Co-localization of the dihydropyridine receptor and the cyclic AMP-binding subunit of an intrinsic protein kinase to the junctional membrane of the transverse tubules of skeletal muscle

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Junctional transverse tubules (TT) isolated from triads of rabbit skeletal muscle by centrifugation in an ion-free sucrose gradient were compared with membrane subfractions, predominantly derived from the free portion of TT, that had been purified from sarcoplasmic reticulum membrane contaminants by three different methods. The markers used were diagnostic membrane markers and the dihydropyridine (DHP) receptor, which is a specific marker of the junctional membrane of TT. Junctional TT have a high membrane density ($B_{\max}$ 60 pmol/mg of protein) of high-affinity (K_d 0.25 nM) DHP-binding sites using [3 H]PN200-110 as the specific ligand. When analysed by SDS/PAGE under reducing conditions and by Western blot techniques, the TT were found to contain a concanavalin A-binding 150 kDa glycoprotein which probably corresponds to the α_2 -subunit of the DHP receptor. This conclusion was supported by correlative immunoblot experiments with a specific antibody. Junctional TT are further distinguished from free TT by the presence of a high number ($B_{\max}$ 20 pmol/mg of protein) of [3 H]cyclic AMP receptor sites, as determined by the Millipore filtration technique of Gill & Walton [(1974) *Methods Enzymol.* **38**, 376–381]. Use of this method means that the number of receptors may have been underestimated. The TT-bound cyclic AMP receptor was identified as a 55 kDa protein by specific photoaffinity labelling with 8-N₃-[3 H]cyclic AMP, and had similar phosphorylation properties and apparent molecular mass to the RII form of the regulatory subunit of cyclic AMP-dependent protein kinase. Co-localization of the intrinsic cyclic AMP-dependent protein kinase and of the DHP receptor complex to the junctional membrane of TT supports the hypothesis that the 170 kDa α_1 -subunit of the receptor is a substrate for the kinase.

INTRODUCTION

Transverse tubules (TT) are invaginations of the plasma membrane of skeletal muscle fibres that are involved in the propagation of the excitatory stimulus to the cell interior and in its transduction to the ryanodine-sensitive Ca²⁺-release channels of the sarcoplasmic reticulum (SR) (Inui *et al.*, 1987; Imagawa *et al.*, 1987b; Lai *et al.*, 1988; Chadwick *et al.*, 1988) to initiate muscle contraction. Freeze-fracture studies of TT membranes in intact muscle fibres (Block *et al.*, 1988) have lent support to the view that dihydropyridine (DHP) receptors, probably acting as voltage sensors (Schwartz *et al.*, 1985; Rios & Brum, 1987) are associated exclusively with the junctional membranes of TT. It is likely that these receptors interact directly or indirectly with the ryanodine receptor complex localized in the facing junctional membrane of SR terminal cisternae (TC) (Knudson *et al.*, 1988; Block *et al.*, 1988).

Early biochemical studies on isolated membrane fractions from skeletal muscles from both avian and mammalian species were aimed mainly at purifying TT from contaminating SR fragments and at defining their basic differences in membrane composition and biochemical properties. There is now fairly general agreement (see Sabbadini & Dahms, 1989, for a review) that TT membranes are distinguished from SR membranes by a high cholesterol content (Lau *et al.*, 1979; Roseblatt *et al.*, 1981; Sumnicht & Sabbadini, 1982), a high Mg²⁺-ATPase activity (Roseblatt *et al.*, 1981; Sabbadini & Okamoto, 1983; Beeler

et al., 1983) and, similar to the plasma membrane, the presence of β -adrenergic receptors (Caswell *et al.*, 1978), adenylate cyclase (Caswell *et al.*, 1978), and ouabain binding and (Na⁺+K⁺)-ATPase activities (Caswell *et al.*, 1976; Lau *et al.*, 1977). Furthermore, DHP-binding studies demonstrated that TT membranes are the richest source of DHP receptors (for reviews, see Hosey & Lazdunski, 1988; Campbell *et al.*, 1988), although with some differences depending on the isolation method (Brandt *et al.*, 1985; Horgan & Kuypers, 1987, 1988; Kanngiesser *et al.*, 1988). It has also been shown that TT membranes contain GTP-binding proteins (G-proteins) (Toutant *et al.*, 1988) and enzymes for the metabolism of phosphatidylinositol 4,5-bisphosphate (Hidalgo *et al.*, 1986).

The procedures used for isolating TT from skeletal muscle fall into two main categories, depending on the membrane source. TT membranes derived from a total microsomal fraction (Scales & Sabbadini, 1979) can be separated from contaminating SR fragments by centrifugation on a sucrose-density gradient, after active loading with either calcium oxalate (Sabbadini & Okamoto, 1983) or calcium phosphate (Roseblatt *et al.*, 1981). The characteristically low buoyant density of TT membranes has likewise been used to advantage in fractionation studies of muscle membranes of predominantly SR origin (Beeler *et al.*, 1983; Saito *et al.*, 1984; Salviati *et al.*, 1989). Alternatively, junctional TT have been dissociated from isolated triads by mechanical shearing with a French press (Caswell *et al.*, 1976), treatment at high ionic strength (Gilbert & Meissner, 1983),

Abbreviations used: TT, transverse tubules; CS, calsequestrin; SR, sarcoplasmic reticulum; TC, terminal cisternae; DHP, dihydropyridine; ConA, concanavalin A; PMSF, phenylmethanesulphonyl fluoride; PTX, pertussis toxin; CTX, cholera toxin; G-protein, GTP-binding protein.

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osmotic shock (Mitchell *et al.*, 1983), or centrifugation in an ion-free sucrose gradient (Horgan & Kuypers, 1987).

This study aimed to define a whole set of membrane markers specific to either junctional or free TT. As a specific marker of the junctional membrane we used the DHP receptor. Our direct comparative study of TT membranes isolated from rabbit fast-twitch muscle by several methods supports the validity of this assumption and, furthermore, it provides evidence for colocalization to the same membrane area of a 55 kDa cyclic AMP-binding phosphoprotein, probably equivalent to the regulatory subunit of an intrinsic type II cyclic AMP-dependent protein kinase. These aspects of membrane specificity are contrasted with the diffuse distribution in TT membranes of β -adrenergic receptor sites, G-proteins and [3 H]ouabain receptor sites, as well as with the enrichment in Mg^{2+} -ATPase activity of free TT obtained by different methods.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany) and Carlo Erba (Milano, Italy). Pyruvate dehydrogenase, lactate dehydrogenase, cholesterol oxidase, cholesterol esterase and peroxidase were purchased from Boehringer (Mannheim, Germany). Phenylmethanesulphonyl fluoride (PMSF), protein kinase II, Stains All {1-ethyl-2-[3-(1-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]-naphtho-[1,2-*d*]thiazolium bromide}, *Bordetella pertussis* toxin (PTX) and *Vibrio cholerae* toxin (CTX) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Molecular mass standards for SDS/PAGE (phosphorylase *b*, 97 kDa; BSA, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21 kDa; lysozyme, 14 kDa) were from Bio-Rad (Richmond, CA, U.S.A.). Sucrose (Ultra-pure grade) was purchased from Beckman Instruments (Palo Alto, CA, U.S.A.). [21,22- 3 H]Ouabain (15.1 Ci/mmol), L-[2,3-propyl- 3 H]-dihydroalprenolol (57 Ci/mmol), [γ - 32 P]ATP (5000 Ci/mmol) and [8- 3 H]cyclic AMP (30 Ci/mmol) were from Amersham International (Amersham, Bucks., U.K.). [3 H]PN200-110, 8-azidoadenosine 3':5'-monophosphate (8-N $_3$ -[3 H]cyclic AMP) (15 Ci/mmol) and [adenylate- 32 P]NAD $^+$ (800 Ci/mmol) were purchased from New England Nuclear (Du Pont De Nemours, Germany).

Isolation of muscle membranes

Predominantly fast-twitch muscles (back and hind limb muscles) from New Zealand male adult rabbits (body weight approx. 2.5 kg) were used. Three different procedures (A, B and C) were used for isolating SR-derived and TT-derived membranes. All muscle homogenates contained PMSF (2 mg/l).

Procedure A. TT were purified from a mixed microsomal fraction by active loading with calcium oxalate (Sabbadini & Okamoto, 1983), followed by centrifugation on a discontinuous sucrose-density gradient (25–40% sucrose) at 140 000 *g* for 2 h.

Procedure B. The isolation procedure, derived from that of Saito *et al.* (1984), has been described previously (Salviati *et al.*, 1989). Membranes banding at the 10–27% sucrose interface of the gradient, corresponding to the TT-enriched fraction R1 of Saito *et al.* (1984), were extracted once with 0.6 M-KCl and were purified further by loading with calcium oxalate, as for procedure A, followed by centrifugation at 140 000 *g* for 1 h.

Procedure C. The procedure was essentially that described by Horgan & Kuypers (1987). TT, referred to as free TT and

corresponding to fraction B3 of Horgan & Kuypers (1987), were obtained by centrifuging a crude microsomal fraction on continuous sucrose-density gradients (0.74–1.4 M-sucrose) in the presence of 20 mM-Tris/maleate, pH 7.0. Triads banding at 1.2 M-sucrose in the same gradient (B1) were dissociated into heavy SR (TC) vesicles and a light membrane fraction (fraction B1.3), consisting of junctional TT, by centrifuging in an ion-free sucrose gradient. The actual dissociation of triads by this treatment was verified by measuring the binding of [3 H]ryanodine by this fraction (3.5 pmol/mg of protein) compared with TC (4.1 pmol/mg of protein) and TT (0.093 pmol/mg of protein).

Protein concentration was determined according to Lowry *et al.* (1951), using BSA as standard.

Biochemical assays

Mg^{2+} -ATPase and Ca^{2+} -ATPase activities were measured at 37 °C as described by Sabbadini & Okamoto (1983). Endogenous protein kinase activity was assayed at 30 °C in 0.1 ml of 20 mM-histidine (pH 7.0)/2.5 mM-MgCl $_2$ /1 mM-dithiothreitol/2.5 mM-EGTA/20 mM-NaF containing about 40 μ g of membrane protein, in the absence and in the presence of 2 μ M-cyclic AMP. The concentration of [32 P]ATP ranged from 0.2 nM to 0.5 nM. The reaction was terminated after 5 min by pipetting the reaction mixture on to a filter paper as described by Corbin & Reimann (1974). For autoradiography the reaction was stopped with 10% trichloroacetic acid and pellets were resuspended in SDS buffer. Cholesterol content was measured as described by Salviati *et al.* (1982).

Gel electrophoresis, autoradiography and Western blot

SDS/PAGE was carried out according to Laemmli (1970) under reducing conditions. Gels were stained with Coomassie Brilliant Blue, destained and then restained with Stains All. Densitometric scanning of slab gels was done using a dual-wavelength Chromato Scanner CS-930 (Shimadzu, Japan). Autoradiography was carried out at –70 °C using Beta-max films (Amersham). Electrophoretic transfer on to nitrocellulose was carried out as described by Barhanin *et al.* (1987).

Immunological procedures

Immunoblotting of slab gels with antibodies to α_2 - δ complex was carried out as described by Barhanin *et al.* (1987).

Concanavalin A binding to SR and TT proteins

ConA binding to proteins transferred on to nitrocellulose was carried out as described previously (Damiani *et al.*, 1989).

Binding assays

Measurement of [3 H]dihydroalprenolol binding to TT membranes was carried out according to Caswell *et al.* (1978). [3 H]PN200-110 binding was determined as described by Barhanin *et al.* (1987), and [3 H]ouabain binding according to Jones *et al.* (1980). [3 H]Cyclic AMP binding was measured by the method of Gill & Walton (1974), and [3 H]ryanodine binding was determined essentially as described by Fleischer *et al.* (1985) in a medium containing 3 mM-ATP.

Photolabelling with 8-azidoadenosine 3':5'-monophosphate

Photoaffinity labelling was carried out in a medium containing, in a final volume of 0.2 ml, 50 mM-potassium phosphate buffer (pH 6.5), 8 mM-theophylline, 10 mM-MgSO $_4$, 2.75 μ M-cyclic AMP, 0.3 μ M-8-N $_3$ -[3 H]cyclic AMP and about 100 μ g of protein. Samples were placed in wells of a refrigerated porcelain plate and then preincubated in the dark for 10 min. U.v. irradiation was performed at 254 nm for 10 min with the lamp (8 W) kept at a distance of about 5–6 cm from the wells. After rinsing the wells

with 100 µl of incubation buffer, membranes were precipitated with 20 % trichloroacetic acid. Pellets after centrifugation were resuspended in SDS buffer.

Membrane protein ADP-ribosylation

PTX- and CTX-catalysed ADP-ribosylation was carried out essentially as described by Ribeiro-Neto *et al.* (1984). Toxins were activated by incubation for 30 min at 37 °C in 50 mM-dithiothreitol. Membranes (0.25 mg of protein/ml) were incubated in 100 µl of a medium containing 25 mM-Tris/HCl (pH 7.0), 1 mM-EDTA, 1 mM-ATP, 0.1 mM-GTP, 10 µg of PTX/ml (or 100 µg of CTX/ml) and 10 µCi of [³²P]NAD. In the case of CTX-catalysed ADP-ribosylation, the medium also contained 1 mM-MgCl₂ and 0.3 M-potassium phosphate buffer. Incubation was carried out for 60 min at 37 °C, and the reaction was terminated by adding 900 µl of ice-cold 20 % trichloroacetic acid. Membranes were sedimented by centrifuging at 15000 rev./min (*r*_{av.} = 53 mm) for 15 min. Pellets were washed once in diethyl ether and resuspended in SDS buffer.

RESULTS

Protein composition of TT membrane fractions and extent of contamination with SR fragments

As shown in Fig. 1, TT membranes isolated from rabbit skeletal muscle by different procedures (see the Materials and methods section), when analysed by SDS/PAGE and stained with Coomassie Blue, share a relatively common, complex, protein pattern. However, junctional TT (lane 4) were clearly distinguishable from free TT membranes (lanes 1–3) as a whole by the presence of a much smaller amount of protein material at about 100 kDa. Parallel measurements of Ca²⁺-independent/Mg²⁺-ATPase activities in the various membrane preparations (Table 1) indicated an overall correlation between the level of this activity and the amount of 100 kDa protein present. This protein appears to be most prominent in free TT obtained by procedure A (Fig. 1, lane 1), in agreement with earlier observations on isolated TT from chicken muscle using the same procedure (Damiani *et al.*, 1987). Junctional TT were also distinguishable on the basis of a more heterogeneous peptide

Fig. 1. SDS/PAGE of TT isolated by different procedures

SDS/PAGE (7.5 % gels) was carried out according to Laemmli (1970). Gels were stained with Coomassie Brilliant Blue. Lanes: 1, TT obtained by procedure A; 2, TT obtained by procedure B; 3 and 4, free and junctional TT respectively obtained by procedure C [fraction B3 and B1.3 respectively according to the nomenclature of Horgan & Kuypers (1987)]. About 30 µg of protein was loaded/lane.

pattern in the 70–64 kDa range of molecular mass (Fig. 1, lane 4).

The glycoprotein compositions of free and junctional TT, as obtained by the general procedure of Horgan & Kuypers (1987), were investigated using a very sensitive ConA binding assay (Damiani *et al.*, 1989). As shown in Fig. 2, junctional TT (lane 5) contained a prominent ConA-binding protein of about 150 kDa. The same protein was present in free TT (Fig. 2, lane 2), as well as in triads (lane 1), but in much lesser amounts. It was found to be totally absent from SR subfractions consisting of either TC or longitudinal SR fragments (lane 4). This 150 kDa

Table 1. Comparison of the biochemical properties of free and junctional TT membranes

Free TT membranes were obtained as described in the Materials and methods section by procedures A, B and C (fraction B3 according to the nomenclature of Horgan & Kuypers, 1987). Junctional TT membranes were obtained by procedure C (Fraction B1.3). Assays were carried out as described in the Materials and methods section. Values are given as means ± S.E.M. (*n*). The data related to fraction B1.3 were statistically evaluated with respect to the data related to the other fractions by Student's *t* test for mean comparisons (**P* < 0.05; ***P* < 0.01). DHA, dihydroalprenolol.

Assay	Procedure ...	Free TT			Junctional TT
		A	B	C	
Cholesterol (µmol/mg of protein)		0.83 ± 0.13* (6)	0.42 ± 0.01 (4)	0.34 ± 0.05 (7)	0.41 ± 0.04 (6)
Mg ²⁺ -ATPase (µmol/min/mg)		3.01 ± 0.20** (7)	1.06 ± 0.31 (3)	2.01 ± 0.69 (3)	0.42 ± 0.08 (3)
Ouabain binding (pmol/mg)		20.0 (2)	18.7 ± 4.2 (4)	10.4 ± 1.6** (5)	30.8 ± 5.3 (4)
DHA binding (pmol/mg)		0.45 (2)	0.35 ± 0.07 (4)	0.32 ± 0.03 (4)	0.58 ± 0.12 (3)
DHP binding (pmol/mg)		13.4 ± 2.8** (3)	7.4 ± 2.5** (4)	13.5 ± 1.9** (4)	58.8 ± 7.7 (6)
Cyclic AMP binding (pmol/mg)		3.22 (1)	2.79 (1)	2.58 ± 0.38** (6)	14.2 ± 2.4 (6)

Fig. 2. Identification of ConA-binding proteins

SR and TT membrane proteins, obtained according to Horgan & Kuypers (1987), were resolved in a 5–15% polyacrylamide linear gradient gel and then transferred on to nitrocellulose. Blots were incubated with ConA-biotin (1 µg/ml) followed by avidin-alkaline phosphatase. A 20 µg portion of protein was loaded per lane. Lanes: 1, triads (fraction B1); 2, free TT (B3); 3, TC (B1.1); 4, longitudinal SR (B1.2); 5, junctional TT (B1.3).

protein which strongly binds ConA probably corresponds to the α_2 -subunit of the DHP receptor complex, which is known to be heavily glycosylated (Hosey & Lazdunski, 1988; Campbell *et al.*, 1988). In contrast, SR fragments (lanes 3 and 4) were characterized by the presence of a 53 kDa ConA-binding protein component, already shown to be a specific marker of longitudinal SR (Damiani *et al.*, 1989). Trace amounts of the same component could be detected in isolated TT (Fig. 2, lanes 2 and 5).

We tried to assess the extent of contamination with SR fragments of the various TT membrane fractions by using specific markers of the Ca^{2+} -pump membrane and of the junctional membrane of the SR, such as Ca^{2+} -ATPase activity on the one hand, and CS and [^3H]ryanodine binding activity (procedure C), on the other. The Ca^{2+} -ATPase activity, measured at 37 °C and in the presence of Ca^{2+} ionophore A23187, was virtually undetectable in TT membrane preparations obtained by procedure A; this activity was 0.26 ± 0.08 ($n = 3$) and 1.02 ± 0.11 ($n = 9$) µmol of P_i /min per mg of protein (mean values $\pm$ S.E.M.) for free TT obtained by procedures B and C respectively. The corresponding value for junctional TT was 0.53 ± 0.12 ($n = 9$) µmol of P_i /min per mg of protein. Under similar assay conditions, Ca^{2+} -ATPase values for purified SR fragments from rabbit fast skeletal muscle were approx. 10 µmol of P_i /min per mg of protein (Salviati *et al.*, 1982). Thus our present results indicate that the contamination by SR fragments derived from longitudinal SR, although being negligible in the case of free TT obtained by procedures A and B (both involving a calcium oxalate purification step), is however high for the corresponding fraction when isolated by the original procedure of Horgan & Kuypers (1987) (procedure C; extent of contamination about 10%). Conversely, the membrane content of cholesterol, a specific marker of TT membranes versus SR membranes (Sumnicht & Sabbadini, 1982), was found to be highest in TT obtained by procedures A and B and lowest in TT obtained by procedure C (Table 1).

Junctional SR contaminants, because of their high buoyant density (see Saito *et al.*, 1984), represented a minor contaminant

Fig. 3. Stains All staining of SR and TT proteins

SDS/PAGE of SR and TT membranes isolated by procedure C (Horgan & Kuypers, 1987) was carried out according to Laemmli (1970), using a 5–15% polyacrylamide linear gradient gel. Gels were stained with Coomassie Blue, destained and restained with Stains All. About 30 µg of protein was loaded per lane. Lanes: 1, junctional TT (fraction B1.3); 2 and 5, longitudinal SR (B1.2 and B2); 3, TC (B1.1); 4, free TT (B3); 6, triads (B1); 7, molecular mass markers.

of TT. The degree of contamination was independent of the isolation procedure, as predicted by the presence of trace amounts of CS in Stains-All-stained SDS/gels (Fig. 3, lanes 1–4).

DHP receptor

Previous studies (Brandt *et al.*, 1985; Kanngiesser *et al.*, 1988) indicated that junctional TT isolated after mechanical disruption of triads had a DHP receptor density significantly lower than that reported for TT isolated as free membranes (Fosset *et al.*, 1983; Burdett *et al.*, 1987; O'Callahan *et al.*, 1988; Toutant *et al.*, 1988). Table 1 and Fig. 4 show that junctional TT prepared by procedure C (Fig. 4d) contained a high number of high-affinity (K_d 0.25 nM) binding sites for the DHP analogue [^3H]PN200-110, used here as a specific ligand. The B_{max} value for junctional TT, while similar to that reported by Horgan & Kuypers (1988), was found to be 4–8 fold higher than that for free TT, which had the lowest value when prepared by procedure B (Table 1). On the other hand, K_d values were found to be in the same range for both junctional and free TT (Fig. 4).

When blots of SDS gels (after electrophoresis under reducing conditions) were probed with polyclonal antibodies against the α_2 and δ subunits of the DHP-sensitive Ca^{2+} -channel (Barhanin *et al.*, 1987), two intensely stained peptides of about 150 kDa and 23 kDa were detected in junctional TT (Fig. 5, lane 3). In agreement with the DHP-binding measurements, the immunostaining of the same peptides appeared to be much weaker for free TT obtained by either procedure A (lane 4) or procedure B (lane 5). By comparison, only trace amounts of the two immunoreactive peptides could be detected in SR fragments (Fig. 5, lanes 2 and 6), as expected.

[^3H]Ouabain binding

Wide differences have been reported in the content of ($\text{Na}^+ + \text{K}^+$)-ATPase between TT isolated from a chicken muscle microsomal fraction according to Sabbadini & Okamoto (1983)

Fig. 4. Binding of [^3H]PN200-110 to free and junctional TT

The binding of [^3H]PN200-110 to TT membranes was performed as described by Barhanin *et al.* (1987). Values are the means of duplicate determinations. B_{max} and K_d values were obtained by Scatchard plot analysis (insets). (a) Free TT prepared by procedure A (B_{max} 12 pmol/mg; K_d 0.34 nM); (b) free TT prepared by procedure C (fraction B3) (B_{max} 11 pmol/mg; K_d 0.16 nM); (c) free TT prepared by procedure B (B_{max} 16 pmol/mg; K_d 0.19 nM); (d) junctional TT prepared by procedure C (fraction B1.3) (B_{max} 82 pmol/mg; K_d 0.25 nM).

and junctional TT obtained from mechanically disrupted triads of rabbit muscle (Caswell *et al.*, 1976). This has been discussed in relation to their different membrane origin (Martonosi, 1986). Since the estimation of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, as well as that of [^3H]ouabain binding activity, is influenced by the sidedness of vesicles (Seiler & Fleischer, 1982; Mitchell *et al.*, 1983), we re-investigated the binding of [^3H]ouabain to membranes in the presence of alamethicin, in order to estimate the total number of binding sites. The results in Table 1 show relatively small differences between TT prepared by procedure A and procedure B, and a significant ($P < 0.01$) difference in [^3H]ouabain binding sites only between free TT and junctional TT, as obtained by procedure C (Table 1). This difference, rather than depending on the intrinsic differences in composition between the free membranes and the junctional membranes of TT, was more likely attributable to the different extent of contamination of the latter two fractions with SR membranes derived from longitudinal SR.

β -Adrenergic receptor

In agreement with earlier findings by Caswell *et al.* (1978), and using the same [^3H]dihydroalprenolol binding assay, we found a relatively high level of β -adrenergic receptor sites in our junctional

TT preparations (Table 1). As shown (Table 1), the membrane density of β -adrenergic receptors appeared to be lowest in free TT prepared by procedure C. However, that again probably reflected the relatively greater extent of contamination of this fraction with SR fragments.

Intrinsic protein kinase and phosphorylatable protein substrates of TT

At variance with the results of Caswell *et al.* (1978) on junctional TT isolated from triads by mechanical disruption, we were able to detect an endogenous phosphorylating activity in the purified fraction as obtained by the method of Horgan & Kuypers (1987), and using similar assay conditions (i.e. in the presence of EGTA). The amount of ^{32}P from [$\gamma\text{-}^{32}\text{P}$]ATP incorporated into protein was about 0.7 nmol of ^{32}P /mg of protein at saturating concentrations of ATP (0.5 mM). Under these conditions the degree of incorporation was unaffected or only slightly stimulated by the addition of 2 μM -cyclic AMP. However, a large stimulatory effect of cyclic AMP on TT membrane phosphorylation by the endogenous protein kinase was observed at low concentrations of ATP, i.e. between 0.2 nM- and 1 μM -[^{32}P]ATP.

As these results could be explained by the presence of a high

Fig. 5. Immunoreactivity of TT proteins with antibody raised against the α/δ subunit of the DHP-sensitive Ca^{2+} channel

Proteins were resolved in a Laemmli (1970) 5–20% polyacrylamide linear gradient gel, and were transferred on to nitrocellulose and incubated with a polyclonal antibody (10 $\mu\text{g}/\text{ml}$) against the α_2/δ -subunit of DHP-sensitive Ca^{2+} channel (Barhanin *et al.*, 1987). After washing, the blot was incubated with ^{125}I -Protein A. Autoradiography was carried out for 6 h. About 50 μg of protein was loaded per lane. Lanes: 1, triads; 2, TC; 3, junctional TT prepared by procedure C; 4, free TT obtained by procedure A; 5, free TT obtained by procedure B; 6, longitudinal SR.

Fig. 6. Scatchard plot analysis of $[^3\text{H}]$ cyclic-AMP binding to TT membranes

Membranes (60 μg) were incubated for 5 min at 30 $^{\circ}\text{C}$ in 50 mM-potassium phosphate buffer (pH 6.5)/10 mM- MgSO_4 /8 mM-theophylline in the presence of the concentrations of $[^3\text{H}]$ cyclic AMP indicated on the abscissa. The reaction was started by addition of $[^3\text{H}]$ cyclic AMP (50000 d.p.m./pmol) and stopped by diluting the samples in 5 ml of ice-cold washing buffer (25 mM-Tris/HCl/10 mM- MgSO_4 , pH 7.0). Samples were immediately filtered on Millipore filters (0.45 μm pore size), and filters were washed with 3×5 ml of washing buffer. Non-specific binding was measured in the presence of a 100-fold excess of unlabelled cyclic AMP. B_{max} , 21.3 pmol/mg of protein; K_d 97.8 nM.

Fig. 7. Photoaffinity labelling of TT proteins by 8-azidoadenosine 3':5'-monophosphate

Membranes were isolated according to Horgan & Kuypers (1987), and 100 μg of membrane protein was incubated with 8- N_3 - $[^3\text{H}]$ cyclic AMP and u.v.-irradiated as described in the Materials and methods section, in the absence (lanes 1, 3 and 5) or in the presence (lanes 2, 4 and 6) of 275 μM unlabelled cyclic AMP. SDS/PAGE (10% gels) was carried out according to Laemmli (1970), and autoradiography was performed as described in the Materials and methods section. Lanes: 1 and 2, free TT; 3 and 4, junctional TT; 5 and 6, triads.

level of endogenous cyclic AMP-dependent protein kinase activity in these TT membrane preparations, coupled to the presence of rate-limiting concentrations of endogenous protein substrates for phosphorylation, we determined the membrane density of cyclic AMP receptors by the Millipore filtration method of Gill & Walton (1974). The results reported in Table 1 show that junctional TT are distinguished from free TT (whether prepared by procedure A, B or C) by the high content of cyclic AMP receptors. Scatchard plot analysis (Fig. 6) demonstrated that junctional TT contain a very high density of receptor sites (B_{max} , 21.3 pmol/mg of protein), having an apparent binding constant (K_d) for cyclic AMP of about 100 nM. This is one order of magnitude higher than that obtained for the purified type II cyclic AMP-dependent protein kinase using the same method, but it is however comparable with earlier reported values at protein kinase concentrations similar to those found in muscle (Rosen & Erlichman, 1975; Hofmann *et al.*, 1975).

In agreement with these results, specific photoaffinity labelling of TT membranes with 8- N_3 - $[^3\text{H}]$ cyclic AMP [i.e. inhibition by an excess of unlabelled cyclic AMP included in the incubation medium (Fig. 7) followed by SDS/PAGE and autoradiography] revealed the presence in junctional TT of a single peptide of about 55 kDa, i.e. having the same size as the regulatory (R_{II}) subunit of cyclic AMP-dependent protein kinase (lane 3). By comparison, much lower amounts of the cyclic AMP-binding protein could be detected in free TT (lane 1) and in triads (lane 5).

Junctional TT that had been phosphorylated in the presence of EGTA, and at low (0.2 nM and 1 μM) concentrations of radioactive ATP, without and with 2 μM -cyclic AMP, were analysed by SDS/PAGE and autoradiography (Fig. 8a). Under all conditions, the main ^{32}P -labelled protein was detected at about 55 kDa. At low ATP concentrations, the labelling was also markedly stimulated by cyclic AMP (Fig. 8a). Both of these findings, together with the evidence of covalent incorporation of 8- N_3 - $[^3\text{H}]$ cyclic AMP into a 55 kDa peptide, thus suggested that this peptide may be equivalent to the R_{II} form of the regulatory subunit of cyclic AMP-dependent protein kinase (Rosen & Erlichman, 1975). In agreement with this interpretation, co-

Fig. 8. Pattern of TT membrane proteins phosphorylated by endogenous cyclic AMP-dependent protein kinase of TT

(a) Junctional TT (fraction B1.3) were incubated for 5 min at 30 °C with 0.2 nM (lanes 5 and 6) or with 1 μM (lanes 3 and 4) [^{32}P]ATP, in the absence (lanes 3 and 5) or in the presence (lanes 4 and 6) of 2 μM-cyclic AMP, as described in the Materials and methods section. The reaction was terminated by the addition of SDS solubilization buffer. TT proteins were resolved using a 5–15% polyacrylamide linear gradient gel. Phosphorylated proteins were revealed by autoradiography (2-day exposure). About 50 μg of protein was loaded per lane. Lanes: 1, Coomassie Blue-stained junctional TT; 2, molecular mass standards; 3–6, autoradiography of junctional TT. (b) Junctional (lanes 1 and 2) and free (lanes 3 and 4) TT membranes obtained by procedure C were incubated as in (a) in the absence (lanes 2 and 4) and in the presence (lanes 1 and 3) of 2 μM-cyclic AMP. Electrophoresis and autoradiography were carried out as in (a). About 50 μg of protein was loaded per lane.

electrophoresis with commercial type II protein kinase from bovine heart demonstrated that the regulatory subunit of the protein kinase and the main phosphorylated protein of junctional TT are closely similar to each other in electrophoretic mobility (results not shown).

Membrane fractions consisting mainly of free TT prepared by three different methods all displayed a low content of phosphorylated protein material at about 55 kDa, and were further distinguishable from junctional TT by the lack of evidence of any other phosphorylated protein band following incubation with radioactive ATP at different concentrations and in the

Fig. 9. Pattern of ADP-ribosylated proteins of TT

TT membrane proteins were ADP-ribosylated in the absence (lanes 1–4) or in the presence (lanes 5–8) of CTX and were electrophoresed using a SDS/10% polyacrylamide gel, as described in the Materials and methods section. About 30 μg of protein was loaded per lane. Lanes: 1 and 5, free TT (fraction B3); 2 and 6, junctional TT (fraction B1.3); 3 and 7, free TT (procedure B); 4 and 8, free TT (procedure A).

absence or in the presence of cyclic AMP (Fig. 8b). On the other hand, in the presence of cyclic AMP, additional phosphoproteins could be detected in junctional TT, banding at about 170 kDa, 130 kDa and 80 kDa (Fig. 8b, lane 1). On considering evidence from previous work on membrane protein phosphorylation in isolated triads, and on phosphorylation of TT by the catalytic subunit of cyclic AMP-dependent protein kinase (Hosey *et al.*, 1986), the protein band of about 170 kDa could correspond to the α_1 subunit of the DHP receptor.

G-proteins

G-proteins that are substrates for ADP-ribosylation catalysed by either CTX (Scherer *et al.*, 1987) or PTX (Toutant *et al.*, 1988) have recently been detected in isolated TT from cardiac and skeletal muscle. We found that when TT membranes were incubated with [^{32}P]NAD in the absence of added toxins, and then analysed by SDS/PAGE and autoradiography, a single peptide of about 55 kDa became radiolabelled, irrespective of the membrane isolation procedure (Fig. 9, lanes 1–3), indicating the presence of an endogenous ADP-ribosylating activity and of a common substrate in all of these preparations. When incubation was carried out in the presence of CTX, a 44 kDa peptide was ADP-ribosylated, but there were no appreciable differences between junctional and free TT (Fig. 9). Similar results were obtained in the case of the 40 kDa substrate of PTX (results not shown).

DISCUSSION

DHP receptors have been implicated both as Ca^{2+} channels and as voltage sensors in excitation–contraction coupling, depending on their state of phosphorylation (Campbell *et al.*, 1988; Hosey & Lazdunski, 1988). However, there is now rather compelling evidence from several laboratories that coupling in skeletal muscle, unlike that in cardiac muscle, involves a voltage-dependent charge movement, rather than a voltage-dependent Ca^{2+} influx through L-type Ca^{2+} channels, across the junction between T-tubules and the terminal cisternae of the SR at the triad (Rios & Brum, 1987). An integral part of the hypothesis that DHP receptors in skeletal muscle act as voltage sensors for transmission of the signal to the Ca^{2+} -release channels of the SR

is that these receptors are selectively localized in junctional membranes of TT. There is overwhelming evidence for this (Campbell *et al.*, 1988; Hosey & Lazdunski, 1988; Leung *et al.*, 1988; Block *et al.*, 1988), except for some recent immuno-electron microscopic evidence (Jorgensen *et al.*, 1989).

Our present results with isolated TT from rabbit fast-twitch muscle, while confirming the existence of areas of membrane specificity within the TT system in relation to the distribution of the DHP receptor, show the co-localization of a cyclic AMP-dependent protein kinase to the same membrane areas, i.e. to junctional TT.

Previous studies (Hosey *et al.*, 1986; Imagawa *et al.*, 1987a) demonstrated that both the DHP-binding α_1 subunit and the β -subunit of the DHP receptor complex of TT from rabbit skeletal muscle are phosphorylated by the catalytic subunit of exogenous cyclic AMP-activated protein kinase. In particular, Imagawa *et al.* (1987b), working with isolated triads, showed that the same subunits of the DHP receptor complex are substrates for an intrinsic protein kinase, which they assumed to be similar to their TT component and maintained to be independent from both Ca^{2+} and cyclic AMP.

We have tried to clarify the problem of the topological relationship to the DHP receptor, as well as to characterize the properties of membrane-bound protein kinase of TT, by a more direct approach. Based on binding studies with [^3H]PN200-110 and on the detection of the α_2 subunit of the DHP receptor, either by immunoblotting with specific antibody or by selective binding to ConA, the DHP receptors appear to be highly concentrated in TT obtained from triads by centrifugation in an ion-free sucrose gradient compared with free TT obtained by three different methods. We have found that junctional TT are highly enriched in a protein kinase, which however has properties different from those described by Imagawa *et al.* (1987b), in that our results show that it is stimulated by cyclic AMP.

Characterization of the intrinsic protein kinase of junctional TT as a cyclic AMP-dependent type II protein kinase relies on selective covalent labelling with 8- N_3 -[^3H]cyclic AMP [a photoactivatable analogue of cyclic AMP (Kerlavage & Taylor, 1982)] of a 55 kDa membrane protein component, i.e. having the same size as the regulatory (R_{II}) subunit of cardiac protein kinase (Rubin *et al.*, 1972). Furthermore, we have demonstrated that the same protein was readily phosphorylated when junctional TT were incubated with [γ - ^{32}P]ATP and that phosphorylation was enhanced by cyclic AMP, as expected from the early findings of Rosen & Erlichman (1975).

The substrate specificity of the intrinsic Ca^{2+} -independent cyclic AMP-activated protein kinase of junctional TT appears to be more restricted when compared with the pattern of protein phosphorylation of triads by intrinsic Ca^{2+} -independent protein kinase (Imagawa *et al.*, 1987a). This is illustrated by the presence in these triad preparations of additional phosphorylatable substrates which are due to the junctional SR component of the triads (Campbell & Shamoo, 1980). Under the conditions of our experiments, phosphorylation of the 55 kDa cyclic AMP-binding protein seems to account for much of the radioactivity incorporated into TT membranes. However, the presence of additional phosphorylated proteins in the same range of electrophoretic mobility cannot be entirely excluded, in particular the β -subunit of the DHP receptor of 52–62 kDa (Campbell *et al.*, 1988), as shown by Imagawa *et al.* (1987a) using more discriminating electrophoretic conditions. As in the study of Imagawa *et al.* (1987a), we also found phosphorylation of a 170 kDa protein in junctional TT, probably corresponding to the α_1 subunit of the DHP receptor. Furthermore, our results show that phosphorylation of this 55 kDa protein, as well as of a second major protein of about 80 kDa, is enhanced by cyclic

AMP. As the stimulatory effect of cyclic AMP was most evident at concentrations of [γ - ^{32}P]ATP in the micromolar range, such an effect might have gone undetected by Imagawa *et al.* (1987a), given the different phosphorylation assay conditions in their study on triads.

Scatchard plot analysis of [^3H]cyclic AMP binding to junctional TT, as determined by the Millipore filtration assay of Gill & Walton (1974), yields approximately 10 cyclic AMP-binding sites for every 3 DHP-binding sites available on the same membrane. Since the binding stoichiometry of cyclic AMP to the type II protein kinase holoenzyme is, however, underestimated by this method compared with other methods (see Kerlavage & Taylor, 1982), the maximum number of cyclic AMP-receptor sites may be actually twice the calculated value.

The idea that junctional and free TT differ basically in membrane composition, as supported by our present results, is not new and has already been considered and discussed, although in an entirely different context, in comparative biochemical studies of TT membrane preparations putatively derived from either the free or the junctional portion of the T system, depending on the particular isolation method (Horgan & Kuypers, 1987, 1988; Sabbadini & Dahms, 1989; see also Martonosi, 1986). With regard to the previously noted high level of Ca^{2+} -independent Mg^{2+} -activated ATPase activity in muscle membrane preparations enriched in free TT, such as those obtained by the method of Sabbadini & Okamoto (1983) in particular, our present findings support the contention that this activity is not due to SR contaminants, without however contributing any new information as to the real nature and physiological meaning of this activity. Concerning the suggested differential distribution of the (Na^+ - K^+)-ATPase in free and junctional TT (Martonosi, 1986), our [^3H]ouabain binding studies seem to argue against this premature conclusion. In retrospect, a methodological problem that might have been overlooked in the past is the influence of membrane-sidedness of TT vesicles on binding of [^3H]ouabain *in vitro* (Mitchell *et al.*, 1983). Significantly, the levels of [^3H]ouabain receptors in isolated TT measured here in the presence of alamethicin (Jones *et al.*, 1980) (i.e. all receptor sites are available to the ligand) were found to be about 10-fold greater, on average, than some of the quoted values of Horgan & Kuypers (1987, see their Table V). An analogous, relatively uniform distribution of β -adrenergic receptors and of G-proteins are displayed by free and junctional TT. However, differences have been recently noted with regard to the binding of dystrophin to the TT membrane (Salviati *et al.*, 1989), as well as in the distribution of the glucose transporter (Plough *et al.*, 1989).

The picture which is emerging of the membrane specificity of junctional TT from the present study has interesting implications for the functional specialization of this portion of the T-system in relation to the excitation-contraction coupling process in skeletal muscle and its possible control by the β -adrenergic system. The present finding that type II cyclic AMP-dependent protein kinase is a specific major component of the junctional membrane of TT fits particularly well with the hypothesis that phosphorylation of the α and β subunits of the DHP receptor may serve to regulate its physiological function, even though direct evidence is still lacking that the coupling process is affected by β -adrenergic stimulation in skeletal muscle. Co-localization to the junctional membrane of TT of the DHP receptor and of cyclic AMP-activated protein kinase, which in itself argues for more than a functional interaction between them, might also have a bearing on the influence of the β -adrenergic system on TT-membranes during differentiation, as indicated by the increased membrane density and changes in properties of the DHP receptor in developing skeletal muscle upon long-term β -adrenergic stimulation (Schmid *et al.*, 1985). Further work along

these lines could thus offer interesting new prospects in relation to the still unresolved problem of the actual role played by the β -adrenergic system in excitation-contraction coupling of skeletal muscle fibres from early development to the adult stage.

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