THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 37, Issue of September 13, pp. 22759–22763, 1996

Printed in U.S.A.

Role of Malignant Hyperthermia Domain in the Regulation of Ca^{2+} Release Channel (Ryanodine Receptor) of Skeletal Muscle Sarcoplasmic Reticulum*

(Received for publication, May 29, 1996, and in revised form, July 10, 1996)

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A fusion protein encompassing Gly^{341} of the skeletal muscle ryanodine receptor was used to raise monoclonal antibodies; epitope mapping demonstrates that monoclonal antibody 419 (mAb419) reacts with a sequence a few residues upstream from Gly^{341}. The mAb419 was then used to probe ryanodine receptor (RYR) functions. Our results show that upon incubation of triads vesicles with mAb419 the Ca^{2+}-induced Ca^{2+} release rate at *p*Ca 8 was increased. Equilibrium evaluation of [^{3}H]ryanodine binding at different [Ca^{2+}] indicates that mAb419 shifted the half-maximal [Ca^{2+}] for stimulation of ryanodine binding to lower value (0.1 *versus* 1.2 μM). Such functional effects may be due to a direct action of the Ab on the Ca^{2+} binding domain of the RYR or to the perturbation by the Ab of the intramolecular interaction between the immunopositive region and regulatory domain of the RYR. The latter hypothesis was tested directly using the optical biosensor BIAcore (Pharmacia Biotech Inc.): we show that the immunopositive RYR polypeptide is able to interact with the native RYR complex. Ligand overlays with immunopositive digoxigenin-RYR fusion protein indicate that such an interaction might occur with a calmodulin binding domain (defined by residues 3010–3225) and with a polypeptide defined by residues 799-1172. In conclusion our results suggest that the stimulation by the mAb419 of the RYR channel activity is due to the perturbation of an intramolecular interaction between the immunopositive polypeptide and a Ca^{2+} regulatory site probably corresponding to a calmodulin binding domain.

The skeletal muscle sarcoplasmic reticulum is an intracellular membrane compartment that controls the myoplasmic Ca^{2+} concentration, thereby playing an important role in the excitation-contraction coupling mechanism (Endo, 1985; Franzini-Armstrong, 1980). Skeletal muscle contraction is triggered by release of Ca^{2+} from sarcoplasmic reticulum terminal cisternae (Somlyo *et al.*, 1985) via a Ca^{2+} release channel which is believed to be the ryanodine receptor (RYR)[1] (Rios and Pizzaro, 1991), a large homotetrameric oligomer made up of four 565-kDa subunits (Pessah *et al.*, 1986; Inui *et al.*, 1987; Smith *et al.*, 1988; Lai *et al.*, 1988; Takeshima *et al.*, 1989; Zorzato *et al.*, 1990; Nakai *et al.*, 1990). Recently it has been found that calmodulin both activates and inhibits the RYR Ca^{2+} channel, depending upon the free [Ca^{2+}]; at submicromolar Ca^{2+} calmodulin activates the channel, while at micromolar Ca^{2+} calmodulin inhibits channel activity (Tripathy *et al.*, 1995; Buratti *et al.*, 1995). The activation of the RYR Ca^{2+} channel by calmodulin is accompanied by the binding of calmodulin to the domain encompassed between residues 3010–3225 (Buratti *et al.*, 1995). An Arg to Cys substitution at position 615 in the primary structure of the RYR Ca^{2+} channel from malignant hyperthermia (MH)-susceptible pigs has been correlated with abnormalities in the sensitivity of Ca^{2+} channel activity, indicating that mutations in the RYR are potential molecular defects underlying MH (Mickelson *et al.*, 1988; Fill *et al.*, 1990, 1991; Fujii *et al.*, 1991). Since the description of the $Arg^{615} \rightarrow$ Cys mutation in the MH pigs, eight other mutations in the RYR have been shown to cosegregate with the MH phenotype in human pedigrees. Six out of these eight mutations are clustered between Arg^{163} and Tyr^{522}. However, except for the mutation $Arg^{615} \rightarrow$ Cys, to date no biochemical evidence has been obtained linking these mutations to altered functional properties of the human RYR.

In the present report we raised a monoclonal antibody (mAb419) against a skeletal muscle RYR domain encompassing Gly^{341}, a residue that has been shown to mutate in 10% of human MH pedigrees (Quane *et al.*, 1994). We found that this mAb increases both Ca^{2+}-induced Ca^{2+} release rate and shifted the Ca^{2+} dependence ryanodine binding to lower values. Biospecific interaction analysis revealed that the immunopositive polypeptide interacts with the native RYR complex. Ligand overlay experiments indicate that this interaction might occur via the calmodulin binding domains of the RYR defined by residues 3010–3225.

EXPERIMENTAL PROCEDURES

Materials

Nitrocellulose was from Schleicher and Schuell; restriction enzymes, DNA-modifying enzymes, calmodulin, digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester, anti-digoxigenin peroxidase-conjugated Ab, and BM chemiluminescence were from Boehringer Mannheim; anti-mouse IgG, protein molecular weight markers, and dansyl-calmodulin were from Sigma; the bluescript cloning vector was from Stratagene; $^{45}Ca^{2+}$ and [^{3}H]ryanodine were from DuPont NEN; all ther chemicals were reagent grade.

Methods

Preparation of Sarcoplasmic Reticulum Fractions—Terminal cisternae fraction were obtained from the white skeletal muscle of New Zealand rabbits as described by Saito *et al.* (1984). Triads were purified according to Marty *et al.* (1994).

DNA Manipulation and Production of Fusion Proteins—DNA manip-

* This work was supported by grants from Telethon (Italy), Ministero d'Università e della Ricerca Scientifica e Tecnologica 60%, and Association Française contre les Myopathies. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RYR, ryanodine receptor; MH, malignant hyperthermia; mAb, monoclonal antibody; MES, 4-morpholineethanesulfonic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

ulations were carried out according to standard protocols as described in Maniatis *et al.* (1989). To cover the entire RYR coding sequence we constructed several fusion proteins (Koerner *et al.*, 1991). The details of the construction of RYR fusion proteins have been described previously (Treves *et al.*, 1993; Menegazzi *et al.*, 1994). The *Exo*III/mung nuclease deletion of the 3′ end of the cDNA encoding the RYR fusion protein PC28 was carried out as described previously (Zorzato *et al.*, 1990).

SDS-Polyacrylamide Gel Electrophoresis and Immunological Techniques—Slab gel electrophoresis was carried out as described by Laemmli (1970). Western blots of bacterial extracts were carried out overnight as described by Gershoni *et al.* (1985). Indirect immunoenzymatic staining of Western blots was carried out as described by Young *et al.* (1985) and detailed by Treves *et al.* (1993). mAb419s were extracted from culture medium first by $(NH_4)_2SO_4$ precipitation and further purified by using DEAE-cellulose anion-exchange column chromatography (Harlow and Lane, 1988). The immunoglobulin fraction was then dialyzed overnight against phosphate-buffered saline at 4 °C.

$^{45}Ca^{2+}$ Release and Ryanodine Binding Assay—Triad vesicles (1 mg/ml) were incubated in the presence or in the absence of mAb419 (Ab/HSR ratio of 1) for 2 h at 37 °C. $^{45}Ca^{2+}$ loading was carried out for 30 min at room temperature in the presence of 2.5 mM $CaCl_2$, 75 mM KCl, 25 mM MES, pH 7.0. Rapid kinetic $^{45}Ca^{2+}$ release was measured by using a rapid filtration apparatus (Biologic, Claix, France) as described previously (Moutin and Dupont, 1988). [^{3}H]Ryanodine binding was measured as described previously (Marty *et al.*, 1994) at an Ab/heavy sarcoplasmic reticulum ratio of 1 in a solution containing 25 mM MES, pH 7.0, 0.15 M KCl, 2 mM EGTA, and 50 nM [^{3}H]ryanodine, and various concentration of Ca^{2+}.

Real Time Surface Plasmon Resonance Recording—For real time binding experiments a BIAcore biosensor system (Pharmacia Biotech. Inc.) was used. All experiments were performed at 25 °C. Between injections the sensor chip was continuously washed with 10 mM HEPES, pH 7.5, 150 mM NaCl, 3.4 mM EDTA, 0.005% BIAcore surfactant P20 (Pharmacia). The fusion protein 19E was directly coupled through its amino groups to the sensor surface activated by *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide according to the manufacturer's instruction. The remaining *N*-hydroxysuccinimide groups were then inactivated with 1 mM ethanolamine.

Ligand Overlays—Fusion protein 19E was electroeluted from SDS-polyacrylamide gel and then labeled with digoxigenin-3-*O*-methylcarbonyl-ϵ-aminocaproic acid *N*-hydroxysuccinimide ester according to manufacturer's instructions. Overlays with digoxigenin-fusion protein 19E and -calmodulin were carried out as described by Menegazzi *et al.* (1994). $^{45}Ca^{2+}$ overlay was carried out as described by Maruyama *et al.* (1984).

RESULTS AND DISCUSSION

Characterization of mAb419—We raised monoclonal Abs (mAb419) against a fusion protein (number 19) encompassing residues 281–620 of the rabbit skeletal muscle RYR protomer. The monoclonal Ab 419 reacted strongly with the protein band corresponding to the RYR protomer and with its own Ag (Fig. 1*A, lanes 1b and 2b*, respectively). To map the RYR immunopositive peptide within fusion protein 19, we made a set of constructs and probed the respective fusion proteins with mAb419. Fig. 1*B* shows that the immunological reactivity of the monoclonal antibody was retained only by fusion proteins 19A and 19E. On the contrary the Ab did not recognize fusion proteins 19C and 19D, suggesting that the sequence surrounding the break point between 19C and 19D is crucial to confer the immunoreactivity to mAb419. Of interest, the break point, which abolishes immunological reactivity occurs in correspondence of Met^{335}, *i.e.* few residues upstream Gly^{341}.

Effect of mAb419 on the Functional Properties of the RYR Ca^{2+} Channel—Triad vesicles were incubated with mAb419 and then passively loaded with $^{45}Ca^{2+}$. Rapid $^{45}Ca^{2+}$ release kinetics were monitored at *p*Ca 8. As shown by Fig. 2*A*, in the presence of mAb419 the rate constant of Ca^{2+} release (k = 32.1 s^{-1}) was increased with respect to control experiments (k = 0.12 s^{-1}). Activation of the Ca^{2+} release rate (k = 4.2 s^{-1} *versus* k = 72.4 s^{-1}) was also observed when the experiments were carried out at *p*Ca 4.5 (Fig. 2*B*). To further confirm the effect of

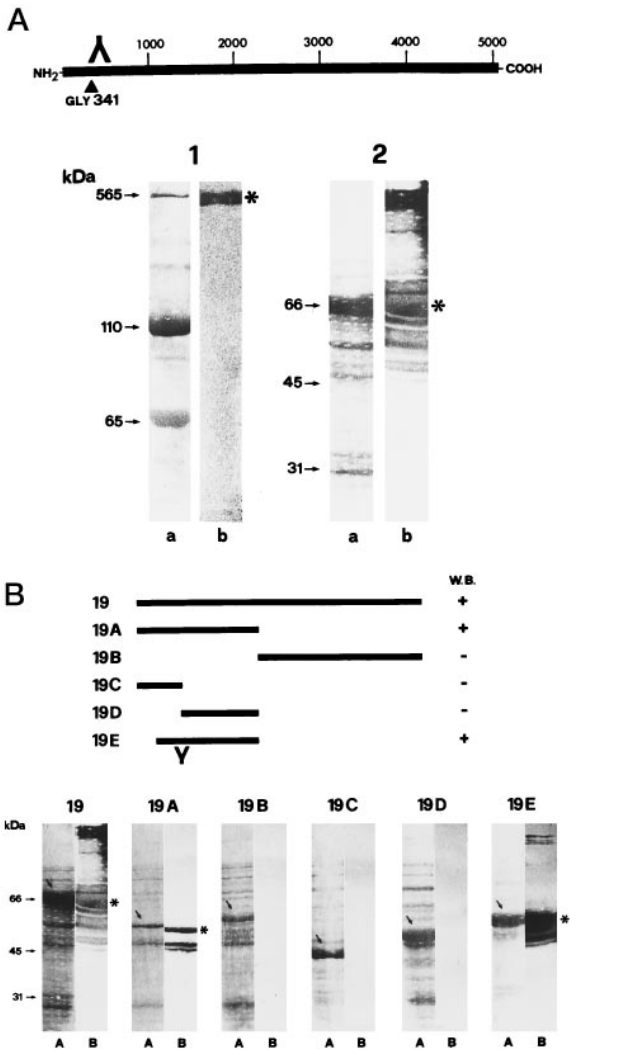

FIG. 1. **Immunological reactivity of mAb 419.** *A*, twenty micrograms of rabbit skeletal muscle terminal cisternae (*panel 1*) or 50 μl of bacterial extract containing fusion protein 19 (*panel 2*) were separated on 7.5% and 10% SDS-PAGE, respectively, and blotted onto nitrocellulose. *Lane a*, Ponceau red staining; *lane b*, indirect immunoenzymatic staining of Western blots (mAb419 was used at a final concentration of 1 μg/ml; secondary Ab was anti-mouse IgG conjugated to alkaline phosphatase). The *top portion* of the figure shows where the immunopositive region is on the planar RYR; *B*, epitope mapping of mAb419. Fusion protein 19 or several constructs covering the whole fusion protein were separated on a 10% SDS-PAGE, blotted onto nitrocellulose, and subjected to indirect immunoenzymatic staining using mAb419 (as described in the legend of Fig. 1). *Lanes A,* Ponceau red staining; *lanes B,* indirect immunoenzymatic staining (50 μl bacterial extracts were loaded per lane).

the mAb419 on the functional properties of RYR, we measured the equilibrium binding of [^{3}H]ryanodine at different *p*Ca. Fig. 2*C* shows that the Ca^{2+} dependence of [^{3}H]ryanodine binding displays a bell-shaped curve both in the presence and in the absence of mAb419. However, the half-maximal [Ca^{2+}] for stimulation of [^{3}H]ryanodine binding was significantly lower in the presence of mAb419 (0.1 *versus* 1.2 μM). No significant change for inhibition of ryanodine binding by high [Ca^{2+}] was observed, indicating the specificity of the effect of mAb419. The increase of Ca^{2+} sensitivity of both $^{45}Ca^{2+}$ release and ryanodine binding rates in the presence of mAb419 could be due to a direct interaction with a domain involved in Ca^{2+} binding or to a modification of a Ca^{2+}-dependent regulatory site, which is adjacent in the three-dimensional structure but may be distant in the linear sequence. To discriminate between these two

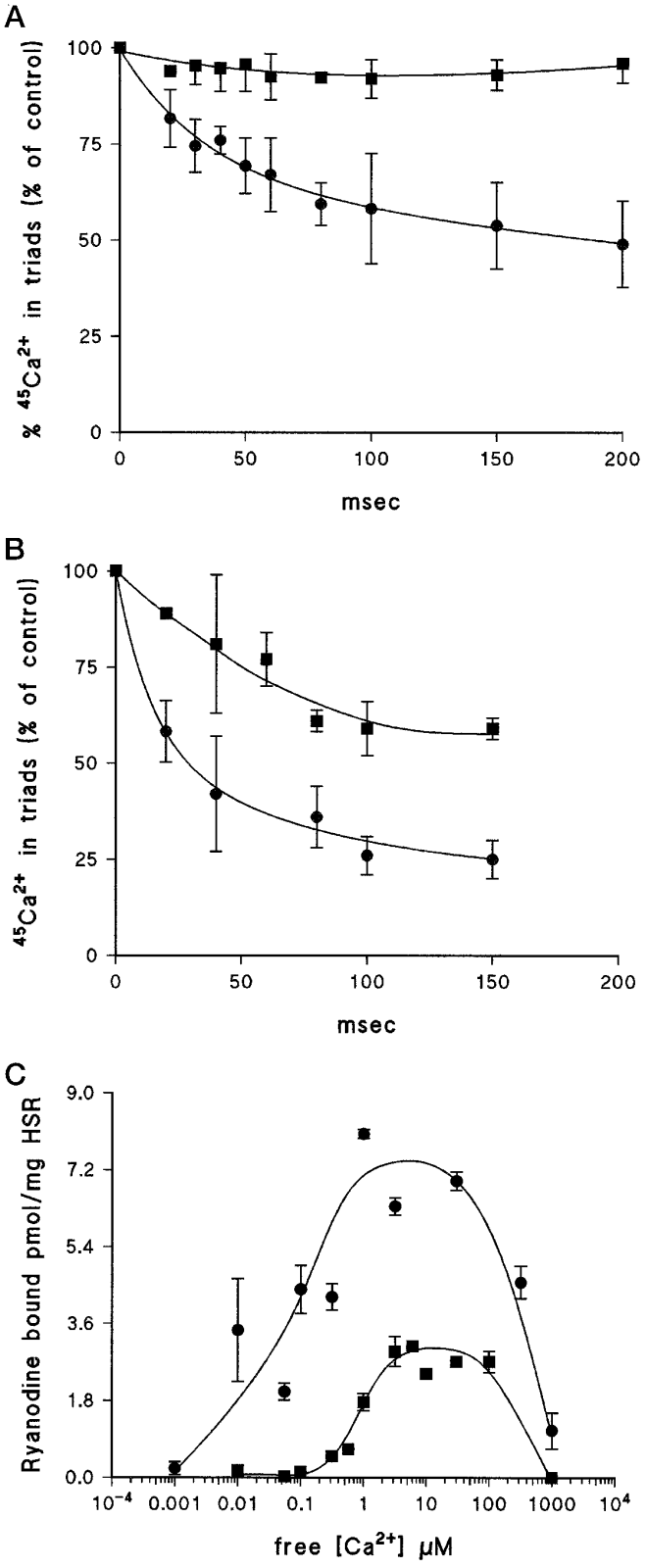

FIG. 2. **Effect of mAb419 on the functional activity of the RYR.** Triad vesicles (1 mg/ml) were incubated in the absence (*filled squares*) or presence (*filled circles*) of mAb419 (Ab: membrane proteins = 1, w/w). Mean + S.D. (n = 3) are shown. Ca^{2+}-induced Ca^{2+} release experiments: triad vesicles were then loaded with ^{45}Ca and Ca^{2+}-induced Ca^{2+} release measured at pCa 8 (*A*) and pCa 4.5 (*B*) as described under "Materials and Methods." $^{45}Ca^{2+}$ loading: 48 + 7 and 65 + 8 nmol/mg p (*A*), 62+7 and 58+13 nmol/mg protein (*B*), in the absence and in the presence of mAb419, respectively. *C*, [^{3}H]ryanodine binding on triad vesicles: Ca^{2+} dependence of equilibrium [^{3}H]ryanodine binding was measured as described under "Materials and Methods."

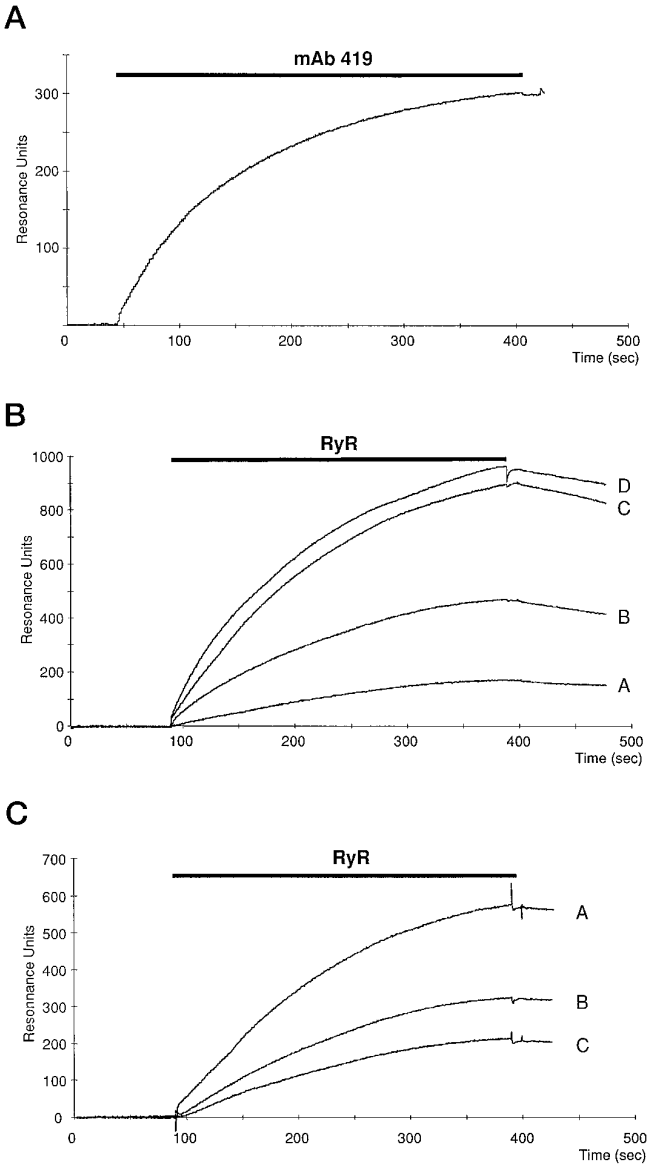

FIG. 3. **Characterization of the binding of fusion protein 19E with the purified RYR using surface plasmon resonance detection.** Fusion protein 19E was immobilized on the sensor chip surface as described under "Materials and Methods." The traces are representative of four different experiments. *A* represents the interaction of mAb419 (5.12 μg) with the immobilized fusion protein 19E. *B* represents the interaction of the purified RYR with immobilized fusion protein 19E. Curves are as follows: *curve A*, 0.45 μg of RYR; *curve B*, 1.1 μg of RYR; *curve C*, 2.25 μg of RYR; *curve D*, 4.5 μg of RYR. The *bar* indicates the injection of 50 μl of RYR complex at a flow rate of 10 μl/min. After each binding experiment, the sensor chip was regenerated as described under "Materials and Methods." The specific binding signal shown was obtained by subtracting the signal measured in the absence of immobilized fusion proteins from the signal in the presence of the fusion protein. *C*, effect of preincubation of RYR with fusion protein 19E. The RYR complex was first preincubated for 15 min at room temperature with RYR complex and then its interaction with the fusion protein coupled to sensor chip was monitored as described in the legend for *B*. Curves are as follows: *curve A*, 1.6 μg of the RYR; *curve B*, 1.6 μg of RYR plus 1.5 μg of fusion protein 19E; *curve C*, 7.5 μg of RYR plus 15 μg of fusion protein 19E.

possibilities, we examined whether the set of RYR fusion proteins we produced encompass Ca^{2+} binding sites. In agreement with previous results, $^{45}Ca^{2+}$ overlay of fusion proteins 19, 19A-19E indicates that none of them contain Ca^{2+} dependent allosteric sites (not shown). Thus, the effect of the mAb on Ca^{2+} dependence of ryanodine binding could be due to the perturba-

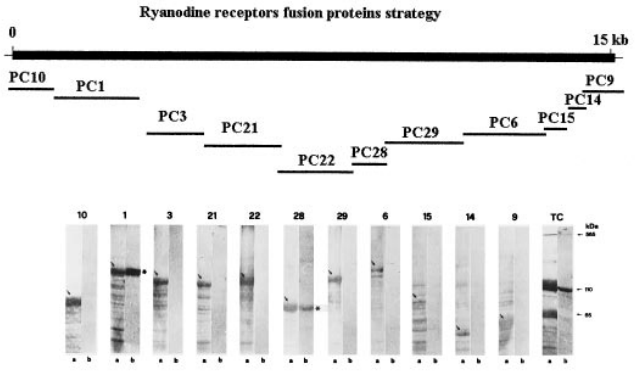

FIG. 4. **Identification of the RYR domains involved in the interaction with fusion protein 19E.** Ligand overlay was carried out at 100 nM digoxigenin-fusion protein 19E. Terminal cisternae or fusion proteins covering the entire length of the RYR were separated on 7.5 or 10% SDS-PAGE, blotted onto nitrocellulose, and colored with Ponceau red (*lanes a*) or subjected to ligand overlay (*lanes b*). Fifty μl of bacterial extracts were loaded per lane. *Arrows* indicate the RYR fusion proteins. *Upper panel*, fusion protein production strategy. The numbering is positive beginning the first nucleotide of initiator methionine. The underlying segments indicate the cDNA fragments cloned into the bacterial expression vectors.

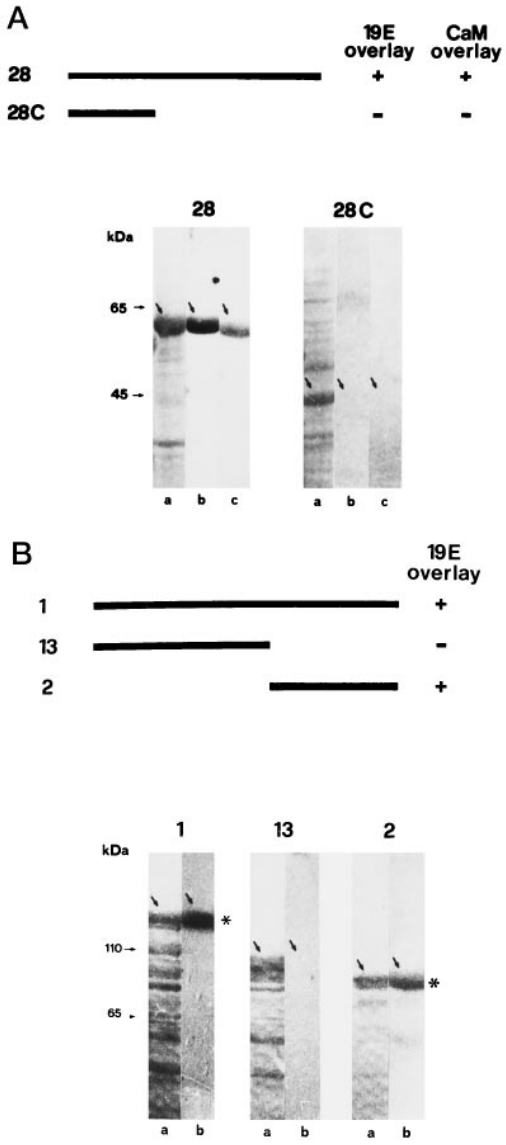

FIG. 5. **Identification of the domain of fusion proteins 28 and 1 involved in the interaction with fusion protein 19E.** *A*, conditions and experiments are the same as in Fig. 5, except that the overlay was carried out on the fusion proteins 28 and with a fusion protein 28 carring a deletion at its COOH terminus. *Lanes: a*, Ponceau red staining of bacterial extracts; *b*, fusion protein overlay; *c*, calmodulin overlay. *Arrows* indicate fusion protein; *B*, conditions and experiments are the same as in *A*. Overlays were carried out on different domains of fusion protein 1. *Lanes: a*, Ponceau red staining of bacterial extracts; *b*, fusion protein 19E overlay. *Arrows* indicate fusion proteins.

tion by the Ab of a regulatory sequence adjacent to the Ab's epitope in the three-dimensional structure. Such a regulatory domain could be localized in the RYR itself or on a protein associated with the RYR complex.

Molecular Interaction of Immunopositive RYR Fusion Protein 19E—To investigate potential molecular interactions of the immunopositive fusion protein 19E with protein components of the Ca^{2+} release machinery, we immobilized fusion protein 19E onto the dextran matrix coating the gold sensor chip, and its interaction with the purified RYR was monitored using the optical biosensor BIAcore (Pharmacia). Fig. 3*A* shows the sensorgram representing real time binding of mAb419 to the immobilized 19E RYR fusion protein; the signal increased and reached a plateau in few hundred seconds, indicating that the immobilization step did not modify the immunological properties of the fusion protein. In the next set of experiments, we analyzed the interaction of the purified RYR complex with the RYR fusion protein 19E. To prevent nonspecific binding of the purified RYR complex to the sensor chip we first injected 10 μl of 1 mg/ml bovine serum albumin and then the purified RYR complex into the flow cell. Injection of bovine serum albumin produced no signal (not shown). The sensorgrams in Fig. 3*B* shows the real time interaction of the immobilized 19E RYR fusion protein with increasing concentrations of purified RYRs. As can be seen, the resonance unit response of the 19E-coupled sensor chip is dose-dependent and approaches saturation after injection of 4.15 μg of purified RYR complex. The interaction between the RYR and the immobilized fuion protein could be inhibited by preincubation of the RYR complex with free fusion protein 19E (Fig. 3*C*). These results clearly indicate that a specific binding between the purified RYR complex and fusion protein 19E takes place.

To identify the domain(s) of the RYR involved in the interaction with fusion protein 19E, we performed overlays on Western blots of fusion proteins covering the entire sequence of the RYR protomer, using digoxigenin-labeled fusion protein 19E as the ligand (Fig. 4). Out of the ten fusion proteins tested, only two, fusion proteins 1 and 28, defined by residues 281-1172 and 2937–3225, respectively, displayed an interaction with digoxigenated 19E. Of the two fusion proteins capable of binding 19E, protein 28 was particularly interestingly, since it encompasses a sequence (residues 2937–3225) which has been reported previously to bind calmodulin even at low [Ca^{2+}] (Menegazzi *et al.*, 1994; Buratti *et al.*, 1995). Thus, we examined whether the binding domain of fusion protein 28 for fusion protein 19E overlaps with that of calmodulin. Fig. 5 shows that a RYR fusion protein 28 carrying a deletion at its COOH terminus did not bind either calmodulin or fusion protein 19E, suggesting that the binding of both calmodulin and RYR fusion protein 19E is specific and requires the COOH-terminal sequence defined by residues 3010–3225. Using a similar approach we found that the presence of the COOH-terminal portion of RYR fusion protein 1 (residues 799-1172, defined by protein 2) is essential to confer binding with fusion protein 19E. Fig. 4 also shows that in the terminal cisternae fraction of rabbit skeletal muscle, two proteins are able to bind digoxigenin-fusion protein 19E. As expected binding was observed in correspondence of a band having a molecular weight identical to that of the RYR

protomer. In addition, a protein with an apparent molecular mass of 110 kDa also exhibited strong binding.

In conclusion the data obtained in this study demonstrate that an anti-RYR mAb directed against an epitope localized few residues upstream Gly^{341} activates both Ca^{2+} release and [^{3}H]ryanodine binding activity. Interestingly, this apparent increase of the Ca^{2+} sensitivity of the RYR in the presence of mAb419 is similar to what has been observed previously with triad vesicles from MH-susceptible pigs (Mickelson *et al.*, 1988). Nevertheless Ca^{2+} overlay experiments indicate that the fusion proteins 19 and 19A-19E do not display Ca^{2+} binding. Therefore the effect of mAb419 suggests that the sequence corresponding to fusion protein 19E interact with another region of the RYR directly involved in the regulation of the channel by Ca^{2+}. Real time surface plasmon resonance (BIAcore technique) indicates that the fusion protein 19E is able to interact with the purified native RYR complex. As to the sequence(s) involved in the intramolecular interaction between the fusion protein 19E and the RYR complex two polypeptides were identified: the first is localized at the NH_2-terminal portion of the RYR protomer and is defined by residues 799-1172, the second is a calmodulin binding domain that is able to interact with calmodulin at low [Ca^{2+}] (Menegazzi *et al.*, 1994). We also show that a modification of the calmodulin binding site, which leads to the loss of calmodulin binding, also brings about the loss of the interaction of fusion protein 19E with its target sequence. These results represent the first evidence for a functional role for the region of the RYR encompassing Gly^{341}; it is tempting to think that this residue plays a crucial role in the intramolecular link between sequences encompassed by fusion protein 19E and calmodulin binding domains of the RYR. The abnormalities of the RYR in MH susceptible individuals could be due to a modification of this intramolecular interaction, which may in turn lead to an increase in Ca^{2+} sensitivity. However, one should also consider that 19E interacts with another region of the RYR defined by residues 799-1172 as well as with a 110-kDa peptide present in terminal cisternae vesicles. These interactions could also play an important role in the regulation of Ca^{2+} release from the RYR.

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J. Biol. Chem. 1996, 271:22759-22763.
doi: 10.1074/jbc.271.37.22759