Functional Association Between Nicotinic Acetylcholine Receptor and Sarcomeric Proteins Via Actin and Desmin Filaments

Takao Mitsui,[1]* Masakazu Kawajiri,[1] Makoto Kunishige,[1] Takenori Endo,[1] Masashi Akaike,[1] Kenji Aki,[2] and Toshio Matsumoto[1]

[1]First Department of Internal Medicine, School of Medicine, University of Tokushima, Tokushima 770-8503, Japan
[2]Institute for Enzyme Research, University of Tokushima, Tokushima 770-8503, Japan

Abstract   By affinity chromatography utilizing α-cobrotoxin from digitonin-solubilized fractions of rabbit skeletal muscle, we found that many proteins are associated with the nicotinic acetylcholine receptor (AChR). In addition to the proteins we previously reported to bind to AChR (including dystrophin-dystrophin-associated protein (DAP) complex, utrophin, rapsyn, and actin; Mitsui et al. [1996] Biochem. Biophys. Res. Commun.224:802–807), α-actinin, desmin, myosin, tropomyosin, troponin T, and titin are also identified to be associated with AChR. Alkaline treatment or Triton X-100 solubilization released dystrophin-DAP complex, utrophin, and rapsyn from the AChR fraction, while actin and desmin remained associated. These findings demonstrate that AChR is supported primarily by a submembranous organization of actin and desmin filaments, and is linked to sarcomeric proteins via these filaments. To further investigate whether the association has any functional role, we studied the effect of acetylcoline on ATPase activity of the AChR fraction. Acetylcholine (0.5–4 μM) significantly activated Mg^{2+}-ATPase activity of digitonin-solubilized AChR fraction ($P < 0.05$). Furthermore, we found that desmin as well as actin activated myosin Mg^{2+}-ATPase activity. From these findings, it is suggested that desmin and actin form a submembranous organization in the postsynaptic region, and function as mediators of excitation of AChR to the sarcomeric contraction system. J. Cell. Biochem. 77: 584–595, 2000.   © 2000 Wiley-Liss, Inc.

Key words: acetylcholine receptor; sarcomeric protein; actin; desmin; ATPase

In neuromuscular junctions, nicotinic acetylcholine receptors (AChRs) of vertebrate skeletal muscle are densely packed at the crests of junctional folds, which are precisely aligned with presynaptic active zones [review in Hall and Sanes, 1993]. The highly specialized organization of postsynaptic apparatus, which enables efficient synaptic transmission, suggests that AChR is anchored, directly or indirectly, to both the underlying cytoskeleton and the overlying basal lamina. Over the past two decades, considerable effort has been focused on the functional relationship between AChR and the components of the submembranous cytoskeletal network in the postsynaptic region. Cytoskeletal proteins seem to participate in the clustering and/or stabilization of AChR [review in Froehner, 1991; Hall and Sanes, 1993]. Under electron microscope, postsynaptic folds are supported by a submembranous meshwork of fine filaments connected to underlying filaments [Heuser and Salpeter, 1979]. Immunocytochemical studies have demonstrated that a variety of cytoskeletal molecules are colocalized with AChR in various developmental stages of cultured muscle fibers. The search for cytoplasmic events during development has highlighted the machinery of AChR clustering rather than that of stabilization. The synthesis and aggregation of AChR are controlled by neuromuscular transmission of acetylcholine during the development of muscle cells, and are altered by denervation. In denervated muscle, AChR is expressed in both extrajunctional

and junctional sarcolemma [Edwards, 1979], when AChR mRNA is substantially increased immediately after an increase in myogenin and MyoD mRNAs [Goldman et al., 1988; Eftimie et al., 1991]. If AChR is linked to some cytoskeletons, they develop at extrajunctional sarcolemma together with AChR after denervation. Therefore, denervation studies should give us a clue to clarify the mechanism of AChR stabilization.

Recently, we purified AChR from the digitonin-solubilized fraction of rabbit skeletal muscle by affinity chromatography, and found that many proteins were linked to AChR, including dystrophin, utrophin, dystrophin-associated proteins (DAPs), rapsyn, and actin (Mitsui et al., 1996). The present study was undertaken to identify the additional AChR-associated proteins, and to clarify the functional roles of these proteins in AChR stabilization in denervated skeletal muscles. Furthermore, it was suggested that the association between AChR and cytoskeletal proteins was important in excitation-contraction coupling at synaptic transmission.

MATERIALS AND METHODS

Antibodies

The following primary antibodies were used: anti-dystrophin rabbit polyclonal antibody [Ab 6-10; Byers et al., 1991], anti-utrophin rabbit polyclonal antibody [Khurana et al., 1990], anti-actin rabbit polyclonal antibody (Sigma Chemical Co., St. Louis, MO), anti-myosin rabbit polyclonal antibody (Sigma), anti-tropomyosin rabbit polyclonal antibody (Sigma), anti-desmin rabbit polyclonal antibody (Sigma), anti-spectrin rabbit polyclonal antibody (Sigma), anti-agrin sheep polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY), anti-filamin goat polyclonal antibody (Sigma), anti-AChR subunit rat monoclonal antibody [mAb 3; Lennon and Lambert, 1980], anti-AChR subunit rat monoclonal antibody [mAb 111; Ratnam et al., 1986], anti-rapsyn mouse monoclonal antibody [mAb 1234; Peng and Froehner, 1985], anti-merosin M chain mouse monoclonal antibody (Chemicon International., Inc., Temecula, CA), anti-α actinin mouse monoclonal antibody (Sigma), anti-myosin heavy chain (fast) mouse monoclonal antibody (Sigma), anti-titin mouse monoclonal antibody (Sigma), anti-troponin T mouse monoclonal an-

tibody (Sigma), anti-vimentin mouse monoclonal antibody (Nichirei Corp., Tokyo, Japan), anti-vinculin mouse monoclonal antibody (Sigma), anti-talin mouse monoclonal antibody (Sigma), and anti-β1 integrin mouse monoclonal antibody (Biohit OY, Helsinki, Finland). Rabbit polyclonal antibodies against the respective sequences of β-dystroglycan and α-sarcoglycan (adhalin) were affinity-purified and characterized as previously described [Kawajiri et al., 1996; Mitsui et al, 1996]. In addition, polyclonal antisera against synthetic polypeptide NKMPILISKIFKGLAADQTQAL, which corresponds to the common syntrophin β1 sequence in human, mouse, and rabbit [Ahn et al., 1994], were raised in New Zealand White rabbits and affinity-purified.

Preparation of AChR

Digitonin (1%)-solubilized membranes from rabbit skeletal muscle were loaded and circulated overnight on an α-cobrotoxin-linked Sepharose 4B column in the presence of 0.5 M NaCl [Mitsui et al., 1996]. The column was washed and eluted with buffer A (50 mM Tris-HCl, pH 7.4, 0.1% digitonin) containing 0.1 M hexamethonium bromide. The eluent solution was again circulated overnight on the toxin-Sepharose column and DE52 column directly attached to the toxin-Sepharose column. The DE52 column was washed extensively, and then eluted with buffer A containing linear gradient 0–0.5 M NaCl. Twenty-five eluted fractions were concentrated and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 4–20% gradient gel), followed by silver staining. Protein peak fraction was concentrated to 1 ml, and layered on an 11-ml linear 2–10% (W/V) sucrose gradient containing 0.5 M NaCl in buffer A. Gradients were centrifuged at 4°C for 20 h at 200,000g. Twenty fractions were collected from the top of the gradients, concentrated, and then subjected to SDS-PAGE. The protein peak fraction was subjected to immunoblotting [Carr et al., 1989]. Control sucrose gradient sedimentation was performed at pH 11 [Ervasti et al., 1991]. All procedures were carried out in the presence of proteinase inhibitors [Ervasti and et al.l, 1991]. AChR complex was also isolated from two control preparations: alkaline treatment at pH 11 [Neubig, 1979] and 1% Triton X-100 solubilization. For alkaline treatment, heavy microsomes were suspended in water at 4°C, and the

pH was adjusted to pH 11 with 0.2 N NaOH. After 1 h at pH 11, membranes were pelleted and then resuspended in 1% digitonin.

Denervation of Skeletal Muscles From *mdx* Mice

mdx mice (3–4 months old) were anesthetized with sodium pentobarbital, and the sciatic nerve was cut unilaterally in the midthigh. Mice were killed 2 or 10 days later, and the excised planter extensor group of the lower hind muscles was used for immunohistochemical study, immunoblotting [Carr et al., 1989; Mitsui et al., 1996], or mRNA analysis.

Reverse Transcription (RT)-PCR Analysis of Dystroglycan mRNA

To test the effect of denervation on dystroglycan mRNA levels, mRNAs for the AChR α subunit and dystroglycan were semiquantified by RT-PCR analysis, according to our previous report [Kawai et al., 1995]. Three sets of primers were designed to amplify mouse mRNAs for dystroglycan, AChR α subunit, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) as an internal control, according to their respective cDNA sequences [Boulter et al., 1985; Górecki et al., 1994; Sabath et al., 1990]. The primers used were as follows: for dystroglycan, 5′-GGATGCTGACCTCACCAAGA-3′ and 5′-TTTGGGGAGAGTGGGCTTCT-3′, corresponding to nt.585–604 and nt.905–924, respectively; for AChR α subunit, 5′-ACGG-CGACTTTGCCATTGTC-3′ and 5′-CAGGCG-CTGCATGACGAAGT-3′, corresponding to nt.316–335 and nt.637–656, respectively; and for G3PDH, 5′-TGAAGGTCGGTG-TGAAC-GGATTTGGC-3′ and 5′-CATGTAGGCCATG-AGGTCCACCAC-3′, corresponding to nt.51-76 and nt.1010-1033, respectively. First-strand cDNA was synthesized from 1 μg of RNA sample, and PCR was carried out by 20 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min), and extension at 72°C (1 min). Linearity of the PCR reaction was tested by amplification of 1 μg of total RNA per reaction from 15-40 cycles, and linear range of reaction was found from 15-30 cycles. These fragments of PCR products were separated by electrophoresis in 2% agarose gel.

Immunohistochemical Study

Cryosections (6-μm thickness) from control and *mdx* mouse skeletal muscles (gastrocnemi-us) were preincubated for 30 min with 5% normal bovine serum albumin in phosphate-buffered saline (PBS), and incubated with a primary antibody overnight at 4°C. After extensive washing in PBS, the sections were labeled with biotin-labeled anti-rabbit IgG, anti-goat IgG, anti-rat IgG, or anti-mouse IgG. The biotin label was detected with an ABC kit (Vector Laboratories, Burlingame, CA).

Assay for ATPase Activity

Mg^{2+}-ATPase activity was assayed by incubating, for 10 min at 25°C, 1 ml of medium containing 20 mM imidazole buffer, pH 7.0, 50 mM KCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 1 mM ATP with proteins and various concentrations of acetylcholine chloride (0.5-16 μM). Ca^{2+} ATPase assay was performed in the buffer containing 5 mM $CaCl_2$ instead of $MgCl_2$. Starting protein concentrations were 100 μg/ml of digitonin-solubilized AChR fraction without or with alkaline treatment, Triton X-100-solubilized AChR fraction, or a mixture of 75 μg/ml of digitonin-solubilized AChR fraction with alkaline treatment and 25 μg/ml of rabbit skeletal muscle myosin (Sigma). The reaction was terminated with 200 μl of 25% trichloroacetic acid, and the amount of Pi released was measured by the method of Murphy and Riley [1962]. Mg^{2+}-ATPase activity of rabbit skeletal muscle myosin (100 μg/ml) was also assayed under the presence of rabbit muscle actin or desmin (0-25 μg/ml, Progen Biotechnik, Heidelberg, Germany). The ATPase activities were assayed 3 times. Statistical significance was determined by unpaired Student's *t*-test.

Overlay Binding of Desmin to AChR Complex

AChR complexes (10 μg) from three preparations, desmin (0.5 μg, Progen Biotechnik), and actin (0.5 μg, Progen Biotechnik) were subjected to 4-20% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked overnight at room temperature in PBS containing 0.1% Tween–20 and 5% bovine serum albumin (BSA) and incubated for 2 h at room temperature in buffer A (0.1% Tween–20, 0.1% BSA, 0.2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM ATP in PBS) with 20 μg/ml desmin. The membranes were washed twice for 30 min with PBS containing 0.1% Tween–20, and processed for im-
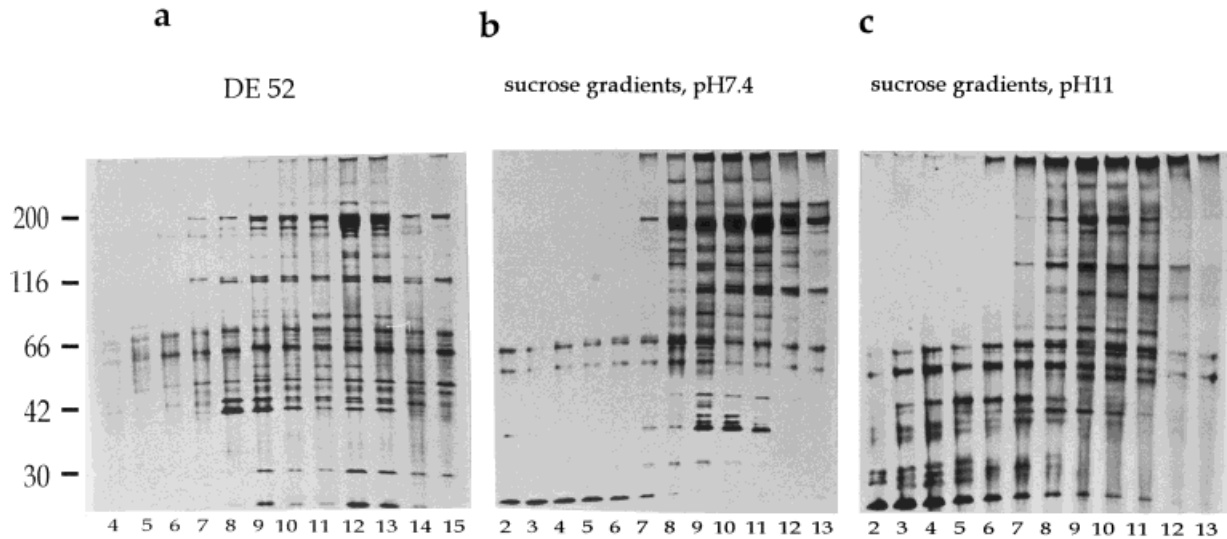
Fig. 1. Purification of AChR complex by DE52 chromatography and 2–10% linear sucrose gradient sedimentation. Silver-stained 4–20% SDS-polyacrylamide gels of DE52 fractions 4-15 (**a**), and sucrose gradient fractions in two conditions at pH 7.4 (**b**) and pH 11 (**c**). The molecular weight standards ($\times$ 10^3) are indicated at left.

munoblotting with anti-desmin antibody. Control membranes were incubated in buffer A without desmin, followed by immunoblotting with anti-desmin antibody.

RESULTS

AChR Is Associated With Many Proteins in Digitonin-Solubilized Membranes

As reported previously [Mitsui et al., 1996], AChRs and associated proteins were obtained by chromatographic methods utilizing an α-cobrotoxin-linked Sepharose 4B column, followed by a DE52 column. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of eluted fractions from DE52 column demonstrated that many proteins were copurified with AChR (Fig. 1a). Peak protein fraction (fraction 12) was further purified by sucrose density gradient centrifugation. At pH 7.4, major proteins were cosedimented in fractions 9-11 (Fig. 1b). Immunoblotting study revealed that these fractions contained AChR α and β subunits (not shown). Sucrose gradients in alkaline condition (pH 11) dissociated relatively small proteins which contained AChR subunits from larger proteins (Fig. 1c). The peak fraction in sucrose gradients at pH 7.4 (fraction 9) contained the following protein bands: ~1,500 kDa, ~400 kDa, 240 kDa, 200 kDa, 160 kDa, 130 kDa, 100 kDa, 90 kDa, 64 kDa, 60 kDa, 56 kDa, 52 kDa, 48 kDa, 45 kDa, 44 kDa, 42 kDa,

39 kDa, 38 kDa, 36 kDa, 31 kDa, and 30 kDa (Fig. 2a). However, the fraction electrophoresed as a large single band in native PAGE (Fig. 2). The purified AChR complex was subjected to an immunoblotting study, and the following proteins were identified: ~1,500 kDa as titin, ~400 kDa as dystrophin and utrophin, 200 kDa as myosin heavy chain, 100 kDa as α-actinin, 90 kDa as merosin M chain, 60 kDa as syntrophin, 56 kDa as desmin, 52 kDa as α-sarcoglycan (adhalin), 48 kDa as AChR β subunit, 45 kDa as rapsyn, 44 kDa as β-dystroglycan and actin, 42 kDa as AChR α subunit, 39 kDa and 36 kDa as tropomyosin, and 38 kDa as troponin T (Fig. 2). These proteins were major components even of the eluent from the α-cobrotoxin-Sepharose 4B column. However, the majority of these proteins were not detected in the eluent from toxin-unbound Sepharose 4B. Monoclonal antibody against myosin heavy chain (fast) detected a 240-kDa protein band in addition to a 200-kDa protein band. This suggests that the AChR complex contains isozymes of myosin heavy chain (fast), and densitometric analysis of the Coomassie blue-stained gel demonstrated that myosin heavy chain comprised 22% of the total protein. Polyclonal antibody against myosin also recognized four protein bands at 15-26 kDa, corresponding to myosin light chain (not shown). Antibodies against
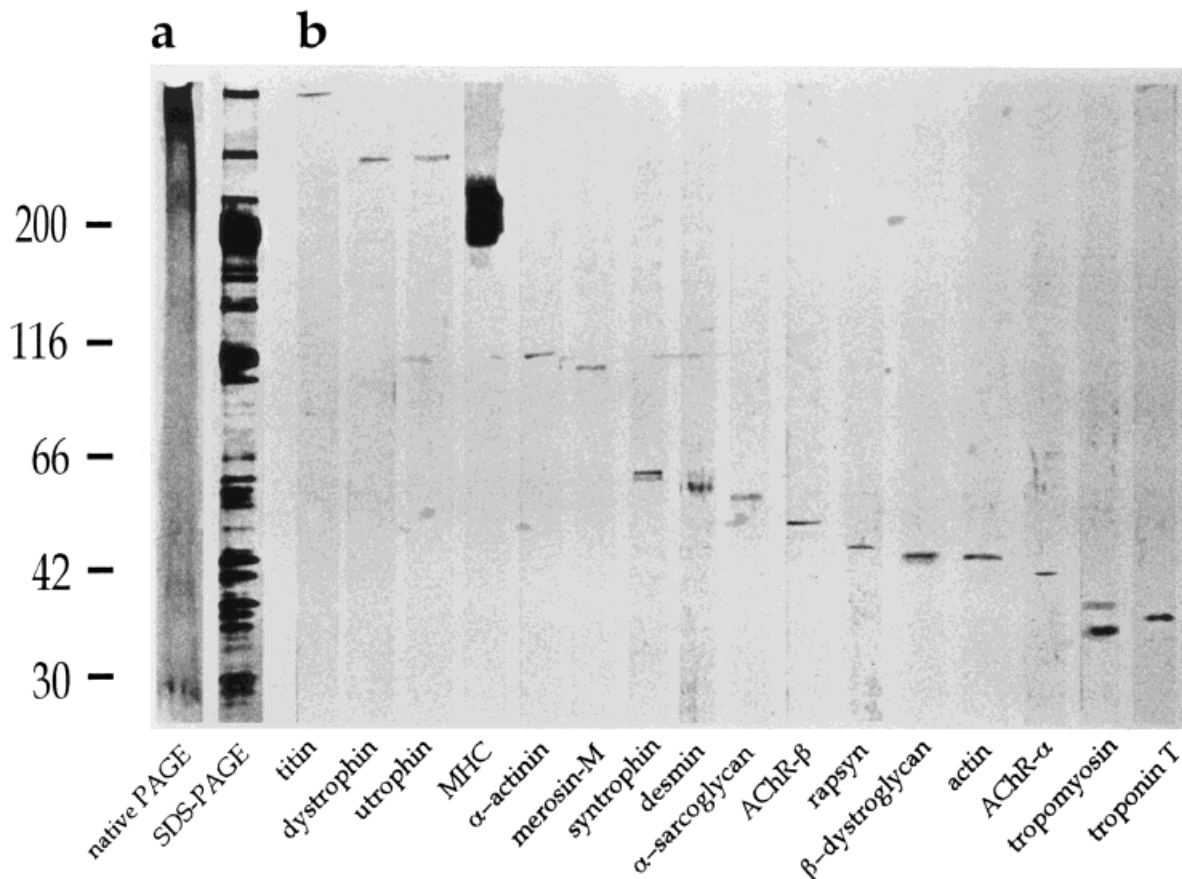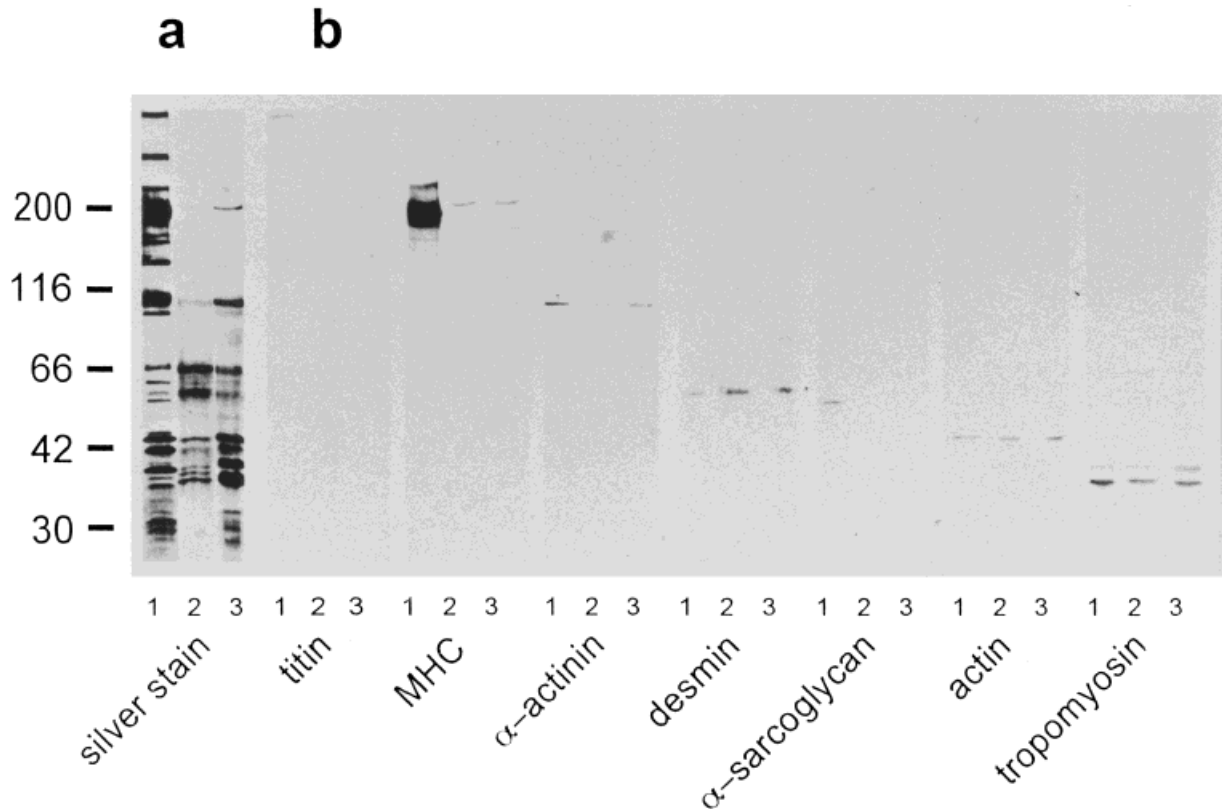
Fig. 2. SDS-PAGE of affinity column-purified AChR complex and immunoblot analysis. **a:** Silver-stained 4–20% native- or SDS-polyacrylamide gels. **b:** Immunolabelling with antibodies to titin, dystrophin, utrophin, myosin heavy chain (MHC), α-actinin, merosin M chain (merosin M), syntrophin, desmin, α-sarcoglycan, AChR β subunit (AChR-β), rapsyn, β-dystroglycan, actin, anti-AChR α subunit (AChR-α), tropomyosin, and troponin T. The molecular weight standards ($\times 10^3$) are indicated at left.

agrin, vinculin, vimentin, talin, β-spectrin, filamin, and β1-integrin did not recognize these protein bands.

AChR Is Associated With Actin and Desmin in the Absence of Rapsyn and Dystrophin-DAP Complex

Rapsyn and dystrophin-DAP complex can be removed by alkaline treatment before digitonin solubilization (Fig. 3, lane 2) or Triton-solubilization.(Fig. 3, lane 3). These treatments released the following proteins; ~1,500 kDa (titin), ~400 kDa (dystrophin and utrophin), 90 kDa (merosin M chain), 60 kDa (syntrophin), 52 kDa (α-sarcoglycan), 45 kDa (rapsyn), and 44 kDa (β-dystroglycan), which were verified by immunoblotting (not shown). Protein bands at 200 kDa, 100 kDa, 56 kDa, 48 kDa, 44 kDa, 42 kDa, 39 kDa, 38 kDa, and 36 kDa were also

recognized in all three preparations (Fig. 3a). Immunoblotting recognized these proteins as myosin heavy chain at 200 kDa, α-actinin at 100 kDa, desmin at 56 kDa, AChR β subunit at 48 kDa, actin at 44 kDa, AChR α subunit at 42 kDa, tropomyosin at 39 and 36 kDa, and troponin T at 38 kDa. The protein band at 44 kDa was reduced after alkaline treatment, but apparently detected by anti-actin antibody. The protein band at 240 kDa which reacted to anti-myosin heavy chain (fast) antibody was not detected in the alkaline or Triton preparations. The intense and massive protein band of myosin heavy chain at 200 kDa was reduced, and the titin band was not detected in either preparation (Fig. 3a). In some alkaline preparations, when a faint titin band was detected, the myosin band was relatively preserved (not shown). These results indicate that AChR is

Fig. 3. SDS-PAGE of AChR complex from three preparations, and immunoblot analysis. **a:** Silver-stained 4–20% SDS-polyacrylamide gels. **b:** Immunolabelling with antibodies to titin, myosin heavy chain (MHC), α-actinin, desmin, α-sarcoglycan, actin, and tropomyosin. **Lane 1,** digitonin-solubilized membrane fraction without alkaline treatment. **Lane 2,** digitonin-solubilized membrane fraction with alkaline treatment. **Lane 3,** Triton X-100-solubilized membrane fraction. The molecular weight standards ($\times$ 10^3) are indicated at left.

associated with actin and desmin even in the absence of dystrophin-DAP complex and rapsyn.

Denervation Induces Extrajunctional Localization of DAPs in *mdx* Mouse Skeletal Muscle by Linking to AChR

In *mdx* mouse skeletal muscle, syntrophin, α-dystroglycan (adhalin) and β-dystroglycan were abundantly and diffusely localized on the sarcolemma of muscle cells 2 days after denervation (Fig. 4). Utrophin was also increased at extrajunctional sarcolemma (not shown), as reported previously [Takemitsu et al., 1991]. At 10 days after denervation, intense staining of DAPs was still observed even in atrophic fibers (not shown). In serial sections, the extrajunctional localization of DAPs was always accompanied by AChR α-subunit and β-subunit, as previously reported [Mitsui et al., 1996]. To investigate

whether or not extrajunctional localization of DAPs is caused by an increase in the protein synthesis, we analyzed mRNA levels of dystroglycan in denervated skeletal muscle. RT-PCR analysis demonstrated that fragments of 340 bp, 343 bp, and 983 bp, corresponding to dystroglycan, AChR α-subunit, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH, internal control), respectively, were amplified (Fig. 5a). The level of AChR α subunit mRNA was prominently increased 2 days after denervation, while there was no apparent change in that of dystroglycan mRNA. However, an immunoblotting study demonstrated that the protein level of β-dystroglycan in *mdx* mouse muscle was prominently increased 2 days after denervation (Fig. 5b). These findings suggest that the extrajunctional localization of DAP seems to depend on AChR localization due to linking to AChR, rather than to transcriptional up-
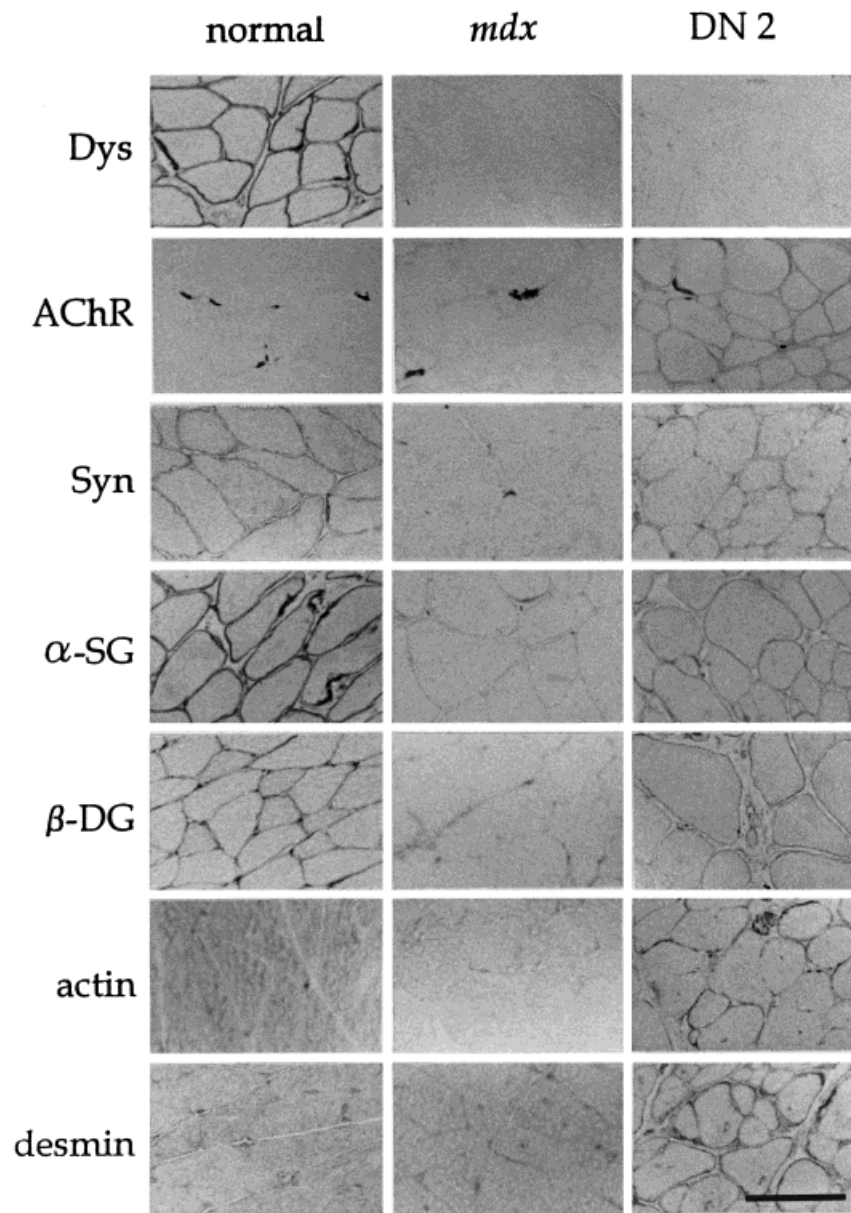
Fig. 4. Immunohistochemical staining of dystrophin (Dys), AChR α subunit (AChR), syntrophin (Syn), α-sarcoglycan (α-SG), β-dystroglycan (β-DG), actin, and desmin in denervated skeletal muscle from *mdx* mouse and normal mouse muscle. AChR α subunit (AChR), syntrophin (Syn), α-sarcoglycan (α-SG), β-dystroglycan (β-DG), actin, and desmin are increased at extrajunctional sarcolemma 2 days (DN2) after denervation in *mdx* mouse muscle, compared with nondenervated *mdx* mouse muscle (*mdx* mouse). Bar, 100 μm.

regulation. Since it has been reported that rapsyn may function as a link between AChR and DAP complex [Apel et al., 1995], some proteins, such as rapsyn and other cytoskeletal proteins, seem to participate in anchoring the AChR complex even in denervated skeletal muscle.

Denervation Also Induces the Extrajunctional Localization of Actin and Desmin in *mdx* Mouse Skeletal Muscle

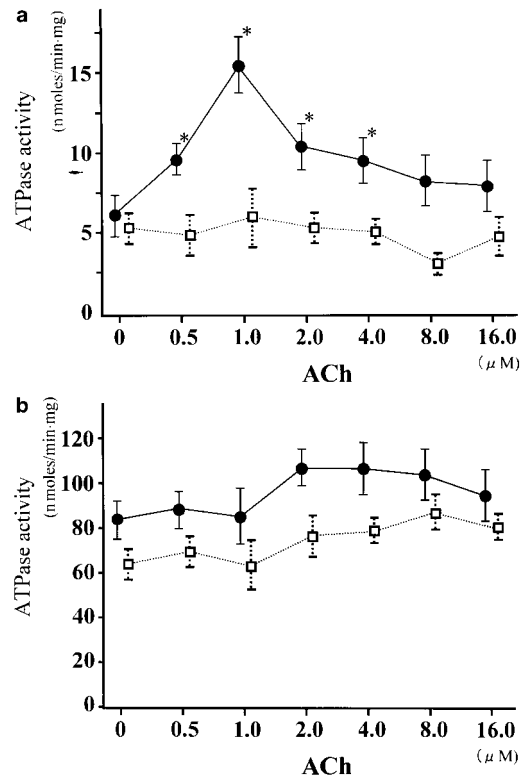Although desmin is predominantly located at the outer circumference of the Z-band in fixed muscle tissues [Granger and Lazarides, 1979], we did not find preferential localization in unfixed muscle samples. In denervated skeletal muscles from *mdx* mice, actin and desmin were abundantly and diffusely localized on the sarcolemma of muscle cells, and AChR and DAP were also diffusely localized (Fig. 4). On the other hand, other copurified proteins, such as α-actinin, myosin, tropomyosin, troponin T, and titin, were not increased at sarcolemma after denervation (not shown). These findings indicate that denervation induces the submembranous organization of actin and desmin filaments.
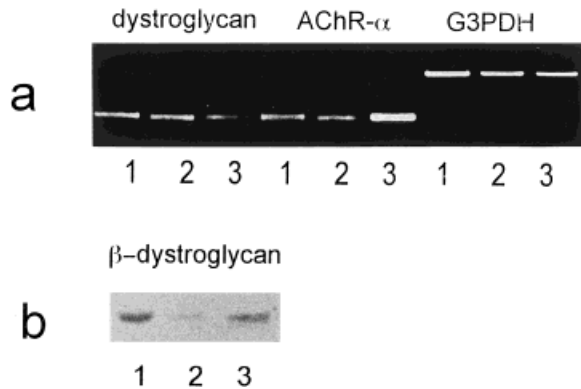
Fig. 5. **a:** RT-PCR analysis of mRNAs for dystroglycan, AChR α-subunit, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH, an internal control) in denervated skeletal muscle from *mdx* mouse. **b:** Immunoblot analysis of β-dystroglycan in denervated skeletal muscle from *mdx* mouse. **Lane 1,** control mouse. **Lane 2,** nondenervated *mdx* mouse. **Lane 3,** denervated *mdx* mouse (day 2). The level of mRNA AChR α-subunit is prominently increased 2 days after denervation, but the level of dystroglycan mRNA is not apparently increased (a). The protein level of β-dystroglycan in *mdx* mouse muscle is prominently increased 2 days after denervation (b).

Fig. 6. Mg^{2+}-ATPase (**a**) and Ca^{2+}-ATPase (**b**) activities of AChR in the presence of various ACh concentrations. Mean ± SE. Solid circle, digitonin-solubilized AChR fraction without alkaline treatment. Open square, myosin plus digitonin-solubilized AChR fraction with alkaline treatment. *$P < ñ$.

## Acetylcholine Activates the Mg^{2+}-ATPase Activity of AChR Fraction

The result that AChR is associated with myosin via actin and desmin filaments prompted us to investigate whether the association has any functional significance. Since it is well known that actin and isolated microfilaments containing actin, α-actinin, and tropomyosin activates Mg^{2+}-ATPase activity of skeletal muscle myosin [Lin et al., 1984], we speculated that AChR-associated actin and desmin might play a role in excitation-contraction coupling of striated muscle cells, and studied the effect of acetylcoline on ATPase activity of the AChR fraction in a cell-free system. As shown in Figure 6, acetylcholine (ACh; 0.5–4 μM) significantly activated Mg^{2+}-ATPase activity of digitonin-solubilized AChR fraction ($P < 0.05$). Although any Mg^{2+}-ATPase activities were not detected in digitonin-solubilized AChR fraction with alkaline treatment or Triton X-100 solubilized fraction, the activities of alkaline-treated AChR plus myosin could be assayed, and were not significantly activated by ACh (0.5–16 μM). Ca^{2+}-ATPase activity was not significantly activated in any sample (Fig. 6). These findings suggest that Mg^{2+}-ATPase activity of the AChR fraction is mainly derived from that of myosin, and that ACh could induce

AChR to activate myosin Mg^{2+}-ATPase activity. Because the artificial mixture of myosin and alkaline-treated AChR fraction which involved actin and desmin did not have ACh-sensitive Mg^{2+}-ATPase activity, the association between AChR and sarcomeric protein via actin and desmin filament seems essential for the activation of ATPase activity. Since actin is known to activate skeletal muscle myosin Mg^{2+}–ATPase activity, we studied whether desmin also has the ability to activate the activity or not. The mean value of Mg^{2+}-ATPase activity of skeletal muscle myosin was 4.15 nmoles/mg · min, which was increased to 6.05, 6.72, 7.85, and 8.31 nmoles/mg · min in the presence of 2.5, 5.0, 10, and 20 μg/ml desmin, respectively. The activation by desmin was dose–dependent, but lower than that by actin (7.28, 9.79, 11.4, and 13.1 nmoles/mg · min in the presence of 2.5, 5.0, 10, and 20 μg/ml actin, respectively).
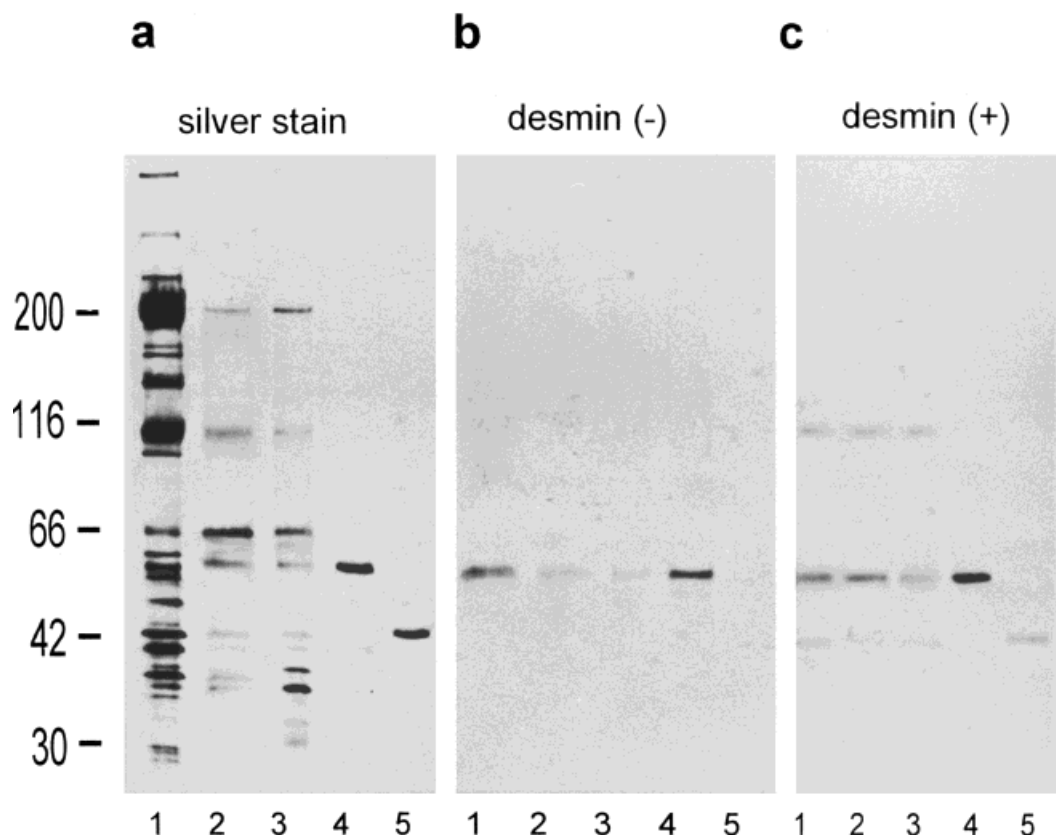
Fig. 7. Overlay binding of desmin to AChR complex. **a:** Silver-stained 4–20% SDS-polyacrylamide gels. **b:** Control experiment in which desmin incubation was omitted. A 56-kDa protein is reacted to anti-desmin antibody. **c:** Desmin overlay assay. Two additional protein bands at 100 kDa and 44 kDa are stained, corresponding to α-actinin and actin, respectively. **Lane 1,** digitonin-solubilized membrane fraction without alkaline treatment. **Lane 2,** digitonin-solubilized membrane fraction with alkaline treatment. **Lane 3,** Triton X-100-solubilized membrane fraction. **Lane 4,** purified desmin. **Lane 5,** purified actin. The molecular weight standards ($\times 10^3$) are indicated at left.

Desmin Binds to Actin and α-Actinin From AChR Complex In Vitro

The present study demonstrated that not only actin but also desmin enhances Mg^{2+}-ATPase of myosin. It seems likely that acetylcholine–bound AChR enables actin and desmin to activate the myosin ATPase. To investigate the relationship between actin and desmin, we analyzed the binding properties of desmin to each component of the AChR complex. The purified AChR complex was separated by 4–20% SDS–PAGE, transferred to polyvinylidene difluoride membranes, and overlaid with purified desmin. The desmin label was detected by anti–desmin antibody. Control experiments, in which desmin incubation was omitted, demonstrated that a 56-kDa protein band was reacted to anti–desmin antibody (Fig. 7). With desmin–overlay, two addi-

tional protein bands at 100 kDa and 44 kDa were stained, corresponding to α–actinin and actin, respectively (Fig. 7).

DISCUSSION

We purified AChR from the digitonin-solubilized fraction of rabbit skeletal muscle by affinity chromatography and found that many proteins were associated with AChR, including dystrophin–DAP complex, utrophin, merosin, rapsyn, actin, α-actinin, desmin, myosin, tropomyosin, troponin T, and titin. Of these proteins, rapsyn [Froehner et al., 1981; Burden et al., 1983], syntrophin [Froehner et al., 1987], dystrophin [Carr et al., 1989; Sealock et al., 1991], desmin [Sealock et al., 1989], and actin [Hall et al., 1981; Gotti et al., 1982] have been copurified mainly by sucrose gradient sedimentation. Utrophin [Ohlendiek et al., 1991], other

DAPs [Matsumura et al., 1992], merosin [Sanes et al., 1990], α-actinin [Bloch and Hall, 1983], and tropomyosin 2 (37-kDa tropomyosin isoform; Marazzi et al., 1989] were shown to be immunocytochemically colocalized with AChR, but have not been copurified with AChR.

Actin, which is tightly attached to AChR even under Triton X-100 treatment [Gotti et al., 1982], forms an underlying meshwork of 5-6-nm filaments at the earliest stage of AChR-clustering and cytoplasmic specialization [Peng and Phlan, 1984]. Immunofluorescence microscopy has revealed that microfilaments in nonmuscle cells are composed of several proteins in addition to actin, such as myosin [Weber and Groeschel-Stewart, 1974], tropomyosin [Lazarides, 1975], α-actinin [Lazarides and Burridge, 1975], and filamin [Wang et al., 1975]. In particular, actin-binding proteins such as tropomyosin, filamin, and α-actinin nonrandomly bind the sides of actin filaments, and participate in regulating the length of such actin filaments and the cellular motility [Prulière et al., 1986]. On the other hand, myosin (heavy and light chain), titin, α-actinin, actin, tropomyosin, and troponin T, which are copurified with AChR, are the major structural components of the sarcomere. In denervated skeletal muscle from *mdx* mice, actin and desmin were dramatically increased at extrajunctional sarcolemma, while myosin, α-actinin, titin, tropomyosin, and troponin T were not. These findings suggest that myosin, titin, α-actinin, tropomyosin, and troponin T, which are copurified with AChR, do not participate in the formation of a meshwork of actin filaments, and that these proteins are derived from sarcomeric structures. Affinity-purified actin may be derived from both microfilaments and sarcomeric structures. Alkaline treatment or Triton solubilization released dystrophin-DAP complex, utrophin, and rapsyn from the AChR complex, while actin and desmin remained associated with the complex. Therefore, the extrajunctional development of AChR seems to be supported primarily by the submembranous organization of actin and desmin filaments. Dystrophin and DAPs are not essential for the AChR-sarcomeric protein linkage, but participate in the submembranous organization after denervation, indicating a loose association with the AChR complex. Since dystrophin is stabilized to F-actin at the N-terminal region [Hemmings et al., 1992], it may take

part in postsynaptic filament formation by linking both to F-actin and DAPs in normal skeletal muscle at denervation.

Desmin, an intermediate filament protein specific to skeletal muscle, is believed to link the Z-lines of adjacent myofibrils and also the outermost myofibrils to the plasma membrane, and may provide mechanical integration to myofibrils, thereby ensuring that they maintain their proper alignment during the contraction-relaxation cycle [Lazarides, 1980]. From the results that desmin was copurified with sarcomeric proteins by affinity chromatography of AChR, that Triton X-100 solubilization and alkaline treatment released dystrophin, utrophin, DAPs, and rapsyn from AChR complex while leaving actin and desmin, and that actin and desmin were prominently increased at denervated mouse skeletal muscle, it appears that desmin may form a meshwork connecting AChR to the sarcomeric apparatus together with actin. These findings also support previous reports that intermediate filaments may serve to influence AChR distribution by stabilizing an actin-based cytoskeleton under the crests [Ellisman et al., 1976], and that an intermediate filament protein at 51 kDa is concentrated at synaptic sites and develops at extrasynaptic sarcolemma in denervated muscle [Burden, 1982].

It is well-known that actin induces the activation of myosin Mg^{2+}-ATPase activity and that the activation is important for force generation between the myofilaments [Stephenson et al., 1989]. Actin forms microfilaments with tropomyosin or α-actinin, and these filaments also have the ability to activate the skeletal muscle myosin ATPase [Lin et al., 1984]. The present results that ACh activates Mg^{2+}-ATPase activity of the AChR fraction suggest that ACh-bound AChR induces actin and desmin to participate in the excitation-contraction coupling by activating the myosin ATPase. In fact, we found that myosin Mg^{2+}-ATPase was activated not only by actin but also by desmin, and that desmin potentially bound to actin and α-actinin from the AChR complex. It is reported that desmin seems essential for the maintainance of the tensile strength and integrity of muscle fibers, and not for myofibrillogenesis, because desmin knockout mice develop normally but fertile [Li et al., 1997]. These findings demonstrate that desmin, as well as actin, forms submembra-

nous organization in the postsynaptic region, and suggest that these proteins function as a mediator of excitation from AChR to the sarcomeric contraction system.

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