zielinski, 1998). Purified CaM was dansylated as previously described (Klee, 1977; Vorherr *et al.*, 1990).

Native gel electrophoresis

Purified CaM (0.5 mmol·L^{-1}) in 20 mmol·L^{-1} Tris pH 7.0, 1 mmol·L^{-1} dithiothreitol, 1.5 mmol·L^{-1} $CaCl_2$ was incubated with the RyR2 peptide (referred to in this report as RyRF) at stoichiometries of Ca^{2+}CaM : RyRF 1:0.25, 1:0.5, 1:1 and 1:3. Complex formation was examined by native Tris-Tricine gels stained with Coomassie brilliant blue (Schagger and von Jagow, 1987). Increasing concentrations of suramin were added to the pre-formed complex (Ca^{2+}CaM : RyRF at a 1:3 molar ratio). After 30 min, samples were analysed by native gel electrophoresis as above. Gel bands were scanned using a

Biorad GS-800 calibrated densitometer and band density calculated using Biorad Quantity One v4.5.2 1D analysis software.

Steady-state tryptophan fluorescence spectroscopy

RyRF (1.5 μmol·L^{-1}) was incubated at room temperature with increasing concentrations of suramin (0–40 μmol·L^{-1}) in buffer containing 100 mmol·L^{-1} KCL, 10 mmol·L^{-1} Tris 0.1% w/v β-octylglucopyranoside and 1 mmol·L^{-1} $CaCl_2$. The excitation wavelength was set at 295 nm and the emission spectra acquired between 310–450 nm. Spectra were collected on a JASCO-750 Spectrofluorimeter. Quartz cuvettes (path length 10 mm; 150 μL) were used. The contribution of suramin to the fluorescence spectra collected under these conditions was not significant. To determine the interaction of suramin with Ca^{2+}CaM alone, a dansylated CaM (d-CaM) derivative was employed as described above. Spectra of the d-CaM were recorded between 425 and 600 nm after excitation at 340 nm with increasing [suramin].

Cardiac myocyte isolation and measurement of Ca^{2+} waves and sparks in permeabilized rat myocytes

Rat ventricular myocytes were isolated using a protease and collagenase Langendorff perfusion technique as previously described (Eisner *et al.*, 1989). Cells were incubated with 0.1 mg·mL^{-1} escin for 1 min to permeabilize the surface membrane as previously described (Smith and O'Neill, 2001). Following permeabilization, cells were treated in two different ways: either treated for 10 min in a low-[Ca^{2+}] medium (no added Ca^{2+}, EGTA 10 mmol·L^{-1}) and then returned to a mock cytoplasmic solution containing 10 μmol·L^{-1} Fluo-5F or exposed to 100 μmol·L^{-1} suramin for 10 min (to remove CaM from RyR2) in a low-[Ca^{2+}] medium (no added Ca^{2+}, EGTA 10 mmol·L^{-1}) before returning to a mock cytoplasmic solution containing 10 μmol·L^{-1} Fluo-5F without suramin for experimentation. After suramin incubation, we then add back a physiological concentration of CaM (50 nmol·L^{-1}) to the mock cytoplasmic solution.

The free [Ca^{2+}] was set to one of two levels in the mock cytoplasmic solution: 100 nmol·L^{-1} (EGTA 500 μmol·L^{-1}) to study sparks and 215 nmol·L^{-1} l (EGTA 100 μmol·L^{-1}) to study waves of Ca^{2+}-induced Ca^{2+}-release (CICR). Both sparks and waves were measured on a Bio-Rad MRC 1024. The wave amplitude and velocity were measured using ImageJ software (http://rsb.info.nih.gov/ij/). These values were divided by the control values to allow us to calculate the relative ratio of velocity/amplitude before and after application of CaM. Sparks were automatically detected using the SparkMaster algorithm 18 (Picht *et al.*, 2007): *n* values refer to the number of cells.

Statistics

Where appropriate, Student's *t*-test, a paired *t*-test or ANOVA followed by a modified *t*-test was used to assess the difference between treatments. A *P*-value of 0.05 was taken as significant.

Materials

CaM and escin were obtained from Sigma-Aldrich (Dorset, UK). Suramin was from Calbiochem (Nottingham, UK). Fluo-5F was obtained from Invitrogen (Paisley, UK). Other chemicals were AnalaR or the best equivalent grade from BDH (Poole, UK) or Sigma-Aldrich (Dorset, UK). All solutions were made in deionized water and those for use in bilayer experiments were filtered through a Millipore membrane filter (0.45 μm pore). Autocamtide-2-related inhibitory peptide (AIP) was from Calbiochem (Nottingham, UK).

Results

Ca^{2+}CaM and RyR2 channel gating

All single-channel experiments were performed in the absence of Mg^{2+} and ATP. This prevents the activation of kinases, such as CaM kinase II (CaMKII) that could incorporate into the bilayer along with RyR2. Additionally, we maintained the free cytosolic [Ca^{2+}] at 100 μmol·L^{-1} for two reasons: (i) to ensure that CaM would be predominantly fully Ca^{2+}-bound (Burger *et al.*, 1984); and (ii) because during EC-coupling, the free [Ca^{2+}] in the cleft region in the immediate vicinity of the RyR2 channels has been calculated to reach high levels close to 100 μmol·L^{-1} (Langer and Peskoff, 1996). Unexpectedly, we found that Ca^{2+}CaM caused inhibition in some experiments, but activation in others. Typically, activation was more likely to occur with low concentrations of Ca^{2+}CaM (50 and 100 nmol·L^{-1}). Figure 1 shows an experiment, where sequential concentrations of Ca^{2+}CaM were added to the cytosolic channel side. It can be seen that the

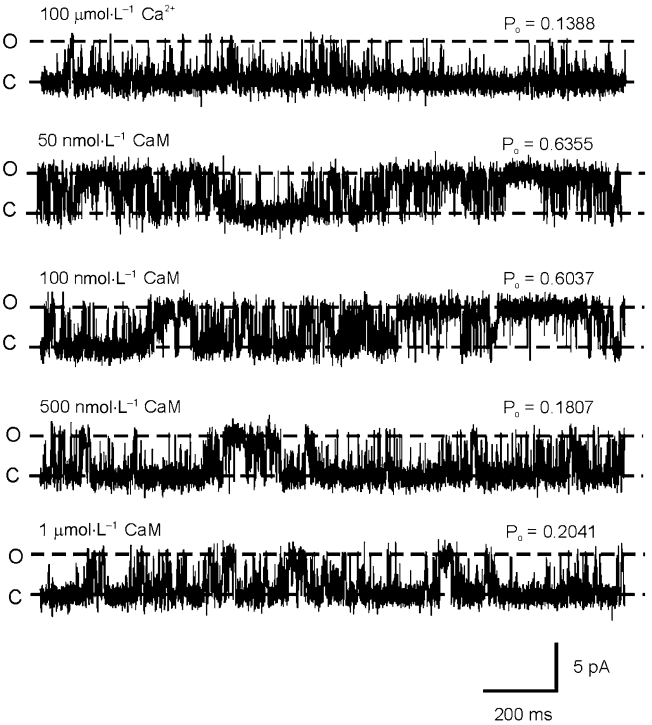

Figure 1 The effects of Ca^{2+}-calmodulin (Ca^{2+}CaM) on RyR2 channel gating. Current fluctuations through a single RyR2 channel in the presence of 100 mol·L^{-1} Ca^{2+} (top trace) and after sequential additions of 50, 100, 500 and 1000 nmol·L^{-1} Ca^{2+}CaM are shown. The holding potential is 0 mV. The dotted lines mark the open (O) and closed (C) channel levels.

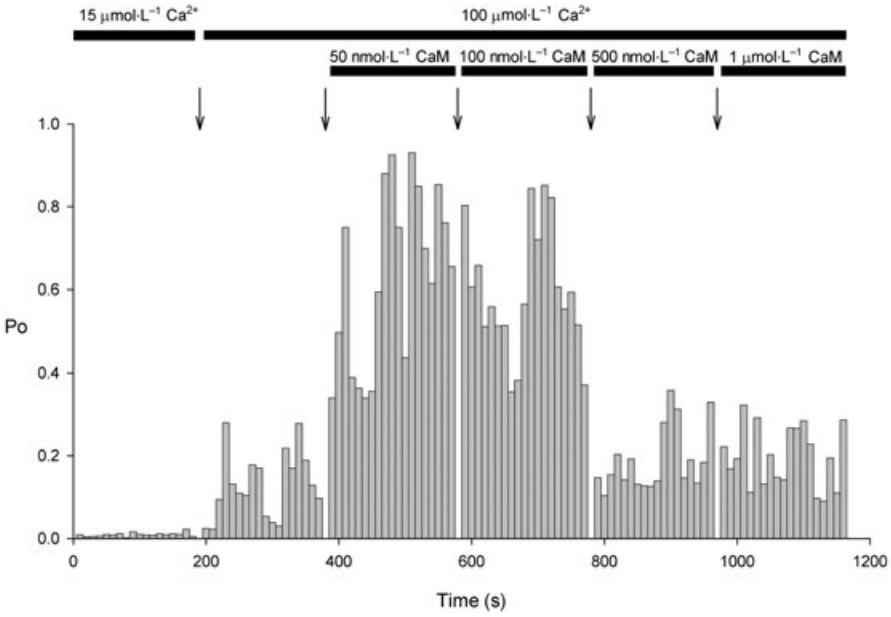

Figure 2 Time dependence of the effects of Ca^{2+}-calmodulin (Ca^{2+}CaM). The plot of P_o against time is illustrated for the channel shown in Figure 1 and shows typical examples of the shifts in P_o (modal gating) that occur with time. The cytosolic [Ca^{2+}] was first raised from 15 to 100 μmol·L^{-1} and this was followed by increasing concentrations of Ca^{2+}CaM (shown by arrows) while maintaining free [Ca^{2+}] at 100 μmol·L^{-1}. Segment duration is 10 s. P_o was recorded for 3 min continuously for each intervention. The arrows mark the 3 min periods period where changes to the solutions on the cytosolic channel side were made followed by 10 s stirring of the chamber. Abrupt changes in gating can be observed following the raising of cytosolic [Ca^{2+}] from 15 to 100 μmol·L^{-1} and following the additions of 50 and 500 nmol·L^{-1} CaM.

low concentrations of Ca^{2+}CaM (50 and 100 nmol·L^{-1}) caused channel activation whereas the high concentrations (≥500 nmol·L^{-1}) reversed the activation caused by the lower doses. To investigate the time dependence of Ca^{2+}CaM-induced changes in P_o, the steady-state single-channel recordings were divided into segments of 10 s in duration as shown in Figure 2. The figure illustrates that large fluctuations in P_o occur. This is normal behaviour for a Ca^{2+}-activated RyR2 channel, which displays spontaneous shifts between high- and low-P_o gating modes and is termed 'modal gating' (Saftenku *et al.*, 2001). The time dependence of all experiments was investigated in this manner, but there was no evidence for any particular time dependence or run-down of activity that could explain the CaM-induced changes in P_o. Ca^{2+}CaM produced changes in P_o that were apparent immediately following the period where Ca^{2+}CaM was added to the cytosolic chamber and stirred for 10 s. The mean steady-state P_o changes for all channels are shown in Figure 3A. It can be seen that, on average, steady-state P_o increased with 50 and 100 nmol·L^{-1} CaM, but decreased with [CaM] ≥500 nmol·L^{-1}. The average P_o, however, in the presence of 500 and 1 μmol·L^{-1} CaM was similar to control values (at 100 μmol·L^{-1} cytosolic Ca^{2+}). Inspection of individual experiments demonstrates that the mean P_o changes shown in Figure 3A mask the fact that some channels appear to be activated by CaM whereas others appear to be inhibited, although in many experiments no large change in P_o was apparent. Inhibition was rare at low doses of CaM, but occurred frequently in experiments with the high concentration of 1 μmol·L^{-1} CaM. This is illustrated in Figure 3B, where the percentage of channels where P_o was increased above control levels by CaM at the various concentrations is shown. At 50 nmol·L^{-1} CaM, the P_o of 90% (9/10 experiments) of channels was increased by 50 nmol·L^{-1} CaM and the P_o of only 10% (1/10 experiments) of channels was reduced. It can be seen that the percentage of channels where P_o is increased by CaM decreases in a dose-dependent manner with only 53% (8/15 channels) of channels exhibiting an increase in P_o above that of control levels at 1 μmol·L^{-1} CaM. In Figure 2 we have eliminated the possibility that Ca^{2+}CaM inhibition is a time-dependent run-down effect, but there are two other likely explanations for the dual effects of CaM. First, it is possible that there is more than one population of RyR2 channels in our SR membrane preparation (one population that is activated by CaM and one population that is inhibited by CaM). Alternatively, more than one CaM molecule may bind to each RyR2 channel and the sequential binding gives rise to activation followed by inhibition.

RyR channels are widely regarded to gate in a heterogeneous manner and there are numerous reports that this is dependent on the presence of different populations of channels. For example, RyR channels have been grouped into separate populations based on P_o: 'high' P_o channels and 'low' P_o channels (Copello *et al.*, 1997). As has been previously suggested, variations in RyR P_o could be due to differences in the stoichiometry of binding of the many proteins that are closely associated with individual RyR2 channels or to changes in the levels of channel phosphorylation (Copello *et al.*, 1997; Marx *et al.*, 2000; Marks *et al.*, 2002). We therefore examined the idea that CaM can activate or inhibit RyR2 because two populations of RyR2 channels exist. Figure 3 shows that the overall P_o values after addition of 1 μmol·L^{-1} CaM are not different from control values in the presence of 100 μmol·L^{-1} cytosolic Ca^{2+} alone; however, 1 μmol·L^{-1} CaM

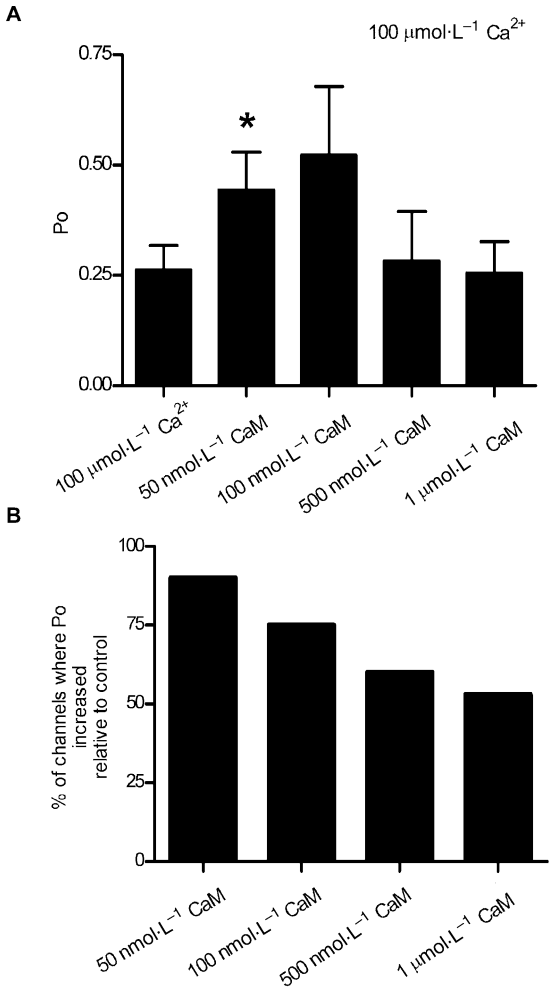

Figure 3 Ca^{2+}-calmodulin (Ca^{2+}CaM) activates RyR2 at low concentrations, but reverses the inhibition at higher concentrations. A. Histogram showing the mean changes in P_o with various Ca^{2+}CaM concentrations. Mean values ± SEM are shown for $n = 5–20$. B. Histogram showing the percentage of channels where CaM increases P_o relative to control levels ($n = 5–20$). * indicates $P < 0.05$.

appeared to activate some channels and appeared to inhibit others. The data were therefore split into two groups: (i) those channels where the average P_o in the 3 min recording period following addition of 1 μmol·L^{-1} CaM was higher than in the control period; and (ii) those channels where the average P_o in the 3 min recording period following addition of 1 μmol·L^{-1} CaM was lower than in the control period. Figure 4A illustrates examples of channels that appeared to be activated or inhibited by 1 μmol·L^{-1} Ca^{2+}CaM and Figure 4B shows histograms of the data when grouped in this manner.

Activation of RyR2 by cytosolic Ca^{2+} gives very variable P_o values at high cytosolic [Ca^{2+}] (Sitsapesan and Williams, 1994; Copello *et al.*, 1997; Saftenku *et al.*, 2001), so could the effect of CaM be dependent on the starting P_o of the channel? We found that the average control P_o levels were not significantly different (Figure 4B) between the two groups and inspection of individual traces also suggested that the control P_o was not an essential determinant of whether 1 μmol·L^{-1} CaM increased or decreased P_o. Figure 5 shows four examples of the P_o changes with time after the addition of 1 μmol·L^{-1} CaM. The figure illustrates two points: (i) addition of 1 μmol·L^{-1} CaM to RyR2 in the presence of 100 μmol·L^{-1} cytosolic Ca^{2+} does not produce an obvious change in gating that can be distinguished from the normal fluctuating gating of the channel (Saftenku *et al.*, 2001); and (ii) the apparent slight increases or decreases in P_o that follow 1 μmol·L^{-1} CaM addition happen irrespective of whether starting P_o is relatively high or low. These experiments demonstrate that although we cannot dismiss the idea of two populations of RyR2 channel, the data provide no evidence to support this hypothesis.

In some of our experiments, only one channel incorporated into the bilayer. Lifetime analysis could then be performed to examine the mechanisms underlying changes in P_o. Figure 6 shows two typical experiments; one where P_o is increased by 50 nmol·L^{-1} CaM and one where the high P_o is reversed by 1 μmol·L^{-1} CaM. With Ca^{2+} as the permeant ion and with cytosolic Ca^{2+} as the only channel activator, the open and closed lifetime distributions are best described by two open states and three closed states (top histograms, Figure 6A) (Hill *et al.*, 2004). After addition of 50 nmol·L^{-1} CaM, it can be seen (lower histograms, Figure 6B) that the main effect is an increase in the duration of the open times. Fewer openings occur to the shortest open state and a third, long open state is detected. A slight reduction in closed event durations was also observed, although there was no change in the percentage of events occurring to each state. Figure 6B demonstrates typical changes in lifetime distributions that were observed when CaM reduces P_o. In the top histograms, P_o has already been increased by 50 nmol·L^{-1} CaM and three open and three closed states are observed. Raising [CaM] to 1 μmol·L^{-1} (lower histograms) has a major effect on the closed lifetime durations, causing a marked increase in the durations of all three closed states. In addition, a reduction in the duration of open lifetimes is also obvious with a shift in the distribution towards shorter openings.

Complex formation between Ca^{2+}CaM and a fragment of RyR2 a.a. 3581–3612 (RyRF) that can be dissociated by the addition of suramin

Suramin shows selectivity in removing CaM from RyR rather than other proteins (Klinger *et al.*, 2001). We have also shown: (i) that suramin has a 10-fold higher affinity for the cardiac over the skeletal isoform of RyR (Sitsapesan and Williams, 1996); (ii) that the effects of suramin are completely reversible (Hill *et al.*, 2004); and (iii) that suramin displaces CaM from RyR2 in SR vesicles (Hill *et al.*, 2004). To explore whether suramin actually binds to the CaM binding site on RyR2, we used a peptide of the cardiac CaM binding domain (Yamaguchi *et al.*, 2003) and examined if suramin could dissociate CaM from this peptide. An earlier report by Hamilton and co-workers (Papineni *et al.*, 2002) using this methodology showed that suramin inhibited the binding of CaM to the RyR1 CaM binding domain peptide. We used the RyR2 CaM binding domain (Yamaguchi *et al.*, 2003) human sequence 3581–3612: RSKKAVWHKLLSKQRKRAVVACFRMAPLYNLP. This peptide differs from the RyR1 CaM binding domain by three amino acids (RyR2 3581, 3596 and 3606).

Purified CaM (0.5 mmol·L^{-1}) was incubated with the RyR2 peptide (RyRF) at stoichiometries of Ca^{2+}CaM : RyRF 1:0.25,

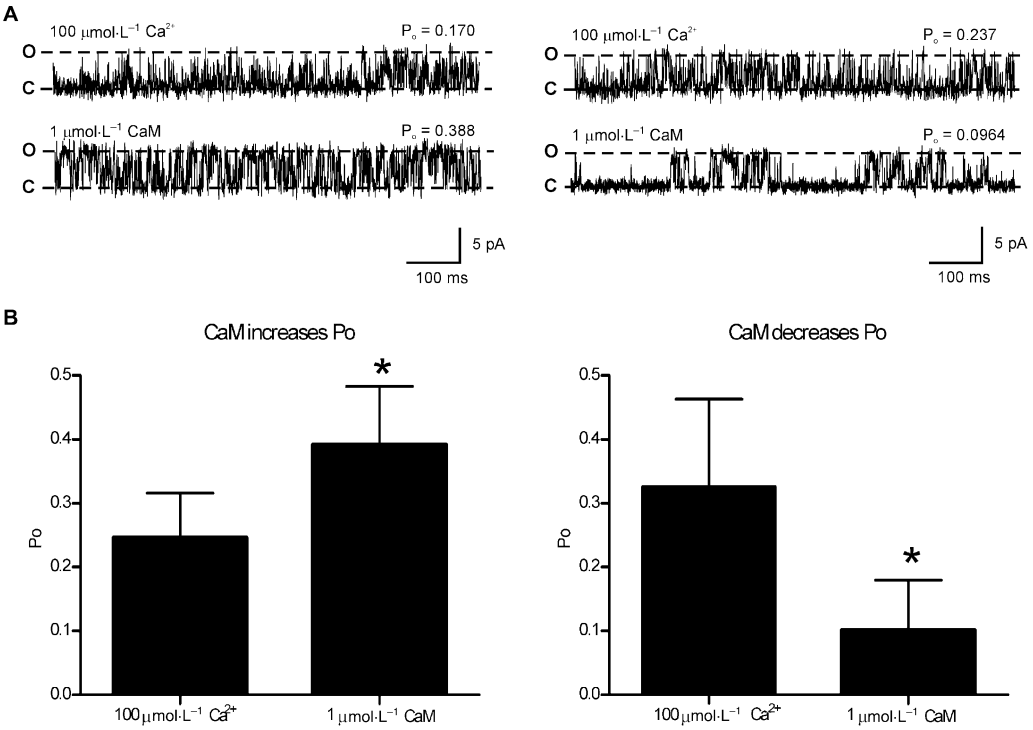

Figure 4 Some channels appear to be slightly activated by 1 μmol·L^{-1} Ca^{2+}-calmodulin (Ca^{2+}CaM) whereas others appear to be slightly inhibited. The experiments are split into two groups: those where P_o is higher after addition of 1 μmol·L^{-1} CaM and those where P_o is lower. Typical examples of this are shown in (A) whereas (B) illustrates the mean data for both groups of channel (P_o is higher than controls in eight experiments and lower than controls in seven experiments).

1:0.5, 1:1 and 1:3 and were analysed by gel electrophoresis. We then added increasing concentrations of suramin to the pre-formed complex (Ca^{2+}CaM : RyRF at a 1:3 molar ratio) before analysis by gel electrophoresis. To examine the effect of suramin on the conformation of the RyRF, steady-state tryptophan fluorescence spectroscopy was employed. To determine the interaction of suramin with Ca^{2+}CaM alone, tryptophan fluorescence spectroscopy of a d-CaM derivative was used.

Figure 7A demonstrates that addition of the RyRF peptide to Ca^{2+}CaM leads to the formation of a higher-molecular-weight band corresponding to a complex formed by Ca^{2+}CaM and RyRF. Thus, the peptide binds to Ca^{2+}CaM forming a higher-molecular-weight complex that can be distinguished from the individual components by native gel-shift analysis and thus is a suitable method for examining this type of interaction. Figure 7B shows that suramin causes a dose-dependent dissociation of the complex. With a ratio of suramin : RyR2/CaM complex of 60:1, dissociation of the complex is essentially complete (in subsequent, permeabilized cell experiments the ratio used was ~500:1 to reduce the time required). Figure 7C demonstrates that suramin addition to RyRF causes quenching and alteration to the tryptophan emission profile. For comparative purposes the two curves have been normalized to illustrate that in the presence of suramin, a shoulder (λ_{max} = 330 nm) forms with the appearance of an underlying peak at the tail of the spectrum (λ_{max} = 412 nm). In contrast, addition of suramin to d-CaM (data not shown) did not elicit an effect upon the fluorescence profile regardless of the suramin concentration. These data show that RyRF is the binding partner for suramin and that suramin does not bind to CaM. Hence, suramin appears to bind to RyR2 at the suggested CaM binding domain.

The effects of Ca^{2+}CaM on SR Ca^{2+}-release in permeabilized cardiac myocytes

As physiological free [CaM] is close to 50 nmol·L^{-1} in cardiac cells (Wu and Bers, 2006) and as this CaM concentration tends to activate RyR2 incorporated into bilayers, we attempted to examine the effects of 50 nmol·L^{-1} CaM on RyR2 channel function *in situ*. We used permeabilized rat cardiac myocytes bathed in a solution mimicking cytoplasmic conditions with [Ca^{2+}] set to 215 nmol·L^{-1} to induce spontaneous waves of CICR. As CaM binds so tightly to RyR2 that it can be immunoprecipitated (Ai *et al.*, 2005), many of the CaM binding sites on RyR2 in the permeabilized cells will already be occupied by CaM. Simply adding CaM to the perfusate would not, therefore, be expected to show the normal, physiological consequences of CaM binding to RyR2. It is first necessary to remove the CaM already bound to RyR2 before adding it back to the cytosolic solution. As suramin displaces CaM from RyR2 (Hill *et al.*, 2004) and we have shown above that the likely mechanism is competition with CaM for its binding site on RyR2, we therefore pre-treated the permeabilized cells with 100 μmol·L^{-1} suramin for 10 min [in a low-[Ca^{2+}] medium (no added Ca^{2+}, EGTA 10 mmol·L^{-1}] to remove CaM bound to RyR2. The suramin and CaM were then washed from the cells before adding back CaM to the perfusate. Suramin increases the P_o and conductance of isolated

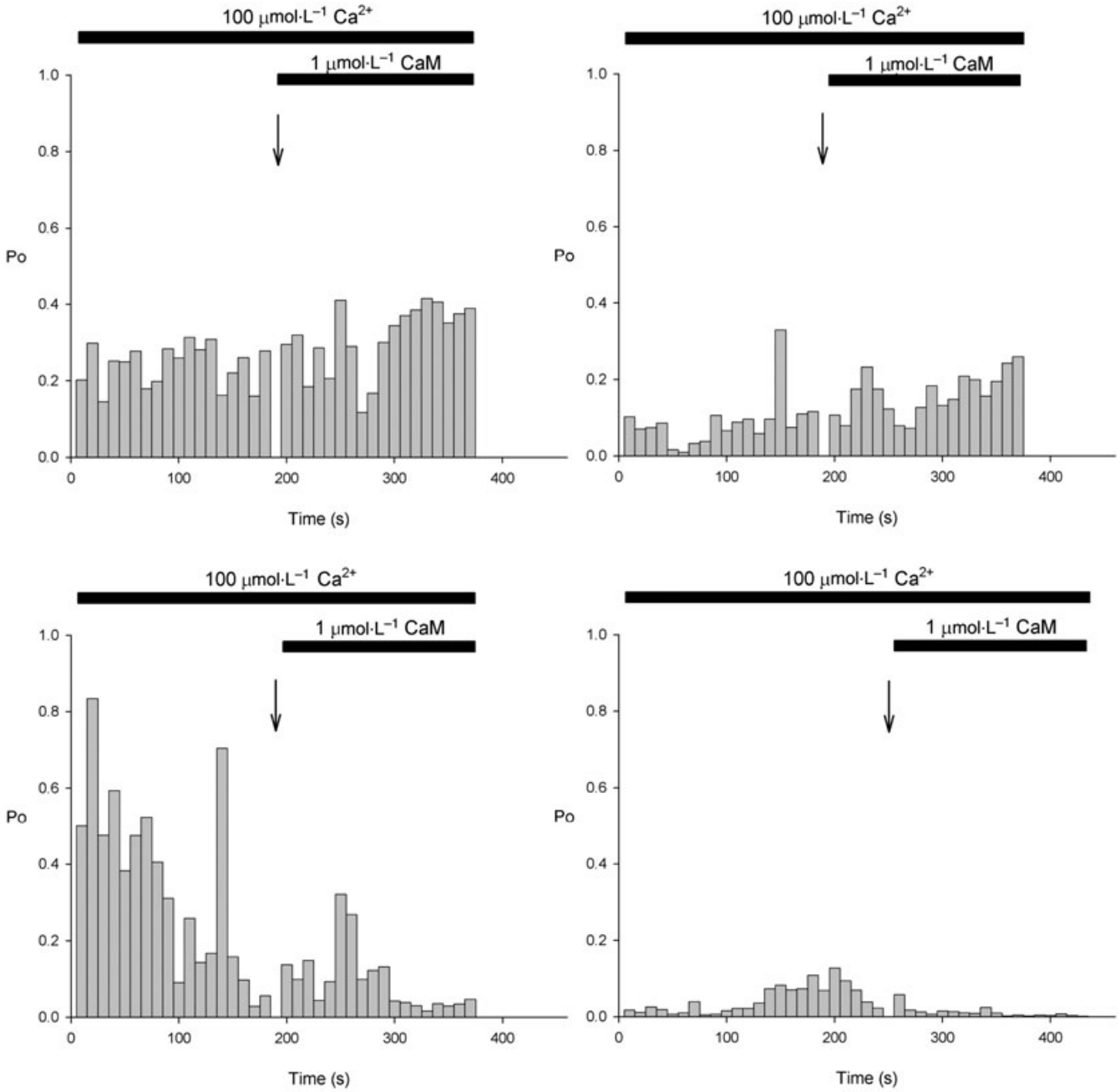

Figure 5 Time dependence of the effects of 1 μmol·L^{-1} calmodulin (CaM) illustrating that this concentration of CaM increases or decreases P_o irrespective of whether P_o is relatively high or low. The upper two plots of P_o against time are typical examples of channels where addition of 1 μmol·L^{-1} CaM produces an overall increase in P_o over the 3 min of recording. CaM increases P_o when P_o is relatively high (upper left) and when P_o is relatively low (upper right). The lower two plots of P_o against time are typical examples of channels where addition of 1 μmol·L^{-1} CaM produces an overall decrease in P_o over the 3 min of recording. CaM decreases P_o when P_o is relatively high (lower left) and when P_o is relatively low (lower right).

RyR2 and these effects are completely reversible (Sitsapesan and Williams, 1996; Hill *et al.*, 2004). Hence, in the permeabilized cells, once suramin is washed out there will be no long-lasting effects on the RyR2 complex (other than loss of CaM). Following washout of CaM and suramin from the cells, the [Ca^{2+}] was raised to 215 nmol·L^{-1} and waves were measured in each cell for a minimum of 3 min to ensure stability before addition of exogenous CaM. The frequency of waves in this period was taken as the control value and compared with the frequency after CaM addition. This protocol was used in both suramin pre-treated and untreated cells. Addition of 50 nmol·L^{-1} CaM to suramin pre-treated cells caused a near 50% increase in wave frequency ($P < 0.001$; $n = 14$) that took place over several minutes. A representative experiment is shown in Figure 8. The two linescan montages in Figure 8A are from the same cell and show wave activity: before (top) and 10 min after (bottom) application of 50 nmol·L^{-1} CaM. CaM causes a clear increase in wave frequency, in this case of

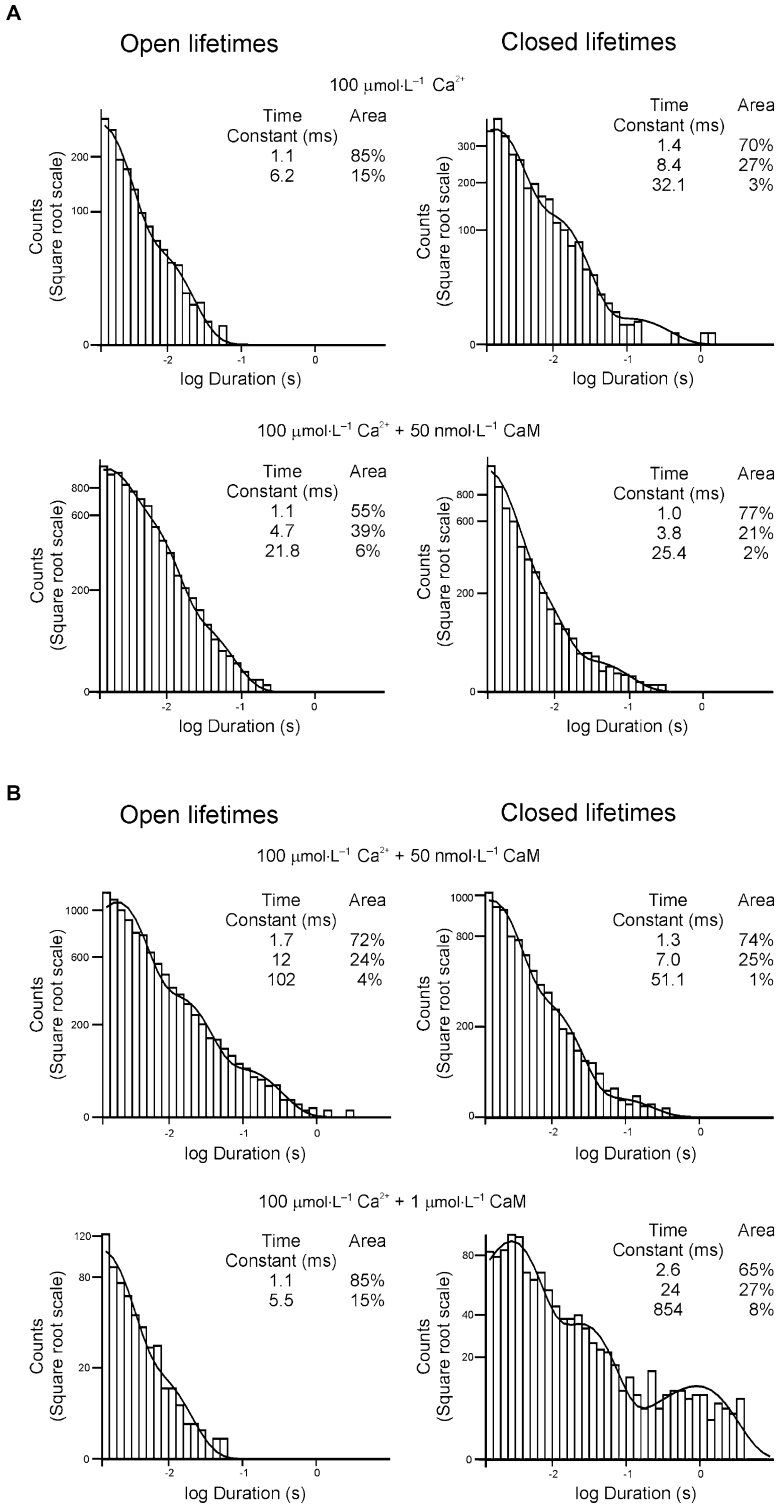

Figure 6 The effects of calmodulin (CaM) on open and closed lifetime distributions. In (A) the lifetime distributions and probability density functions from a typical single channel activated by 100 mol·L^{-1} Ca^{2+} alone (upper panel) and 50 nmol·L^{-1} CaM (lower panel) are shown. In (B) another single channel is shown where P_o has been raised by 100 mol·L^{-1} Ca^{2+} and 50 nmol·L^{-1} CaM (upper panel) and then CaM levels are further increased to 1 mol·L^{-1} CaM (lower panel). Best fits to the data were obtained by maximum likelihood fitting. Resulting time constants and percentage areas are shown. Similar results were obtained in seven further channels.

nearly 40%. In addition, the traces in Figure 8B show that waves in CaM are of lower amplitude. The effects of CaM application on wave characteristics in suramin pre-treated cells are summarized in Table 1.

As would be expected from the above results, suramin pre-treatment itself leads to a 45% reduction in wave frequency from 17.0 ± 0.5 min^{-1} ($n = 8$) in control cells to 9.3 ± 0.9 min^{-1} ($n = 22$; $P < 0.001$).

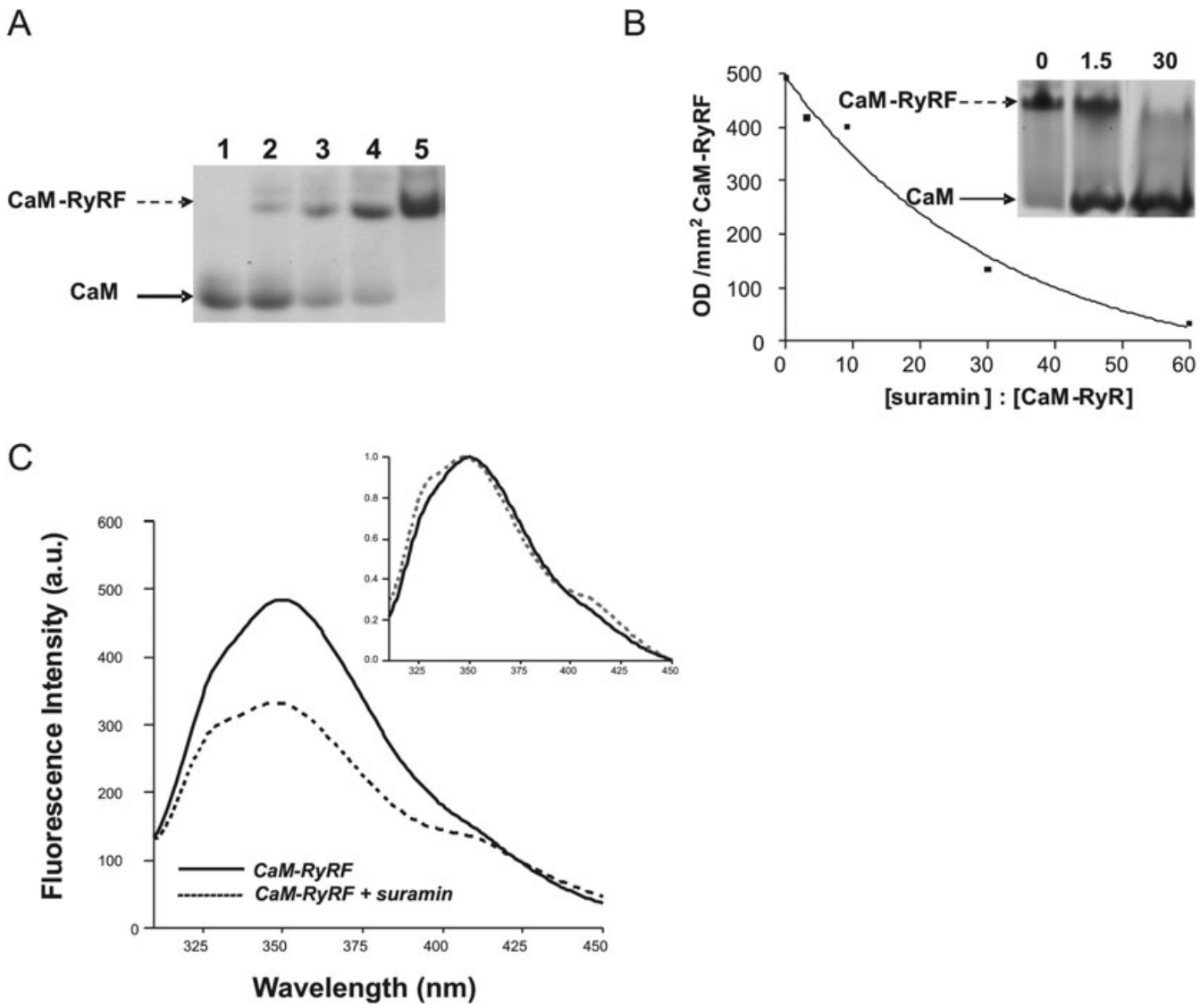

Figure 7 Complex formation between Ca^{2+}-calmodulin (Ca^{2+}CaM) and RyR2 fragment a.a. 3581–3612 (RyRF), which can be dissociated by the addition of suramin. A. Coomassie-stained gel demonstrating complex formation between Ca^{2+}CaM and RyRF. Ca^{2+}CaM was maintained at a concentration of 0.5 mmol·L^{-1}. Lanes 1–5 show complex formation with the addition of the RyR2 peptide at concentrations of 0.25, 0.5 1.0 and 1.5 mmol·L^{-1} respectively. B. Densitometric analysis of a Coomassie-stained gel of a preformed complex of Ca^{2+}CaM–RyRF (1:3) with increasing additions of suramin. An excess of suramin (30 mmol·L^{-1}) leads to complete complex dissociation. *Inset* shows the Coomassie gel at the indicated proportions of suramin. (C) Steady-state tryptophan fluorescence spectrum of the RyRF peptide. The addition of an equimolar amount of suramin causes fluorescence quenching. *Inset:* Normalization of the two spectra illustrates a change to the emission spectrum profile of the RyRF–suramin mix compared with the peptide alone.

Unlike the single-channel experiments, it is possible that the effects of CaM on permeabilized cells could be due to activation of CaMKII. In suramin pre-treated cells we therefore added an inhibitor of CaMKII, AIP to the perfusate. We used 1 µmol·L^{-1} AIP, a high concentration (IC_{50} for inhibition of CaMKII ≈ 40 nmol·L^{-1}) and one that has been shown to be more than sufficient to inhibit CaMKII in permeabilized cardiac cells (Currie *et al.*, 2004; Guo *et al.*, 2006; MacQuaide *et al.*, 2007). Perfusion with AIP (1 µmol·L^{-1}) itself caused a slight, non-significant decrease in wave frequency, which may indicate a very low level of CaMKII activity under our experimental conditions (215 nmol·L^{-1} Ca^{2+}). A similar conclusion was made by Guo *et al.* (2006) and MacQuaide *et al.* (2007). The effects of CaM on wave frequency and amplitude were unaffected by the CaMKII inhibitor, AIP (1 µmol·L^{-1}), ruling out a role for activation of CaMKII. CaM increased frequency by 47 ± 9% (n = 16) of control in the absence of AIP and 43 ± 8% (n = 7) in the presence of AIP and decreased wave amplitude to 58.8 ± 8.3% (n = 6) and 74.6 ± 12.8% (n = 7) of control in the absence and presence of AIP respectively.

The smaller waves in the presence of 50 nmol·L^{-1} CaM suggest that SR Ca^{2+} content is lower. We tested this by application of 10 mmol·L^{-1} caffeine to release the SR Ca^{2+} before and after adding CaM. The traces in Figure 9 show mean linescan values of F/F_o measured by confocal microscopy and are typical of the effects of CaM. On the left, in control, the amplitude of the Ca^{2+} transient resulting from application of 10 mmol·L^{-1} caffeine is shown. This is significantly

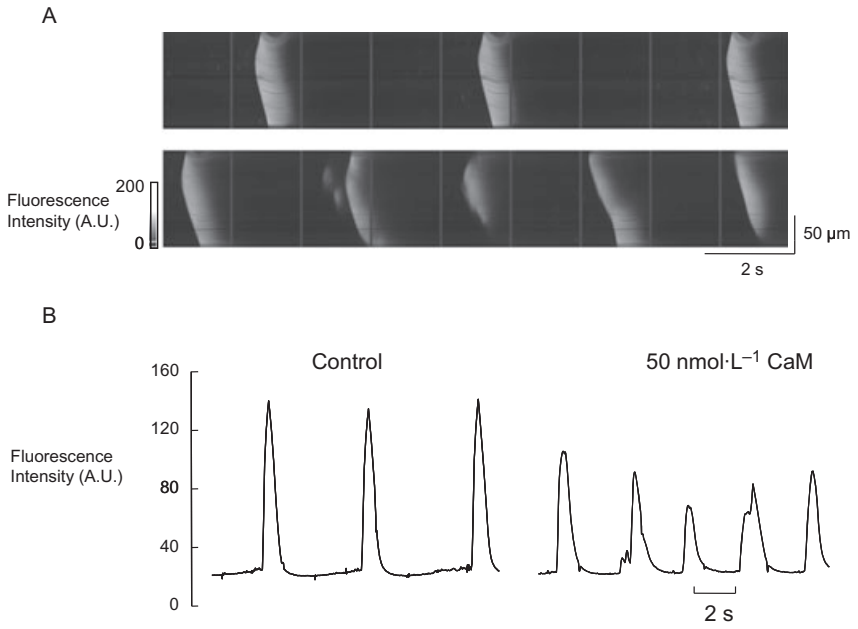

Figure 8 50 nmol·L^{-1} calmodulin (CaM) causes an increase in Ca^{2+}-wave frequency. A. Linescan images collected under control conditions (upper) in cells pre-treated with suramin, and in the presence of 50 nmol·L^{-1} CaM (lower). B. Mean fluorescence records from the linescans in (A).

Table 1 Wave characteristics before and after calmodulin (CaM) application in suramin pre-treated cells

	Suramin pre-treated	*+ 50 nmol·L^{-1} CaM*
Wave frequency (min^{-1}) ($P < 0.001$; $n = 14$)	8.7 ± 1.3	12.7 ± 2.0
Wave amplitude (F/F_o) ($P < 0.0003$; $n = 10$)	5.3 ± 0.3	3.6 ± 0.3
Wave velocity (µm·s^{-1}) ($P < 0.04$; $n = 10$)	188.0 ± 11.6	166.3 ± 6.4
Relative velocity/amplitude ($P < 0.015$; $n = 10$)	1	1.39 ± 0.15

larger than the same transient in the presence of CaM. On average, the caffeine response (in units of F/F_o) was 6.0 ± 0.6 in control and 4.0 ± 0.7 ($P < 0.001$, $n = 5$) in the presence of CaM. Thus, 50 nmol·L^{-1} CaM causes a reduction of SR Ca^{2+} content while increasing the frequency of spontaneous waves of CICR.

The above results show that CaM causes a marked increase in the frequency of Ca^{2+} waves when the free $[Ca^{2+}]_i$ is set to 215 nmol·L^{-1}. We subsequently examined if CaM also affected spark characteristics when the free $[Ca^{2+}]$ is set to physiological levels of 100 nmol·L^{-1}. The effects of CaM on Ca^{2+} sparks in cells pre-treated with suramin are shown in Figure 10. With the free $[Ca^{2+}]$ set at 100 nmol·L^{-1}, sparks were present at a frequency of 7.2 ± 0.5 events per 100 µm s^{-1}. This value was significantly increased by 50 nmol·L^{-1} CaM to 9.4 ± 1.1 events per 100 µm·s^{-1} ($n = 3$; $P < 0.05$).

Discussion and conclusions

Our study demonstrates for the first time that Ca^{2+}CaM can activate and inactivate RyR2 channels in bilayers, with activation most likely at low physiological concentrations (50–100 nmol·L^{-1}). Neither activation nor inhibition of the channels could have arisen from activation of CaMKII, as our experimental conditions did not allow kinase activation. In line with these results, we demonstrated that low levels of CaM (50 nmol·L^{-1}), close to the measured free level in cardiac cells (Wu and Bers, 2006), increased the frequency of Ca^{2+}-sparks and Ca^{2+}-waves. The increased frequency of waves was accompanied by a fall of SR Ca^{2+}-content, suggesting that activation of RyR2 is involved.

Our single-channel studies demonstrate that 90% of channels are activated by a physiological concentration of CaM (50 nmol·L^{-1}), but that increasing [CaM] reverses the activation. How can we explain this dual action of CaM? Although our data provide no evidence for two populations of RyR2 channels, we cannot completely discard the idea of a heterogeneous population of RyR2 channels that respond differently to CaM, as 10% of channels were inhibited even by 50 nmol·L^{-1} CaM. In a previous publication, we also found that low [Ca^{2+}CaM] inhibited RyR2 (Hill *et al.*, 2004). Perhaps some RyR2 channels are more sensitive to Ca^{2+}CaM.

Another possible explanation is that more than one population of CaM molecule exists. At 100 µmol·L^{-1} free $[Ca^{2+}]$, each CaM molecule would be expected to be predominantly bound by four Ca^{2+} molecules, although a small percentage would be bound to three or two Ca^{2+} molecules (Burger *et al.*, 1984); perhaps these CaM molecules induce a different gating response to the CaM molecules bound to four Ca^{2+} molecules.

As almost all channels (90%) are activated by low [Ca^{2+}CaM] (50 nmol·L^{-1}) and exhibit a dual response to CaM (an increase in P_o at low doses with reversal of that effect at high doses), we favour the idea that sequential binding of multiple CaM molecules to one RyR2 tetramer causes the dual functional effects. Possibly, RyR2 possesses two distinct CaM

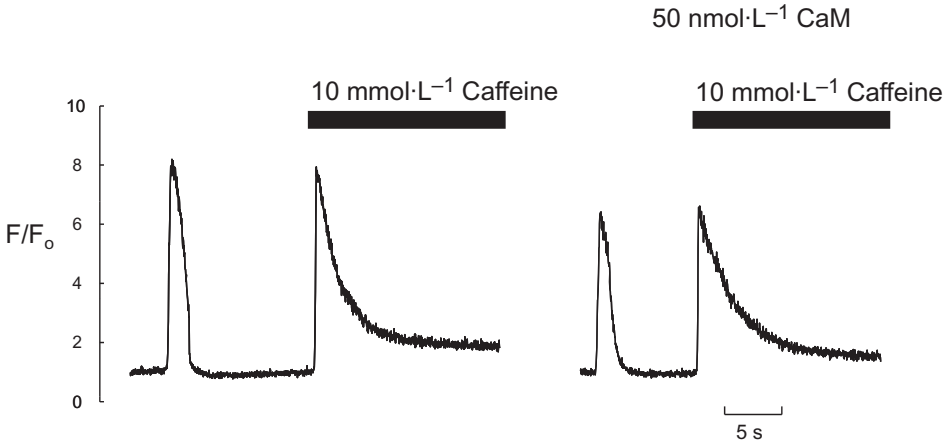

Figure 9 In the presence of calmodulin (CaM), the sarcoplasmic reticulum Ca^{2+} content is reduced. On the left is a typical spontaneous wave of calcium-induced calcium release before application of 10 mmol·L^{-1} caffeine. On the right after application of 50 nmol·L^{-1} CaM, both spontaneous wave amplitude and responses to caffeine are reduced.

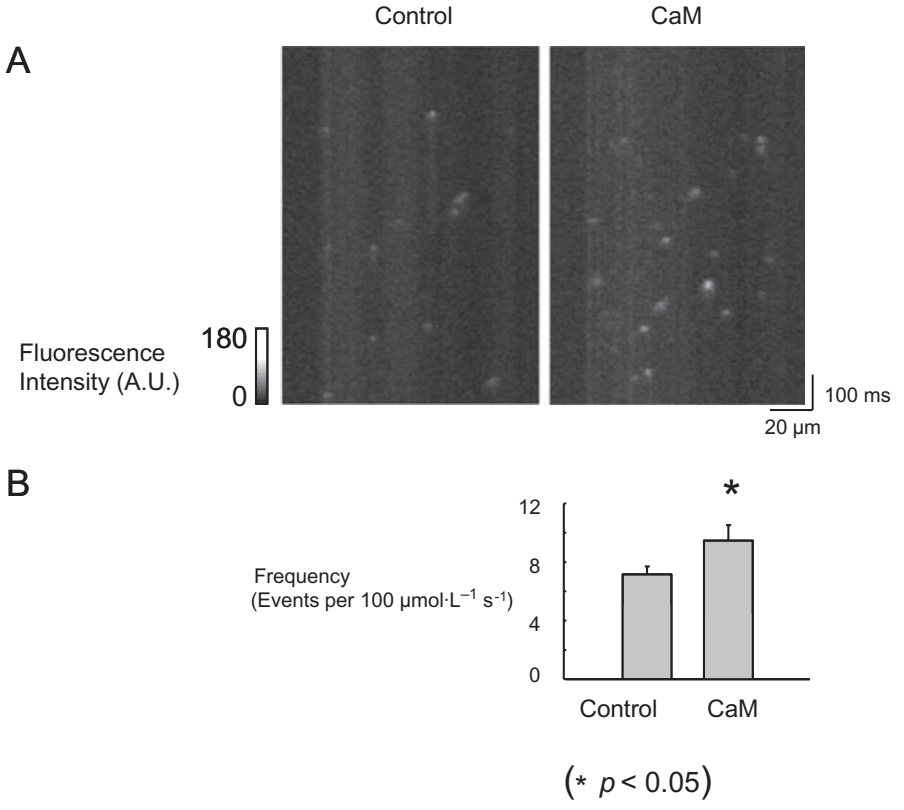

Figure 10 50 nmol·L^{-1} calmodulin (CaM) increases Ca^{2+}-spark frequency. (A) shows typical linescan images in one cell before and after the application of 50 nmol·L^{-1} CaM. Below in (B) is the mean change in frequency ($n = 3$). Sparks were detected automatically by the SparkMaster algorithm of ImageJ image processing software.

binding sites: high-affinity activation and lower-affinity inactivation sites. However, only one binding site for CaM on RyR2 has been agreed and this includes the residues 3581–3612 (Yamaguchi *et al*., 2003; 2004). Since RyR2 is a tetramer, it must possess four identical CaM sites per channel. An alternative hypothesis for the dual actions of CaM, therefore, is that, the binding of the initial molecule/s of Ca^{2+}CaM is a high-affinity interaction/s that leads to an increase in P_o. The subsequent conformational changes that follow CaM/RyR2 binding reduce the affinity of the remaining CaM binding sites for CaM. Higher concentrations of CaM are required for subsequent binding and binding to these sites causes channel inhibition.

Lifetime analysis (Figure 6) also strongly suggested that CaM modulated RyR2 gating by two distinct mechanisms. The predominant mechanism by which CaM increased P_o was

by producing a shift in open lifetimes towards longer openings. An additional third long open state is *always* detected when CaM increases P_o. This is important because cytosolic Ca^{2+}-activation of RyR2 does not change the open lifetime distribution; Ca^{2+} increases P_o by increasing the frequency of channel openings (Sitsapesan and Williams, 1994). We have previously suggested that CaM modulates RyR2 gating by altering the sensitivity of RyR2 to cytosolic Ca^{2+} (Hill *et al.*, 2004), but the generation of a third long open state demonstrates that CaM is doing more than merely sensitizing the channel to cytosolic Ca^{2+}.

Our results run counter to established ideas of Ca^{2+}CaM as an inhibitor of RyR2; however, previous reports have not looked in detail at the effects of physiological levels of CaM. At the single-channel level, the study by Yamaguchi *et al.* (2004) on recombinant RyR2 did investigate the effects of 50 nmol·L^{-1} CaM, however, the free [Ca^{2+}] was only 2 μmol·L^{-1}. These experiments cannot, therefore, be directly compared with our experiments performed at 100 μmol·L^{-1} Ca^{2+} where we will have a greater proportion of fully saturated CaM molecules (four Ca^{2+} molecules per CaM molecule) (Burger *et al.*, 1984) and, importantly, each RyR2 molecule will also have more Ca^{2+} molecules bound.

Many studies have used [^{3}H]ryanodine binding assays to indicate how CaM would affect RyR2 P_o and it is predominantly in these studies that low doses of CaM appear to inhibit RyR2 (Fruen *et al.*, 2000; Balshaw *et al.*, 2001). However, changes in [^{3}H]ryanodine binding do not equate exactly to P_o changes as the conditions of a bilayer experiment and a [^{3}H]ryanodine binding experiment are very different, thus preventing direct comparisons (differences include the luminal [Ca], the presence of associated proteins and the duration of the experiment). Although most published [^{3}H]ryanodine binding work describes CaM only as an inhibitor of RyR2, there are reports from two different laboratories that describe CaM as stimulating [^{3}H]ryanodine binding to cardiac SR (Fruen *et al.*, 2000; Chugun *et al.*, 2007), thus suggesting that perhaps the conditions of the [^{3}H]ryanodine binding experiments are important in determining whether Ca^{2+}CaM stimulates or inhibits ryanodine binding.

The effects of CaM on RyR2 channel gating and on permeabilized cells are consistent. 50 nmol·L^{-1} CaM increases the frequency of both Ca^{2+}-sparks and spontaneous Ca^{2+}-waves and this is associated with a lower SR Ca^{2+}-content. Other agents that increase the P_o of RyR2, for example, caffeine, are also known to decrease SR Ca^{2+}-content and increase the frequency of spontaneous release events (Trafford *et al.*, 2000). Following a spontaneous wave of CICR, the SR is depleted of Ca^{2+} and this lost Ca^{2+} must be recovered before another wave is possible (Smith and O'Neill, 2001). Before initiation of a wave, there is a gradually increasing frequency of sparks (perhaps as SR Ca^{2+} recovers from the previous wave; see Figure 8); each of these sparks could initiate a wave, but not before SR Ca^{2+}-content has recovered sufficiently. If, however, some factor is present to increase the RyR2 P_o (in this case CaM), then a spark will be able to initiate a wave earlier in this recovery process, that is, at a lower SR Ca^{2+}-content. That this is the case can be seen from the ratio of wave velocity to amplitude. These values are higher in CaM by almost 40%, indicating that the threshold for propagation is reduced (Smith and O'Neill, 2001). It follows that the resulting waves will be smaller (SR Ca^{2+}-content is lower) and will deplete the SR less (in absolute terms). Less depletion, of course, means that the SR will require less time to refill to threshold, so waves will be smaller and more frequent. In contrast, higher wave frequency brought about by, for example, increasing activity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase, would not be associated with smaller waves as RyR sensitivity would not be changed.

Suramin was used in this study to remove CaM from RyR2 in the permeabilized cells in an attempt to observe the effects of physiological levels of CaM. However, when CaM was added to cells that had not been treated with suramin we also found an increase of wave frequency [from 15.2 ± 0.8 to 17.9 ± 0.9 min^{-1} ($n = 8$; $P < 0.003$)]. This effect was smaller (a 15% increase in wave frequency) than that following suramin treatment presumably because significant amounts of CaM were already bound to RyR2.

Previous studies have suggested that CaM only increases RyR2 P_o in cardiac cells if CaMKII is activated (Lukyanenko and Gyorke, 1999; Guo *et al.*, 2006). However, in our experiments, the CaMKII inhibitor AIP did not inhibit the ability of CaM to increase the frequency of Ca^{2+}-waves, suggesting that CaMKII is not involved in this effect. Moreover, if the effects of CaM that we observed were related to stimulation of CaMKII, then we would not expect to see a lowering of the SR Ca^{2+}-content; rather, as CaMKII phosphorylation of phospholamban stimulates sarco/endoplasmic reticulum Ca^{2+}-ATPase, we would expect SR Ca^{2+} to remain unchanged as it will remain limited by (the unchanged) RyR2 sensitivity to cytosolic Ca^{2+} (Eisner *et al.*, 2005).

Our single-channel data, gel-shift assays and permeabilized cell experiments provide a consistent story. By using suramin to strip CaM from RyR2 *in situ*, this study is the first to suggest that CaM could be an activator of RyR2 in the cellular setting. The results not only provide insight into normal cardiac function, but also have implications for disease such as heart failure where changes in the levels of CaM immunoprecipitated with RyR2 have been reported (Ai *et al.*, 2005).

Acknowledgements

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Statement of conflicts of interest

None.

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