Modulation of cardiac ryanodine receptors of swine and rabbit by a phosphorylation–dephosphorylation mechanism

Andrew J. Lokuta*, Terry B. Rogers*, W. Jonathan Lederer† and Hector H. Valdivia†‡

*Departments of †Physiology and *Biochemistry, University of Maryland Medical School, Baltimore, MD 21201, USA*

1. The regulation of the cardiac Ca^{2+} release channel–ryanodine receptor (RyR) by exogenous acid phosphatase (AcPh) and purified Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) was studied in swine and rabbit sarcoplasmic reticulum (SR) vesicles using [^{3}H]ryanodine binding and planar bilayer reconstitution experiments.

2. Addition of AcPh (1–20 U ml^{-1}) to a standard incubation medium increased [^{3}H]ryanodine binding in a Ca^{2+}-dependent manner. Stimulation was only readily apparent in media containing micromolar Ca^{2+} concentrations.

3. Scatchard analysis of [^{3}H]ryanodine binding curves revealed that AcPh enhanced binding by increasing the affinity of the receptor for [^{3}H]ryanodine without recruiting additional receptor sites (K_d, 9·8 ± 0·85 and 3·9 ± 0·65 nM; B_{max} (the maximal receptor density), 1·45 ± 0·14 and 1·47 ± 0·12 pmol mg^{-1} for control and AcPh, respectively). The failure of AcPh to increase B_{max} suggested that the number of receptors that were 'dormant' due to phosphorylation in the SR preparation was very small.

4. At the single channel level, AcPh increased the open probability (P_o) of RyR channels by increasing the opening rate and inducing the appearance of a longer open state while having no effect on single channel conductance. Thus AcPh acted directly on RyR channels or a closely associated regulatory protein.

5. CaMKII decreased both [^{3}H]ryanodine binding and P_o of RyRs when added to medium supplemented with micromolar levels of Ca^{2+} and calmodulin (CaM). Addition of a synthetic peptide inhibitor of CaMKII, or replacement of ATP with the non-hydrolysable ATP analogue adenyl[β,γ-methylene]-diphosphate (AMP-PCP), prevented CaMKII inhibition of RyRs, suggesting that CaMKII acted specifically through a phosphorylation mechanism.

6. The inhibition of RyR channel activity by CaMKII was reversed by the addition of AcPh. Thus we showed that an *in vitro* phosphorylation–dephosphorylation mechanism effectively regulates RyRs.

7. The results suggest that intracellular signalling pathways that lead to activation of CaMKII may reduce efflux of Ca^{2+} from the SR by inhibition of RyR channel activity. The Ca^{2+} dependence of CaMKII inhibition suggests that the role of the phosphorylation mechanism is to modulate the RyR response to Ca^{2+}.

In cardiac muscle, depolarization of the surface membrane evokes a small influx of extracellular Ca^{2+} which, although by itself is insufficient to fully activate myofilament contraction, is sufficient to induce a massive release of Ca^{2+} from the junctional sarcoplasmic reticulum (SR). This process, known as Ca^{2+}-induced Ca^{2+} release (CICR), is central to excitation–contraction coupling, the process that links membrane depolarization to mechanical contraction (Bers, 1991). CICR is mediated by the Ca^{2+} release channel–ryanodine receptor (RyR), a homotetramer of ~500 kDa subunits whose cytoplasmic domain protrudes from the surface of the SR to form the 'foot' structure (Block, Imagawa, Campbell & Franzini-Armstrong, 1988). In addition to Ca^{2+}, which is the primary effector of Ca^{2+}

‡ To whom correspondence should be addressed at Department of Physiology, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706, USA.

release *in vivo*, gating of the RyR is controlled by a variety of cytosolic factors such as Mg^{2+}, ATP, fatty acids and calmodulin (CaM) (for a review, see McPherson & Campbell, 1993). Recently, evidence has been accumulating which suggests that phosphorylation is also a relevant mechanism for regulation of RyR activity. Several phosphorylation sites have been identified in cardiac and skeletal RyRs (Otsu, Willard, Khanna, Zorzato, Green & MacLennan, 1990; Zorzato *et al.* 1990; Witcher, Kovacs, Schulman, Cefali & Jones, 1991). β-Adrenergic treatment of cardiac cells stimulates the phosphorylation of RyRs (Yoshida, Takahashi, Imagawa, Shigekawa, Takisawa & Nakamura, 1992), and several protein kinases readily phosphorylate RyRs *in vitro*.

Despite the clear demonstration of RyR phosphorylation *in vivo* and *in vitro*, the functional impact of the phosphorylation reaction has not been clearly established. On one hand, multiple phosphorylation sites in the RyR protein, each specific for a protein kinase, may eventually affect the RyR differently. For example, protein kinase A (PKA) and protein kinase C (PKC) share a common phosphorylation site (Takasago, Imagawa, Furukawa, Ogurusu & Shigekawa, 1991), but Ca^{2+}–CaM-dependent protein kinase II (CaMKII) phosphorylates RyRs with different stoichiometry and at a different phosphorylation site (Witcher *et al.* 1991). On the other hand, the phosphorylation state of the RyR obtained after SR purification is unknown. Therefore, the effect of exogenous kinases at the single channel level may be conspicuous or minimal, depending on the previous phosphorylation state of that particular channel (Hain, Nath, Mayrleitner, Fleisher & Schindler, 1994). Above all, the qualitative effect of a specific protein kinase on the RyR in a cell-free system has only recently begun to be evaluated.

The multifunctional CaMKII is an important regulator of SR Ca^{2+} movement in cardiac muscle. Upon elevation of cytoplasmic [Ca^{2+}] and in the presence of CaM, CaMKII phosphorylates phospholamban, which in turn increases the efficiency of the SR Ca^{2+} pump (Tada & Inui, 1983). Upon fragmentation of cardiac cells, CAMKII co-purifies with markers specific for the junctional SR (Hohenegger & Suko, 1993). The intimate association of CaMKII with regions critical for excitation–contraction coupling, the modulation of Ca^{2+} release by CaMKII in ventricular myocytes (Anderson & Braun, 1994), and the presence of putative CaMKII phosphorylation consensus sites in the cardiac RyR (Witcher *et al.* 1991) strongly suggest that CaMKII also regulates SR Ca^{2+} movement in heart via phosphorylation of RyRs. In support of this hypothesis, modulation of [^{3}H]ryanodine binding (Takasago *et al.* 1991) and RyR activity (Witcher *et al.* 1991) by CaMKII has been reported.

In the present work, we tested the functional consequences of CaMKII phosphorylation on cardiac RyRs at the single channel and SR membrane levels under well-defined conditions of substrate, cofactor and enzyme concentrations. We found that CaMKII inhibited RyRs in a Ca^{2+}-dependent manner through a specific phosphorylation mechanism. The inhibitory effect of this phosphorylation reaction could be reversed by a protein phosphatase that acted on Ca^{2+}-activated RyRs. We suggest that phosphorylation–dephosphorylation is a relevant mechanism to modulate the response of RyRs to Ca^{2+}. Part of this work has been published as an abstract (Lokuta, Fuentes, Lederer, Rogers & Valdivia, 1994).

METHODS

Materials

[^{3}H]Ryanodine (67 Ci mmol^{-1}) was purchased from Du Pont New England Nuclear (Wilmington, DE, USA). Phosphatidylserine (PS) and phosphatidylethanolamine (PE) were from Avanti Polar Lipids (Birmingham, AL, USA). Acid phosphatase, extracted from potato, was from Calbiochem (San Diego, CA, USA). Bovine heart calmodulin (phosphodiesterase 3′,5′-cyclic nucleotide activator), ATP (adenosine 5′-triphosphate) and AMP-PCP (adenylyl[β,γ-methylene]-diphosphate) were from Sigma. Purified rat brain Ca^{2+}–calmodulin-dependent protein kinase II was a generous gift from Dr Howard Schulman (Stanford, CA, USA).

Preparation of SR

New Zealand adult rabbits were anaesthetized by an intraperitoneal injection of pentobarbitone (100 mg kg^{-1}). Yorkshire adult pigs were anaesthetized by an intramuscular injection of ketamine (10 mg kg^{-1}). For both animals, a sternostomy was performed and the heart was exposed. The aorta was then cross-clamped and cardioplegia solution (mM: 154 NaCl, 20 KCl, 2 $CaCl_2$, 16 $MgCl_2$, 2 EGTA and 5 Na^+–Pipes; pH 7·2) was retrogradely perfused until asystole. The heart was rapidly excised and placed in ice-cold saline solution for isolation of SR.

Heavy SR-enriched microsomes were isolated by differential centrifugation from rabbit or pig heart by modification of a method described previously (Tate, Brick, Chu, VanWinkle & Entman, 1985). Briefly, freshly dissected hearts were drained free of blood in ice-cold saline solution (0·9% NaCl, 10 mM Tris–HCl; pH 6·8), minced to small pieces, weighed, and homogenized with 3 volumes of saline solution in a Waring blender for 2 min at high speed. The saline solution contained the following protease inhibitors: leupeptin (12 μM), phenylmethylsulphonyl fluoride (PMSF; 100 μM), benzamidine (10 μM) and aprotinin (10 μM). The blender homogenate still contained fine lumps of muscle which were thoroughly disintegrated with a Brickmann Polytron (20 μm probe, three times for 15 s each at setting 2). The Polytron homogenate was centrifuged at 4000 *g* for 20 min and the supernatant was filtered through four layers of cheesecloth and further centrifuged at 8000 *g* for 20 min. The 8000 *g* pellet was kept on ice and the supernatant centrifuged at 40 000 *g* for 30 min. The 8000 and 40 000 *g* pellets were resuspended in saline solution containing 0·3 M sucrose, aliquoted, and tested for [^{3}H]ryanodine receptor density. In saturation binding curves, the 40 000 *g* pellet of both pig and rabbit hearts invariably yielded higher B_{max} (maximal receptor density) than the 8000 *g* pellet and was used for all subsequent experiments.

[^{3}H]Ryanodine binding assay

[^{3}H]Ryanodine binding to rabbit and pig cardiac microsomes was carried out as described previously (Valdivia, Hogan & Coronado, 1991*b*; Valdivia, Kirby, Lederer & Coronado, 1992). Briefly, the

standard incubation medium contained 0·2 M KCl, 10 mM Na^+–Hepes (pH 7·2), 1 mM EGTA, and sufficient $CaCl_2$ to set free Ca^{2+} in the range of 1 nM to 100 μM. Ca^{2+}/EGTA ratios were calculated using the stability constants of Fabiato (1981). [^{3}H]Ryanodine was diluted directly into the incubation medium to achieve a final concentration in the saturable range of 1–40 nM. Protein concentration was in the range 0·4–0·6 mg ml^{-1} and was determined by the Bradford method. Unless otherwise indicated, incubations lasted 90 min at 36 °C. Samples (0·1 ml) were always run in duplicate, filtered onto glass fibre filters (Whatman GF/B or GF/C) and washed twice with 5 ml cold water using a Brandel M-24R cell harvester (Gaithersburg, MD, USA). The filters were placed in scintillation vials containing 7 ml of liquid scintillation cocktail, and the retained radioactivity was measured in a Beckman LS-5000 TD β-counter. The specific binding was defined as the difference between the binding in the absence (total binding) and in the presence (non-specific binding) of 10 μM unlabelled ryanodine. Under these conditions, the average value of non-specific binding to the high-affinity site amounted to 10–20% of the total binding of 7 nM [^{3}H]ryanodine. Equilibrium binding data from saturation curves were fitted to a one-site model, and the dissociation constant (K_d) and maximal receptor density (B_{max}) were determined by non-linear regression analysis using the NFIT computer program (Island Products, Galveston, TX, USA).

Planar bilayer technique

A phospholipid bilayer of PE–PS (1 : 1 dissolved in *n*-decane to a final concentration of 20 mg ml^{-1}) was 'painted', with a Teflon rod, across an aperture of ~300 μm diameter in a delrin cup. The *cis* chamber was the voltage control side connected to the head stage of a 200A Axopatch amplifier, while the *trans* side was held at virtual ground. The capacitance of the bilayer was constantly monitored with a +5 mV, 10 ms square pulse. Incorporation of RyRs more often occurred in bilayers of 150–300 pF capacitance. Agar–KCl bridges were used to connect the chambers to Ag–AgCl electrodes immersed in 0·2 M KCl. The *cis* (600 μl) and *trans* (900 μl) chambers were initially filled with 50 mM caesium methanesulphonate and 10 mM Tris–Hepes, pH 7·2. After bilayer formation, an asymmetrical caesium methanesulphonate gradient (300 mM *cis*/50 mM *trans*) was established. A Ca^{2+}–EGTA admixture was then added to both chambers from a ×100 stock solution to a final concentration 0·994 : 1·0 mM, respectively. The calculated free [Ca^{2+}] was 10 μM, which was verified with a Ca^{2+}-sensitive electrode using the Molecular Probes (Eugene, OR, USA) Ca^{2+} calibration kit (range, 1 nM to 1 mM). Addition of CaM (3 μM) alone or in combination with CaMKII (1 μg ml^{-1}), caused negligible changes in free [Ca^{2+}] due to the presence of a comparatively large EGTA concentration. The cardiac microsomes were added to the *cis* chamber, which corresponded to the cytoplasmic side of the SR, while the *trans* side corresponded to the luminal side. After visualization of channels, Cs^+ in the *trans* chamber was raised to 300 mM to collapse the chemical gradient. This manoeuvre greatly improved bilayer stability and prevented further vesicle insertion, thus allowing the manipulation of the same channel(s) over a long time period (20–40 min). For each condition, single channel data were collected at steady voltages (+40 and −40 mV) for 2–5 min. The bilayer was then 'rested' at 0 mV to improve stability while adding reagents or perfusing the *cis* chamber. Channel activity was recorded with a 16-bit VCR-based acquisition and storage system at a 10 kHz sampling rate. Signals were analysed after filtering with an 8-pole Bessel filter at a sampling frequency of 1·5–2 kHz. Data acquisition and analysis were done with Axon Instruments (Burlingame, CA, USA) software and hardware (pCLAMP v 5.7, Digidata 200 AD/DA interface). The mean duration and amplitude of open events were defined by two threshold detectors placed between the baseline and the mean open current. One detector was placed 1 S.D. above the mean of the baseline current, and the second detector was placed at 1 S.D. below the mean current of the open channel. Open and close time intervals were obtained from idealized records. Dwell time distributions were logarithmically binned with equally spaced time intervals (6–8 bins $decade^{-1}$) and fitted to probability density functions by the maximum likelihood method (correlation coefficients > 0·96).

Preparation of AcPh

AcPh is obtained in a crystalline suspension of 3·2 M $(NH_4)_2SO_4$ and 1·0% BSA. This high salt concentration interferes with the binding of [^{3}H]ryanodine to cardiac SR vesicles. To avoid phosphatase-independent effects, the enzyme was dialysed overnight at 4 °C against 1000 volumes of 0·2 M KCl–10 mM Hepes (pH 7·2) in Spectrapore Cellulose Ester dialysis tubing (MW cut-off, 1000) (Spectrum, Houston, TX, USA). Dialysis equilibrium was checked with a vapour pressure osmometer (Wescor Inc., Logan, UT, USA).

All values are given as the mean ± S.E.M.

RESULTS

There is generalized agreement that [^{3}H]ryanodine, at nanomolar concentrations, binds with high affinity to the open conformational state of the RyR (Buck, Zimany, Abramson & Pessah, 1992; Meissner & El-Hashem, 1992). This conclusion stems from the fact that ligands which stimulate Ca^{2+} release from SR (micromolar Ca^{2+}, ATP, caffeine) also stimulate [^{3}H]ryanodine binding, whereas ligands that inhibit Ca^{2+} release (nanomolar Ca^{2+}, Ruthenium Red, Mg^{2+}) also inhibit [^{3}H]ryanodine binding (Pessah, Stambuk & Casida, 1987; Lattanzio, Schlatter, Nikar, Campbell & Sutko, 1987; Michalak, Dupraz & Shoshan-Bartmatz, 1988). Thus under well-defined conditions of ligand concentration and receptor site density, the [^{3}H]ryanodine binding assay may be used as an index of RyR channel gating (Valdivia, Fuentes, El-Hayek, Morrissette & Coronado, 1991*a*; Meissner & El-Hashem, 1992).

Nucleotide-stimulated effect is independent of protein kinase cofactors

Several reports have suggested that endogenous protein kinases remain associated to junctional SR after muscle homogenization (Seiler, Wegener, Whang, Hathaway & Jones, 1984; Chu, Sumbilla, Inesi, Jay & Campbell, 1990; Hohenegger & Suko, 1993). To test the potential contribution of endogenous PKA, PKC, and CaMKII to [^{3}H]ryanodine binding, we supplemented the incubation medium with 1 mM MgATP and the cofactors necessary for activation of each of these kinases, i.e. cyclic adenosine monophosphate (cAMP), the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and CaM. cAMP, TPA, and CaM were also added to a separate set of samples containing 1 mM of the non-hydrolysable ATP analogue, AMP-PCP, which interacts directly with ryanodine receptors (Meissner, 1986), but is a poor substrate for

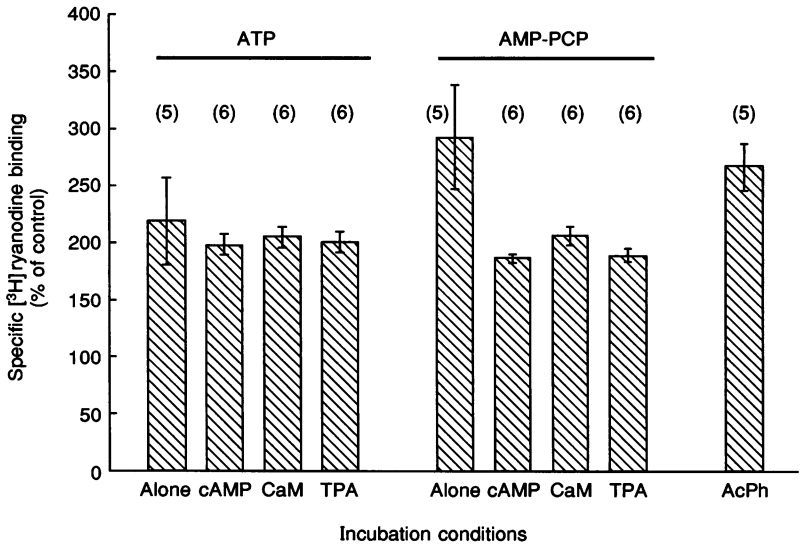

Figure 1. Effect of protein kinase cofactors on nucleotide-stimulated [^{3}H]ryanodine binding

[^{3}H]Ryanodine (7 nM) was incubated at 36 °C with 60 μg rabbit cardiac SR in 0·2 M KCl, 1 mM Na_2EGTA, 10 mM Na^+–Hepes (pH 7·2) and 0·994 mM $CaCl_2$ (incubation medium). The free [Ca^{2+}] was 10 μM. The incubation volume was 100 μl. At time 0, 10 μl of incubation medium containing MgATP or MgAMP-PCP plus indicated protein kinase cofactors was added and the reaction stopped after 90 min. The final concentration of nucleotides and cofactors is indicated in the text. AcPh was extensively dialysed in incubation medium as described in Methods and added in the absence of nucleotides. Non-specific binding, determined in the presence of 10 μM unlabelled ryanodine, has been subtracted in all graphs. Numbers in parentheses indicate the number of independent measurements conducted in duplicate and the means ± S.E.M. are plotted.

protein kinases. The standard incubation medium consisted of 0·2 M KCl, 1 mM EGTA, 0·994 mM $CaCl_2$ (free [Ca^{2+}], 10 μM), 10 mM Hepes (pH 7·2), 7 nM [^{3}H]ryanodine, and 0·6 mg ml^{-1} of cardiac SR protein. Assays were carried out at a [^{3}H]ryanodine concentration approximately equal to the K_d of the [^{3}H]ryanodine receptor complex (Imagawa, Smith, Coronado & Campbell, 1987; Valdivia *et al.* 1991*b*; see also Fig. 5) to ensure a relatively linear relationship between the number of occupied receptors and the concentration of free ligand. Under these conditions, we could clearly distinguish between the effect of an activator from that of an inhibitor. Figure 1 shows that ATP increased binding to 218 ± 38% of control. There was a small but statistically insignificant decrease in binding

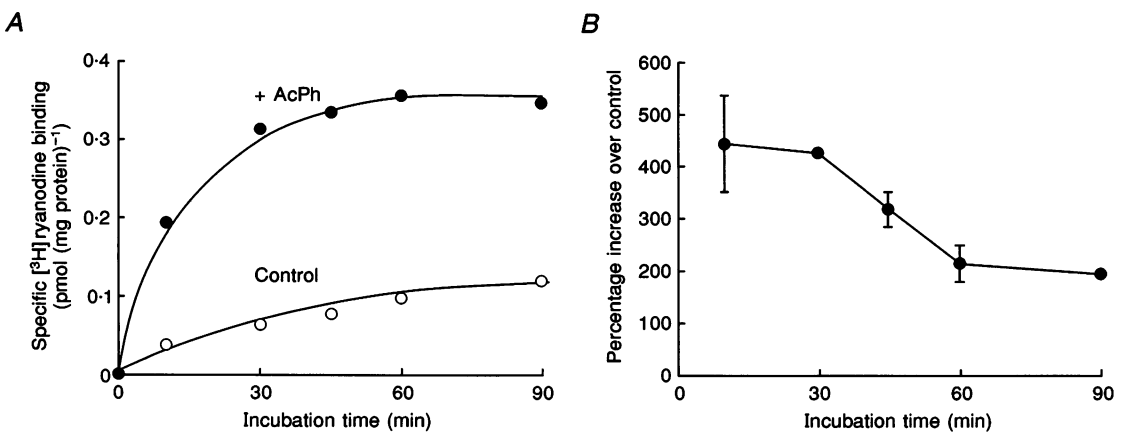

Figure 2. Time course of AcPh effect

A, the specific binding of [^{3}H]ryanodine was measured as described in Methods and Fig. 1 at indicated times in the absence (Control) and in the continuous presence of 10 U ml^{-1} AcPh (+ AcPh). *B*, the AcPh-stimulated binding from four experiments was pooled and plotted as a percentage of increase over control.

when ATP was combined with 20 μM cAMP (197 ± 9%), or 100 nM TPA (205 ± 8%), or 5 μM CaM (200 ± 9%). Thus under conditions favourable for phosphorylation, no major changes are detected in binding density. On the other hand, AMP-PCP increased binding to 293 ± 45% of control. Binding decreased slighty when AMP-PCP was combined with cAMP (187 ± 4%), or TPA (207 ± 9%), or CaM (190 ± 6%). Since cAMP, TPA, and CaM produced the same effect under conditions permissive and non-permissive for channel phosphorylation, the stimulation of binding by ATP and AMP-PCP may be ascribed to a direct effect of the nucleotides on RyRs as observed previously by others.

Stimulation of ryanodine receptors by AcPh

A stimulation of [^{3}H]ryanodine binding, comparable to that induced by the nucleotides, was obtained with acid phosphatase (AcPh), a serine/threonine phosphatase that affects a broad spectrum of protein substrates (Papavassiliou & Bowman, 1992). Figure 1 shows that 15 U ml^{-1} AcPh increased the binding of [^{3}H]ryanodine to 268 ± 20% over control. Figure 2*A* shows the time course of the effect of AcPh (10 U ml^{-1}). The stimulation of binding by AcPh was evident at early times of incubation and reached a plateau at ~60 min. Figure 2*B* shows that the percentage increment of binding actually decreases as the incubation progresses. The percentage increase over control induced by AcPh was 443 ± 80, 425 ± 15, 317 ± 20, 215 ± 15 and 196 ± 8% at 10, 30, 45, 60 and 90 min, respectively ($n = 4$). Poor binding signals due to intrinsically slow association kinetics of [^{3}H]ryanodine precluded the accurate estimation of AcPh effects at earlier times, but the substantial effect detected at 10 min suggested a fast activation of RyRs.

To rule out the possibility of a contaminant in the AcPh solution as being responsible for the increase in [^{3}H]ryanodine binding, we incubated SR vesicles without (control) and with 15 U ml^{-1} of AcPh for 20 min at 25 °C. Vesicles were then washed free of AcPh and incubated with [^{3}H]ryanodine for different times. Without further phosphorylation, the stimulating effect of AcPh is expected to persist for prolonged times while that of a contaminant would be eliminated with the washing. Figure 3 shows that AcPh-pretreated vesicles consistently showed higher [^{3}H]ryanodine binding than control vesicles. The percentage increase over control was similar to that obtained in vesicles incubated in the continuous presence of AcPh (Fig. 2*B*). Thus the stimulatory effect of AcPh persisted even in the absence of AcPh itself. Assuming that the [^{3}H]ryanodine binding assay followed changes in channel gating, the stimulatory effect of AcPh suggests that the enzyme dephosphorylated the RyR or a closely associated regulatory protein and increased the channel open probability. A direct demonstration of such an effect by AcPh has been documented using skinned skeletal muscle fibres (Wang & Best, 1992). Our results imply that cardiac RyRs are also effective substrates for AcPh.

A dose–response relationship (not shown) was also constructed to determine the effective range over which AcPh was able to increase [^{3}H]ryanodine binding. After 90 min of incubating SR vesicles with 7 nM [^{3}H]ryanodine in standard incubation medium (pCa 5) in the absence and presence of AcPh, little effect was detected at 0·1 U ml^{-1} AcPh. As the AcPh concentration was increased beyond 1·0 U ml^{-1}, the stimulatory effect on binding became

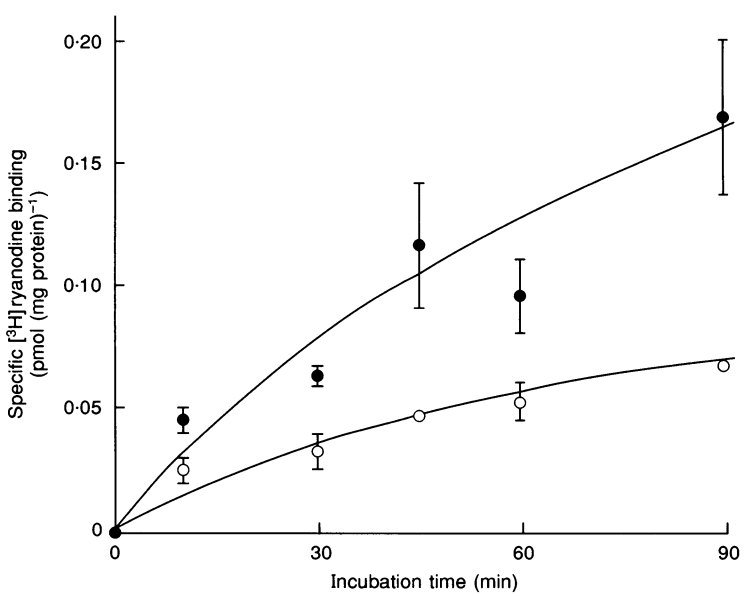

Figure 3. Pretreatment of SR vesicles with AcPh increases [^{3}H]ryanodine binding

SR vesicles were incubated with 10 U ml^{-1} of AcPh (●), or without AcPh (○) for 20 min at 25 °C. Vesicles were then centrifuged at 5200 *g* for 10 min and resuspended in standard incubation medium. This wash was repeated twice. At time 0, 7 nM [^{3}H]ryanodine was added and binding measured at the indicated times. Non-specific binding was similar in both sets of vesicles.

apparent. Briefly, 10 U ml^{-1} AcPh induced an increment of binding (195 ± 10% of control) ($n = 3$) while 30 U ml^{-1} induced a slightly higher increment of binding (225 ± 18% of control). These concentrations of AcPh are similiar to those required for activation of skeletal RyRs (Wang & Best, 1992) and are in the typical range of concentrations achieved after dialysing overnight to remove the storage buffer.

An open conformational state of the RyR is required for AcPh effect

In cardiac muscle, CICR may be smoothly graded by the influx of external Ca^{2+} (Cannel, Berlin & Lederer, 1987; Beuckelmann & Wier, 1988). In rabbit cardiac SR vesicles, Ca^{2+} was critical for the binding of the alkaloid to the receptor and the Ca^{2+} dependence of [^{3}H]ryanodine binding was also graded. Figure 4 shows the Ca^{2+} dependence of [^{3}H]ryanodine binding and the effect of AcPh. Specific binding in the absence of AcPh (control, ○) had a threshold for detection at 100 nM [Ca^{2+}] (pCa 7) and was maximal at 100 μM [Ca^{2+}]. Thus the binding curve shows a smooth and graded dependence of [Ca^{2+}] just as photolysis-dependent increases in [Ca^{2+}] smoothly activate contraction (Niggli & Lederer, 1990). Binding of [^{3}H]ryanodine in the presence of 15 U ml^{-1} of AcPh (●) had a threshold for detection equal to control, but increased as [Ca^{2+}] in the incubation medium increased. At Ca^{2+} concentrations of 1, 10 and 100 μM, the AcPh-stimulated binding was 0·046 ± 0·02, 0·135 ± 0·02 and 0·109 ± 0·03 pmol mg^{-1} ($n = 4$), respectively (shaded area), i.e. binding was 125, 205 and 170% of control. Thus the augmentation of [^{3}H]ryanodine binding produced by AcPh was dependent on [Ca^{2+}]. However, the Ca^{2+} requirement of activation could have resulted from two different processes. One is the Ca^{2+}-dependent formation of a Ca^{2+}–AcPh complex that would activate the enzyme; the second is the Ca^{2+}-dependent formation of the RyR conformational state on which AcPh acts. To discern between these two possible processes, we bound [^{3}H]ryanodine at pCa 7 and tested the effect of AcPh in the absence and the presence of caffeine and AMP-PCP. Both agonists directly open RyRs by increasing the sensitivity of the receptor to Ca^{2+} (Valdivia *et al.* 1991*b*). Binding was 0·016 ± 0·004 pmol mg^{-1} ($n = 3$) in the absence of AcPh and remained essentially unchanged in the presence of 10 U ml^{-1} AcPh (0·013 ± 0·002 pmol mg^{-1}). Binding increased to 0·062 ± 0·002 in the presence of 10 mM caffeine and to 0·068 ± 0·005 in the presence of 5 mM AMP-PCP. When AcPh was combined with either agonist, a synergistic effect was observed. Binding was 0·191 ± 0·04 and 0·231 ± 0·05 pmol mg^{-1} in the presence of AcPh plus caffeine and AcPh plus AMP-PCP, respectively. Since at low [Ca^{2+}] the effect of AcPh was apparent in the presence of modulators that drive the channel open, these results suggested that AcPh required an open RyR on which to exert its enzymatic effect.

AcPh increases K_d without affecting B_{max}

To determine if the stimulation by AcPh resulted from higher binding affinity or recruitment of additional receptor sites, we carried out saturation binding curves for

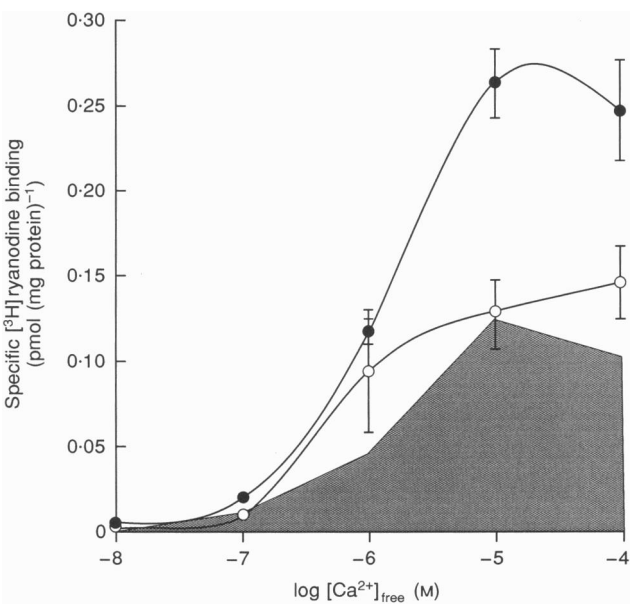

Figure 4. Ca^{2+} dependence of the AcPh effect

[^{3}H]Ryanodine (7 nM) was incubated for 90 min at 36 °C with 60 μg of cardiac SR in incubation medium containing Ca^{2+}–EGTA admixtures set to yield the indicated concentration of free Ca^{2+}. ○, specific binding of [^{3}H]ryanodine in the absence of AcPh; ●, specific binding in the presence of 15 U ml^{-1} AcPh added at the beginning of the incubation period. The shaded area represents the AcPh-stimulated binding. It is defined as the difference, in pmol (mg protein)$^{-1}$, of binding in the presence of AcPh minus binding in control.

[^{3}H]ryanodine in the absence and presence of AcPh (Fig. 5*A*). In the absence of AcPh, [^{3}H]ryanodine saturated a single class of non-interacting binding sites with a maximal density (B_{max}) of 1·45 ± 0·04 pmol mg^{-1} and apparent affinity constant (K_d) of 9·87 ± 0·85 nM ($n = 5$; Fig. 5*B*). In the presence of 10 U ml^{-1} AcPh, B_{max} remained unchanged (1·47 ± 0·02 pmol mg^{-1}) but the K_d decreased to 3·9 ± 0·65 nM, i.e. an almost 3-fold increase in affinity. The selective increment of K_d by AcPh is expressed in the binding isotherm as high stimulation of binding at low [^{3}H]ryanodine concentrations and almost null effect at saturating concentrations. The lack of AcPh effect on B_{max} indicates that AcPh stimulates binding by exclusively increasing the affinity of receptors to [^{3}H]ryanodine without making available an additional pool of binding sites. This effect is similar to that of caffeine (Valdivia *et al.* 1991*b*) or ATP (Pessah *et al.* 1987) and is reflected in single channel recordings of RyRs as an increase in channel open probability.

Direct effect of AcPh on RyRs

The latter conclusion was independently confirmed by testing the effect of AcPh on RyRs reconstituted in lipid bilayers in the absence of ryanodine. Incorporation of channels was assisted as previously described (Valdivia *et al.* 1991*b*, 1992). Solutions in both chambers bathing the planar bilayer were 300 mM caesium methanesulphonate, 10 μM free Ca^{2+} (1 mM EGTA and 0·994 mM $CaCl_2$), and 10 mM Tris–Hepes (pH 7·2). Cs^+, instead of Ca^{2+}, was chosen as the charge carrier to precisely control [Ca^{2+}] around the channel, to increase the channel conductance ($g_{Cs^+}/g_{Ca^{2+}} = 2$; Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1987) and to avoid interference from K^+ channels present in the SR membrane. Cl^- channels were blocked by replacing chloride with the impermeant anion methanesulphonate. Figure 6*A* shows traces from continuous recordings at a holding potential of −40 mV in the absence and presence of 6 U ml^{-1} AcPh. Channel activity was monitored over 80 s in each condition and had a mean open probability (P_o) of 0·203 ± 0·07 in control which increased to 0·616 ± 0·12 following AcPh addition ($n = 3$). The most significant kinetic effects of AcPh were an increase in the burst time (Fig. 6*B*) and an increase in the mean open time (Fig. 6*C*). No changes in unitary channel conductance or mean closed time were detected. As indicated by the open time histogram (Fig. 6*C*), AcPh produced a significant increase in the number of long openings which resulted in sweeps with high P_o (Fig. 6*B*). The length of each bar in Fig. 6*B* represents P_o during consecutive sweeps. Each sweep had a duration of 1 s. Empty spaces corresponded to null sweeps in which no openings were seen. Successive sweeps containing long openings gave rise to bursts (Fig. 6*B*, control) which were substantially increased by AcPh (Fig. 6*B*, + AcPh). Figure 6*C* shows the open time histogram of control (open area) overlapped on that of AcPh (shaded area). The best fit to the histograms could be accomplished by using single and double exponential models (see below). The presence of ultrafast openings was likely (Tinker, Lindsay & Williams, 1992), but our temporal resolution limited the detection of open events to those with durations of 0·66 ms and longer. In control, 1538 events collected during 80 s were fitted by a single exponential, τ_{open1}, of 1·32 ms. After addition of AcPh, the number of events collected during a similar period increased to 2529 and τ_{open1} increased to 2·43 ms. More striking was

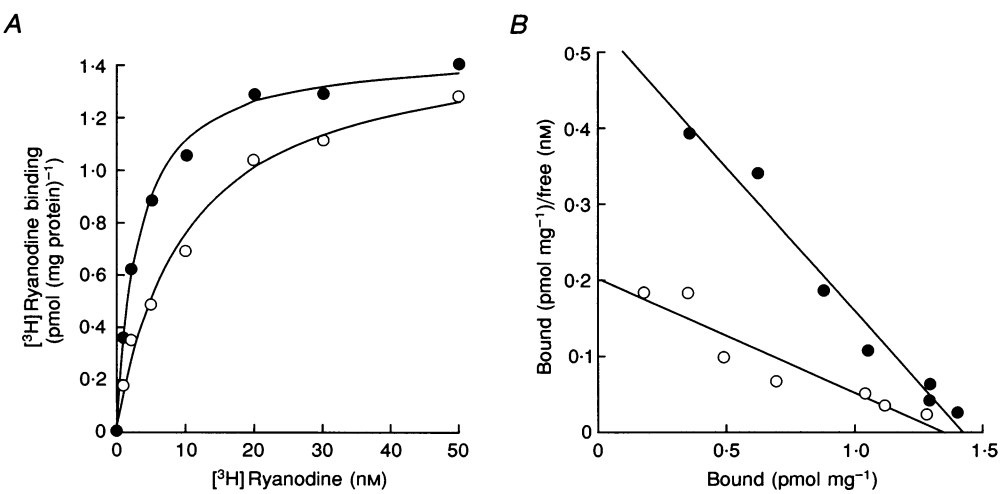

Figure 5. Saturation binding curves of [^{3}H]ryanodine in the absence (○) and the presence (●) of 10 U ml^{-1} AcPh

Indicated concentrations of [^{3}H]ryanodine were incubated with 60 μg of cardiac SR in incubation medium as described in Methods. Data points were fitted with the computer program NFIT using the equation Bound = $B_{max} \times [L]/([L] + K_d)$, where [L] represents the concentration of [^{3}H]ryanodine. *B*, Scatchard plot of specific binding. K_d values of the apparent dissociation constant for the [^{3}H]ryanodine–receptor complex, and B_{max}, the maximal density of binding sites, are given in the text.

the appearance of a longer time constant (τ_{open2}) of 10·97 ms that was virtually absent in control. The contribution of τ_{open2} to the total number of openings was 21%. Similar activation was seen when 5 mM Ca^{2+} was added to the *trans* chamber to establish a Ca^{2+} gradient equivalent to that present across the SR membrane during constant Ca^{2+} pump activity. We thus concluded that AcPh stabilized the RyR in an open conformational state. This activating effect would explain the increased affinity of the receptor for [^{3}H]ryanodine in the presence of AcPh because open channels have a much higher affinity for the alkaloid than closed channels.

Inhibition of [^{3}H]ryanodine binding by CaMKII

The modulation of RyR activity by AcPh suggests that in the intracellular environment there is a balance of influences by protein kinases on the RyR that leads to a low P_o of RyRs. The low opening rate of the RyR suggested by the measurement of 'Ca^{2+} sparks' in intact rat heart cells (Cheng, Cannel & Lederer, 1993) is consistent with this possibility and our results below. Several studies have shown that RyRs are major substrates for CaMKII (Witcher *et al.* 1991; Takasago *et al.* 1991; Witcher, Strifler & Jones, 1992). We tested the functional impact of CaMKII phosphorylation

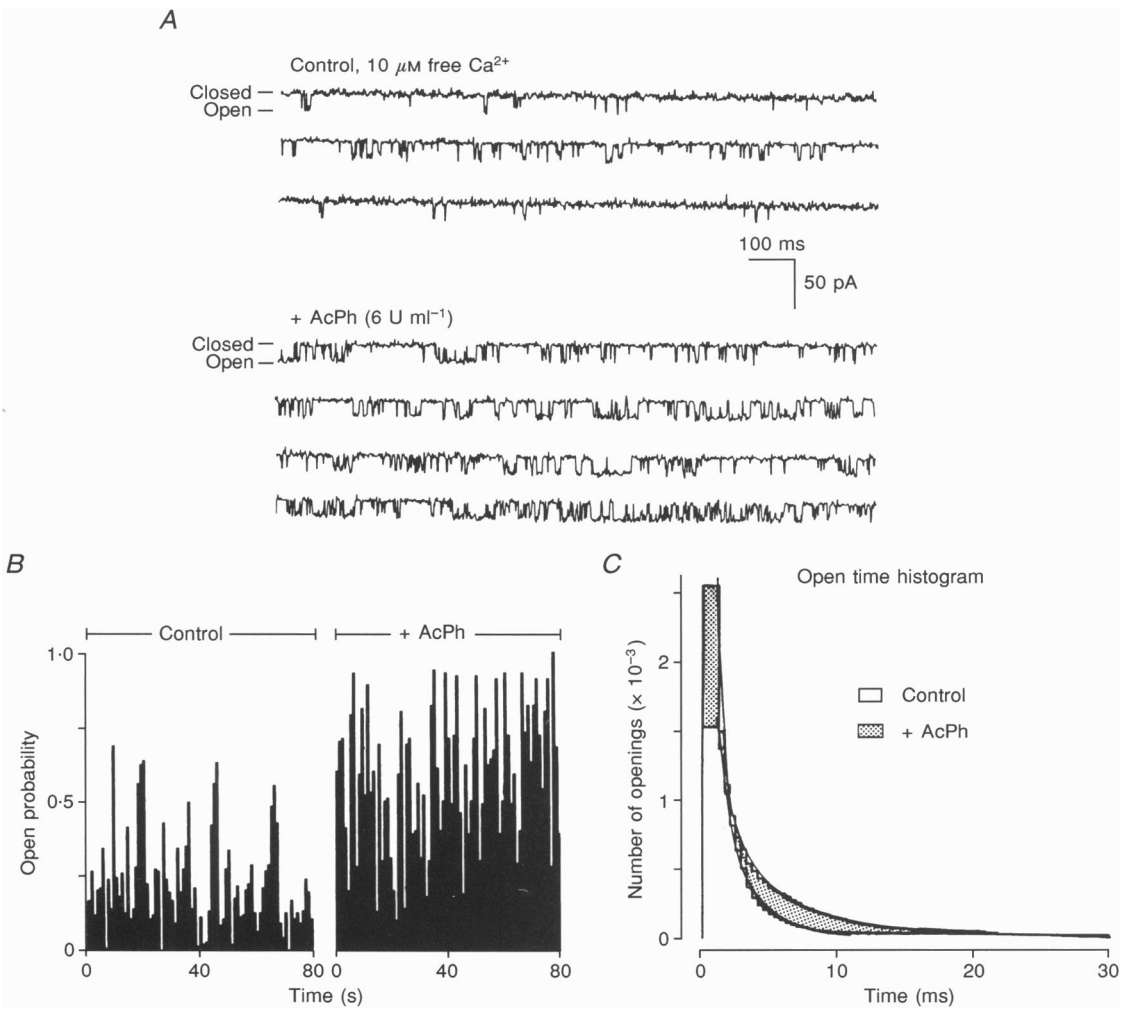

Figure 6. Activation of RyR by AcPh

A, pig cardiac SR vesicles were incorporated in planar lipid bilayers as described in Methods. Single channel currents, shown as downward deflections, were recorded at holding potential −40 mV in symmetrical 300 mM caesium methanesulphonate, 10 mM Na^+–Hepes (pH 7·2), 1 mM EGTA and 0·994 mM $CaCl_2$. The calculated free [Ca^{2+}] was 10 μM, which was sufficient to elicit RyR activity, as shown in control recordings. Bottom traces: same channel after *cis* addition of 6 U ml^{-1} of acid phosphatase. Recordings were filtered at 1·5 kHz and digitized at 3 kHz. *B*, 80 s of continuous records in control and after addition of AcPh were divided into intervals of 1 s; P_o in each interval is plotted as a bar of length 0–1. Average P_o for the channel shown was 0·241 in control and 0·689 after addition of AcPh. *C*, cumulative open time histogram. Number of open events of duration time, *t*, or longer are plotted as a function of open time, *t*. Number of observations collected during an 80 s period were 1538 (Control) or 2529 (+ AcPh).

on RyR. Figure 7*A* shows the effect of purified CaMKII on the binding of [^{3}H]ryanodine to cardiac SR vesicles. Our standard incubation medium was supplemented with 1 mM MgATP and the binding activity was measured at equilibrium (90 min incubation at 36 °C). Under these conditions, specific binding was 0·389 ± 0·034 pmol mg^{-1} ($n = 3$) (Fig. 7*A*). Addition of 2 μM CaM at the beginning of the reaction reduced specific binding to 0·318 ± 0·031 pmol mg^{-1} by a mechanism previously demonstrated to be phosphorylation independent (see Fig. 1 and Fuentes, Valdivia, Vaughan, Coronado & Valdivia, 1994). In the combined presence of MgATP and CaM, purified CaMKII (0·3 μg per reaction tube) produced an additional inhibition of [^{3}H]ryanodine binding activity (0·15 ± 0·02 pmol mg^{-1}). Figure 7*B* shows the time course of the effect of CaMKII. CaMKII decreased binding at every time tested and the inhibition increased as the incubation progressed. The percentage of inhibition by CaMKII relative to 'control' (defined as the specific binding obtained in the presence of MgATP and CaM) was 13 ± 4, 23 ± 6 and 45 ± 7% at 5, 40 and 90 min, respectively. Longer periods of incubation, however, failed to result in any further inhibition.

CaMKII induces the appearance of a longer closed state

The kinetic consequences of CaMKII incubations are shown in Fig. 8. Figure 8*A* shows the activity of a single RyR at −40 mV in the presence of 10 μM free [Ca^{2+}] and 1 mM MgATP, and the same channel after successive additions of 3 μM CaM and 1 μg ml^{-1} of CaMKII. At this concentration, which was the highest tested in [^{3}H]ryanodine binding experiments, CaMKII produced a dramatic decrease in P_o.

To describe the kinetic changes and the onset of CaMKII-induced effect, channels were monitored for relatively long periods (100–200 s) during control and after addition of modulators. The data showed that the decrease in P_o caused by CaMKII was due to a combination of a conspicuous increase in the total time the channel spent closed and an increase in the mean closed time. Both effects are clear in the open and closed time histograms of Fig. 8*B* and the diary of events of Fig. 8*C*. Under control conditions, defined as the activity in the presence of Ca^{2+}, MgATP, and CaM (Fig. 8*A*, middle traces), open event histograms could be fitted with a single exponential, τ_{open1}, with value of 1·32 ms (2037 events). One-and-a-half minutes after addition of CaMKII, open events could still be fitted with a single exponential of 1·19 ms (1391 events, Fig. 8*B*, shaded area). Thus CaMKII did not change the mean open time. On the other hand, 1334 closed events in control were monoexponential with $\tau_{close1} = 8{\cdot}3$ ms (Fig. 8*B*, bottom panel). After addition of CaMKII, 552 events collected over 60 s were best fitted with two exponentials of values, $\tau_{close1} = 9{\cdot}4$ ms and $\tau_{close2} = 32{\cdot}5$ ms. Number of events were normalized in the closed time histogram of Fig. 8*B* to display fully the second closing component. Figure 8*C* summarizes the channel activity during the 60–80 s covered by each analysis. The bars of length 0 to 1 indicate P_o on a sweep-by-sweep basis (1 s sweep^{-1}). There was a steady activity in control and in the presence of CaM that, after addition of CaMKII, was interrupted by the random appearance of clusters of sweeps with no activity. Events recorded during the first 90 s after addition of CaMKII showed no difference to those recorded in the presence of CaM (not shown). Indeed, the average lag time for CaMKII effect was ~1–2 min ($n = 3$). In summary, the most significant kinetic effects of CaMKII were an increase in mean closed time and a reduction in the total number of events per unit time. Thus the effects of CaMKII are clear

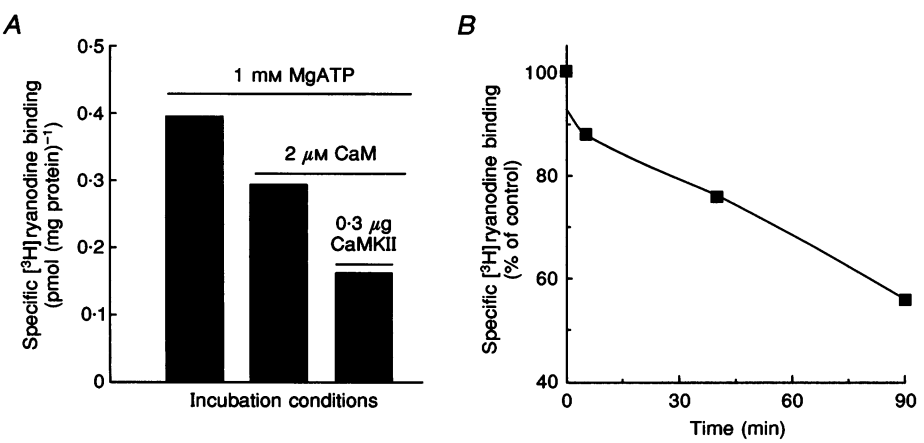

Figure 7. Inhibition of [^{3}H]ryanodine binding by CaM and CaMKII

A, [^{3}H]ryanodine was dissolved to a final concentration of 7 nM in 100 μl of incubation medium containing 10 μM free [Ca^{2+}]. MgATP, CaM and CaMKII were added to the reaction tubes from ×100 stock solutions. Incubation time was 90 min. *B*, time course of CaMKII inhibition of binding. The incubation medium contained 1 mM MgATP and 2 μM CaM. The reaction was stopped at the indicated times and the specific binding in the absence of CaMKII (control) was normalized to 100%.

and could occur by modification of a single kinetic step (see Discussion). The average P_o computed from three separate recordings showed a consistent change from $P_o = 0{\cdot}318 \pm 0{\cdot}071$ in control to $0{\cdot}212 \pm 0{\cdot}045$ in the presence of CaM, to $0{\cdot}053 \pm 0{\cdot}031$ after addition of CaMKII. We also detected an inhibitory effect of CaMKII (0·3 μg ml^{-1}) on [^{3}H]ryanodine binding to RyR purified by the sucrose gradient procedure (Lai, Erickson, Rousseau, Liu & Meissner, 1988; Fuentes *et al.* 1994), from 180 to 110 pmol mg^{-1} (mean of two independent experiments).

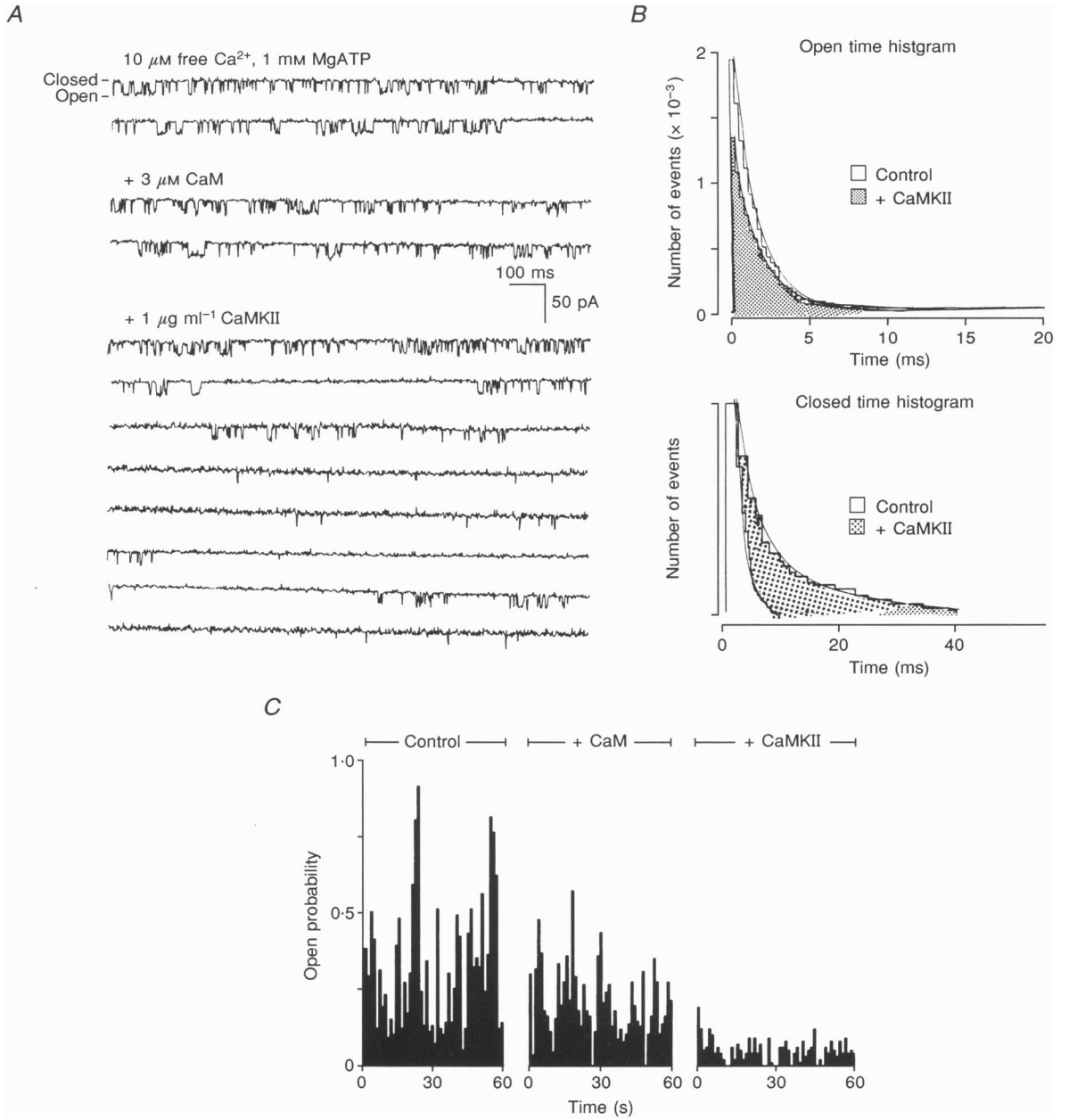

Figure 8. Inhibition of RyRs by CaMKII

A, top traces: single RyR channel activity recorded at a holding potential of −40 mV in the presence of *cis* 10 μM free Ca^{2+} and 1 mM MgATP. Recording solutions were the same as described in the legend to Fig. 6. Middle traces: the same channel recorded 2 min after *cis* addition of 3 μM CaM. Bottom traces: continuous recording of the same channel taken 1·5 min after *cis* addition of 1 μg ml^{-1} CaMKII. The first trace shows gating characteristics similar to those in the presence of CaM and was included to show the transition to the CaMKII-induced gating mode. *B*, open and closed time histograms. Events of duration time t or longer are plotted as a function of time, t. Number of events and fitting values are described in the text. *C*, diary of openings for the complete period of analysis. Continuous records in control and after addition of CaM and CaMKII were divided into 1 s intervals and P_o in each interval is plotted as a bar of length 0–1. Breaks in the horizontal axis represent pauses for addition and mixing of CaM and CaMKII. Average P_o for each condition was 0·351 (Control), 0·232 (+ CaM) and 0·036 (+ CaMKII).

Thus we interpret the changes of RyR kinetics as the result of direct phosphorylation of the RyR protein by CaMKII. Two critical experimental controls ensured that the exogenously added CaMKII specifically reduced RyR activity by a phosphorylation mechanism: (i) addition of 10 μM of a synthetic peptide inhibitor of CaMKII (residues 290–309 of the CaMKII autophosphorylation domain sequence) to the phosphorylation cocktail prevented the effect of CaMKII; and (ii) replacement of MgATP with an equimolar concentration of AMP-PCP abolished the inhibitory effect by CaMKII (not shown).

Activation of CaMKII-phosphorylated channels by AcPh

To determine if the kinetic changes induced by CaMKII could be effectively reversed by AcPh, experimental conditions were initially set that presumably enable CAMKII to phosphorylate RyRs. After a period of recording in these conditions, AcPh was mixed into the *cis* chamber. Figure 9 shows representative traces from a RyR recorded during control (*cis* 8 μM Ca^{2+}, 1 mM MgATP and 3 μM CaM) and the same channel after successive application of CaMKII and AcPh. CaMKII produced the kinetic changes described above that culminated in a marked decrease of channel activity from $P_o = 0{\cdot}183$ in control to $P_o = 0{\cdot}039$ in the presence of 1 μg ml^{-1} CaMKII. Again, the onset of CaMKII effect was ~1 min. Extensive washout of CaMKII with the bath solution resulted in persistent blockade of channel activity (middle traces). P_o after perfusion was 0·042. Remarkably, addition of 10 U ml^{-1} AcPh (bottom traces) reverted P_o to a level that was above the initial control P_o, producing again the appearance of a new time constant (τ_{open2}) and increasing the number of events per unit time. Thirty seconds after addition of AcPh, P_o averaged 0·640 and the histogram of 1194 opening events recorded during a 80 s time period was best fitted with two exponentials of 2·15 and 11·39 ms (not shown). The mean P_o ($\pm$ S.D.) values of three RyRs recorded separately were 0·211 $\pm$ 0·06 (control), 0·046 $\pm$ 0·02 (+ CaMKII) and 0·58 $\pm$ 0·12 (+ AcPh).

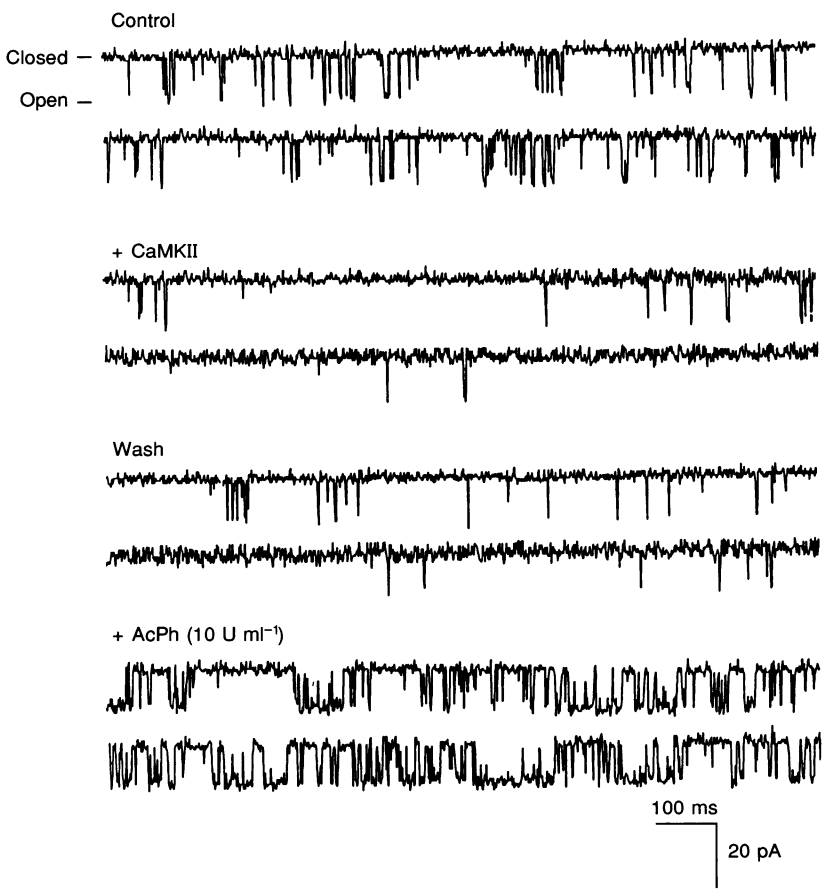

Figure 9. Inhibition of RyRs by CaMKII and activation by AcPh

Recording solutions were similar to those described in the legend to Fig. 6 except that the *cis* chamber also contained 1 mM MgATP and 3 mM CaM (control solution). The calculated free [Ca^{2+}] was 8 μM. Middle traces were recorded 2 min after addition of 1 mg ml^{-1} CaMKII to the *cis* chamber (labelled + CaMKII) and immediately after perfusion of the *cis* chamber with control solution (Wash). Bottom traces show the effect of *cis* 10 U ml^{-1} AcPh. P_o for each condition was 0·183 (Control), 0·039 (+ CaMKII), 0·042 (Wash) and 0·640 (+ AcPh). All traces correspond to the same channel.

DISCUSSION

We have shown that dephosphorylation of the RyR or of a closely associated regulatory protein by AcPh increases channel activity while phosphorylation by CaMKII inhibits it. An important implication of these findings is that phosphorylation–dephosphorylation of RyRs is a relevant mechanism for regulating SR Ca^{2+} release and maintaining Ca^{2+} homeostasis in cardiac myocytes. The results also point out the need to establish the phosphorylated state of the RyR when it is being characterized by detailed physiological and biophysical experiments using planar lipid bilayers, [^{3}H]ryanodine binding assays, and whole-cell studies.

Phosphorylation–dephosphorylation modulates the RyR response to Ca^{2+}

We found a strong stimulating effect of AcPh on [^{3}H]ryanodine binding that was paralleled with an increase of RyR channel activity. A stimulating effect by AcPh on RyRs had been previously observed by Wang & Best (1992) in frog skeletal muscle. In their study, dephosphorylation by AcPh increased RyR activity in excised patches of SR membranes and this effect could be reversed by activation of an endogenous CaMKII. Based on these observations, the authors raised the interesting possibility that a phosphorylation–dephosphorylation reaction could provide the molecular basis for activation–inactivation of Ca^{2+} release in skeletal muscle. In cardiac muscle, Ca^{2+} influx via sarcolemmal Ca^{2+} channels opens RyRs (Beuckelmann & Wier, 1988; Näbauer, Callewart, Cleeman & Morad, 1989; Niggli & Lederer, 1990) but the mechanism that inactivates RyRs and counters the intrinsically positive feedback of the CICR process is yet to be determined. By analogy to the results of Wang & Best (1992), it is tempting to propose that phosphorylation of RyRs inactivates Ca^{2+} release in heart. However, although we observed a qualitatively similar modulatory effect of AcPh and CaMKII on cardiac RyRs, the quantitative and Ca^{2+}-dependent response suggests a more limited participation of the phosphorylation reaction. In our hands, CaMKII was incapable of inducing a total inactivation of RyRs (Figs 8 and 9) and AcPh could not act as the sole activator of [^{3}H]ryanodine binding (Fig. 4). Moreover, the Ca^{2+} dependence of AcPh and CaMKII effect indicates that an increase in $[Ca^{2+}]_i$ must occur before modulation of channel activity by AcPh and CaMKII may occur. Therefore, a direct control of RyR gating by Ca^{2+}, modulated by the phosphorylation state of the channel, would be a more plausible mechanism for activation and possible inactivation of SR Ca^{2+} release in heart. Once phosphorylation occurs in a cellular environment, the net effect would be a change in the 'sensitivity' of the RyR to be activated by Ca^{2+} and a change in the SR Ca^{2+} efflux associated with activation.

Kinetic effects of AcPh and CaMKII

The modification of single channel kinetics by AcPh encompassed an increase in the frequency of events per unit time and the appearance of a second, longer open state (Fig. 4). Both effects resulted in an increase in P_o. This activation mechanism is consistent with the following simple scheme in which the channel gates normally in a two-state model, from:

$$C_1 \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O_1, \quad (1)$$

where C_1 and O_1 represent closed and open states of the channel and α and β represent steady-state rate constants obtained under a defined concentration of Ca^{2+} and other relevant modulators of channel activity, to:

$$C_1 \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O_1 \underset{\beta'}{\overset{\alpha'}{\rightleftharpoons}} O_2, \quad (2)$$

where α' and β' represent rate constants governing open and close transitions to and from the AcPh-stimulated open state O_2 (Fig. 6).

Following the same argument, the inhibition of P_o by CaMKII, which at the single channel level arose from the appearance of a second, longer closed state and a decrease in the frequency of open events per unit time, may be explained by transitions from scheme 1 above to:

$$C_2 \underset{\beta''}{\overset{\alpha''}{\rightleftharpoons}} C_1 \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O_1, \quad (3)$$

where α'' and β'' represent rate constants governing open and close transitions to and from the CaMKII-induced state C_2 (Fig. 8).

Accordingly, we expected that treatment of CaMKII-phosphorylated channels with AcPh (Fig. 9) would simply eliminate the CaMKII-promoted state C_2 in scheme 3 above to the initial gating mechanism (scheme 1). This transition would be compatible with dephosphorylation by AcPh of a CaMKII-phosphorylated channel. Instead, we found that AcPh not only removed C_2 but induced the appearance of O_2 that drove P_o to a value that was higher than the initial control P_o. Such mechanism of action is compatible with the hypothesis that AcPh acted on two different types of phosphorylation sites, one phosphorylated by the exogenously added CaMKII, and the second phosphorylated by an endogenous kinase. Since the effect of AcPh on native SR did not require pre-phosphorylation of RyRs, this suggests that AcPh acted on RyRs or a closely associated regulatory protein that remained phosphorylated through the isolation of SR. Two lines of experimental data support the contention that the population of RyRs used in this study were phosphorylated before our interventions began. First, AcPh was capable of increasing P_o of RyRs present in native, non-protein kinase-treated SR vesicles (Fig. 6); second, AcPh increased [^{3}H]ryanodine binding by increasing K_d while leaving B_{max} unchanged (Fig. 5), indicating that essentially the whole population of receptors was available as substrate for AcPh. It is conceivable then that the removal of C_2 and the

appearence of O_2 represent the combined effect of AcPh acting on two different phosphorylation sites.

Comparison with other results

An activating effect of CaMKII on Ca^{2+} release channels has been described in canine SR (Witcher *et al.* 1991). In their study, 1 μg of CaMKII could open RyRs from $P_o \approx 0{\cdot}15$ to $P_o \approx 1{\cdot}0$ with a lag time of ~1 min at a cytosolic free Ca^{2+} of ~10 μM. These results differ substantially from those presented in Figs 8 and 9. Essentially, we found that CaMKII inhibited RyR activity in a phosphorylation reaction that was Ca^{2+} and CaM dependent. However, there are differences in recording solutions that should be pointed out. Witcher *et al.* (1991) used 50 mM *trans* (luminal) Ba^{2+} as the charge carrier whereas we kept *trans* Ca^{2+} at 10 μM. We have found that millimolar concentrations of divalent ions in the luminal side increase P_o when the cytoplasmic Ca^{2+} is maintained in the micromolar range (Thedford, Lederer & Valdivia, 1994), a finding that is consistent with the results in intact cells (Cheng *et al.* 1993). Therefore it could be argued that the functional impact of CaMKII phosphorylation depends on the occupancy of intraluminal Ca^{2+} binding sites that modulate channel activity. Although this is an attractive hypothesis in the light of the postulated role for CaMKII phosphorylation mentioned above, we considered it unlikely because we could not reverse the inhibition caused by CaMKII when 5 mM Ca^{2+} was added to the luminal side (not shown). Another important difference between our study and that of Witcher *et al.* (1991) was the source of RyRs, i.e. swine *versus* canine. Although we are not aware of major differences in the CICR process between these two animal species, we noted a different response of single RyRs to classical modulators of channel activity. For instance, in our hands, 3 μM CaM always produced a modest, but significant, decrease of channel activity (Figs 7 and 8) and 3 mM cytosolic free Mg^{2+} totally abolished channel openings (not shown). In contrast, Witcher *et al.* used 3 mM Mg^{2+} in their starting recording solution ($P_o \approx 0{\cdot}26$) and 3 μM CaM profoundly inhibited P_o. In any event, there seem to be more factors involved in the differing effects of CaMKII than can be ascribed to animal species only, because Takasago *et al.* (1991) found that activation of an endogenous CaM kinase decreased [^{3}H]ryanodine binding to canine SR and we could reproduce our results using rabbit SR.

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