

## Characteristics of Irreversible ATP Activation Suggest that Native Skeletal Ryanodine Receptors Can Be Phosphorylated via an Endogenous CaMKII

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**ABSTRACT** Phosphorylation of skeletal muscle ryanodine receptor (RyR) calcium release channels by endogenous kinases incorporated into lipid bilayers with native sarcoplasmic reticulum vesicles was investigated during exposure to 2 mM cytoplasmic ATP. Activation of RyRs after 1-min exposure to ATP was reversible upon ATP washout. In contrast, activation after 5 to 8 min was largely irreversible: the small fall in activity with washout was significantly less than that after brief ATP exposure. The irreversible activation was reduced by acid phosphatase and was not seen after exposure to nonhydrolyzable ATP analogs. The data suggested that the channel complex was phosphorylated after addition of ATP and that phosphorylation reduced the RyR's sensitivity to ATP, adenosine, and  $\text{Ca}^{2+}$ . The endogenous kinase was likely to be a calcium calmodulin kinase II (CaMKII) because the CaMKII inhibitor KN-93 and an inhibitory peptide for CaMKII prevented the phosphorylation-induced irreversible activation. In contrast, phosphorylation effects remained unchanged with inhibitory peptides for protein kinase C and A. The presence of CaMKII $\beta$  in the SR vesicles was confirmed by immunoblotting. The results suggest that CaMKII is anchored to skeletal muscle RyRs and that phosphorylation by this kinase alters the enhancement of channel activity by ATP and  $\text{Ca}^{2+}$ .

### INTRODUCTION

Mammalian skeletal and cardiac muscles contract when  $\text{Ca}^{2+}$  is released from the terminal cisternae (TC) of their sarcoplasmic reticulum (SR) through ryanodine receptor (RyR) calcium release channels.  $\text{Ca}^{2+}$  release in vivo occurs during excitation-contraction coupling (EC-coupling) following activation of an L-type  $\text{Ca}^{2+}$  channel (dihydropyridine receptor, DHPR), which senses T-tubule depolarization and transmits an activating signal to the RyR via a protein-protein interaction. The II-III loop of the DHPR is critical for activation of skeletal RyRs by the DHPR during EC coupling (Tanabe et al., 1990). The degree of activation of RyRs during EC coupling depends on the strength of the signal from the DHPR, which is added to activity imposed by other regulatory factors such as ATP,  $\text{Mg}^{2+}$ , FKBP12, and calmodulin (i.e., the background activity of the RyR). The resting background activity of the calcium release channel can be modulated by a variety of cytoplasmic factors including  $\text{Ca}^{2+}$  and ATP (Meissner, 1994), by co-proteins such as FKBP12 (Jayaraman et al., 1992; Timerman et al., 1993; Ahern et al., 1994), by covalent modification with oxidation or nitrosylation (see review by Dulhunty et al., 2000; Hart, 2000; Eu et al., 2000), and by phosphorylation

(Wang and Best, 1992; Hermann-Frank and Varsanyi, 1993; Hain et al., 1994; Sonnleitner et al., 1997).

ATP can alter RyR activity either by directly binding to a specific adenine nucleotide binding site on the cytoplasmic domain of the protein (Meissner, 1984; Chan et al., 2000), or by phosphorylation when it provides a substrate for kinases that are free in the cytoplasm or anchored to the SR membrane (Wang and Best, 1992; Hermann-Frank and Varsanyi, 1993; Hain et al., 1994; Sonnleitner et al., 1997). As a ligand, ATP is a strong activator of the RyR channel under a variety of conditions (Meissner, 1984). Phosphorylation in the presence of ATP, however, can have several different effects on channel activity. For example, Wang and Best (1992) found that amphibian RyRs were inhibited following phosphorylation by endogenous calcium calmodulin kinase II (CaMKII) when ATP was added to their cytoplasmic side. Addition of calmodulin was not required for kinase activity. Mammalian RyRs were similarly inhibited when phosphorylated by endogenous CaMKII after addition of ATP, although added calmodulin was required; conversely, RyRs were activated when phosphorylated by exogenous protein kinase A (PKA) or protein kinase C (PKC) (Hain et al., 1994). Phosphorylation via PKA, PKC, or endogenous membrane-associated kinases can enhance RyR activity by increasing the channel's sensitivity to  $\text{Ca}^{2+}$  and to ATP (Hermann-Frank and Varsanyi, 1993; Sonnleitner et al., 1997). It is normally assumed that ATP added to RyRs, in the absence of added kinases or calmodulin, activates the channel via the adenine nucleotide binding site (Chan et al., 2000). However, ATP can phosphorylate RyRs if endogenous kinases and their activators are present.

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We briefly reported that ATP in the *cis* solution prevents activation of pig skeletal RyR channels by the 20-amino acid basic peptide A corresponding to the Thr<sup>671</sup>-Leu<sup>690</sup> sequence in the II-III loop of the skeletal dihydropyridine receptor (DHPR) (Gallant et al., 2001). Activation of pig RyRs by peptide A was significant when experiments were done in the absence of ATP and activation was substantially greater in RyRs from malignant hyperthermia (MH)-susceptible pigs than in RyRs from normal pigs (Gallant et al., 2001). MH is an inherited disorder which leads to enhanced Ca<sup>2+</sup> release from the SR in the presence of a variety of activating substances and during EC coupling (Ohta et al., 1989; Mickelson and Louis, 1996; Dietz et al., 2000), leading to increased force production (Gallant et al., 1980).

The reason for the reduced ability of peptide A to activate RyRs in the presence of ATP was not clear. It was possible that either ATP binding to the RyR prevented activation by the peptide or that phosphorylation altered the RyRs response to the peptide. If phosphorylation occurred, an endogenous kinase anchored to the SR membrane and to the RyR must have been involved because exogenous kinases were not added. Indeed, cardiac RyRs are associated with a PKA and an A-kinase anchoring protein (AKAP) (Marx et al., 2000), which must be embedded in the junctional face membrane close to the RyR.

The aim of the present study was to examine the effects of ATP on skeletal RyR channel activity to determine whether channels were phosphorylated and, if so, which kinase was associated with the RyR. The results showed that exposure to ATP for more than 5 min caused strong activation of RyRs that could not be reversed by removal of ATP, but could be reversed by acid phosphatase. This strong irreversible activation was not seen with the nonhydrolyzable AMP-PNP or AMP-PCP and did not occur in the presence of a CaMKII-inhibitory peptide or the CaMKII inhibitor KN-93. Phosphorylation reduced the sensitivity of RyRs to activation by Ca<sup>2+</sup> and the degree of activation by ATP and its competitive inhibition by adenosine. The presence of CaMKII $\beta$  in the SR vesicle preparations was confirmed by immunoblotting. Thus we conclude that skeletal RyRs are phosphorylated by an associated CaMKII upon addition of ATP to their cytoplasmic side. This phosphorylation alters the ability of the RyR to respond to some activating ligands.

## MATERIALS AND METHODS

### Materials

Chemicals and biochemicals were from Sigma-Aldrich (Castle Hill, Australia). DHPR II-III loop peptide synthesis has been described previously (Dulhunty et al., 1999). Peptides were synthesized with purification to 98 to 100% using HPLC, tested by mass spectroscopy and NMR. Stock peptide solutions (~2 mM) were prepared in H<sub>2</sub>O and frozen in 20- $\mu$ l aliquots. Precise stock solution concentrations were determined by Auspep Pty Ltd. Peptides used in this study were 1) PKCi-(19–31)—a conserved region of the PKC family and

a potent pseudosubstrate blocker of PKC (Malinow et al., 1989); <sup>9</sup>Arg Phe Ala Arg Lys Glu Ala Leu Arg Gln Arg Asn Val<sup>31</sup>; 2) PKAi-(6–22)—the active portion of the heat stable inhibitor protein of PKA (Glass et al., 1989); <sup>6</sup>Thr Tyr Ala Asp Phe Ile Ala Ser Gln Arg Thr Gln Arg Arg Asn Ala Ile<sup>22</sup>; 3) CaMKIi-(273–302)—a peptide containing the autoinhibitory domain of CaMKII (Malinow et al., 1989); <sup>273</sup>His Arg Ser Thr Val Ala Ser Cys Met His Arg Gln Glu Thr Val Asp Cys Leu Lys Lys Phe Asn Ala Arg Arg Lys Leu Lys Gln Ala<sup>302</sup>; and 4) CaMKIc-(284–302)—a control peptide lacking the arginine essential for inhibition of the catalytic site (Malinow et al., 1989); <sup>284</sup>Gln Glu Thr Val Asp Cys Leu Lys Lys Phe Asn Ala Arg Arg Lys Leu Lys Gln Ala<sup>302</sup>.

### Biological material and caffeine-halothane contracture test for MH susceptibility

Skeletal muscle was obtained from New Zealand White rabbits and normal and malignant hyperthermia-susceptible Landrace and Belgium Landrace Pigs. The methods for genetic testing of the pigs, anesthetic techniques, muscle dissection, caffeine-halothane contracture testing, preparation of SR vesicles, and single channel recording have been described previously (Otsu et al., 1992; Laver et al., 1997; Owen et al., 1997). Muscle and blood samples were obtained from three homozygous normal pigs (one Belgium Landrace and two Landrace) and three homozygous MH pigs (two Belgium Landrace and one Landrace) aged ~4 months. Each animal was genetically tested for normal or MH RyR allele (containing either Arg<sup>615</sup> or Cys<sup>615</sup>). The SR preparations were from the same animals as those used in Laver et al. (1997), Haarmann et al. (1999), and Gallant et al. (2001). All fiber bundles from the three homozygous normal animals failed to respond to halothane or 2 mM caffeine, while all fiber bundles from the three homozygous MH animals developed tension in response to both drugs.

### Isolation of SR vesicles

The preparation of crude SR vesicles was based on Meissner (1984) and Ma (1995). Freshly dissected back and leg muscle was washed in cold phosphate-buffered saline containing 2 mM EGTA (pH 7.0), trimmed of fat and connective tissue, cut into cubes, and either frozen in liquid N<sub>2</sub> and stored at -70°C or freshly processed. The fresh or thawed muscle cubes were suspended in (mM): 5 Tris maleate; 100 NaCl; 2 EDTA; 0.1 EGTA; pH 6.8 (5 ml/g of tissue). The muscle was homogenized in a Waring Blendor with four 15-s high-speed bursts. The homogenate was centrifuged at 2600  $\times$  g for 30 min and the supernatant filtered through cotton gauze and centrifuged at 10,000  $\times$  g for 30 min. The pellet (P2) was collected and the supernatant was centrifuged again at 35,000  $\times$  g and the pellet (P3) collected. Pellets P2 and P3 were resuspended in (mM): 5 Tris-2-(*N*-morpholino)ethanesulfonic acid (Tris-MES); 300 sucrose; 100 KCl; 2 DTT; pH 6.8. Aliquots of the suspensions were frozen in liquid nitrogen and stored at -70°C. All buffers contained the protease inhibitors phenylmethylsulfonyl fluoride (0.7 mM), leupeptin (1  $\mu$ g/ml), pepstatin A (1  $\mu$ M), and benzamide (1 mM). SR vesicles were isolated from the back and leg muscles of New Zealand White rabbits using methods described by Saito et al. (1984) with minor modifications (Laver et al., 1995).

### Electrophoresis and immunoblotting

SDS-PAGE gels (6.5%, 12%, 5–17%, or 3–12%) were stained with Coomassie Brilliant Blue or silver stain, as appropriate. Proteins were transferred onto Immobilon-P PVDF transfer membranes. Either an alkaline phosphatase or horseradish peroxidase development system was used for immunodetection.

### Lipid bilayer techniques

The lipid bilayer and single channel recording technique are described in Laver et al. (1995) and Dulhunty et al. (1999). Bilayers were formed from

phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (5:3:2 w/w/w) (Avanti Polar Lipids, Alabaster, AL) across an aperture with a diameter of 200–250  $\mu\text{m}$  in the wall of a 1.0 ml Delrin cup (Cadillac Plastics, Canberra, Australia). TC vesicles (final concentration, 10  $\mu\text{g/ml}$ ) were added to the *cis* chamber and stirred until vesicle incorporation was observed. The cytoplasmic side of channels incorporated into the bilayer faced the *cis* solution. The bilayer potential was controlled, and single channel activity recorded, using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). For experimental purposes, the *cis* chamber was held at ground and the voltage of the *trans* chamber controlled. Bilayer potential is expressed in the conventional way as  $V_{\text{cis}} - V_{\text{trans}}$ , (i.e.,  $V_{\text{cytoplasm}} - V_{\text{lumen}}$ ).

Bilayers were formed and vesicles incorporated into the bilayer using *cis* solutions containing (mM): 230 cesium methanesulfonate (CsMS); 20 CsCl; 1.0  $\text{CaCl}_2$ ; 10 *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES); pH 7.4 adjusted with CsOH; and a *trans* solution that was same except that CsMS was 30 mM. The *cis* solution sometimes contained 500 mM mannitol to aid SR vesicle fusion. Following incorporation, 1) the *cis* solution was replaced (by perfusion) with an identical solution, except that  $[\text{Ca}^{2+}]$  was varied among 0.3, 10, and 100  $\mu\text{M}$  ( $\text{Ca}^{2+}$  in the 0.3 and 10  $\mu\text{M}$   $\text{Ca}^{2+}$  solutions was buffered by 2 mM BAPTA); and 2) 200 mM CsMS was added to the *trans* chamber to establish symmetrical conditions.

## Drug addition and solution exchange

For the majority of experiments, drugs were added to the *cis* chamber in appropriate aliquots of stock solutions and removed from the *cis* chamber by perfusion of the chamber with 10 volumes of *cis* solution using back-to-back syringes. In some experiments a local perfusion system was used in which the solution covering the *cis* face of the bilayer was exchanged by flowing solutions from a vinyl tube directly onto the bilayer surface (Laver and Curtis, 1996).

## Recording and analysis of single channel activity

Currents were filtered at 1 kHz (8-pole low-pass Bessel,  $-3$  dB) and digitized at 5 kHz. Analysis of single channel records (using *Channel 2*, developed by P. W. Gage and M. Smith, JCSMR) yielded channel open probability ( $P_o$ ), open times, closed times, and mean open ( $T_o$ ) and closed ( $T_c$ ) times, as well as mean current ( $I'$ ).  $I'$  measurements were used to assess activity in all bilayers containing one, two, or three channels. The single channel parameters  $P_o$ ,  $T_o$ , and  $T_c$  were measured in records showing openings only to the maximum single channel conductance. The event discriminator for single channel analysis was set above the baseline noise at  $\sim 20\%$  of the maximum current, rather than the usual 50%, so that openings to both subconductance and maximum conductance levels were included in the analysis. Channel activity was analyzed over two 30-s periods of continuous activity at  $+40$  mV and two 30-s periods at  $-40$  mV.

## Statistics

Average data are given as mean  $\pm$  SEM. The significance of the difference between control and test values was tested using either 1) a Student's *t*-test, either one- or two-sided and either for independent or paired data, as appropriate (numbers i or ii, used to indicate significance from control or a preceding condition, respectively); or 2) using the nonparametric "sign" test (Minium et al., 1993) (numbers I or II used to indicate significance from control or a preceding condition, respectively). The results of both tests are not shown when significance was indicated by the Student's *t*-test, because those differences were usually also significant according to the sign test.

## RESULTS

### Long-term effects of exposure to ATP

RyR activity with  $0.3 \times 10^{-6}$  M *cis*  $\text{Ca}^{2+}$  increased substantially after addition of 2 mM *cis* ATP (Fig. 1). Surprisingly, when ATP was perfused from the *cis* chamber after 5 to 8 min, with 10 volumes of *cis* solution, channel activity remained substantially greater than control. Re-exposure to ATP after perfusion caused a further increase in activity, but the increase was usually less than the increase after the first exposure to ATP. Channel activity is shown, and full analyses were performed, only on data at  $-40$  mV. However, changes in activity at  $+40$  mV were very similar to those at  $-40$  mV under all conditions described here. Experiments were terminated by exposing channels to 30  $\mu\text{M}$  ruthenium red to confirm the identity of the channels as RyRs.

The history plots (Fig. 1 *E*) show 1) that high activity after washout of ATP was maintained for 5 to 6 min; 2) that the largely irreversible increase in activity after several minutes' exposure to ATP was similar in channels from normal pigs (RyR<sub>N</sub>), from malignant hyperthermia-susceptible pigs (RyR<sub>MH</sub>), and from rabbit muscle (RyR<sub>R</sub>); and 3) a small increase in activity when ATP was added for a second time. The second observation shows that the irreversible action of ATP was not species- or preparation-dependent and was not altered by the MH mutation. The fact that  $I'$  did not increase substantially with the second addition of ATP suggests that the degree of channel activation by ATP may have been reduced during the experiment.

On average, the single channel parameters  $I'$ ,  $P_o$ , and  $T_o$  increased, while  $T_c$  decreased significantly after the first ATP addition, and all parameters then remained significantly different from control for the remainder of the experiment (Fig. 1, *F--I*). The decreases in  $I'$ ,  $P_o$ , and  $T_o$  when ATP was perfused out of the *cis* chamber were small when compared with the increase after ATP addition, and were insignificant with the sample size in Fig. 1 (see also Fig. 7 below). Thus a large fraction of the increase in activity with ATP was irreversible. Indeed, activity actually increased in 4 of 13 channels upon washout. Although all data are included in the averages in Fig. 1 and later figures, there were clearly two populations of channels, a major population that showed a large component of irreversible activation after long exposures to ATP (58 of 75 channels, 77%), and a smaller population in which activation was reversible (17 of 75 channels, 23%).

The results obtained with prolonged exposure to ATP were very different from those obtained after briefer exposures to the adenine nucleotide, where the increase in channel activity was fully reversible. Similar increases and decreases in activity were seen with sequential addition and washout of ATP in RyR<sub>N</sub> and RyR<sub>MH</sub> (Fig. 2), and in RyR<sub>R</sub> (see Fig. 9 *B*). Curiously, the mean current after a 2-min exposure to ATP was much the same as the mean current after a 5–6-min exposure (Fig. 1). This can be explained if

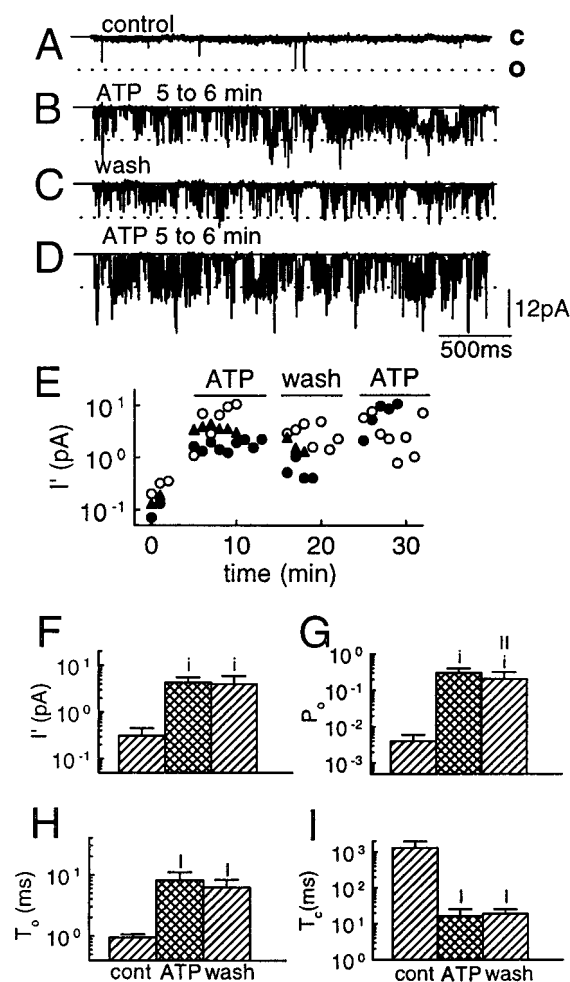


FIGURE 1 Irreversible activation of RyRs by prolonged exposure to 2 mM ATP. (A–D) RyR<sub>N</sub> channel activity is shown at –40 mV with 300 nM *cis* Ca<sup>2+</sup>. Channel opening is downward from the closed (c), to the maximum single channel conductance (o), and to higher current levels when a second channel opens in D. Each record shows 3 s of activity under control conditions (A), 6 min after addition of 2 mM *cis* ATP (B), 4 min after perfusion of ATP from the chamber (C), and 5 min after a second addition of 2 mM *cis* ATP (D). The plot in E shows the average mean current,  $I'$ , during continuous 30-s recordings at –40 mV taken every 60 s during the experiment with RyR<sub>N</sub> (filled circles), RyR<sub>MH</sub> (open circles), and RyR<sub>R</sub> (filled triangles), where the bilayer broke before the second exposure to ATP, under control conditions (first three points), during exposure to ATP (ATP), after perfusion of the *cis* chamber (wash), and then a second addition of ATP (ATP). Because all channel types responded in the same way, data were combined for average values (F–I). F shows average  $I'$  (mean current (pA)) for all experiments ( $n = 13$ , for bilayers showing openings of 1 or 2 channels) with RyR<sub>N</sub> ( $n = 5$ ), RyR<sub>MH</sub> ( $n = 5$ ), and RyR<sub>R</sub> ( $n = 3$ ). G–I show average single channel parameters,  $P_o$ ,  $T_o$ , and  $T_c$ , respectively ( $n = 9$ ), for bilayers showing single channel openings only. The bins show data for control conditions (cont), after ~5 min exposure to 2 mM *cis* ATP (ATP) and ~5 min after washout of ATP (wash). The scale on the vertical axes, here and in subsequent figures, is logarithmic to display activity over the wide range seen in these experiments. The vertical bars show 1 SEM. Data were significantly different 1) from control, assessed either by Student's *t*-test (i) or sign test (I); or 2) from the preceding condition, assessed by Student's *t*-test (ii) or sign test (II) (see Methods).

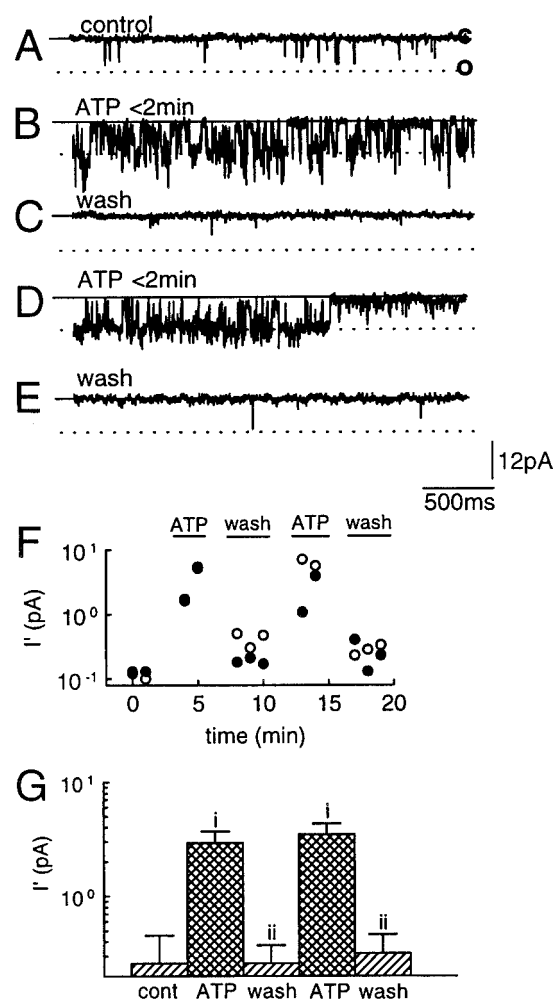


FIGURE 2 Reversible activation of RyRs after brief exposure to 2 mM ATP. (A–E) activity from RyR<sub>MH</sub> channels at –40 mV with 300 nM *cis* Ca<sup>2+</sup>. Channel opening is downward from the closed level (c), to the single channel conductance (o), and to higher current levels when a second channel opens in B. Each record shows 3 s of channel activity; under control conditions (A), 1 min after addition of 2 mM ATP (B), 2 min after perfusion of ATP from the *cis* chamber (C), 1 min after a second addition of 2 mM *cis* ATP (D), and 2 min after re-perfusion (E). The history plot in E shows the average mean current,  $I'$ , during continuous 30-s recordings at –40 mV taken every minute during the experiments with RyR<sub>N</sub> (filled circles) and RyR<sub>MH</sub> (open circles). Because the two channel types responded in the same way, data were combined for averages  $I'$  (G) ( $n = 7$ ; RyR<sub>N</sub> = 3, RyR<sub>MH</sub> = 4). F and B show data for control conditions (first 2 points or cont, respectively), and then after exposure to 2 mM *cis* ATP (ATP), after washout of ATP (wash), re-exposure to *cis* ATP (ATP), and re-perfusion (wash). Data in G were taken after ~1 min exposure to ATP or ~2 min after washout. The vertical bars in G show 1 SEM. Data were significantly different from control (i) or from the preceding condition (ii), assessed by Student's *t*-test.

the activation by ATP declined as activation by phosphorylation increased, so that overall channel activity did not change much during exposure to ATP (see below).

The irreversible action of prolonged exposure to ATP could have been due 1) to failure to remove ATP from the



*cis* chamber during perfusion; or 2) to phosphorylation by an endogenous kinase. The first possibility seemed unlikely because perfusion of the *cis* chamber with 10 volumes of solution causes a 1000-fold dilution of drugs present before perfusion (Haarmann, unpublished observation). Thus ATP was diluted to  $\sim 2 \mu\text{M}$ , which would not significantly activate the RyR (Meissner, 1984; Chan et al., 2000). In addition, irreversible activation was also seen when ATP was added and removed using local perfusion techniques (see Fig. 7) in which the solution change is essentially complete. We examined the possibility that the irreversible effect of ATP was due to phosphorylation of RyRs by an endogenous kinase located in the SR membrane.

### Effect of acid phosphatase on ATP-activated RyR channels

In this experiment channels were exposed to ATP and then exposed to  $\sim 10$  units of acid phosphatase (Fig. 3). Channel activity increased after adding ATP, then fell following addition of phosphatase and fell further when phosphatase and ATP were perfused out of the *cis* chamber, with all single channel parameters after perfusion achieving values that were similar to control values. This was in marked contrast to the maintained high activity when ATP without phosphatase was removed (see Fig. 1). The channels were then exposed to ATP for a second time and phosphatase was not added. Channel activity increased with the second addition of ATP, and fell again with subsequent perfusion. Unlike the first perfusion, channel activity remained substantially higher than control after the second perfusion.

Average combined data in Fig. 4, for normal channels ( $\text{RyR}_\text{N}$  and  $\text{RyR}_\text{R}$ ) and for channels from MH pigs ( $\text{RyR}_\text{MH}$ ), show the usual increase in  $I'$ ,  $P_o$ , and  $T_o$  and decrease in  $T_c$  after adding ATP. This was followed by a significant decline in  $I'$ ,  $P_o$ , and  $T_o$ , and increase in  $T_c$  when phosphatase was added. Activity fell further in six of seven channels when ATP and phosphatase were perfused out of the *cis* chamber and there was a significant increase in  $T_c$  in normal RyRs.

Curiously, the mean current in the presence of phosphatase (Figs. 3 and 4) was substantially lower than the mean current after brief exposure to ATP (Figs. 1 *E* and 2). This was unexpected because phosphatase should have returned activity to the same dephosphorylated level as that seen after brief exposure to ATP. In addition, although washout of ATP plus phosphatase reduced activity to control levels (Figs. 3 and 4), the magnitude of this fall in mean current was small compared to that seen when ATP was washed out after a short exposure (Fig. 2). These results suggest that the degree of activation of RyRs by ATP was reduced upon phosphorylation (see below) and was maintained for several minutes after dephosphorylation. Sensitivity to ATP clearly returned by the time that the channel was re-exposed to ATP (Fig. 3).

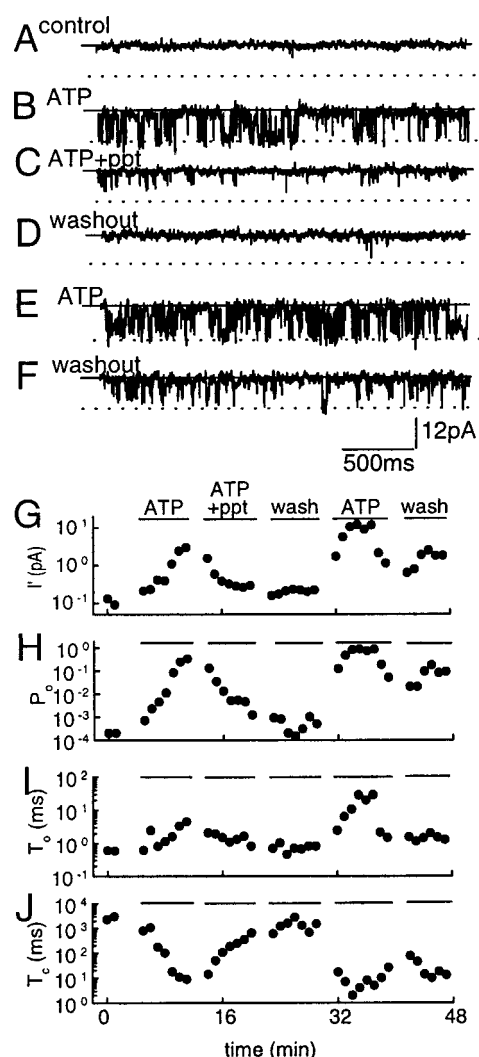


FIGURE 3 Acid phosphatase reduces activation of RyRs during prolonged exposure to ATP. Activity is shown from an  $\text{RyR}_\text{N}$  channel at  $-40$  mV with  $300 \text{ nM}$  *cis*  $\text{Ca}^{2+}$ . Channel opening is downward. *A–F* each show a 3-s recording of channel activity under control conditions (*A*), 5 min after addition of  $2 \text{ mM}$  *cis* ATP (*B*), 5 min after addition of 10 units of acid phosphatase to the *cis* chamber (*C*), 4 min after perfusion of ATP from the *cis* chamber (*D*), 5 min after a second addition of  $2 \text{ mM}$  *cis* ATP (*E*), and 5 min after reperfusion of the *cis* chamber (*F*). The history plots in *G–J* show average  $I'$ ,  $P_o$ ,  $T_o$ , and  $T_c$  measured during continuous 30-s recordings taken every minute during the experiment. The first two points were obtained under control conditions, then *cis* ATP was added (ATP) followed by phosphatase (ATP + ppt), ATP and phosphatase were washed out of the *cis* chamber (wash), ATP was then added alone to the *cis* chamber (ATP), and finally washed out (wash).

The results in Figs. 3 and 4 provide strong evidence that RyRs were phosphorylated in the presence of ATP. However, it could be argued that the decline in activity with phosphatase was due to dephosphorylation of residues that were phosphorylated in the native vesicles before addition of ATP. This was not the case because there was no fall in activity when 10 units of phosphatase were added to chan-

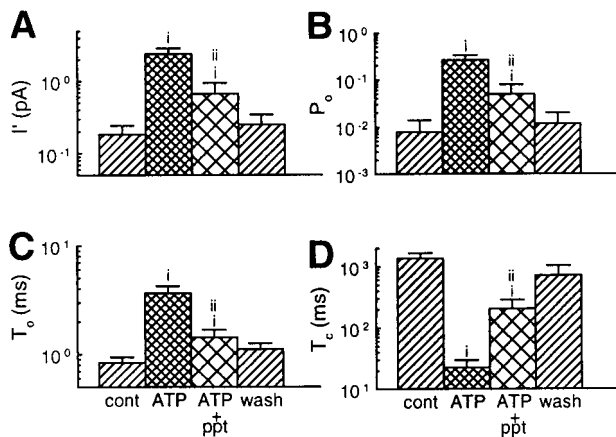


FIGURE 4 Average effects of acid phosphatase on ATP-activated RyRs. The graphs show average combined data for RyR<sub>N</sub> and RyR<sub>MH</sub>. A shows average  $I'$  (mean current (pA)) for all experiments (i.e., for bilayers showing openings of 1 or 2 channels) with RyR<sub>N</sub> ( $n = 8$ ), RyR<sub>MH</sub> ( $n = 6$ ). B–D show average single channel parameters,  $P_o$ ,  $T_o$ , and  $T_c$ , respectively, for records displaying single channel openings only, RyR<sub>N</sub> ( $n = 8$ ), RyR<sub>MH</sub> ( $n = 5$ ). The bins show data for control conditions (cont), after ~5 min exposure to 2 mM *cis* ATP (ATP), ~3–5 min after addition of 10 units of acid phosphatase to the *cis* chamber (ATP + ppt), and ~5 min after a perfusion of ATP and phosphatase from the *cis* chamber (wash). The vertical bars show 1 SEM. Data were significantly different (i) from control, assessed either by Student's *t*-test (i) or sign test (I); or 2) from the preceding condition, assessed by Student's *t*-test (ii) or sign test (II).

nels that were activated by 10  $\mu$ M *cis* Ca<sup>2+</sup> in the absence of ATP; if anything, there was a small increase in activity in four of four channels (RyR<sub>N</sub>,  $n = 2$ ; RyR<sub>MH</sub>,  $n = 2$ ).

### AMP-PCP and AMP-PNP do not cause a large irreversible increase in channel activity

Further evidence that an endogenous kinase phosphorylated the RyR in the presence of added ATP was obtained with two nonhydrolyzable analogs of ATP (AMP-PCP and AMP-PNP). In contrast to the results with ATP, channel activity fell substantially when AMP-PCP and AMP-PNP were perfused out of the bath. An example of results from one RyR<sub>N</sub> channel exposed to AMP-PCP is shown in Fig. 5, A–D. Similar results were obtained in five other RyR<sub>N</sub> channels exposed to AMP-PNP (average data Fig. 5 E) and two RyR<sub>MH</sub> channels exposed to AMP-PCP. Since activation was reversible with the nonhydrolyzable analogs, it was likely that the irreversible activation with ATP was due to phosphorylation.

### Effects of PKC, CaMKII, and PKA inhibitory peptides, and KN-93, on the response of RyRs to ATP

To establish which kinase was responsible for phosphorylation in the presence of ATP, inhibitory peptides for PKC

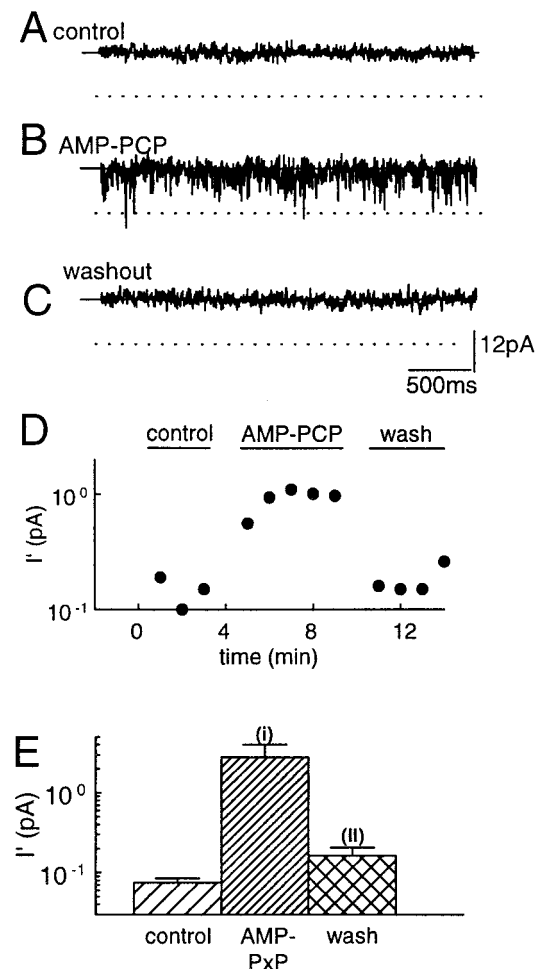


FIGURE 5 Addition of AMP-PCP to the *cis* solution reversibly activates RyRs. Activity is shown from an RyR<sub>N</sub> channel at  $-40$  mV with 300 nM *cis* Ca<sup>2+</sup>. Channel opening is downward. A–E each show a 3-s recording of channel activity under control conditions (A), 4 min after addition of 2 mM *cis* AMP-PCP (B), 3 min after perfusion of AMP-PCP from the *cis* chamber (C). The history plot in D shows average  $I'$  (pA) for the same channel measured during continuous 30-s recordings taken every minute during the experiment, under control conditions (control), during exposure to AMP-PCP (AMP-PCP) and after perfusion of the *cis* chamber (wash). E shows average data for exposure to AMP-PNP ( $n = 6$ ). The bins show data for control conditions (cont), after ~5-min exposure to 2 mM *cis* AMP-PNP (ATP), and ~5 min after a perfusion of AMP-PNP from the *cis* chamber (wash). The vertical bars show 1 SEM. Data was significantly different (a) from control (i), or from the preceding condition (ii), assessed by the student's *t*-test.

(PKC 19–31), PKA (PKA inhibitory peptide 6–22), or CaMKII (CaMKII 273–302) (see Methods) were added to the *cis* solution before addition of ATP. Average results for mean current only are shown in Fig. 6. Changes in  $P_o$ ,  $T_o$ , and  $T_c$  with addition of ATP followed the same trends as those shown above (i.e., an increase in  $P_o$  accompanied the increase in  $I'$ , with concomitant increases in  $T_o$ , and decreases in  $T_c$ ). A more detailed examination of changes in  $T_o$  and  $T_c$  with phosphorylation is shown for a larger dataset in

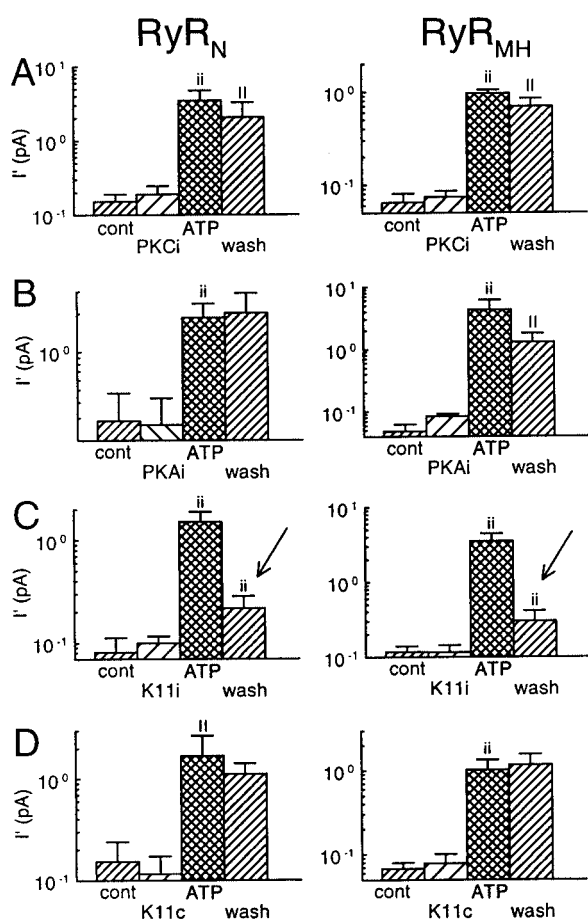


FIGURE 6 The irreversible activation of RyRs during exposure to ATP is due to phosphorylation by endogenous CaMKII. Average mean current,  $I'$  (pA), is shown for RyR<sub>N</sub> (left-hand graphs) and RyR<sub>MH</sub> (right-hand graphs). Two mM ATP were added to the *cis* chamber 2 min after inhibitory peptides for PKC (PKCi, A), PKA (PKAi, B), CaMKII (K11i, C) or a control peptide for CaMKII (K11c, D). The numbers of experiments were (A) (PKCi, 10  $\mu$ M), RyR<sub>N</sub> = 5, RyR<sub>MH</sub> = 8; (B) (PKAi, 1  $\mu$ M), RyR<sub>N</sub> = 8, RyR<sub>MH</sub> = 5; (C) (K11i, 100 nM–10  $\mu$ M), RyR<sub>N</sub> = 11, RyR<sub>MH</sub> = 10; (D) (K11c, 1–10  $\mu$ M), RyR<sub>N</sub> = 7, RyR<sub>MH</sub> = 8. The bins show  $I'$  for control conditions (cont), after 2 min with inhibitory peptide (e.g., PKCi), ~5 min after exposure to 2 mM *cis* ATP (ATP), ~5 min after washout of ATP (wash). The vertical bars show 1 SEM. Data were significantly different from the preceding condition, assessed by Student's *t*-test (ii) or sign test (II) (see Methods).

Fig. 10. PKC 19–31 and PKAi 6–22 (Fig. 6, A and B) had no effect on channel activity when added at 10  $\mu$ M to the *cis* side of either RyR<sub>N</sub> or RyR<sub>MH</sub>, and changes in activity after addition of ATP were similar to those seen in the absence of the inhibitory peptides (Fig. 1).

CaMKII 273–302 (100 nM–10  $\mu$ M) did not alter channel activity when added alone (Fig. 6 C), or the increase in  $I'$  with ATP addition, but in marked contrast to results with the PKC and PKA inhibitory peptides, channel activity fell significantly when ATP and CaMKII 273–302 were perfused out of the *cis* chamber. Similar results were obtained

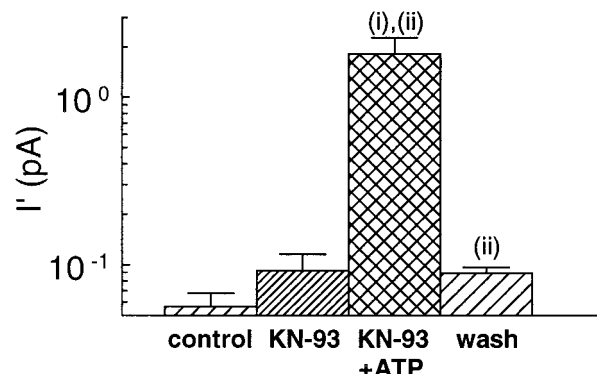


FIGURE 7 The CaMKII inhibitory drug KN-93 also abolishes irreversible activation during long exposure to ATP. The bins show average  $I'$  ( $n = 12$  experiments) for control conditions (control), after 2 min with 1  $\mu$ M or 10  $\mu$ M KN-94 (KN-94), ~5 min after exposure to 2 mM *cis* ATP (KN-94 + ATP), ~5 min after washout of KN-93 and ATP (wash). The vertical bars show 1 SEM. Data were significantly different from control (i) or the preceding condition (ii), assessed by Student's *t*-test (see Methods).

with RyR<sub>N</sub> and RyR<sub>MH</sub>. Therefore, the irreversible increase in activity with ATP was largely abolished by CaMKII inhibition.

To ensure that the effects of CaMKII 273–302 were not nonspecific effects of the peptide, the inactive inhibitory peptide CaMKII 284–302 was tested (Fig. 6 D). The increase in channel activity in RyR<sub>N</sub> and RyR<sub>MH</sub> with ATP in the presence of the control peptide (CaMKII 284–302) was largely irreversible. The possibility that CaMKII phosphorylated the RyR complex after adding ATP was further explored by adding another specific inhibitor of CaMKII, KN-93 to the *cis* chamber, before adding ATP. Activation by ATP was essentially reversible in the presence of 1–10  $\mu$ M KN-93 (Fig. 7). These results with CaMKII 273–302 and KN-93 suggest that a CaMKII kinase in the SR membrane is closely associated with the RyR complex, possibly attached to the membrane by a CaMKII anchoring protein, because CaMKII is a soluble enzyme.

### CaMKII is present in rabbit and pig SR membranes used in bilayer studies

Immunodetection was used to confirm the presence of CaMKII in the SR vesicle preparations. The immunoblots in Fig. 8 show that CaMKII  $\beta$  is present at ~72 kDa in both the rabbit and pig SR vesicle preparations that were used in the bilayer experiments (Fig. 8). A very small amount of PKA is also apparent in both vesicle preparations. These results suggest that CaMKII is associated with the SR/terminal cisternae membrane.

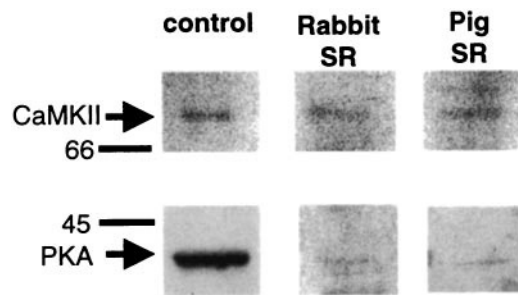


FIGURE 8 Immunodetection of CaMKII in terminal cisternae and SR membranes. (A) Western blots using anti-CaMKII $\beta$  of 10  $\mu$ l whole cell homogenate (10  $\mu$ g protein; control, lane 1), 20  $\mu$ l rabbit SR (20  $\mu$ g protein; lane 2), and 20  $\mu$ l pig SR ( $\mu$ g protein; lane 3) (prepared as described in the Methods section). The position of the 66 kDa markers is shown next to lane 1. The arrow points to an immunostaining band at 72 kDa, equivalent to the SR CaMKII $\beta$  (Bayer et al., 1998). (B) Western blot using anti-PKA on purified PKA (Auspep, control, lane 1), and the same quantities of rabbit SR and pig SR as in A. The position of the 45 kDa marker is shown next to lane 1. The arrow points to an immunostaining band at 42 kDa equivalent to PKA.

### Phosphorylation decreases the amount of RyR activation by ATP

The relatively small increase in activity when channels were exposed to ATP for a second time after exposure to ATP under phosphorylating conditions (Fig. 1) suggested that phosphorylation reduced the activation of RyRs by ATP. Further evidence to support this hypothesis was that the fall in activity when ATP was removed after 5–8 min was less than the increase when ATP was added (Fig. 9 A). The increase in channel activity during the first 2 min after ATP addition was reversible upon washout and attributed solely to activation of RyRs by ATP (Fig. 2). Longer exposure to ATP (with phosphorylation) increased  $I'$  by a further 0.7 pA. If irreversible activation by phosphorylation was added to the maintained, but reversible, activation by ATP, then 1) the fall in activity upon washout (due to reversal of ATP activation) should be equal to the initial increase in activity with exposure to ATP; and 2) the irreversible fraction of activation remaining after washout (phosphorylation-induced activation) should equal the increase in activity seen during phosphorylation (between 2 and 5 min exposure to ATP). However, 1) the fall in activity on washout (0.7 pA) was significantly smaller than the initial increase of 2.2 pA; and 2) activity after washout, 2.2 pA (above control), was significantly greater than the 0.7 pA increase induced by phosphorylation (Fig. 9 A). These results are explained if ATP-induced activation declined as phosphorylation-induced activation developed.

Reduced ATP activation is also indicated by the fact that the increase in activity caused by AMP-PCP or -PNP (Fig. 5), or by ATP in the presence of the CaMKII inhibitory peptide (Fig. 6) (conditions in which phosphorylation did not occur), was much the same as that

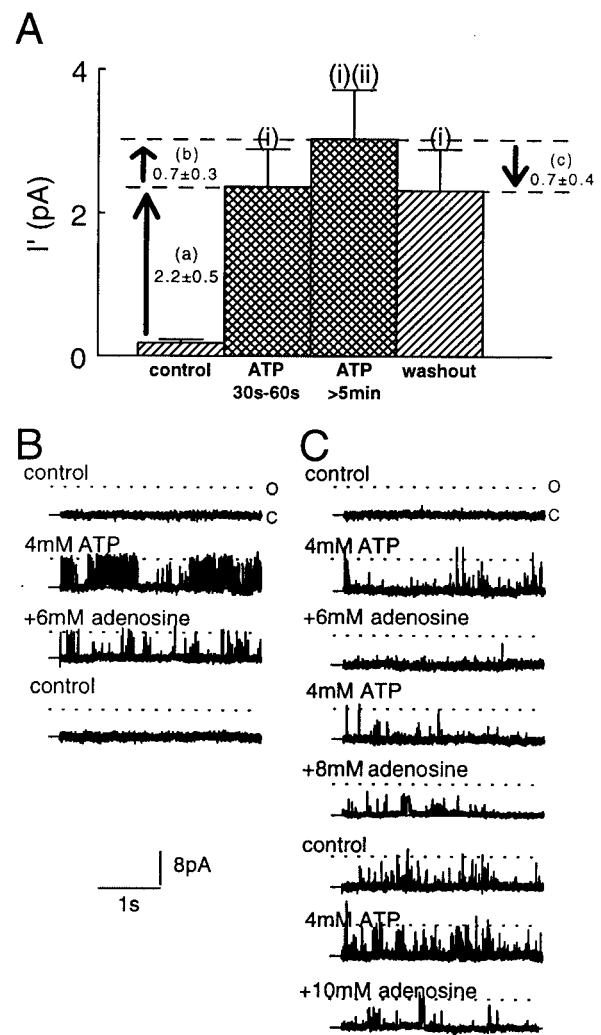


FIGURE 9 Phosphorylation reduces the sensitivity of RyRs to activation by ATP. A shows average mean current,  $I'$  (pA), for 44 experiments ( $RyR_N = 20$ ;  $RyR_{MH} = 24$ ) under control conditions (control), 30–60 s after exposure to ATP (ATP 30 s–60 s), between 5 and 8 min after exposure to ATP (ATP > 5 min), and after ATP washout (washout). Addition of ATP initially increased activity by  $2.2 \pm 0.5$  pA (a), with a further significant increase of  $0.7 \pm 0.3$  pA between 30 s and 5 min after exposure to ATP (b). Activity then fell significantly by  $0.7 \pm 0.4$  pA when ATP was perfused out of the *cis* chamber (c). Vertical bars show 1 SEM. Data were significantly different 1) from control (i) or 2) from the preceding condition (ii), assessed by Student's *t*-test. B and C show 3-s records from  $RyR_R$  during exposure to ATP and adenosine, applied by local perfusion of the *cis* side of the bilayer. B shows sequential recordings; under control conditions (100 nM *cis*  $Ca^{2+}$ ), during a 30-s application of 4 mM ATP, a 30-s application of 4 mM ATP + 6 mM adenosine, and then return to control conditions. C shows sequential recordings; under control conditions, during a 1-min 20-s application of 4 mM ATP, a 1-min 40-s application of 4 mM ATP + 6 mM adenosine, a return to 4 mM ATP alone for 2 min, exposure to 4 mM ATP plus 8 mM adenosine for a further 1-min 30-s (total 6-min 30-s exposure to ATP), then (sequentially) returned to control conditions, exposed to 4 mM ATP, and then to 4 mM ATP + 10 mM adenosine. Loss of RyR regulation by ATP and adenosine is apparent in the lower four records after a 5-min exposure to ATP.



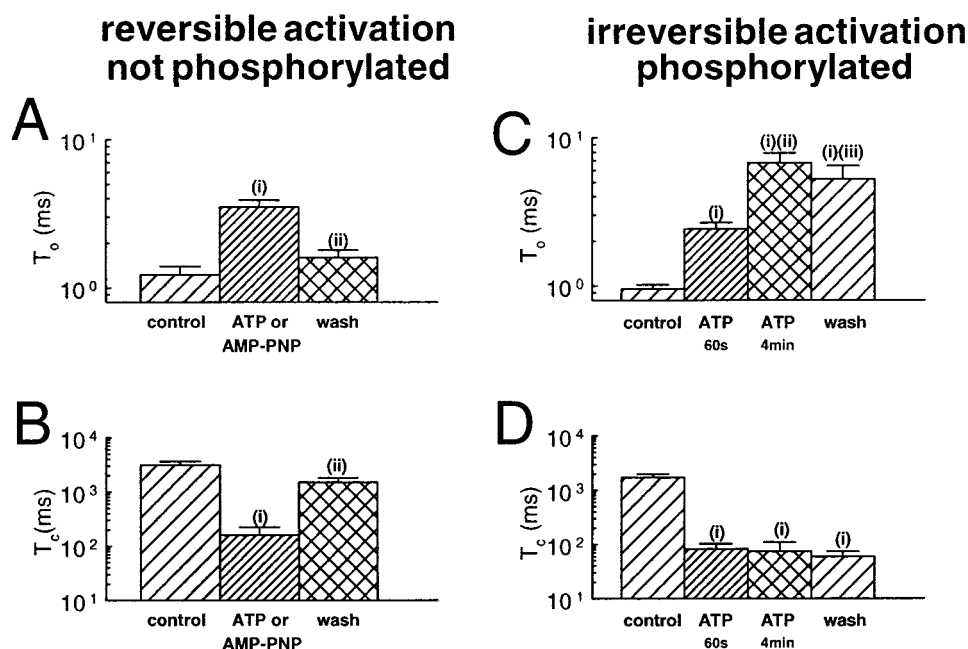


FIGURE 10 Mean open times are increased and mean closed times reduced by ATP and by phosphorylation. Data from all appropriate experiments (see text) have been collected into nonphosphorylated (*A* and *B*) and phosphorylated (*C* and *D*) groups. In *A* and *B*, bins show average ( $n = 39$ ) mean open time ( $T_o$ ) and mean closed time ( $T_c$ ) obtained under control conditions (*control*), after exposure to nucleotide (*ATP* or *AMP-PNP*) for appropriate times, and then after perfusion of the *cis* chamber (*wash*). In *C* and *D*, the bins show average ( $n = 55$ )  $T_o$  and  $T_c$  obtained under control conditions (*control*), between 30 s and 1 min exposure to ATP (*ATP 1 min*) and after 4-min exposure to ATP (*ATP 4 min*) and then after perfusion (*wash*). The vertical bars show 1 SEM. Data were significantly different from control (i), or the preceding condition (ii), assessed by Student's *t*-test (see Methods). In *C*, (iii) indicates that  $T_o$  after perfusion (last bar) was significantly greater than  $T_o$  after brief exposure to ATP (second bar).

seen under phosphorylating conditions. Additional evidence that RyR sensitivity to adenine nucleotides is reduced by phosphorylation was obtained in separate experiments (shown in Fig. 9 *B*), which were further examining competition between adenosine and ATP at the adenine nucleotide binding site (Chan et al., 2000; Laver et al., 2000, 2001). Addition of adenosine to ATP-activated channels caused a reduction in RyR activity when exposure to ATP was brief (Fig. 9 *B*). A similar inhibition by adenosine is seen in the initial part of the experiment in Fig. 9 *C*, when the channel had been exposed to ATP for <2 min. Initially in the experiment in Fig. 9 *C*, 6 mM adenosine reversed the activation seen upon addition of 4 mM ATP (*traces 1–3*). However, after 4 min exposure to ATP, 8 mM adenosine caused less inhibition (*trace 4*) than that seen with 6 mM adenosine (*trace 3*) and channel activity failed then to return to control levels when adenosine and ATP were removed (*trace 6*). Subsequent exposure to 4 mM ATP caused only a small increase in activity (*trace 7*) and then 10 mM adenosine produced a small reduction in activity (*trace 8*). Thus the channel became less sensitive to both ATP and adenosine after a 4-min exposure to ATP.

These results suggest that phosphorylation occurring during the longer exposure to ATP reduced the sensitivity of

the channel to both activation by ATP and to the competitive inhibition of ATP activation by adenosine.

### Phosphorylation increases the mean open time of RyR channels and reduces the mean closed times

To dissect the effects of ATP and phosphorylation on RyR gating, the mean open times and mean closed times from all experiments were collected into two groups, one for conditions that led to reversible activation by ATP (i.e., no phosphorylation: brief exposures to ATP; AMP-PNP or AMP-PCP; KN-93 and CaMKII 273–302;  $n = 39$ ) and a second group for conditions that led to irreversible activation by ATP (i.e., phosphorylation: long exposures to ATP; CaMKII 284–302; PKC 19–31 and PKAi 6–22,  $n = 55$ ). Under nonphosphorylating conditions the increase in  $T_o$  and decrease in  $T_c$  were fully reversed upon washout of the nucleotide (Fig. 10, *A* and *B*). A biphasic increase in  $T_o$  occurred during long exposure to ATP, under phosphorylating conditions (Fig. 10 *C*), with an initial increase to the same level as that seen under nonphosphorylating conditions, with a further significant increase during the subsequent 3 to 4 min (Fig. 10 *C*).  $T_o$  fell slightly after perfusion

of ATP from the *cis* chamber, but remained significantly greater than control and greater than  $T_o$  in the absence of phosphorylation.

In contrast to the mean open time,  $T_c$  in the phosphorylated channels (Fig. 10 *D*) was not significantly different from  $T_c$  in nonphosphorylated channels. The closed times remained at the same short values after perfusion of the *cis* chamber, again showing that  $T_c$  was similar in phosphorylated channels and nonphosphorylated channels in the presence of ATP.

We assume 1) that the mean open and closed times in the presence of nucleotides under nonphosphorylating conditions (Fig. 10, *A* and *B*), or after brief exposure to ATP under phosphorylating conditions (Fig. 10, *C* and *D*), show the effects of adenine nucleotide binding alone on RyR gating; and 2) the mean open and closed times, after perfusion of the *cis* chamber when ATP had been applied under phosphorylating conditions (Fig. 10, *C* and *D*), are indicative of the effect of phosphorylation alone on channel gating. Data after long exposure to ATP under phosphorylating conditions is complicated by effects of phosphorylation on ATP activation (above) and is thus difficult to interpret.

The  $T_o$  of phosphorylated channels after perfusion of ATP from the *cis* chamber was significantly higher than  $T_o$  after brief exposure of the same channels to ATP (Fig. 10 *C*), showing that the increase in  $T_o$  due to phosphorylation was significantly greater than the increase in  $T_o$  due to the adenine nucleotide ligand effect.  $T_c$  in phosphorylated channels was much the same as  $T_c$  in channels influenced only by adenine nucleotide ligand effects. The fact that  $T_c$  was much the same in the presence and absence of ATP following phosphorylation is again consistent with a reduced sensitivity to ATP after phosphorylation.

### Phosphorylation by endogenous CaMKII reduces $\text{Ca}^{2+}$ sensitivity of RyRs

Channel activity as a function of cytoplasmic  $[\text{Ca}^{2+}]$  was examined under control conditions during prolonged exposure to AMP-PNP or to ATP. Under control conditions, channel activity increased significantly with an increase in  $[\text{Ca}^{2+}]$  from 100 nM (with a trend toward higher activity at 300 nM  $\text{Ca}^{2+}$ ) (Fig. 11 *A*). Activity was maximal between 100 nM and 100  $\mu\text{M}$  and then decreased significantly at 500  $\mu\text{M}$   $\text{Ca}^{2+}$ . Similar  $\text{Ca}^{2+}$  sensitivity curves have previously been reported with an ionic strength of 250 mM (Laver et al., 1995). Although mean current was greater at all *cis*  $[\text{Ca}^{2+}]$ s with 2 mM AMP-PNP in the *cis* solution, the  $\text{Ca}^{2+}$  sensitivity showed similar activation and inactivation characteristics (Fig. 11;  $\text{Ca}^{2+}$  concentrations in *B* and *C* have been corrected for  $\text{Ca}^{2+}$  binding to AMP-PNP and ATP, assuming similar binding constants for the two adenine nucleotides). In the presence of AMP-PNP, channel activity increased significantly when *cis*  $\text{Ca}^{2+}$  was increased from 100 to 300 nM.

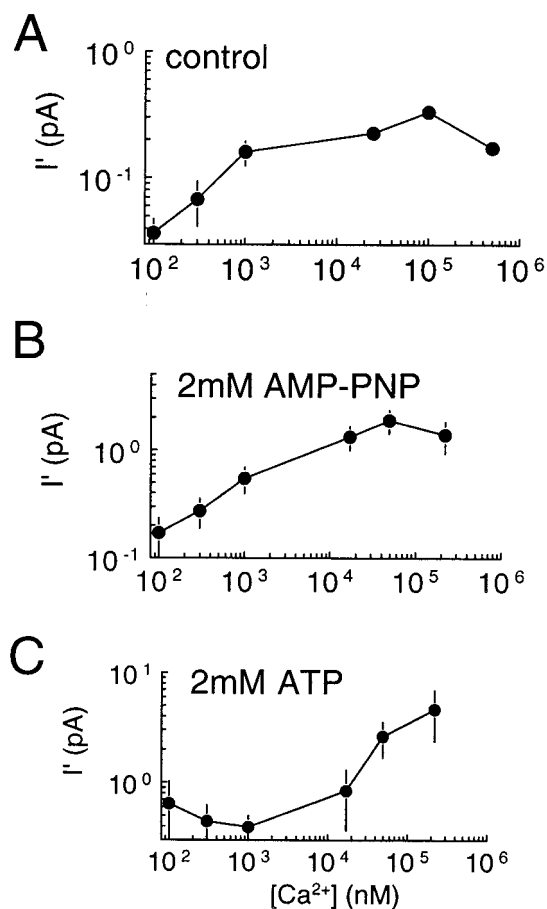


FIGURE 11 Cytoplasmic  $[\text{Ca}^{2+}]$ -sensitivity of RyRs is reduced by phosphorylation. Average mean current ( $I'$ ) ( $n = 5-8$  RyR<sub>N</sub> channels) obtained over a range of *cis*  $[\text{Ca}^{2+}]$  under control conditions (*A*), in the presence of 2 mM *cis* AMP-PNP (*B*), and in the presence of 2 mM *cis* ATP (*C*). Note that the curve is shifted higher  $[\text{Ca}^{2+}]$  in the presence of ATP.

In contrast to data obtained under control conditions and with AMP-PNP, channel activity after exposure to ATP was insensitive to changes in *cis*  $[\text{Ca}^{2+}]$  between 100 nM and 1  $\mu\text{M}$  (Fig. 11 *C*). Activity tended to increase at 10  $\mu\text{M}$  *cis*  $\text{Ca}^{2+}$ , and there was a significant increase only at 50  $\mu\text{M}$  *cis*  $\text{Ca}^{2+}$ . These results suggest that  $\text{Ca}^{2+}$  activation characteristics of the channel were shifted to higher  $\text{Ca}^{2+}$  concentrations after phosphorylation by ATP.

### DISCUSSION

The results show that prolonged exposure to ATP causes an irreversible increase in RyR activity that is attributed to phosphorylation because it could 1) be prevented by addition of acid phosphatase; and 2) did not occur with nonhydrolyzable analogs of ATP. The results further suggest that phosphorylation was due to an anchored endogenous CaMKII because irreversible activation did not occur in the presence of a peptide corresponding to the conserved auto-

inhibitory domain of CaMKII. This is the first report 1) of activation of skeletal RyRs after phosphorylation by endogenous CaMKII; 2) of CaMKII activity in mammalian RyRs in the absence of added calmodulin; and 3) of activation by phosphorylation that is associated with reduced ATP-induced activation and reduced  $\text{Ca}^{2+}$  activation (see Introduction).

### CaMKII in skeletal muscle

Our observations that phosphorylation occurred at low cytoplasmic  $[\text{Ca}^{2+}]$  and in the absence of added calmodulin are consistent with properties of CaMKII in general and skeletal muscle CaMKII in particular. First, the CaMKII isolated from skeletal muscle SR is active at the  $\text{Ca}^{2+}$  concentration used here (100 nM) (Campbell and MacLennan, 1982; Tuana and MacLennan, 1988; Chu et al., 1990). Second, autophosphorylation of CaMKII in the presence of  $\text{Ca}^{2+}$ -calmodulin and ATP renders the kinase  $\text{Ca}^{2+}$  independent, and it remains ~70% active even after calmodulin dissociation (Hanson et al., 1994; Bayer et al., 1998). Thus, if the CaMKII was autophosphorylated in the presence of calmodulin in vivo, the CaMKII should remain active during isolation and incorporation into the bilayer. Our observation that the CaMKII is associated with the RyR in the bilayer suggests that it is anchored to the junctional face membrane and to the RyR. An anchoring protein for CaMKII, i.e., an  $\alpha$  kinase anchoring protein ( $\alpha$ KAP) has been isolated from skeletal muscle SR (Bayer et al., 1998).  $\beta_{\text{M}}$ -CaMKII, one of several CaMKII isoforms isolated from skeletal SR with the  $\alpha$ KAP, is a novel isoform of CaMKII that might be linked to tyrosine kinase pathways (Bayer et al., 1998). The fact that 23% of channels were not irreversibly activated after long exposures to ATP suggests that a fraction of RyRs either did not have associated CaMKII or associated  $\alpha$ KAP and raises the possibility that  $\alpha$ KAP is absent from a fraction of channels in vivo.

In contrast to the increased RyR activity after phosphorylation by constitutively active CaMKII reported here, the endogenous CaMKII previously described with mammalian RyRs required added calmodulin and its phosphorylation reduced RyR activity at 120–150 nM *cis*  $\text{Ca}^{2+}$  (Hain et al., 1994). However, phosphorylation of cardiac RyRs by CaMKII does cause an increase in activity (Witcher et al., 1991). Although it is possible that differences in isolation techniques used with skeletal SR can alter the effects of phosphorylation, we found similar phosphorylation-dependent changes in activity of RyRs from pigs and rabbit, which were prepared in different ways (see Methods). It is more likely that different CaMKII kinases are associated with different phosphorylation sites on the RyR, which produce different effects on RyR activity (Hain et al., 1994). Several isoforms of CaMKII, including  $\delta_{\text{D}}$ -,  $\delta_{\text{A}}$ -, and  $\delta_{\text{D}}$ -CaMKII, are isolated with  $\beta_{\text{M}}$ -CaMKII and associated with the  $\alpha$ KAP in skeletal muscle (Bayer et al., 1998). In agree-

ment with Bayer et al. (1998) we show the presence of a CaMKII $\beta$  in the SR membrane with a molecular mass close to 72 kDa.

### Anchored kinases in muscle

PKA is associated with cardiac RyRs and anchored to junctional face membrane by an A-kinase anchoring protein (Marx et al., 2000). The possibility that skeletal RyRs contain anchored PKA in addition to one or more CaMKII isoforms remains to be explored. Further questions are 1) are kinases anchored directly to the RyR or via other proteins associated with the RyR in the junctional face membrane, e.g., triadin and junctin (Zhang et al., 1997); and 2) is the RyR, or an associated regulatory protein, phosphorylated?

The possibility that kinases are anchored to the RyR further underlines the enormity of the RyR macromolecular complex in skeletal muscle. The complex includes the RyR, triadin, junctin, and calsequestrin in the SR lumen (Zhang et al., 1997), the DHPR, FKBP12, and calmodulin on the cytoplasmic domain (Franzini-Armstrong and Protasi, 1997), and anchored kinases (this study; Wang and Best, 1992; Hain et al., 1994), and a variety of associated glycolytic enzymes in the triad junction (Kim et al., 1990).

### Functional consequences of phosphorylation by CaMKII in skeletal muscle

Phosphorylation by CaMKII had functional implications for two ligand-gated RyR responses that were examined. First, activation by ATP was reduced by >50%. This was in contrast to an increased efficacy of ATP following phosphorylation by exogenous PKA or PKC (Sonnleitner et al., 1997) or membrane-associated kinases (Hermann-Frank and Varsanyi, 1993). The different results again suggest a variety of phosphorylation sites associated with the RyR.

The second functional consequence of phosphorylation was reduced sensitivity of the RyR to cytoplasmic  $[\text{Ca}^{2+}]$  with both activation and inhibition shifted to higher  $[\text{Ca}^{2+}]$ s. This result is again in contrast to reports of enhanced  $\text{Ca}^{2+}$ -activation (Hermann-Frank and Varsanyi, 1993; Sonnleitner et al., 1997).

### Phosphorylation sites on the RyR channel complex

A major phosphorylation site on the skeletal RyR has been identified at serine 2843 (Suko et al., 1993) and, given the size of the cytoplasmic domain of the RyR and the number of serine, threonine, and tyrosine residues, it is possible that more sites will be identified. In addition, phosphorylation of associated proteins (e.g., calsequestrin) could alter RyR activity. Thus we cannot differentiate between effects due to

phosphorylation of the RyR itself in native vesicles and effects due to phosphorylation of an associated regulatory protein.

### Physiological significance of the anchored CaMKII in skeletal muscle

It could be argued that CaMKII would be phosphorylated in vivo because ATP and calmodulin concentrations are high, and thus that ATP sensitivity might be low. However, ATP sensitivity remains high in skinned fibers where ATP activation appears to be a prerequisite for voltage-sensor controlled  $\text{Ca}^{2+}$  release and adenosine strongly inhibits voltage-sensor controlled  $\text{Ca}^{2+}$  release (Blazev and Lamb, 1999a, b). Adenosine is a competitive inhibitor of ATP's direct activation of RyRs (Chan et al., 2000; Laver et al., 2000, 2001). We show here that phosphorylation of RyRs leads to reduced activation by ATP and reduced competitive inhibition by adenosine. If the residual sensitivity to ATP and adenosine in phosphorylated channels is sufficient to explain the effects of these compounds in skinned fibers, then the RyR may be phosphorylated in vivo. However, if the residual activity is insufficient to explain the effects, it is likely either that the channels are not phosphorylated or other modulatory factors, not present in the bilayer, alter the in vivo sensitivity of the RyR to ATP and adenosine.

If the RyR complex is phosphorylated in vivo, phosphorylation-induced activation and ATP-induced activation may together contribute to the background activity of RyRs that is suppressed by  $\text{Mg}^{2+}$  inhibition.  $\text{Mg}^{2+}$  inhibition is relieved during the EC coupling process (Lamb and Stephenson, 1991). In addition to relief of  $\text{Mg}^{2+}$  inhibition, the II-III loop of the DHPR is likely to activate the RyR through either the A or the C regions, or both (Lu et al., 1994, 1995; El-Hayek et al., 1995; Dulhunty et al., 1999; Proenza et al., 2000). Activation by the II-III loop would be added to the background activation of the RyR provided by both ATP and phosphorylation.

The question arises of whether the anchored CaMKII is constitutively active in vivo and the RyR activated by phosphorylation, or whether phosphorylation and dephosphorylation are part of a signaling system that can modulate EC coupling. If RyRs are phosphorylated by the anchored CaMKII in vivo, then they must be dephosphorylated during vesicle isolation, as phosphatase had no effect on the activity of the channels unless they were first exposed to ATP. RyRs could have been exposed to endogenous phosphatases during SR vesicle isolation. Indeed, Hain et al. (1994) found that 60% of RyRs from native SR vesicles were in a dephosphorylated state when first incorporated into bilayers.

In conclusion, we find that phosphorylation of the skeletal muscle RyR channel complex by an endogenous CaMKII causes an increase in RyR activity and alters the response of the RyR to ATP and  $\text{Ca}^{2+}$  activation. This was

not an exhaustive test of the effects of phosphorylation on ligand-induced activation and inhibition of RyRs, and effects on other important ligand-based interactions may well be discovered. It is likely that the phosphorylation by the CaMKII influences EC coupling by an effect on the background activity of the RyR.

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