Calcium/calmodulin-dependent protein kinase IIδ associates with the ryanodine receptor complex and regulates channel function in rabbit heart

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Cardiac ryanodine receptors (RyR2s) play a critical role in excitation–contraction coupling by providing a pathway for the release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol. RyR2s exist as macromolecular complexes that are regulated via binding of Ca^{2+} and protein phosphorylation/dephosphorylation. The present study examined the association of endogenous CaMKII (calcium/calmodulin-dependent protein kinase II) with the RyR2 complex and whether this enzyme could modulate RyR2 function in isolated rabbit ventricular myocardium. Endogenous phosphorylation of RyR2 was verified using phosphorylation site-specific antibodies. Co-immunoprecipitation studies established that RyR2 was physically associated with CaMKIIδ. Quantitative assessment of RyR2 protein was performed by [^{3}H]ryanodine binding to RyR2 immunoprecipitates. Parallel kinase assays allowed the endogenous CaMKII activity associated with these immunoprecipitates to be expressed relative to the amount of RyR2. The activity of RyR2 in isolated cardiac myocytes was measured in two ways: (i) RyR2-mediated Ca^{2+} release (Ca^{2+} sparks) using confocal microscopy and (ii) Ca^{2+}-sensitive [^{3}H]ryanodine binding. These studies were performed in the presence and absence of AIP (autocamtide-2-related inhibitory peptide), a highly specific inhibitor of CaMKII. At 1 μM AIP Ca^{2+} spark duration, frequency and width were decreased significantly. Similarly, 1 μM AIP decreased [^{3}H]ryanodine binding. At 5 μM AIP, a more profound inhibition of Ca^{2+} sparks and a decrease in [^{3}H]ryanodine binding was observed. Separate measurements showed that AIP (1–5 μM) did not affect sarcoplasmic reticulum Ca^{2+}-ATPase-mediated Ca^{2+} uptake. These results suggest the existence of an endogenous CaMKIIδ that associates directly with RyR2 and specifically modulates RyR2 activity.

Key words: calcium, CaMKII (calcium/calmodulin-dependent protein kinase II), cardiac, immunoprecipitation, phosphorylation, ryanodine receptor.

INTRODUCTION

Cardiac muscle contraction depends on a series of tightly regulated subcellular events culminating in Ca^{2+} release from the SR (sarcoplasmic reticulum) via Ca^{2+} release channels or RyR2 (type 2 ryanodine receptors). Synchronous activation of these channels causes a transient increase in cytoplasmic [Ca^{2+}] from approx. 100 nM to approx. 1 μM and a subsequent transient contraction. Tight control over the opening and closing of these channels is essential for normal cardiac excitation–contraction coupling to occur. This is provided via direct actions of Ca^{2+} on both the luminal and cytosolic faces, by direct and/or indirect phosphorylation/dephosphorylation of RyR2 by specific kinases and phosphatases and by interactions with small regulatory 12 kDa FK506-binding proteins (FKBP12.6) that act to stabilize RyR channel function. There are now various studies suggesting that defective release of Ca^{2+} from the SR, as observed in cardiac hypertrophy, may be due to either decreased protein levels of RyR or deficient regulation (via phosphorylation and/or FKBP12.6 binding) of the channels or a combination of both [1–3].

RyRs are tetramers comprising four subunits of approx. 565 kDa each and are the largest ion channels identified to date. Each of the subunits has one FKBP12.6 molecule bound and these exist as part of a macromolecular complex comprising an anchoring protein and signalling molecules that are bound to the cytoplasmic portion of RyR and serve to regulate the channel function. PKA (protein kinase A), PP1 (protein phosphatase 1), PP2A and calcineurin (PP2B) have all been identified as part of the complex, binding to RyR2 via leucine/isoleucine zipper motifs [4,5] and have been shown to regulate RyR phosphorylation and resultant dissociation of FKBP12.6 from RyR2 [6]. Phosphorylation and removal of FKBP12.6 results in increased channel activity associated with destabilization of the tetrameric complex [7]. Although other kinases have been reported to phosphorylate RyR, there is some evidence in the literature to suggest that only PKA phosphorylation induces dissociation of FKBP12.6, which may prove physiologically important [3]. However, effects of protein kinases on RyR function may not necessarily be direct, as demonstrated in a study examining PKA phosphorylation in resting mouse ventricular myocytes [8]. This study suggested that the PKA-dependent increase in Ca^{2+} spark frequency and amplitude was attributable to PLB (phospholamban) phosphorylation and consequent enhancement of SR Ca^{2+} load [8].

Various studies have suggested that CaMKII (calcium/calmodulin-dependent protein kinase II) phosphorylates RyR2. Whether this occurs on the same subset of RyRs that have been identified as associating with PKA and the A-kinase anchoring protein is yet to be established but the existence of multiple

Abbreviations used: AIP, autocamtide-2-related inhibitory peptide; CaMKII, calcium/calmodulin-dependent protein kinase II; FKBP, FK506-binding protein; PKA, protein kinase A; PKC, protein kinase C; PLB, phospholamban; RuR, Ruthenium Red; RyR, ryanodine receptor; SERCA 2a, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase 2a; SR, sarcoplasmic reticulum.

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populations with different signalling components may also be possible. Recent studies with transgenic mice that overexpress the δ_c isoform of CaMKII have shown increased phosphorylation of RyR2 and PLB and physical association of the overexpressed kinase with RyR2 [9]. However, the relationship between endogenous CaMKII and RyR2 and the effect on channel activity following phosphorylation by CaMKII have not been explored and remain somewhat controversial. One early study stated that RyR2 has 16 candidate sites for CaMKII and only five for PKA [10]. Despite this, phosphorylation occurs apparently at only one of these sites, namely Ser^{2809}. This study also suggested that the cardiac form of RyR was a preferred substrate for CaMKII and that phosphorylation at this site served to increase channel activity. This observation has been verified by work performed on canine cardiac SR, where both PKA and CaMKII were shown to phosphorylate and increase RyR2 activity [11]. However, other studies examining phosphorylation/dephosphorylation of RyR2 in swine and rabbit have demonstrated that CaMKII decreased channel activity. These effects were reversed by either a synthetic inhibitor of CaMKII or treatment with acid phosphatase [12]. The apparent contradictory results in the literature may be due to differences in the state and stoichiometry of phosphorylation of the channel and its dependence on assay conditions. Also it may be important to consider whether the kinases that exist endogenously in the non-activated cell phosphorylate RyR2 in a manner different from exogenously added kinases in assays *in vitro*.

Now it is fairly well established that intrinsic SR kinase activity exists in cardiac tissue. At least part of this activity had been attributed to the δ-class of CaMKII enzymes [13]. Four variants of the δ-class are expressed in adult rat heart (δ_B, δ_C, δ_4 and δ_9) and these may be localized to different cellular compartments with δ_B presenting as a good candidate for the nuclear CaMKII and δ_C for the cytoplasmic enzyme [14]. In general, localization of signalling enzymes close to their substrates has important regulatory consequences [15], especially for broad-specificity enzymes such as CaMKII. As a result, proof that CaMKII is subject to intracellular compartmentalization and characterization of its physiological roles are intense areas of investigation. In addition to RyR2, substrates for CaMKII have been suggested to include PLB [16], SERCA 2a (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase 2a) [17] and the L-type Ca^{2+} channel [18]. CaMKII has also been suggested to functionally couple L-type Ca^{2+} channel and RyR2 during cardiac excitation–contraction coupling [19] and shown to co-localize with these proteins in the vicinity of the T-tubule. This highlights the possibility that, as suggested recently for other signalling molecules, CaMKII may be coupled with its substrates through specific anchoring proteins and cytoskeletal elements yet to be identified.

Many studies have examined the effect of exogenously added CaMKII on RyR function. The artificial nature of this approach makes interpretation difficult. The present study has specifically focused on the interaction of endogenous CaMKII with RyR2. To understand this interaction, co-immunoprecipitation studies were used to examine the expression and activity of CaMKII that is directly associated with RyR2 in rabbit cardiac tissue. These experiments were combined with functional measurements of localized Ca^{2+} release or spark activity and [^{3}H]ryanodine binding in freshly isolated rabbit cardiac myocytes to explore RyR2 function and protein expression. This was performed in the absence and increased presence of a specific CaMKII inhibitor to allow RyR2 function to be monitored in the presence and absence of endogenous CaMKII activity. The specificity of modulation of RyR2 function by this inhibitor was confirmed by examining SERCA 2a-mediated SR Ca^{2+} uptake under similar conditions.

EXPERIMENTAL

Materials

CaMKII assay kit, PKI and PKCIP were from Upstate (Milton Keynes, U.K.). AIP (autocamtide-2-related inhibitory peptide, myristoylated) and high-purity ryanodine were from Calbiochem–Merck Biosciences (Nottingham, U.K.). RyR2 mouse monoclonal antibody was from Affinity Bioreagents (Golden, CO, U.S.A.). RyR2 phosphorylation site-specific antibodies and PLB mouse monoclonal antibody were from Phosphoprotein Research (Bardsey, U.K.). CaMKIIδ rabbit polyclonal antibody was a gift from Dr P. Karczewski (Max Delbrück Centre for Molecular Medicine, Berlin-Buch, Germany).

Immunoprecipitation

Freshly isolated left ventricular tissue was weighed and minced with fine scissors on ice in 3 vol. of homogenization buffer (50 mM Hepes, pH 7.4/1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride/0.8 μM aprotinin/20 μM leupeptin/36 μM bestatin/15 μM pepstatin A/1 % glycerol). This was homogenized using an Ultra-Turrax for 3×15 s bursts at 4 °C. The resulting homogenate was centrifuged at 45 000 ***g*** for 10 min at 4 °C. The supernatant from this was discarded and the pellet resuspended in an equal volume of solubilization buffer (50 mM Hepes, pH 7.4/100 mM NaCl/1 % CHAPS/1 % glycerol and protease inhibitors as before). This was left to mix at 4 °C for 20 min before repeating centrifugation as before. The solubilized supernatant was assayed for protein content using the Coomassie Plus Protein Assay Reagent (Perbio Science, Tattenhall, Cheshire, U.K.). Preparations (500 μg of total protein) were incubated in the presence or absence of 1 μg RyR monoclonal antibody (mouse IgG1) (ABR, Golden, CO, U.S.A.) for 3 h at 4 °C. Identical volumes of prewashed Protein G–Sepharose beads (Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, U.K.) were then added to each sample and these were left to mix for a further 1 h. After several washes in rinse buffer (solubilization buffer without CHAPS), RyR was eluted from the Sepharose beads by heating at 70 °C in 4 × Laemmli sample buffer containing 5 mM dithiothreitol, and samples were prepared for SDS/PAGE. For kinase activity measurements, Sepharose beads were washed in the same way and these were left in rinse buffer on ice (see below).

Western blotting

SDS/PAGE was performed as described previously [20] using the NuPAGE system (Invitrogen, Paisley, U.K.) with either 3–8 % Tris/acetate gels and Tris/acetate running buffer (50 mM Tris base, pH 8.24/50 mM tricine/0.1 % SDS) for RyR2 detection or 4–12 % Bis-Tris gels with MES buffer (50 mM MES, pH 7.2/50 mM Tris base/0.1 % SDS/1 mM EDTA) for CaMKII or PLB detection. Proteins were transferred to nitrocellulose membranes using the Transblot semi-dry transfer cell system (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, U.K.) with NuPAGE transfer buffer [25 mM Bicine/25 mM Bis-Tris (free base)/1 mM EDTA/1 mM chlorobutanol/10 % (v/v) methanol] and blocked in wash buffer as described previously [21].

Blots were incubated overnight at 4 °C with 0.25 μg/ml mouse anti-RyR antibody, 0.2 μg/ml rabbit anti-phospho-RyR2 (RYR2-PS2809) or 0.2 μg/ml rabbit anti-dephospho-RyR2 (RYR2-2809deP) (antibodies characterized using specific peptide and phosphopeptide [22]), 1 μg/ml rabbit anti-CaMKIIδ or 0.0625 μg/ml mouse anti-PLB (A1) in blotting buffer. Membranes were then washed and incubated for 1 h at room temperature 21 °C with either goat anti-mouse IgG-horseradish

peroxidase or goat anti-rabbit IgG-horseradish peroxidase diluted 1:4000. Blots were then given a further wash and developed using the ECL® detection system (Amersham Biosciences). For densitometry analysis, films were scanned using a calibrated GS-710 scanner (Bio-Rad Laboratories) and analysed using Quantity One software (Bio-Rad Laboratories).

CaMKII activity assay

Sepharose beads bound with RyR2 were washed several times in rinse buffer (described previously) and most of this buffer removed before the assay. Beads were kept on ice for all pre-incubation steps. For experiments in which AIP was used, beads were preincubated with the appropriate concentration (0.1–5 μM) in assay dilution buffer (4 mM Mops, pH 7.2/5 mM β-glycerol phosphate/0.2 mM Na_3VO_4/0.2 mM dithiothreitol/0.2 mM $CaCl_2$) for 30 min at 30 °C before the kinase assay was performed. CaMKII activity was measured using a CaMKII assay kit and 3000 Ci/mmol [γ-^{32}P]ATP (Amersham Biosciences). Briefly, beads were incubated for 15 min at 30 °C in assay kit dilution buffer in the presence of specific substrate peptide (KKALRRQETVDAL) and inhibitor peptides for PKA (PKI) and PKC (corresponding to residues 19–31 of PKC, termed as pseudosubstrate). The assay is linear for incubation times up to 30 min. CaMKII activity was measured by separating phosphorylated substrate from the residual [γ-^{32}P]ATP using P81 phosphocellulose paper, which was subjected to several high stringency washes and counted using a scintillation counter.

Cardiac myocyte isolation

New Zealand White rabbits (2–2.5 kg) were given an intravenous injection of 500 i.u. heparin, together with an overdose of sodium pentobarbitone (100 mg/kg). The heart was rapidly excised and cannulated on to a Langendorff perfusion column via the aorta. It was then retrograde-perfused at a perfusion rate of 25 ml/min (37 °C), initially with 150 ml Krebs–Henseleit solution. Thereafter, it was perfused with re-circulated Krebs–Henseleit solution supplemented with 1.4 mg/ml collagenase (type I; Worthington Chemical, Twyford, Reading, U.K.), 0.1 mg/ml protease (type XIV; Sigma) for 2 min. At approx. 2 min, 50 μM $CaCl_2$ was added to the re-circulating perfusate. Finally, at approx. 6–7 min, the heart was perfused with 50 ml of 0.1 % Krebs–Henseleit solution containing 0.1 % BSA and 0.075 mM $CaCl_2$. The right and left ventricles were dissected free from the heart, cut into chunks and incubated (37 °C) for 1 h in Krebs–Henseleit solution (no added Ca^{2+}). The cell suspensions obtained at the end of the incubation period were filtered (250 μm mesh) back into Krebs–Henseleit solution at a concentration of approx. 10^5 cells/ml.

Ca^{2+} spark measurement in permeabilized cardiac myocytes

Cardiac myocytes were suspended in a modified Krebs solution containing 1 mM EGTA (no added Ca^{2+}). Cells were allowed to settle on to the coverslip at the base of a small bath. β-Escin (Sigma) was added from a freshly prepared stock solution to the cell suspension to give a final concentration of 0.1 mg/ml for 0.5–1 min and the β-escin subsequently removed by perfusion using a mock intracellular solution with the following composition (mM): 100 KCl, 5 Na_2-ATP, 10 Na_2-phosphocreatine, 5.5 $MgCl_2$, 25 Hepes, 0.05 K_2-EGTA, pH 7.0 (20–21 °C). The [Ca^{2+}] in the perfusing solution was varied by the addition of known amounts of 1 M $CaCl_2$ stock solution (BDH–Merck, Lutterworth, Leicester, U.K.). The fluorescent Ca^{2+} indicator Fluo-3 (free acid) (Molecular Probes, Eugene, OR, U.S.A.) was added to the solution to give a nominal final concentration of 10 μM. Confocal line-scan images were recorded using a Bio-Rad Radiance 2000 confocal system. Fluo-3 in the perfusing solution was excited at 488 nm and measured above 515 nm using the epifluorescence optics of a Nikon Eclipse inverted microscope with a 60 × 1.2 NA water-immersion objective lens (Plan Apochromat; Nikon, U.K.). Iris diameter was set at 1.9 mm providing an axial (z) resolution of approx. 0.9 μm and X–Y resolution of approx. 0.5 μm based on full-width half-maximal amplitude measurements of images of 0.1 μm fluorescent beads (Molecular Probes). Results were acquired in line-scan mode at 2 ms/line; pixel dimension was 0.3 μm (512 pixels/scan; zoom = 1.4). The scanning laser line was orientated parallel to the long axis of the cell and placed approximately equidistant from the outer edge of the cell and the nucleus/nuclei to ensure that the nuclear area was not included in the scan line.

The [Ca^{2+}] in the perfusing solution used to generate Ca^{2+} sparks was measured by ensuring that the line-scan covered a significant region of extracellular space as well as the entire cell length. Under these conditions, the fluorescence signal recorded from the line-scan contains signals from both intracellular and extracellular compartments. Both compartments could be clearly distinguished due to the significantly greater fluorescence signal from the indicator within the cell caused by binding of Fluo-3 [23]. To enable this trace to be converted into [Ca^{2+}], a series of calibration solutions were used at the end of each spark measurement period. Each cardiomyocyte was perfused sequentially with the following solutions: (i) a solution containing nominally 365 nM Ca^{2+}, (ii) a solution containing < 1 nM Ca^{2+} and (iii) a solution containing 35 μM Ca^{2+} (all calibration solutions contained 10 mM total EGTA). On the basis of the extracellular and intracellular signals, the [Ca^{2+}] in the experimental solution can be calculated (assuming the apparent affinity constant of Fluo-3 for Ca^{2+} was 558 nM [23]). In all experiments included in the analysis, the [Ca^{2+}] in the test solution was 150–170 nM; test solutions containing a [Ca^{2+}] outside this range were excluded from the analysis. Ca^{2+} sparks were quantified using an automatic detection and measurement algorithm adapted from a previously published method [24]. The spark activity was measured in permeabilized cells for clear methodological reasons: (i) the object was to investigate the effects of AIP on RyR2 activity independently of effects of other transport systems; (ii) if measurements were made on intact cells, concomitant effects on sarcolemma Ca^{2+} transport processes may alter SR Ca^{2+} content in the rabbit thus indirectly affecting spark activity and it would be technically difficult to compensate for these effects accurately in intact myocytes; and (iii) permeabilized cells can be perfused with standard [Ca^{2+}] (150 nM) accurately (within 10–15 nM), this is impossible to arrange in intact cells. The loss of diffusible mediators of RyR2 activity was not considered to be an issue in this method. Previous work has established that Ca^{2+} spark characteristics (amplitude, time course and frequency) in permeabilized cells are indistinguishable from those observed in intact cells and are regulated by known endogenous modulators of RyR2 activity [25]. Similar confirmatory measurements have been made for the present study (results not shown).

[^{3}H]Ryanodine-binding studies

These studies were performed on freshly isolated cardiac myocytes and RyR2 immunoprecipitates. Cardiac myocytes were resuspended in a solution containing (in mM) 100 KCl, 10 NaCl, 5 Na_2-ATP, 10 Na_2-phosphocreatine, 5.5 $MgCl_2$, 25 Hepes and

1 EGTA (pH 7.2) and were permeabilized as described prior to the binding assay. RyR2 immunoprecipitation was performed as described and Sepharose beads left on ice in wash buffer. This buffer was removed just before the binding assay. Cells (2×10^4) or beads (50 μl) were incubated in binding buffer (total volume, 300 μl) containing 20 mM Hepes (pH 7.4), 1 M KCl, 5 mM caffeine, protease inhibitor cocktail (Roche) and either 1 mM EGTA, 1.1 mM $CaCl_2$ (final free [Ca^{2+}], 100 μM) or 0.711 mM $CaCl_2$ (final free [Ca^{2+}], 0.3 μM). Control incubations were also performed in 0 (< 10 nM) Ca^{2+}. Samples were incubated at 37 °C for 90 min with 5 nM [^{3}H]ryanodine (100 Ci/mmol). Bound [^{3}H]ryanodine was measured by Millipore filtration (GF/B Whatman filters) and liquid-scintillation counting. Non-specific binding was measured in the presence of 50 μM non-radioactive high-purity ryanodine.

Ca^{2+} uptake studies

Cardiomyocytes (approx. 5×10^5/ml) were suspended in a mock intracellular solution (see above), exposed to 0.1 mg/ml β-escin (Sigma) and gently stirred for 2 min. To remove β-escin, cells were spun-down (5 ***g***, 1 min) and the pellet was resuspended in 1 ml of mock intracellular solution. The concentration of permeabilized cardiomyocytes was assessed after staining with Trypan Blue dye in a Neubauer counting chamber. Then, the cardiomyocyte suspension (approx. 2×10^5 cells/ml) was placed in a cuvette and stirred to maintain the cells in suspension. fura 2 (10 μmol/l; Molecular Probes) was used to monitor the [Ca^{2+}] within the cuvette using a dual-wavelength spectrophotometer (Cairn Research, Faversham, Kent, U.K.). Oxalate (10 mmol/l; Sigma) was added to maintain low and constant levels of intra-SR [Ca^{2+}]. RuR (Ruthenium Red, 3 μM) was used to block Ca^{2+} efflux via the RyR [26] and inhibit mitochondrial Ca^{2+} uptake [27]; RuR quenches the fluorescence signal from fura 2 as described previously [28]. However, at 3 μM RuR, the quenching appeared to affect the fluorescence at both excitation wavelengths equally, with no effect on the value of K_d of fura 2 for Ca^{2+}. Additional effects of RuR on SR Ca^{2+}-ATPase are minimal at the concentrations used in the present study. The time course of the decrease in [Ca^{2+}] within the cuvette was monitored in response to the addition of an aliquot of $CaCl_2$ and all measurements were made at room temperature (20–22 °C). At the end of a series of uptake measurements on an aliquot of cells, the fura 2 signal was calibrated using two standard solutions buffered by 10 mM total [EGTA]: (i) < 1 nM Ca^{2+} to provide a minimum ratio value (R_{min}) and (ii) 365 nM Ca^{2+} to provide a second calibration signal (R_{365}). The affinity of fura 2 for Ca^{2+} under our experimental conditions was 110 ± 20 nM, a value close to previous measurements [29]. The time course of Ca^{2+} uptake was used to assess Ca^{2+} uptake capacity to SR by (i) conversion of free [Ca^{2+}] to total [Ca^{2+}] assuming that the major Ca^{2+} buffer in the solution was EGTA and (ii) differentiation of the total [Ca^{2+}] with respect to time and correcting the Ca^{2+} uptake rate for the total number of permeabilized cardiomyocytes in the cuvette. The value of instantaneous Ca^{2+} uptake rate was plotted against the corresponding free [Ca^{2+}] to generate the sigmoid relationship shown in Figure 6(B). The resultant data were fitted to a logistic curve using Origin (Origin Lab Corporation, Northampton, MA, U.S.A.) and the V_{max} and K_d values derived from the best fit of the curve to the data.

Statistics

Results were expressed as means $\pm$ S.E.M. Comparisons were made by using the paired Student's *t* test and differences were considered significant for $P < 0.05$.

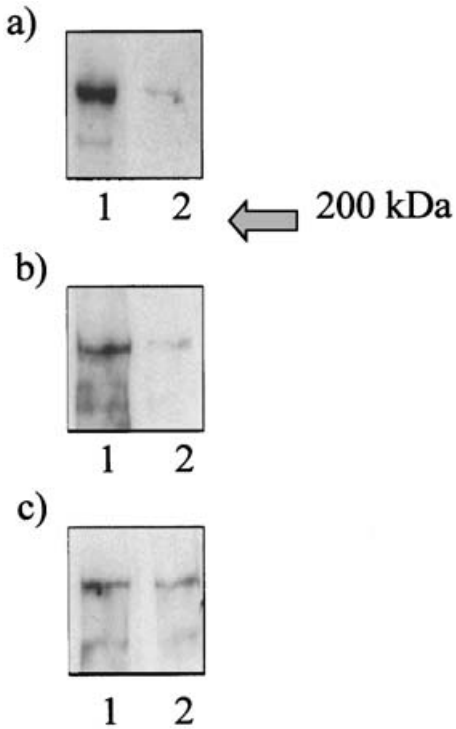

Figure 1 RyR2 phosphorylation on Ser^{2809} after immunoprecipitation and in freshly isolated cardiac myocytes

(**a**) Western blot of identical RyR2 immunoprecipitates from 500 μg of ventricular tissue, which have been probed with (lane 1) an antibody raised against the phosphopeptide sequence YNRTRRIS(PO_4)QT, specific for the Ser^{2809}-phosphorylated form of RyR2 (RyR2-PS2809) and (lane 2) an antibody raised against the phosphopeptide sequence YNRTRRISQT, specific for the dephosphorylated form of RyR2. The arrow incidates the position of the 200 kDa marker, which ran further on the gel than the portion shows. (**b**) A similar Western blot for RyR2 protein identified in cell extracts from 2×10^4 cells per well probed with the same antibodies. (**c**) Cell extracts at the same loads that have been treated with PKA and PKC inhibitors [PKI and PKCIP (19–31) both at 0.4 μM] and immediately prepared for SDS/PAGE. These results are typical of those obtained from three other experiments.

RESULTS

Phosphorylation of RyR2

To determine the basal phosphorylation status of RyR2 in this system, the protein was immunoprecipitated from freshly isolated cardiac ventricular tissue and probed with specific anti-phosphoRyR2 antibodies. These recognized either the phosphorylated form of the protein, where Ser^{2809} was phosphorylated (RYR2-PS2809) or the dephosphorylated form, where Ser^{2809} was dephosphorylated (RYR2-2809deP). Figure 1(a) shows that after immunoprecipitation, a signal is only obtained with RYR2-PS2809 suggesting that RyR2, even in non-stimulated tissue, is phosphorylated. Since functional measurements of RyR2 in the present study were performed on freshly dissociated cardiac myocytes and not on immunoprecipitated protein, we also investigated whether RyR2 was phosphorylated in these preparations. Figure 1(b) shows that a similar profile was obtained from freshly isolated cells. This suggests that phosphorylation of RyR2 is not a by-product of the immunoprecipitation procedure but is an ongoing process in rabbit cardiac tissue. This is hardly surprising given the complexity of RyR2 phosphorylation and the observation that the stoichiometry of phosphorylation of the receptor may be critical in the regulation of function with a

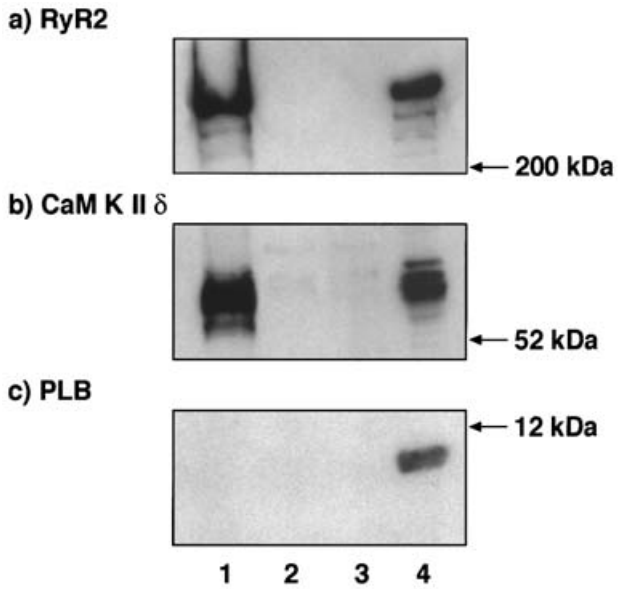

Figure 2 Co-immunoprecipitation of CaMKIIδ protein with the RyR2 protein complex

RyR2 was immunoprecipitated from 500 μg of ventricular tissue as described, and immunoprecipitates were subjected to SDS/PAGE and probed with the appropriate antibodies to detect the presence of (**a**) RyR2, (**b**) CaMKIIδ and (**c**) PLB. Lanes 1–3 show immunoprecipitates each from 500 μg of total solubilized protein. Immunoprecipitations were performed with all the necessary reagents (lane 1), in the absence of RyR2 monoclonal antibody (lane 2) and in the presence of antibody without sample (lane 3). Lane 4 shows a non-immunoprecipitated SR preparation (10 μg of total SR protein) as a positive control. These results are typical of those obtained from two other experiments.

percentage of RyR2 being phosphorylated at any given time [11]. Since Ser^{2809} is phosphorylated by CaMKII, we assume that at least part, if not all, of the phosphorylation observed is due to an endogenously active CaMKII. By treating cell preparations with inhibitors of PKA and PKC, we tested whether we could still detect phosphorylation of RyR2 under basal conditions. Figure 1(c) shows that although the signal is fainter and the dephosphorylated signal stronger, we can still detect RyR2 phosphorylated at Ser^{2809}.

Association of endogenous CaMKIIδ with RyR2

Previous studies by our group have suggested that a CaMKIIδ is present in rabbit cardiac SR [30]. To determine whether this enzyme interacts directly with RyR2 in this system, co-immunoprecipitation studies were performed. As before, RyR2 was immunoprecipitated using the anti-RyR2 antibody (MA3-916) and the presence of CaMKIIδ in the immunoprecipitate was examined by Western-blot analysis using anti-CaMKIIδ antibody. A strong signal at approx. 58 kDa was obtained, corresponding to this class of CaMKII. This antibody is capable of recognizing δ_C, δ_B and δ_9 but does not distinguish between the isoforms. In the present study, we have not specifically determined which δ isoform(s) is/are associated with the SR. In the absence of RyR antibody, with antibody alone or when the immunoprecipitate was probed for another SR-associated protein PLB, this signal was not obtained (Figure 2).

To strengthen these findings, we investigated whether we could perform activity measurements on these immunoprecipitates that would indicate CaMKII activity directly associated with RyR2. Using a specific assay for CaMKII activity and in the presence of

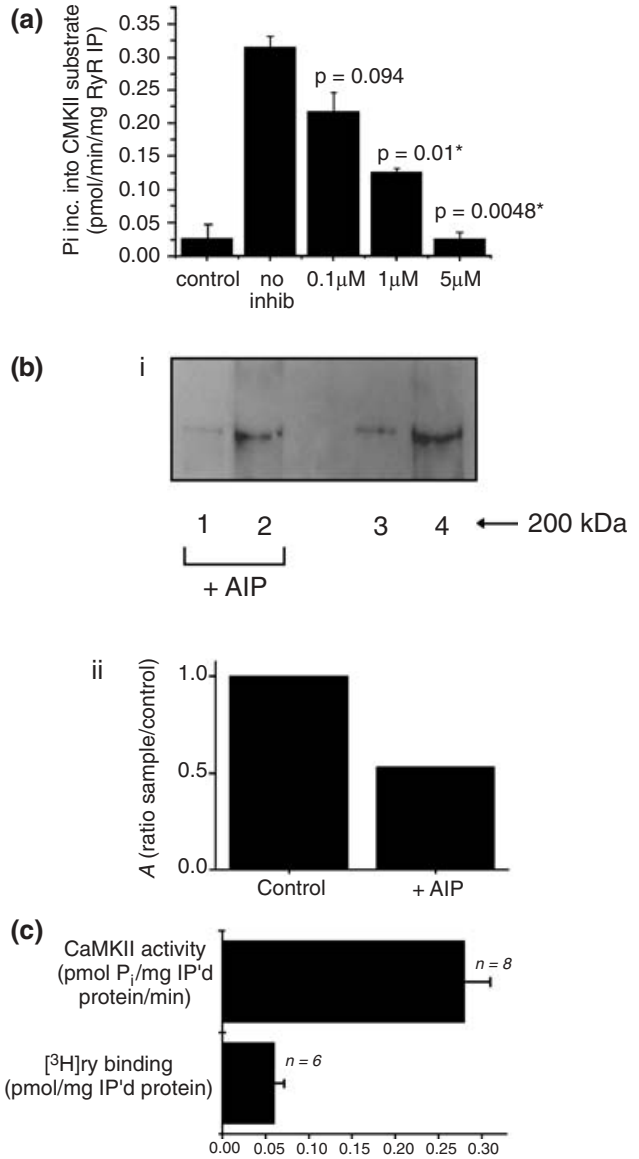

Figure 3 Measurement of CaMKII activity associated with RyR2 immunoprecipitates

The phosphotransferase activity of CaMKII was measured from RyR2 immunoprecipitates as described in the Experimental section. This was assayed in the presence of increasing amounts (0.1–5 μM) of the CaMKII inhibitor AIP. The assay was performed on duplicate samples and results are expressed as means $\pm$ S.E.M. in histogram form as shown in (**a**). Results shown are from one experiment typical of five others. Statistical significance was assessed by ANOVA with $P < 0.05$. The effect of AIP treatment on RyR2 phosphorylation by CaMKII in isolated cardiac myocytes was also assessed by Western blot using the antibody RyR2-PS2809. The effects of pretreatment with 1 μM AIP on the intensity of the signal, where identical amounts were loaded is shown in (**b**,i) Lanes 1 and 3 had 1×10^4 cells loaded and lanes 2 and 4 had 2×10^4 cells loaded. Lanes 1 and 2 were pretreated with AIP. The signal intensity represents the amount of phosphorylated RyR2 as recognized by the phospho-specific antibody. The arrow incidates the position of the 200 kDa marker, which ran further on the gel than the portion shows. Densitometry analysis for these data is shown in (**b**,ii). This is typical of two other blots. (**c**) CaMKII activity (pmol of phosphate) and amount of [^{3}H]ryanodine bound in RyR2 immunoprecipitates from equivalent amounts of starting protein.

selective inhibitors for PKA and PKC, we were able to measure a 5- to 10-fold increase in the incorporation of ^{32}P into a CaMKII-specific peptide substrate during the course of the experiment (Figure 3a). The fact that this activity was due to CaMKII was also demonstrated by a decrease in ^{32}P incorporation selectively

and to increasing extents using the CaMKII-specific inhibitory peptide AIP. This inhibitor binds to the substrate-binding site for autophosphorylation and potently inhibits autophosphorylation and activation ($IC_{50} = 45$ nM) [31,32]. AIP is far more specific than previous CaMKII inhibitors such as KN-62 and KN-93, which have been reported to inhibit other calmodulin-dependent enzymes [33]. At 100 nM, we observed 30 % inhibition of activity, and this increased to close to 100 % inhibition between 1 and 5 μM AIP. We were also able to detect a significant decrease in the level of phosphorylated RyR2 recognized by immunoblotting following AIP treatment (Figure 3bi). This was verified by densitometry analysis (Figure 3bii) and the signal due to phosphorylated RyR2 was shown to decrease by approx. 45 % after treatment with AIP. Taken together, these findings suggest the presence of an endogenous CaMKIIδ that is directly associated with RyR2 and displays significant levels of activity in rabbit cardiac tissue, even under non-stimulated conditions. The effect of endogenous CaMKII on RyR2 is not well documented. Most studies have examined the effects of adding exogenous CaMKII to RyR2 in isolated systems.

To estimate the stoichiometry between CaMKII and RyR2 in our preparations, we quantified the amount of RyR2 protein immunoprecipitated from a known amount of solubilized and immunoprecipitated preparation using [^{3}H]ryanodine binding. This was expressed in relation to the activity (incorporation of radioactive phosphate into peptide substrate) of endogenous CaMKII measured from exactly the same amount and source of the starting material. Results are shown in Figure 3(c). From equivalent amounts of starting material (1 mg of total protein) under exactly the same conditions, we obtain RyR2 immunoprecipitates that have approx. 0.06 pmol of [^{3}H]ryanodine bound specifically with an associated CaMKII activity that gives approx. 0.27 pmol of phosphate/min incorporation into specific peptide substrate under optimal conditions as described previously. Assuming a stoichiometry of ryanodine binding of 1:1, these measurements indicate a CaMKII activity of approx. 4.5 mol of phosphate incorporated $\cdot$ min^{-1} $\cdot$ mol^{-1} RyR2.

Effect of CaMKII inhibition on ryanodine binding

Ryanodine-binding assays were performed on freshly isolated cells pretreated with the appropriate concentrations of AIP. These assays were conducted under three different conditions: (i) with 100 μM free Ca^{2+}; (ii) submaximal conditions with 0.3 μM free Ca^{2+} and (iii) with < 10 nM Ca^{2+} (Figure 4). As expected, specific binding decreased as the [Ca^{2+}] was reduced. At the lowest [Ca^{2+}] (< 10 nM), binding was decreased to background levels. Interestingly, when cells were treated with 1 μM AIP, ryanodine binding was decreased significantly (approx. 10 %) in the presence of 100 μM Ca^{2+}, and was even lower (approx. 20 % decrease) at 0.3 μM Ca^{2+}. This effect was more pronounced at 5 μM AIP. These results suggest that inhibition of CaMKII decreases the activity of RyR2.

Effect of CaMKII inhibition on Ca^{2+} spark activity

Figure 5(B) shows sample line-scan images displayed in pseudocolour of Ca^{2+} sparks recorded from permeabilized cardiomyocytes under control conditions and after incubation with a range of inhibitor concentrations. Mean values of amplitude, width, duration and frequency of Ca^{2+} spark over a range of AIP concentrations studied are shown in Figure 5(A). Increasing doses of AIP had marked effects on Ca^{2+} spark characteristics. At 0.1 μM, a concentration that decreased RyR-associated CaMKII activity by 10 %, spark frequency was significantly increased, but there was no obvious change in any other characteristic. At 1 μM AIP, a concentration that inhibited 75–90 % RyR2-associated CaMKII activity, Ca^{2+} spark amplitude was slightly increased, but all other parameters (spark width, duration and frequency) were significantly decreased. AIP was also tested at 5 μM, a concentration that completely inhibits RyR2-associated CaMKII activity. At this concentration, Ca^{2+} spark amplitude was substantially decreased. This decrease in amplitude was accompanied by a sustained decrease in all other parameters, as was seen for 1 μM AIP. In summary, levels of AIP known to inhibit RyR2-associated CaMKII activity caused a decrease in RyR2 activity as assessed by two independent assays. These results support the hypothesis that CaMKIIδ activity associated with RyR2 increases the activity of RyR2.

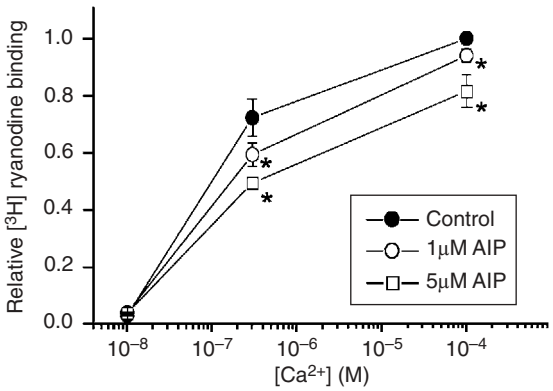

Figure 4 Effect of AIP on [^{3}H]ryanodine binding

Binding assays were performed as described on freshly dissociated cardiac myocytes. Cells were either pretreated for 30 min with AIP at 1 or 5 μM or left untreated (control). Samples were assayed in triplicate at the indicated [Ca^{2+}] and results plotted as relative [^{3}H]ryanodine binding against log [Ca^{2+}]. Results represent means $\pm$ S.E.M. for $n = 5$ experiments. *Values that are significantly different from control values with $P < 0.05$.

Effect of CaMKII on SR Ca^{2+} uptake

To distinguish a possible indirect effect of AIP on Ca^{2+} sparks via the SR Ca^{2+} pump SERCA 2a, the same range of AIP concentrations was studied on a direct assay of SERCA activity. As shown in Figure 6(A), SERCA activity could be monitored in the presence of RuR by measuring oxalate-supported Ca^{2+} uptake. Control experiments established that the Ca^{2+} uptake was completely inhibited by thapsigargin, indicating that the uptake component was attributable to SERCA. Conversion of the free-Ca^{2+} signal to total Ca^{2+} was made, assuming EGTA is the dominant buffer (Figure 6A, thick line). The apparently slower decay of the total Ca^{2+} signal compared with free Ca^{2+} signal is simply the result of the non-linear relationship between these two variables. Differentiation of the total Ca^{2+} signal generates the net flux across the SR. Plotting this signal relative to the free-[Ca^{2+}] signal allows the Ca^{2+} dependence of SERCA activity to be assessed. Each curve was fitted to a sigmoidal (logistic relationship) that estimated the parameters K_d and V_{max}. Despite preincubation with the inhibitor for a length of time similar to that used in the Ca^{2+} spark assay, neither the K_d nor V_{max} value of Ca^{2+} uptake was significantly affected at any of the concentrations tested (control, $K_d = 0.39 \pm 0.006$ μM, $V_{max} = 0.14 \pm 0.01$ nmol $\cdot$ (10^5 cells)$^{-1}$ $\cdot$ s^{-1}; 5 μM AIP, $K_d = 0.35 \pm 0.02$ μM, $V_{max} = 0.14 \pm 0.005$ nmol $\cdot$ (10^5 cells)$^{-1}$ $\cdot$ s^{-1}; Figure 6C). Direct

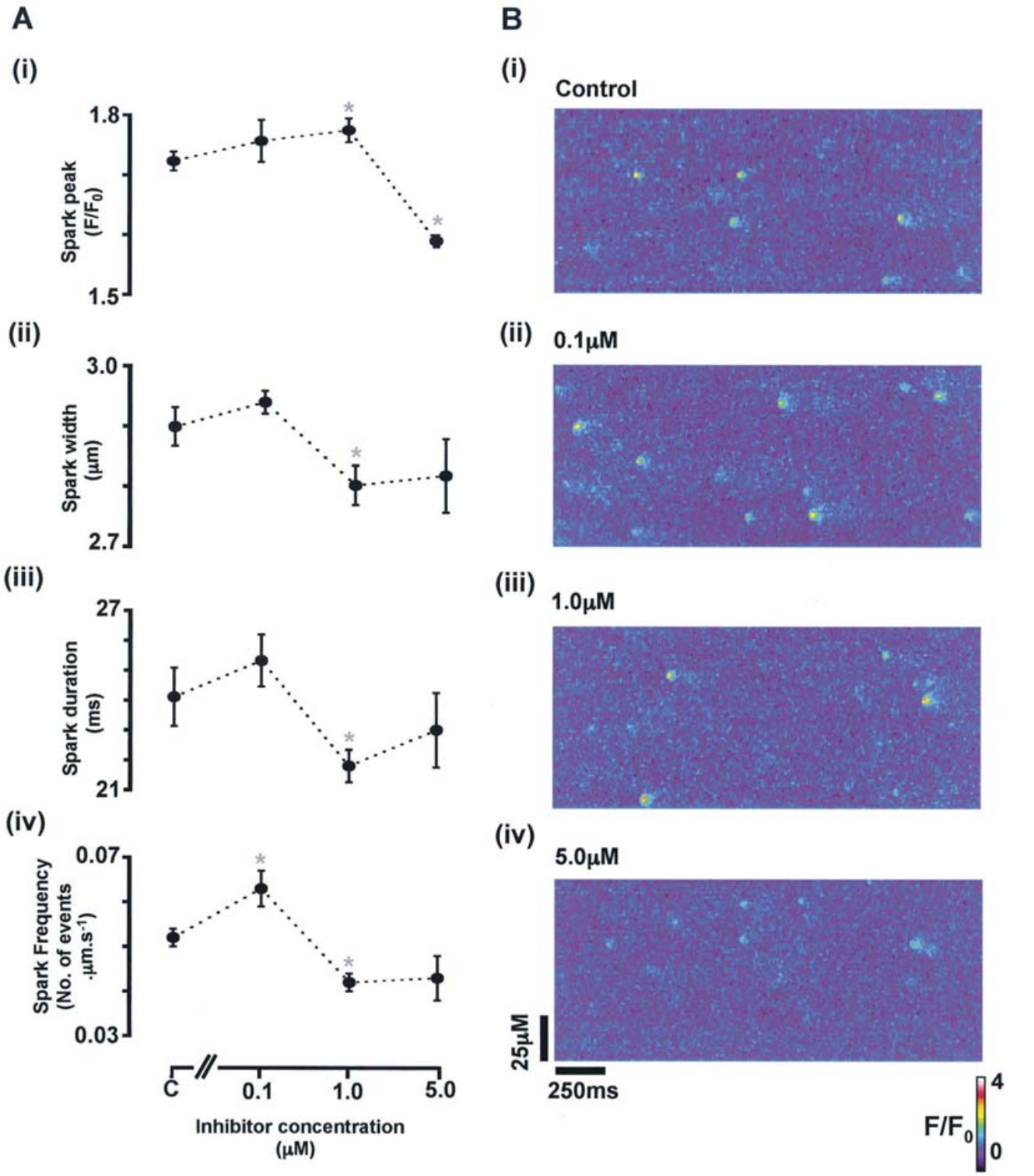

Figure 5 Calcium spark characteristics in permeabilized rabbit ventricular cardiomyocytes perfused with different inhibitor (AIP) concentrations

(**A**) Ca^{2+} spark (i) peak, (ii) width, (iii) duration and (iv) frequency for control rabbit ventricular myocytes (C), and inhibitor (AIP) concentrations of 0.1, 1 and 5 μM. (**B**) Line-scan images of Ca^{2+} sparks taken from permeabilized cardiomyocytes perfused with a mock intracellular solution containing 155 nM free Ca^{2+} (i) and with the addition of AIP at concentrations (ii) 0.1 μM, (iii) 1 μM and (iv) 5 μM.

comparison of Ca^{2+} uptake in AIP-treated cells with the corresponding control cells (using paired *t* test) indicated that up to 5 μM AIP had no significant effect on SERCA activity.

DISCUSSION

The network of signalling molecules that exist and function as part of the RyR2 multimeric complex is still poorly understood.

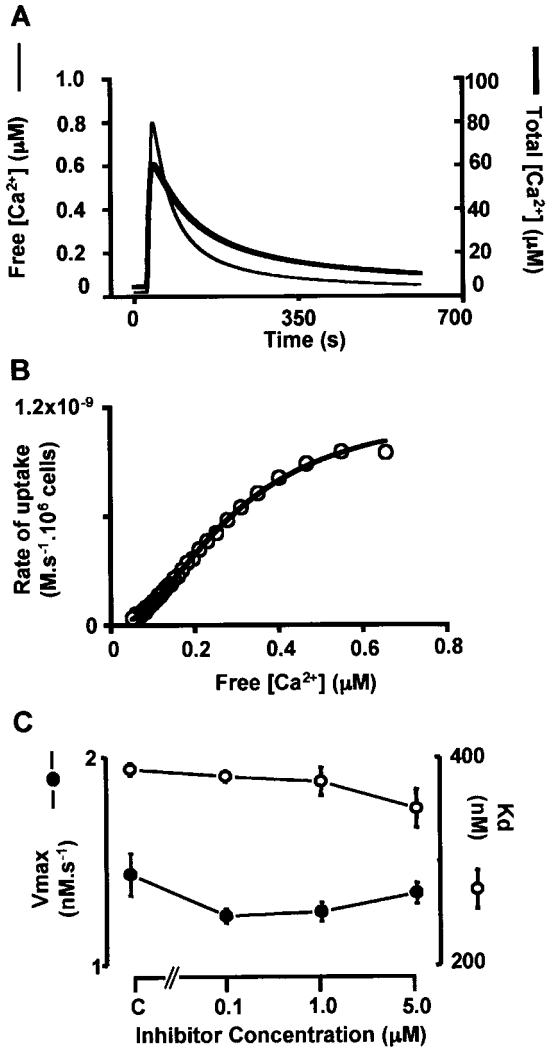

Figure 6 Effects of AIP on SERCA-mediated Ca^{2+} uptake in permeabilized myocytes

(**A**) Time course of $[Ca^{2+}]$ recorded from a cuvette of permeabilized cells (2×10^5 cells/ml) recorded using fura 2 acid (10 μM). Increasing total $[Ca^{2+}]$ within the cuvette by 500 μM caused a rapid increase in free $[Ca^{2+}]$. The subsequent decrease in $[Ca^{2+}]$ is a result of SERCA-mediated uptake into the SR. The superimposed thick-line trace is the time course of the changes in total $[Ca^{2+}]$ calculated from the known Ca^{2+} buffers in the bathing solution. (**B**) Plot of the rate of uptake of Ca^{2+} ($d[Ca^{2+}]_{total}/dt$) plotted against the $[Ca^{2+}]$. The points (○) represent the best-fit line to the following equation: $d[Ca^{2+}]_{total}/dt = V_{max}/\{1 + ([Ca^{2+}]/K_m)^2\}$. The V_{max} and K_d values (along with the least-squares error) are shown in the Results section. (**C**) Means ± S.E.M. of K_d and V_{max} values for $n = 4$ under control conditions (C), and after incubation in CaMKII inhibitor concentrations: 0.1, 1 and 5 μM.

Since RyR2 is such a large protein and contains a number of potential phosphorylation sites, it is probable that its regulation via phosphorylation/dephosphorylation is a complex process and may involve at least several different kinases and phosphatases. The present study has, for the first time, demonstrated direct association of an endogenous CaMKIIδ with RyR2 and shown evidence that the activity of this kinase regulates cardiac SR Ca^{2+} release. In these experiments, other factors that could potentially influence the activity of individual Ca^{2+} release sites were carefully controlled or monitored. These included the Ca^{2+} levels in cytosol, Ca^{2+} buffering and the presence of modulatory agents and the activity of the SR Ca^{2+}-ATPase.

Alterations in individual Ca^{2+} spark parameters following treatment with AIP, as observed in Figure 5, suggest that endogenous CaMKII has a specific effect on RyR2 function. Based on the activity measurements from immunoprecipitated RyR2, we are confident that AIP at 1–5 μM will almost completely inhibit RyR2-associated CaMKII activity. However, AIP will also inhibit any other endogenous CaMKIIs with other subcellular and/or substrate specificities. Therefore we cannot state unequivocally that the altered spark and ryanodine binding is the result of inhibition of RyR2-associated CaMKII alone. One alternative explanation is the modulation of RyR2 activity indirectly by CaMKII by altering SERCA 2a activity. Increased luminal $[Ca^{2+}]$ is known to affect RyR activity [34] and this pathway represents a route for an indirect effect of AIP on RyR2 activity. To investigate this, we studied SERCA-mediated Ca^{2+} uptake under similar inhibitory conditions. None of the concentrations tested (0.1–5 μM) had any significant effect on the rate of SERCA 2a-mediated uptake. These results may indicate the existence of different pools of CaMKII in the SR.

The effect of AIP treatment on RyR2 activity is highlighted in the Ca^{2+} spark parameters and ryanodine-binding measurements. At 1 μM AIP, a concentration known to abolish most of the RyR2-associated CaMKII activity, the frequency, width and duration of sparks are significantly decreased with a slight increase in amplitude. Parallel experiments examining [^{3}H]ryanodine binding show that 1 μM AIP also significantly decreased specific binding. Higher concentrations of 5 μM AIP caused significantly decreased spark amplitude and further decreased [^{3}H]ryanodine binding. This complex pattern of Ca^{2+} spark activity is similar to the effects of other modulators of RyR2 activity; dissociation of FKBP12.6 by rapamycin initially affected Ca^{2+} spark frequency before eventually affecting spark amplitude [35]. These results imply that endogenous CaMKII-mediated phosphorylation increases RyR2 activity. This is in agreement with other studies showing phosphorylation of RyR2 by CaMKII [10,11] and PKA [3,36] increases channel activity. Most recently, studies overexpressing transgenic CaMKIIδ have shown that this leads to increased Ca^{2+} release, reflected in enhanced spark frequency [37]. This effect may be a contributing factor to the accompanying dilated heart failure. None of these studies have examined the relationship between natural endogenously expressed CaMKII and RyR. The overall implications of adding exogenous CaMKII or modifying the expression of the endogenous enzyme have to be considered in these studies. The importance of discriminating endogenous versus exogenous kinase activity has been highlighted previously [11]. It has been suggested that exogenously added CaMK could phosphorylate Ser^{2809} on all four subunits of the homotetrameric RyR2. However, only one Ser^{2809} may be accessible for phosphorylation by endogenous CaMKII [10]. It was suggested that this structural asymmetry might affect allosteric coupling of the four units leading to channel closure. However, results of the present study, demonstrating a stimulatory effect of endogenous CaMKII on RyR2 activity, suggest that this explanation may not be applicable. An explanation for differences in the effects of CaMKII phosphorylation on RyR2 in the literature [11,12] may be that different residues are phosphorylated, depending on the source of the kinase. The cardiac RyR contains 16 possible positive consensus sequences for CaMKII, making this perfectly feasible [38]. One consideration when analysing the Ca^{2+} spark response to AIP treatment is that other components of the RyR2 multimeric complex may be substrates for CaMKII and phosphorylation of these additional substrates may regulate RyR2, in a manner analogous to PLB regulation of SERCA 2a. Since AIP will inhibit all CaMKII activity in the preparations and not just that directly associated with RyR2, specific analysis is difficult.

However, in the present study, the effects of AIP were studied over a concentration range known to decrease CaMKIIδ activity associated with RyR2. Presently, this approach will give the best estimate of the modulatory effects of the associated CaMKIIδ on RyR2.

It would appear from previous reports already highlighted that phosphorylation of RyR2 can act to achieve opposite end effects on channel function, depending on the source, type and specificity of the kinase involved. The large mass of the protein and the number of potential phosphorylation sites both on RyR2 and its associated proteins offer scope for more effectors to be identified. It is intriguing to speculate that under resting conditions, SR Ca^{2+} release may be balanced in part by the concerted actions of at least two intrinsic SR protein kinases (CaMKIIδ and PKA) both of which are localized to the membrane via specific anchoring proteins. It is probable that these kinases will act not only on RyR2, but on other components of the complex and also on other SR proteins. As such, the question then arises whether there may actually be subcompartmentation, i.e. targeting of compartmentalized enzymes to specific substrates. A recent study has used transgenic mice with the novel technique of targeting AIP to longitudinal SR using a truncated PLB transmembrane domain [39]. Specific inhibition of CaMKII-mediated PLB phosphorylation was achieved suggesting that targeted inhibition of CaMKII is possible and may be extrapolated to other SR substrates, notably RyR2.

In the present work new evidence has been presented for specific association with and activation of RyR2 by an endogenous SR-associated CaMKIIδ. Specific inhibition of this kinase decreases SR Ca^{2+} release but has no effect on SR Ca^{2+} uptake. Future experiments will be directed at RyR2-targeted manipulation of this CaMKII to provide clearer insight into the role of this enzyme in the regulation of excitation–contraction coupling in cardiac muscle.

This work was supported by the British Heart Foundation.

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Received 10 July 2003/18 September 2003; accepted 14 October 2003
Published as BJ Immediate Publication 14 October 2003, DOI 10.1042/BJ20031043