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Enhancement of Ca^{2+} release channel activity by phosphorylation of the skeletal muscle ryanodine receptor**

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The Ca^{2+} release channel of rabbit skeletal muscle sarcoplasmic reticulum (SR) can be phosphorylated by membrane associated protein kinase(s) utilizing endogenously synthesized or exogenously added ATP. The channel protein has been enriched in non-phosphorylated and phosphorylated form from heavy SR following solubilization with CHAPS (3-[(3-cholamidopropyl)dimethylammonio-1-propane-sulfonate) and ultracentrifugation on a linear sucrose/CHAPS gradient. Reconstitution of the isolated channels into planar bilayers shows that phosphorylation enhances the open probability by increasing the sensitivity towards micromolar Ca^{2+} and ATP. The phosphorylation induced enhancement of the channel activity can be reversed by purified protein phosphatase 2A.

Skeletal muscle; Ryanodine receptor; Phosphorylation; Ca^{2+}-channel; Sarcoplasmic reticulum; Ca^{2+} release

1. INTRODUCTION

The ryanodine receptor of skeletal and cardiac muscle has been shown to function as Ca^{2+} release channel of sarcoplasmic reticulum (SR) [1]. Both channels are regulated by micromolar Ca^{2+} and millimolar Mg^{2+}; they can be further modulated by a series of ligands like adenosine nucleotides, and by pharmacological agents like caffeine, ryanodine, anaesthetics, etc. [2].

Phosphorylation of the ryanodine receptor in junctional SR from rabbit skeletal and canine cardiac muscle has been reported to occur by Ca^{2+}/calmodulin- [3,4], cAMP- [5,6] and cGMP-dependent protein kinases [5]. One unique phosphorylation site, namely serine-2809, has been localized on the cardiac ryanodine receptor. In parallel to this phosphorylation, the Ca^{2+} channel is activated [7]. Recently, Suko et al. [8] reported phosphorylation of the isolated ryanodine receptor from rabbit skeletal muscle SR by purified cAMP-, cGMP-, and Ca^{2+}/calmodulin-dependent protein kinases. All these protein kinases incorporate phosphate exclusively into serine-2843, a region which is homologous to the phosphorylation site of the cardiac channel. The role of the skeletal muscle channel phosphorylation in the Ca^{2+} release process, however, has not been established.

Recently, we have shown that triads isolated from rabbit skeletal muscle contain besides sarcoplasmic and T-tubular membrane proteins also glycolytic enzymes. After supplying glycerinaldehyd 3-phosphate or fructose 1,6-bisphosphate, these enzymes catalyze substrate level ATP synthesis. This triphosphate is produced transiently and appears to be compartmentalized in the junctional gap [9]. It is consumed mainly for phosphorylation of triadic porteins by endogenously present protein kinases. One of these phosphorylated proteins is the ryanodine receptor [9]. This allowed us to isolate the Ca^{2+} release channel following in situ phosphorylation. Here we show that after reconstitution of the receptor protein into planar lipid bilayers phosphorylation enhances the activity of the Ca^{2+} release channel by increasing its sensitivity towards various ligands.

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Abbreviations: SR, sarcoplasmic reticulum; P_o, open probability; T-tubule, transverse tubule, an infolded extension of the plasma membrane in close proximity to sarcoplasmic reticulum in muscle; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; Pefabloc, 4-(2-aminoethyl)benzylsulfonylfluoride; PIPES, piperazine-*N,N'*-bis[2-ethanesulfonic acid].

2. MATERIALS AND METHODS

2.1. *Preparations of triads/terminal cisternae*

The membranes were prepared from rabbit back skeletal muscle according to [10].

2.2. *Endogenous [γ-^{32}P]ATP synthesis*

[γ-^{32}P]ATP formation employing the triadic membranes (2.1 mg/ml) was assayed at 30°C in 20 mM imidazole, pH 7.0, 20 mM Na-acetate, 1 mM EGTA, 1 mM DTE, 2 mM NAD^+, 20 mM KF, 50 μM P^1,P^5-di(adenosine-5')pentaphosphate (AP_5A), and 1 mM [^{32}P]H_3PO_4 (1 μCi/ml). The reaction was started by addition of a mixture containing

2 mM D-glyceraldehyde 3-phosphate and 6 mM ADP. During incubation three parallel probes were removed and analyzed for [γ-^{32}P]ATP formation as described by [9], for [^{32}P]phosphate incorporation into proteins according to [11] and by SDS-PAGE according to [12]. For this purpose 40–50 μg of the phosphorylated membranes were applied onto a 7.5% SDS-polyacrylamide gel.

2.3. *Phosphorylation by exogenously added [γ-^{32}P]ATP*

Triadic proteins at a protein concentration of ca. 3 mg/ml were incubated with 1 mM [γ-^{32}P]ATP, 3 mM Mg^{2+} (1 mCi/ml) in presence of 1 mM DTE, 1 mM EGTA, 2 μM ocadaic acid, 30 mM NaF in 20 mM imidazole-HCl, pH 7.0, 20 mM Na-acetate. During phosphorylation at 30°C for 2 min two parallel probes were removed and analyzed for [^{32}P]phosphate incorporation and by SDS-PAGE as described above.

2.4. *Preparation of 'heavy' SR fraction*

'Heavy' SR vesicles were isolated from rabbit back muscle as described by [13]. The membranes recovered from the 36–40% region of a linear sucrose gradient were pelleted at 123,000 × *g* for 60 min, resuspended in 0.3 M sucrose, 10 mM K-PIPES, pH 6.8, rapidly frozen in liquid nitrogen and stored at −70°C. Protease inhibitors were added to the muscle homogenate in concentrations of 200 μM Pefabloc, 100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM benzamidine.

2.5. *Enrichment of the Ca^{2+} release channel*

The Ca^{2+} release channel was enriched as the ryanodine receptor mainly following the method of [13]. Heavy SR was solubilized for 1 h at 0°C and subsequently for 30 min at room temperature with 1.6% CHAPS in a buffer containing 1 M NaCl, 100 mM EGTA, 150 μM $CaCl_2$, 20 mM Na-PIPES, pH 7.2, 0.5% phosphatidylcholine, 5 mM AMP, protease inhibitors in identical concentrations as used during SR preparation described above and additionally 1 μM calpain 1 inhibitor (*N*-acetyl-Leu-Leu-norleucinal) and 1 μM calpain 2 inhibitor (*N*-acetyl-Leu-Leu-methioninal). Following ultracentrifugation at 100,000 × *g* the supernatant was applied onto a 10–28% linear sucrose gradient containing 1–0% CHAPS, 0.7 M NaCl, 1 mM DTE, 70 μM EGTA, 100 μM Ca^{2+}, 13 mM Na-PIPES, pH 7.2, 3.3 mM AMP, 0.5% phosphatidylcholine, all protease inhibitors in identical concentrations as used before, and sedimented for 16 h at 92,000 × *g* at 4°C. To localize the receptor in the gradient, a sample of vesicles was labelled with 40 nM [^{3}H]ryanodine during solubilization. For enrichment of the phosphorylated ryanodine receptor, the SR vesicles were phosphorylated with exogenously added [γ-^{32}P]ATP. Phosphorylated membranes were cooled on ice and collected by ultracentrifugation at 100,000 × *g* for 30 min. Pelleted membranes were resuspended in a 5 mM Na-PIPES, pH 7.4, containing 1 μM ocadaic acid, 20 mM NaF and 100 μM Pefabloc. The Ca^{2+} release channel was then solubilized as described above. Three solubilized probes, the unlabelled, the [^{3}H]ryanodine-labelled and the one obtained following phosphorylation of SR membranes, were centrifuged in parallel through the above sucrose/CHAPS gradients. After fractionation of the gradients, aliquots were analyzed for radioactivity and for protein composition on a 7.5% SDS-PAGE according to [12]. Autoradiography of [^{32}P]phosphate labelled probes were performed on Kodak X-ray film at −70°C.

2.6. *Planar lipid bilayer measurements*

The isolated ryanodine receptor was incorporated into planar lipid bilayers of the Müller-Rudin type. Bilayers were formed from a mixture of phosphatidylethanolamine/phosphatidylserine/L-α-diphytanoyl phosphatidyl choline in a ratio of 5:4:1 (20 mg/ml phospholipid in decane) across a 250 μm-aperture in symmetric buffer solutions containing 250 mM KCl, 150 μM Ca^{2+}, 100 μM EGTA, 20 mM PIPES, pH 7.2. Hence K^+ was the charge carrier in our experiments. Aliquots (of <10 μl) from the solubilized receptor were added to one side of the bilayer chamber defined as the *cis* (cytoplasmic) side. The incorporated channels were detected as steplike increases in current. Electrical signals were filtered at 1 kHz through an 8-pole low-pass bessel filter and digitized at 3 kHz. Applied voltages are defined with respect to the *trans* side of the bilayer chamber. Data were acquired, stored, and analyzed on an IBM-compatible AT-computer using Axon Instruments hard- and software. Open probability values (P_o) and time histograms were calculated from representative data segments of 20–40 s length. The total recording time was >5 min for any experimental condition tested.

2.7. *Other methods*

Ca^{2+} concentrations were calculated using a computer program and binding constants published in [14].

Protein was determined by [15] employing bovine serum albumin as standard.

2.8. *Materials*

[γ-^{32}P]ATP and [γ-^{32}P]H_3PO_4 were from NEN DuPont de Nemours (Germany) and ICN Biomedicals (Germany). The molecular weight standards for SDS-PAGE were obtained from Sigma (Germany) and the protease inhibitors were purchased from Boehringer (Germany). Hydroluma cocktail was from Baker Chemicals (The Netherlands). The lipids were obtained from Avanti Polar Lipids (USA). All other reagents used were of p.A. grade.

3. RESULTS

Maximally about 80 pmol of [^{32}P]phosphate are incorporated into proteins during compartmentalized ATP synthesis via substrate level phosphorylation in the triadic junction (not shown; for comparison see [9]). However, adding 1 mM ATP exogenously, this level can be enhanced about 10-fold. Fig. 1A shows that under the conditions chosen about 700 pmol phosphate is incorporated per mg of protein and this level is maintained over a time period of about 15 min. Following SDS-PAGE the radioactively labelled polypeptides are found in a molecular weight range between 370k and 15k (Fig. 1B). For comparison, Fig. 1C shows the pattern of stained proteins. No qualitative differences are observed using endogenously synthesized or exogenously added ATP.

In view of an expected role of ATP compartmentalized in the triadic junction as a possible modulator of Ca^{2+} release, it seems especially interesting to look for the two high molecular weight phosphorylated polypeptides with M_r values of 370k and 320k representing the Ca^{2+} release channel. These labelled polypeptides have been isolated as ryanodine receptor by treatment of the membranes with CHAPS and by ultracentrifugation through a linear sucrose gradient. Fig. 2A shows that [^{3}H]ryanodine binding reaches a maximum in fractions 7 and 8; in these fractions polypeptides with apparent molecular weights of 370k and 320k are enriched (Fig. 2B). Autoradiography shows that both proteins are [^{32}P]phosphate-labelled (Fig. 2C).

The phosphorylated receptor protein present in the peak gradient fractions (see Fig. 2) has been reconstituted into planar lipid bilayers. For comparison also the channel activity of the non-phosphorylated receptor protein has been determined. Reconstitution has been initiated in presence of 50 μM Ca^{2+}. In Fig. 3A single-channel recordings of the non-phosphorylated and the

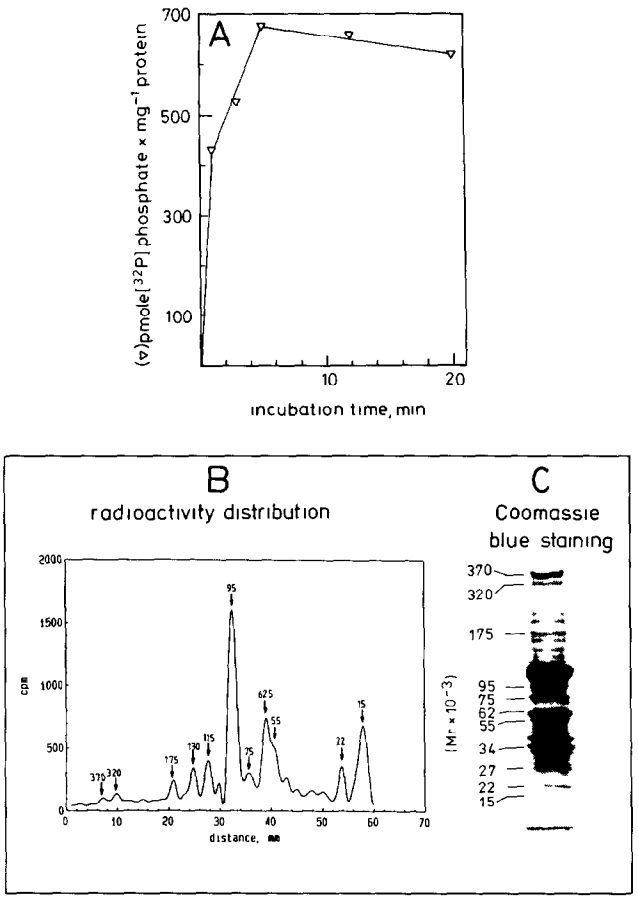


Fig. 1. Protein phosphorylation in skeletal muscle triadic junction by endogenous kinase(s) using exogenously added ATP. (A) Time course of [^{32}P]phosphate incorporation into the triadic proteins using exogenously added ATP. Electrophoretic patterns following phosphorylation. (B) Radioactivity distribution. (C) Coomassie brilliant blue staining. Details for phosphorylation and SDS-PAGE are described in section 2.

phosphorylated receptor are shown at 5 and 50 μM Ca^{2+}; each of the demonstrated parts is representative for the whole recording period. Phosphorylation considerably increases the sensitivity of the channel protein towards (*cis*) Ca^{2+}. At 50 μM Ca^{2+} about 55% of the phosphorylated channels display an increased $P_o > 0.2$. Only these channels have been selected for further statistical and pharmacological analyses. The conductance of the non-labelled channel (567 pS) does not differ from that of the phosphorylated one (516 pS). Fig. 3B compares the open probability of the phosphorylated with that of the non-phosphorylated channel between 1 and 50 μM Ca^{2+}. The phosphorylated channel displays an overall enhanced activity as well as a steeper increase between 1 and 10 μM Ca^{2+}. Analysis of open time histograms (Fig. 3C) shows that the enhanced activity is accompanied by prolonged mean open times. In presence of 50 μM Ca^{2+} mean open times are calculated to be 1.1 ± 0.6 ms (n = 9) for the non-phosphorylated channel and 3.2 ± 1.0 ms (n = 5) for the phosphorylated channel.

Phosphorylation also changes the sensitivity of the channel towards *cis* ATP. Fig. 4A shows representative single-channel recordings of a non-phosphorylated and of a phosphorylated channel. At identical [ATP] the phosphorylated channel exhibits a significantly higher open probability than the non-phosphorylated one. To activate maximally at 0.9 μM Ca^{2+} 1 mM ATP is required for the phosphorylated and more than 4 mM

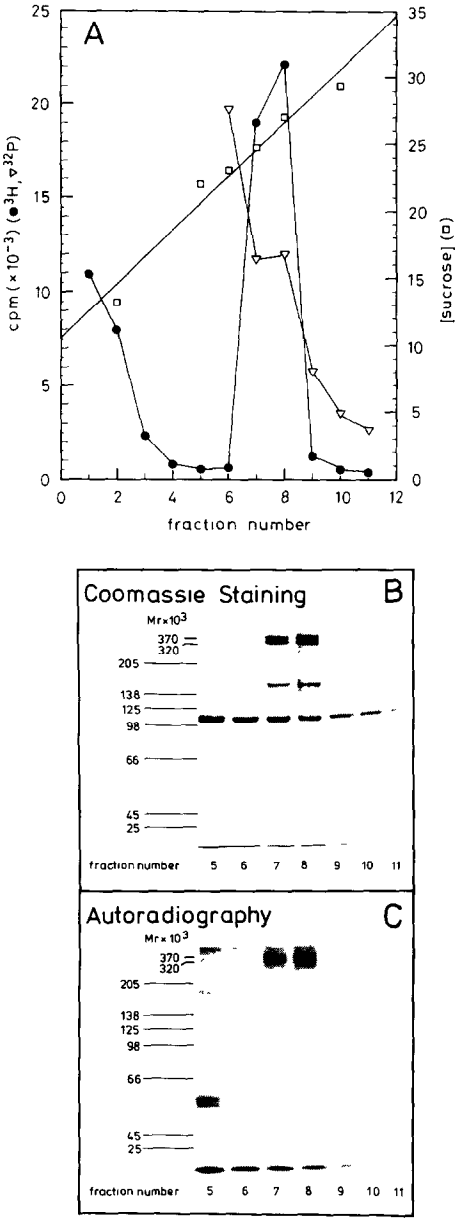
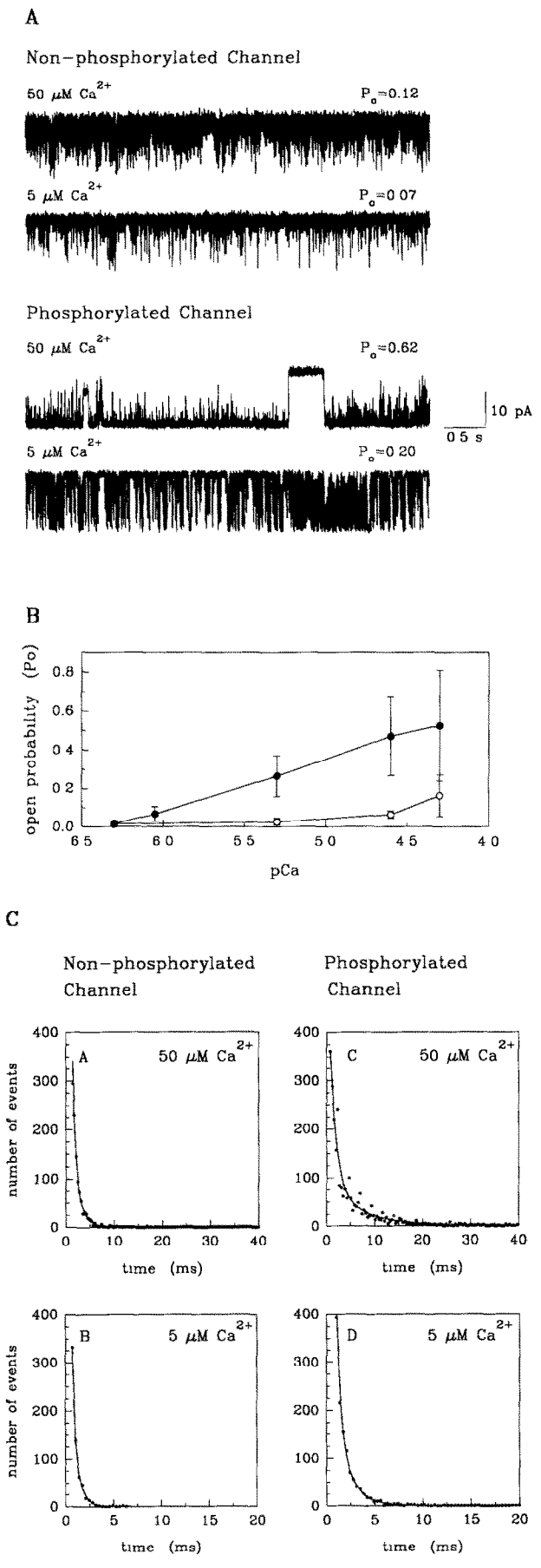


Fig. 2. Enrichment of the phosphorylated skeletal muscle ryanodine receptor. (A) [^{3}H]Ryanodine (●), and [^{32}P]phosphate (▽) distribution profiles of the CHAPS-solubilized heavy SR membranes following centrifugation in a linear sucrose/CHAPS gradient. (B) Coomassie brilliant blue staining of the SDS-polyacrylamide gel obtained from fractions of the [^{32}P]phosphorylated membranes. Molecular weight standards were: myosin (205k), phosphorylase (97.4k), bovine serum albumin (66k), ovalbumin (45k) and carbonic anhydrase (25k) additionally including phosphorylase kinase consisting of α (138.4k), β (125.2k), γ (44.6k) and δ (16.7k) subunits. (C) Corresponding autoradiogram of the SDS gel performed in B.



ATP for the non-phosphorylated channel (Fig. 4B). Fitting the data to a hyperbola shows that the phosphorylated channel yields a K_d value of about 0.5 mM, the non-phosphorylated one of about 2.2 mM. 1 mM ATP also activates the phosphorylated channel maximally at lower (0.4 μM) *cis* Ca^{2+}, however, the absolute P_o remains lower than at 0.9 μM Ca^{2+} (not shown). The K_d [ATP] at 0.4 μM Ca^{2+} is about 0.3 mM.

To prove the reversibility of the phosphorylation induced enhancement of the Ca^{2+} release channel activity, the effect of a highly purified protein phosphatase 2A has been tested. Fig. 5 demonstrates that the protein phosphatase 2A considerably decreases the channel activity. This deactivated channel can, however, still be reactivated by adding ATP on the *cis* side of the channel. Deactivation by phosphatase 2A has been observed in about 85% of the phosphorylated channels tested. Channel activity of the non-phosphorylated protein can not be affected by this protein phosphatase (not shown). Interestingly, the activity of the phosphorylated channel cannot be diminished by protein phosphatase 2A if it has previously been activated by ATP (not shown).

4. DISCUSSION

One of the phosphate acceptors in the junctional gap represents the SR Ca^{2+} release channel [9]. The purified receptor binds 393 ± 65 pmol ryanodine · mg^{-1} receptor protein [16]. The amount of the ryanodine receptor can be calculated from the amount of ryanodine bound per mg of triadic protein. Based on the [^{32}P]phosphate incorporated into the high molecular weight proteins and on the assumption that both the 370k and 320k proteins bind ryanodine, an average phosphorylation degree of 0.3 mol per mol of ryanodine receptor has been calculated, i.e. approximately only one subunit of the holotetrameric receptor protein is phosphorylated under the conditions employed. This situation resembles the ryanodine binding to the channel; in this instance the holotetramer also binds only one mol of this alkaloid [16].

Although all preparation steps of Ca^{2+} release channel are highly standardized, and protease inhibitors are used continuously during the whole purification, the

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Fig. 3. Ca^{2+} sensitivity of the non-phosphorylated and phosphorylated ryanodine receptor channel. (A) Single-channel recordings at 5 and 50 μM free Ca^{2+} on the *cis* side of the channel. The holding potential for the phosphorylated channel was −31 mV, for the non-phosphorylated one −30 mV. Open events are shown as downward deflections. (B) Dependence of open probability on free *cis* [Ca^{2+}]. (●) Phosphorylated channel (n = 10), (○) non-phosphorylated channel (n = 12). (C) Representative open time histograms at different *cis* [Ca^{2+}]. Data are derived from 20 s recordings at given [Ca^{2+}]. Open time histograms were fitted to the sum of a double-exponential function with the following time constants: (A) t_1 = 0.5 ms, t_2 = 1.5 ms; (B) t_1 = 0.3 ms, t_2 = 0.7 ms; (C) t_1 = 1.3 ms, t_2 = 6.8 ms; (D) t_1 = 0.4 ms, t_2 = 1.5 ms.

ratio between the two high molecular weight polypeptides changes from preparation to preparation (for comparison see Fig. 2). The relationship between these peptides is still unknown; however, proteolytic digestion of the protein cannot be excluded.

Our results show that phosphorylation of the ryanodine receptor enhances the sensitivity of the channel towards Ca^{2+}. This observation indicates that Ca^{2+}, in the physiologically relevant range, might play an essential role in initiating the Ca^{2+} release process, but only in the phosphorylated state of the channel. Accordingly, Ca^{2+} release would cease after dephosphorylation of the channel. This is, however, not the unique mode of regulating this channel. As shown here, the phosphorylated receptor protein shows a ca. 4-fold higher sensitivity towards ATP than the non-phosphorylated one (Fig. 4).

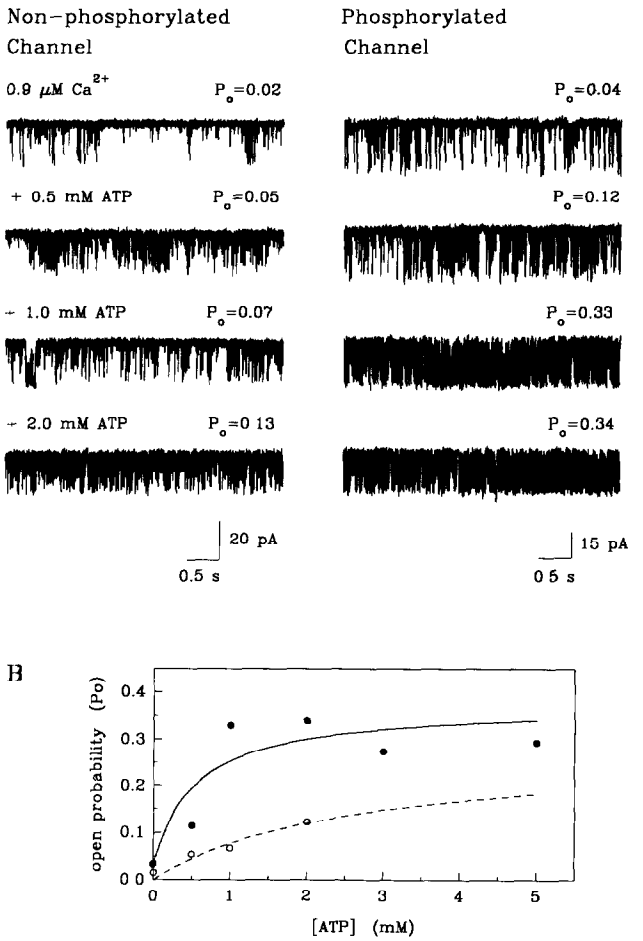

Fig. 4. ATP sensitivity of the non-phosphorylated and phosphorylated ryanodine receptor channel. (A) Single-channel recordings in presence of 0.9 μM *cis* Ca^{2+} and indicated [ATP]. The holding potential was −38 mV for the phosphorylated channel and −32 mV for the non-phosphorylated one. Open events are shown as downward deflections. (B) Dependence of open probability on *cis* [ATP]. Data are taken from representative single channel recordings of the phosphorylated (●) and non-phosphorylated channel (○) in presence of 0.9 μM *cis* Ca^{2+}. Curves are fitted as a hyperbola.

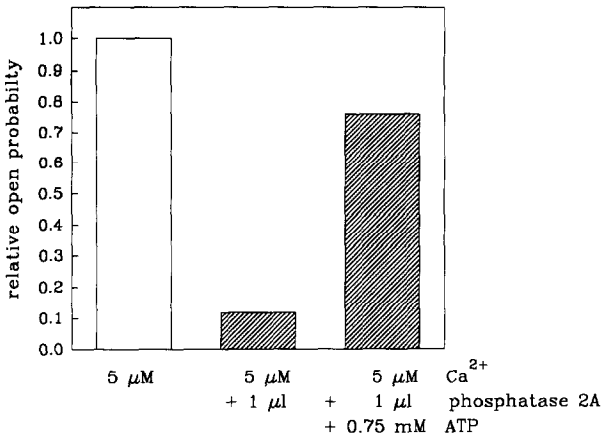

Fig. 5. Reversible deactivation of the phosphorylated channel by protein phosphatase 2A. Bars represent relative open probabilities of phosphorylated channels activated by Ca^{2+}. All modulators were added to the *cis* side of the chamber in the sequence given below. A relative P_o of 1 represents an absolute P_o value of 0.2.

Thus, ATP seems to have a dual role in regulation of Ca^{2+} release: first, being an allosteric effector which enhances the open probability [17]; secondly, being substrate for protein kinase(s) which phosphorylate(s) the channel as demonstrated here. The allosteric activation occurs with the non-phosphorylated channel in a range up to 4 mM ATP. The ATP concentration in the junctional gap seems to depend on the local ATP synthesis and might not be identical with the ATP concentration in the sarcoplasm [9]. Phosphorylation of the channel enhances the ATP sensitivity so much that the junctional concentration is sufficient to fully activate the channel. Vice versa this might indicate that the ATP concentration in the junctional gap is in the range of the sensitivity of the phosphorylated channel, i.e. ca. 0.5 mM. In any case it is only the phosphorylated channel which seems to be relevant for modulation by the low molecular weight effectors, Ca^{2+} and ATP.

Phosphorylation of the channel seems to occur very specifically. As demonstrated here, the phosphorylation induced enhancement of the channel activity can be reversed with protein phosphatase 2A. Thus, the regulation of the phosphatase activity seems to be very important in relation to the modulation of the channel activity.

Recently, Wang and Best [18] reported the inactivation of the skeletal muscle Ca^{2+} release channel recorded in excised patches from SR under conditions favorable for protein phosphorylation. It has not been studied in detail, however, which protein component can be phosphorylated under their conditions. Their results are controversial to our observations and might be explained by phosphorylation of another protein component rather than the ryanodine receptor protein itself. Proteins tightly associated to the SR Ca^{2+} release channel are reported [19] but their role in regulating Ca^{2+}

release, directly or following any covalent modification, has not been clarified till now.

Our results show similarities to the cardiac ryanodine receptor which is also activated upon phosphorylation [7]. However, Ca^{2+} release from the cardiac SR contributes only partially to the cardiac myoplasmic [Ca^{2+}]. This level is influenced mainly by cAMP-dependent phosphorylation of the voltage-dependent Ca^{2+} channel as well as by that of phospholamban (for review see [20,21]). Thus, the role and part that phosphorylation of the cardiac ryanodine receptor plays is presently unclear.

The contribution of Ca^{2+} release from SR to excitation–contraction coupling in skeletal muscle has been discussed controversially (for review see [22–24]). In early studies Blinks et al. [25] have shown that intracellular [Ca^{2+}] remains high for several milliseconds after cessation of tetanic stimulation. Ca^{2+} is also maintained at high level for several milliseconds after repolarization under voltage clamp conditions [26,27]. The delay between repolarization and the decay of the Ca^{2+} transient is further accentuated by caffeine [28]. Thus, also the ryanodine receptor phosphorylation – possibly occurring at high sarcoplasmic [Ca^{2+}] ($\geq$10 μM) – can lead to an enhanced channel activity resulting in an increased Ca^{2+} release from SR. This mechanism offers an alternate explanation for the prolonged high Ca^{2+} level maintained after repolarization. This might be relevant under extreme conditions, like fatigue. A local drop in [ATP] during muscle exercise or fatigue might partially be antagonized by phosphorylation of the SR Ca^{2+} release channel leading to an enhancement of myoplasmic [Ca^{2+}].

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