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Research Articles

Studies of a key protein in the mechanism of the excitation-contraction coupling process of frog skeletal muscle, using phenylglyoxal

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Abstract. The excitation-contraction (E-C) coupling process in single twitch fibres from frog toe muscle was inhibited selectively by phenylglyoxal (PGO), a specific guanidyl modifying reagent. A new protein (31.5 kDa), which has PGO-binding ability and seems to play a key role in the E-C coupling process, was solubilized from transverse tubule membrane-junctional sarcoplasmic reticulum complexes (TTM-JSR) of frog skeletal muscles, using ^{14}C-PGO. The monoclonal antibody against this protein applied extracellularly inhibited the E-C coupling process of the single fibres. This protein appears to constitute the very first step of input for E-C coupling. It is considered to behave as an indispensable part of an 'electrometer' to measure membrane potentials. Therefore, the name 'electrometrin' is suggested for the new protein.
Key words. Frog skeletal single fibres; phenylglyoxal (PGO); E-C coupling; solubilization; PGO-binding protein (PGO-protein); monoclonal antibody; electrometrin.

When the plasma membrane of vertebrate twitch muscle fibres becomes depolarized enough, either actively (action potential) or passively (K-induced depolarization), the fibres contract. For this to take place, depolarization must first spread in the transverse (T) tubular system. The depolarization thus produced at the T-tubular membrane triggers the release of Ca stored in the terminal cisternae of the sarcoplasmic reticulum (SR) into the milieu surrounding the myofibrils, which then contract. In this series of events, the mechanism coupling the electrical event (E) at the T-tubular membrane with the release of the Ca which is indispensable for contraction (C) is still almost unknown. The present paper will deal with this E-C coupling mechanism.

We have already reported[1,2] that the E-C coupling process in single twitch fibres from frog toe muscles is inhibited selectively by the application of phenylglyoxal (PGO), a specific guanidyl modifying reagent[3]. To clarify the mechanism of the E-C coupling process in skeletal muscle, we performed a study on the solubilization of a protein which appears to play a key role in the process. The protein was extracted from transverse tubule membrane-junctional sarcoplasmic reticulum complexes (TTM-JSR) of frog skeletal muscles, using PGO. This protein has not previously been reported.

Materials and methods

1) Materials. Muscle preparations: Single fibres from M. extensor longus digiti IV, whole sartorius muscles and leg muscles of the frog, *R. japonica*, were used. PGO and ^{14}C-PGO ((7-^{14}C)-PGO 25 mCi/m-mol) were from WAKO Pure Chem. Ind., Japan, and from Amersham, the Netherlands, respectively. Ringer's solution contained 110 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 5 $NaHCO_3$ (all mM), pH 6.8. 185 mM NaCl-Ringer is a Ringer's solution in which NaCl concentration was 185 mM, so that the milieu was made slightly hypertonic. In K-Ringer (K or 50 K), 50 mM NaCl in Ringer was replaced by KCl at the same concentration. Caffeine-Ringer (C or caff.): K-Ringer containing 15 mM caffeine.

2) Physiological observations. Observations were made at about 20 °C. According to the purpose of the experiments, tissues were driven by electrical square pulses of 0.3 ms singly for twitch (mostly, once a min) or in train for short tetanus, Tt, (20 times, 100 c/s). Resting and action (AP) potentials were measured intracellularly both before and after conditioning. After conditioning, determinations of contractility were made (tetanus, K-, and caffeine-contractures). To evoke contractures, bathing solutions were replaced by K-Ringer or caffeine-Ringer.

PGO was applied at exactly 3 mM for 3 min to single fibres, and at 5 mM for 10 min in whole muscles. This procedure brought about a rather typical inhibition of E-C coupling; namely, the resting and action potentials and the ability of the contractile apparatus (caffeine contracture) itself remained almost unchanged, while 50 mM K, which would otherwise produce an almost full tension-development, evoked only a weak tension-development (fig. 1).

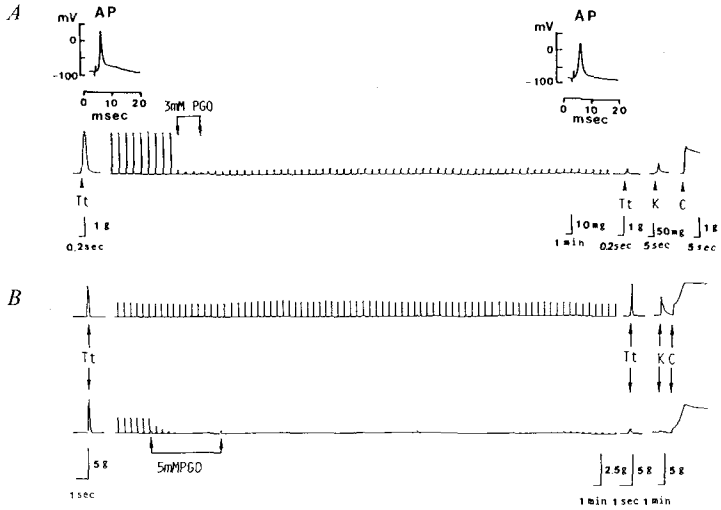


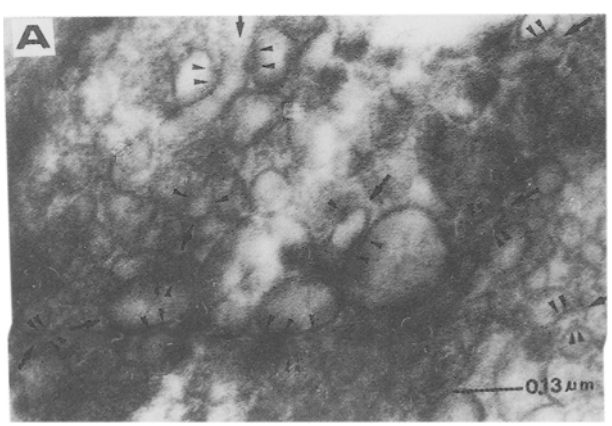
Figure 1. Effect of phenylglyoxal on the E-C coupling process of frog skeletal muscle tissues. *A* Single fibre; *B* whole sartorius muscle (upper trace: control). Tissues were driven electrically (twitch, once per minute, no symbol; short tetanus, Tt). Resting and action (AP) potentials were measured both before and at about 60 min after PGO-administration. About 60 min after the administration, determinations were made on contractility (tetanus, K-, and caffeine-contractures).

For observations of the effects of antibodies (AB), the antibody was dissolved in Ringer's solution at a protein concentration of 10 µg/ml. The preparation of antibodies is described below. For control observations (C in fig. 4B) an uncoupled AB was used. This was obtained by the same procedure as that used for coupled AB, from hybridomas secreting uncoupled AB (SG 856). The protein concentration was adjusted to 10 µg/ml by the addition of appropriate amounts of anti-rabbit immunoglobulin. Determinations of contractility were made about 90 min after the administration of AB solutions.

3) Isolation of TTM-JSR and protein-solubilization. For the isolation of TTM-JSR, the previously described method[4,5] was used. TTM-JSR were separated by centrifugation at 4000–25,000 × g, diluted (1:4) with isolating solution (20 mM histidine-HCl, 80 mM KCl, pH 6.8), and pelleted at 25,000 × g for 30 min.

Figure 2A shows a typical electron micrograph of the TTM-JSR preparations used in the present experiments. Staining shows feet of triads[6]. The pellet was fixed with a solution (pH 7.4) containing 4% tannic acid, 2.5% glutaraldehyde, 3% sucrose, and 0.1 M Na cacodylate for 10 min, left overnight at 0 °C, post-fixed with 1% OsO_4 for 3 h, and embedded in Epon 812. Cut sections were stained with uranyl acetate and lead citrate.

For solubilization and analysis of proteins from TTM-JSR, isolated TTM-JSR were treated with solutions



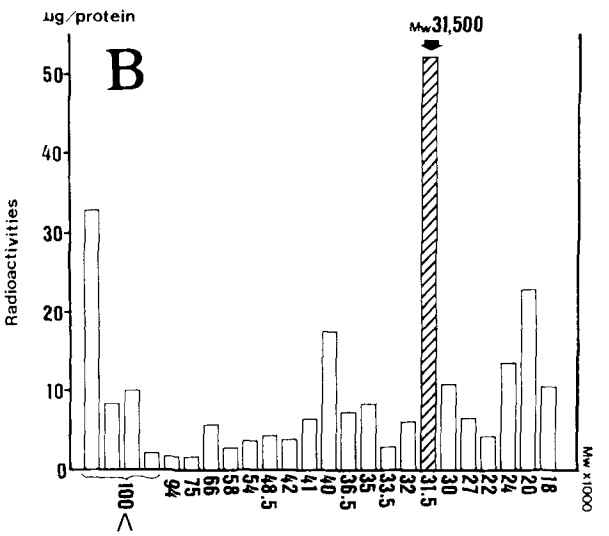


Figure 2. Electron micrograph (A) and radioactivity profile of proteins (B) of used TTM-JSR fractions isolated from whole skeletal muscles of frog. *A* Electron micrograph of TTM-JSR from leg muscles. Arrows and arrowheads indicate the T-tubules and the feet of triads, respectively. *B* Radioactivity profile of proteins contained in TTM-JSR of sartorius muscles after E-C uncoupling had been brought about by PGO-pretreatment.

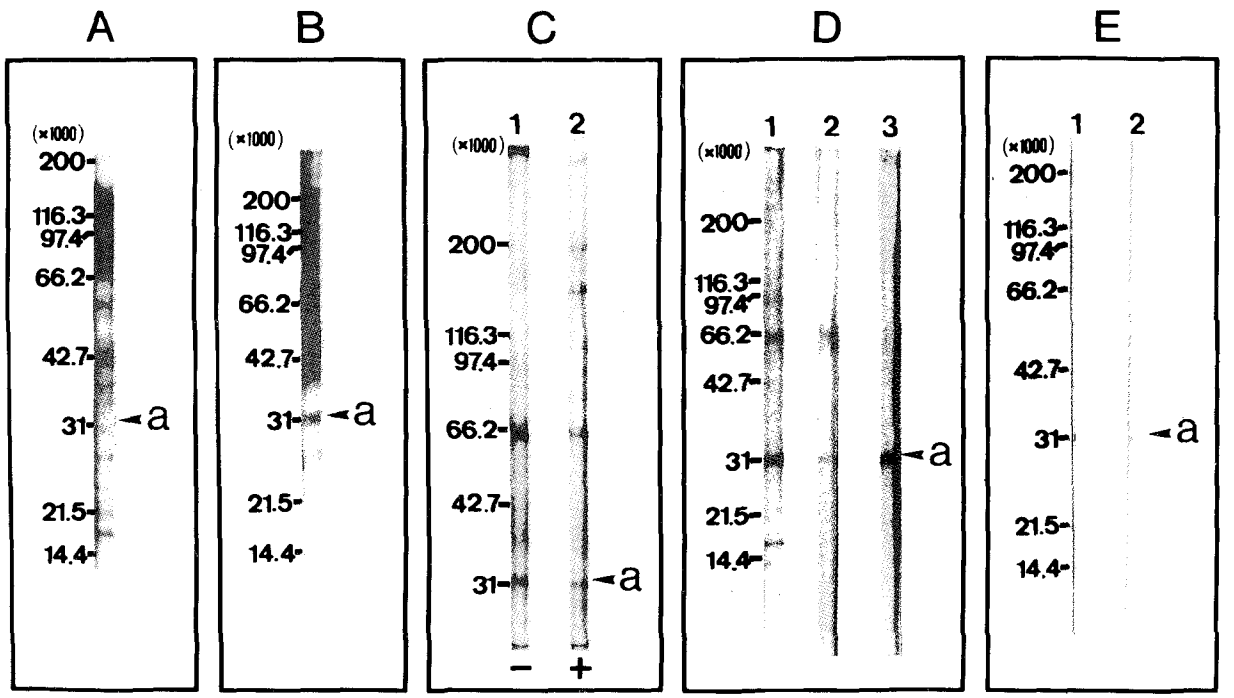


Figure 3. Characteristics and immunoblot analysis of the targeted protein isolated from TTM-JSR of frog skeletal muscles. Frog leg muscles without PGO-administration were used. *A*–*E* 4–20% gel was used for SDS-PAGE. In all panels, the letter a indicates a protein band of 31.5 kDa. *A* and *B* Total TTM-JSR proteins separated by SDS-PAGE. *C*, *D* and *E* Proteins selected by Con A affinity chromatography of solubilized TTM-JSR (Con A-sensitive proteins). Protein content, 20 µg/lane. *A* TTM-JSR proteins stained with CBB. The other procedures used are the same as in figure 2B. *B* TTM-JSR proteins transferred from the SDS-PAGE gel to a nitrocellulose membrane[32], which was stained using the Con A peroxidase method[8], showing that the 31.5 kDa (a) protein is a glycoprotein. *C* SDS-PAGE of Con A-sensitive proteins done under reducing (+) and non-reducing (−) conditions. The SDS-PAGE gel was transferred to a nitrocellulose membrane, which was silver-stained. *D* SDS-PAGE of Con A-sensitive proteins was done first (D-1), the 31.5 kDa protein eluted (for method, see ref. 5), and SDS-PAGE repeated. D-2; elution at pH 7.4. D-3; elution at pH 5.3. Staining was the same as in *C*. *E* Western blot to determine the specificity of the monoclonal AB against the 31.5 kDa protein.

containing CHAPS detergent. Solutions used for solubilization contained the following protease inhibitors: pepstatin A, 0.7 µM; aprotinin, 76.8 nM; iodoacetamide, 1 mM; leupeptin, 1.1 µM; benzamidine, 0.83 nM, and phenylmethylsulphonyl fluoride, 0.23 mM. SDS-PAGE (gel: 4–20%) was then carried out by Laemmli's method[7]. For estimation of molecular weights, standard proteins (Bio-Rad Laboratories) were used.

Concanavalin A (Con A)-peroxidase method[8] (see fig. 3B) was used for glycoprotein identification.

In experiments on the binding of radioactive PGO, twenty frog sartorius muscles were immersed in Ringer containing 5 mM ^{14}C-PGO for 10 min, and then washed out. 1 h after ^{14}C-PGO removal, when the E-C coupling process in the muscles had been selectively inhibited (see fig. 1B), TTM-JSR were isolated, and the ^{14}C-PGO-binding proteins from TTM-JSR were analyzed using SDS-PAGE. Each protein band was first stained with Coomassie brilliant blue (CBB), then cut out under a stereo-microscope[5], and counted[9] (fig. 2B).

For selection of Con A-sensitive proteins from TTM-JSR of frog leg muscles without PGO-administration, Con A affinity chromatography of solubilized TTM-JSR was done prior to SDS-PAGE (see fig. 3).

4) Preparation of the monoclonal antibodies (AB) against the 31.5 kDa-protein. For preparation of the monoclonal AB, 5–6-week-old female BALB/c mice were immunized intraperitoneally with 100 µg of the Con A-sensitive proteins from frog skeletal TTM-JSR in Freund's complete adjuvant. Then the immunization was repeated twice at 2-weekly intervals with 50 µg of the proteins in the adjuvant. Two days after the last immunization, the mice were sacrificed. Spleen cells from the mice were fused with myeloma cells (63-Ag8-6.5.3) according to Köhler and Milstein[10]. 10–14 days after the fusion, hybridomas with the ability to secrete antibodies against the 31.5 kDa protein were selected and cloned by the limiting dilution technique and ELISA. For the production of large quantities of the monoclonal AB, mice were injected i.p. with Pristane and 7 days later with 1×10^7 hybridomas[11]. 10–14 days later, the ascitic fluid was collected and centrifuged at 15,000 × g for 15 min, and the immunoglobulin fraction was precipitated in 33% (w/v) ammonium sulfate (pH 7.4) at 4 °C for 2 h. The precipitate was washed once with the ammonium sulfate solution and then dialysed against 10 mM phosphate buffer containing 110 mM NaCl (pH 7.2). After the dialysis, the immunoglobulin fraction was purified by

protein A-affinity chromatography, freeze-dried, and stored at −90 °C.

The Western blot procedure and ELISA were used to determine the specificity of the monoclonal AB against the 31.5 kDa protein. The Western blot was done as follows.

After SDS-PAGE of Con A-sensitive proteins had been carried out (fig. 3D-1), the 31.5 kDa band was eluted at pH 5.3 and pH 7.4[5], and the proteins separated by SDS-PAGE (fig. 3D-3) were then transferred to a nitrocellulose membrane. One of the two longitudinal half-strips obtained was silver-stained (fig. 3E-1) and the other was stained with the Western blot procedure (fig. 3E-2). Diaminobenzidine was used as the color reagent for developing the immunoblot.

Results

Figure 1 shows that, in single twitch muscle fibres of frog, E-C coupling is inhibited selectively and intensely by treatment with 3 mM PGO for 3 min (A), and in whole sartorius muscles it is inhibited similarly by treatment with 5 mM PGO for 10 min (B). The next step was to isolate the PGO-binding protein. Twenty frog sartorius muscles were immersed in Ringer containing 5 mM ^{14}C-PGO for 10 min, and then washed out. 1 h after the ^{14}C-PGO removal, when the E-C coupling process in the muscles has been selectively inhibited (see fig. 1B), TTM-JSR were isolated. Figure 2A is a typical electron micrograph of the TTM-JSR preparations obtained. It shows the presence of many triadic junctions. The mean values of (Na^+-K^+)- and Ca^{2+}-ATPase activities represented by released inorganic phosphate released from TTM-JSR were 5.34 μmoles/h/mg protein and 1.44 μmoles/h/mg protein, respectively, showing that the fraction used contained abundant amounts both of external membrane- and triadic junction-components.

The proteins from the isolated TTM-JSR were solubilized and separated using SDS-PAGE. The ^{14}C-PGO was predominantly incorporated into a protein with a molecular weight of 31.5 kDa. This 'PGO-protein' was shown to be a glycoprotein by the Con A-peroxidase method[8] (see also fig. 3B). Other observations by us showed that E-C coupling of single muscle fibres of frog is inhibited to a certain extent by the lectin Con A (though not by phytohemagglutinin)[12,13], and that the Con A-inhibition took place as a result of its binding to the 'PGO-protein'[12,14]. Therefore Con A affinity chromatography was used in an attempt to isolate the PGO-protein by a process not involving PGO, in order to understand its physiological role.

The noticeable point in the SDS-PAGE profile of proteins obtained with Con A affinity chromatography (Con A-sensitive proteins) is that a considerable quantity of protein with a molecular weight of 31.5 kDa (fig. 3, C and D-1) is present. This mol.wt coincides exactly with that of the PGO-protein. This indicates that the 31.5 kDa protein in the Con A-sensitive protein preparation is the PGO-protein itself. An extraction of the protein from the 31.5 kDa protein band of the gel was made under two different pH conditions, 7.4 and 5.3. The SDS-PAGE profile of the eluate obtained under acidic conditions showed the presence of a considerable quantity of protein of 31.5 kDa (a in fig. 3D-3). The ratio (mean protein yield) of a- and 66 kDa-bands was 9:1. The SDS-PAGE profile of the pH 7.4 eluate showed two bands (fig. 3D-2) with a ratio of 1:1. These results demonstrate that the 66 kDa protein originates from the 31.5 kDa protein and is a polymer of the latter. The polymerization may well be of physiological significance.

To investigate the physiological role of the 31.5 kDa protein, monoclonal antibodies (AB) against it were developed and purified as described in 'Materials and methods'. Single muscle fibres stimulated electrically once a minute were immersed for 60 min in Ringer's solution containing AB. The NaCl concentration of the Ringer's was 185 mM instead of 110 mM[15], to facilitate the influence of extracellularly applied AB. The fibres were then washed out in normal Ringer (fig. 4A-b). 30 min after AB removal, twitch and 50 mM K-contracture were markedly inhibited, without recognizable alterations in resting or action potentials or caffeine contracture (fig. 4, A-b and B). If AB was given in isotonic Ringer's solution, no significant AB-effect on E-C coupling was observed 30 min after AB removal, though the AB-effect appears to be recognizable during the 60 min after AB-administration. The results obtained showed a decrease of twitch size; however, the decrease was not statistically significant. The situation during the presence of AB was not different between the cases of figure 4, A-a and b. Pretreatment with hypertonic Ringer per se had no effect on contractile responses (twitch, tetanus, and the contractures induced by 50 mM K^+ and caffeine).

The large effect on K-contracture at a moderate depolarization, while tetanus height remained unaffected, could be explained by a shift in the threshold of force activation, i.e., E-C coupling inhibition. An inhibition of tension development due to incomplete depolarization (e.g. induced by 50 mM K) in the presence of intact tension development in tetanus, caffeine contracture, and a complete depolarization contraction, can be considered a sufficient criterion for the inhibition of E-C coupling. It is also accepted that the size of the twitch tension is not always enough, because it is affected not only by the size but also by the form of the action potential. Thus, it follows that these results show a selective inhibition of the E-C coupling process by AB.

Discussion

The important points of the present results are the following: 1) Extracellular AB against a protein pre-

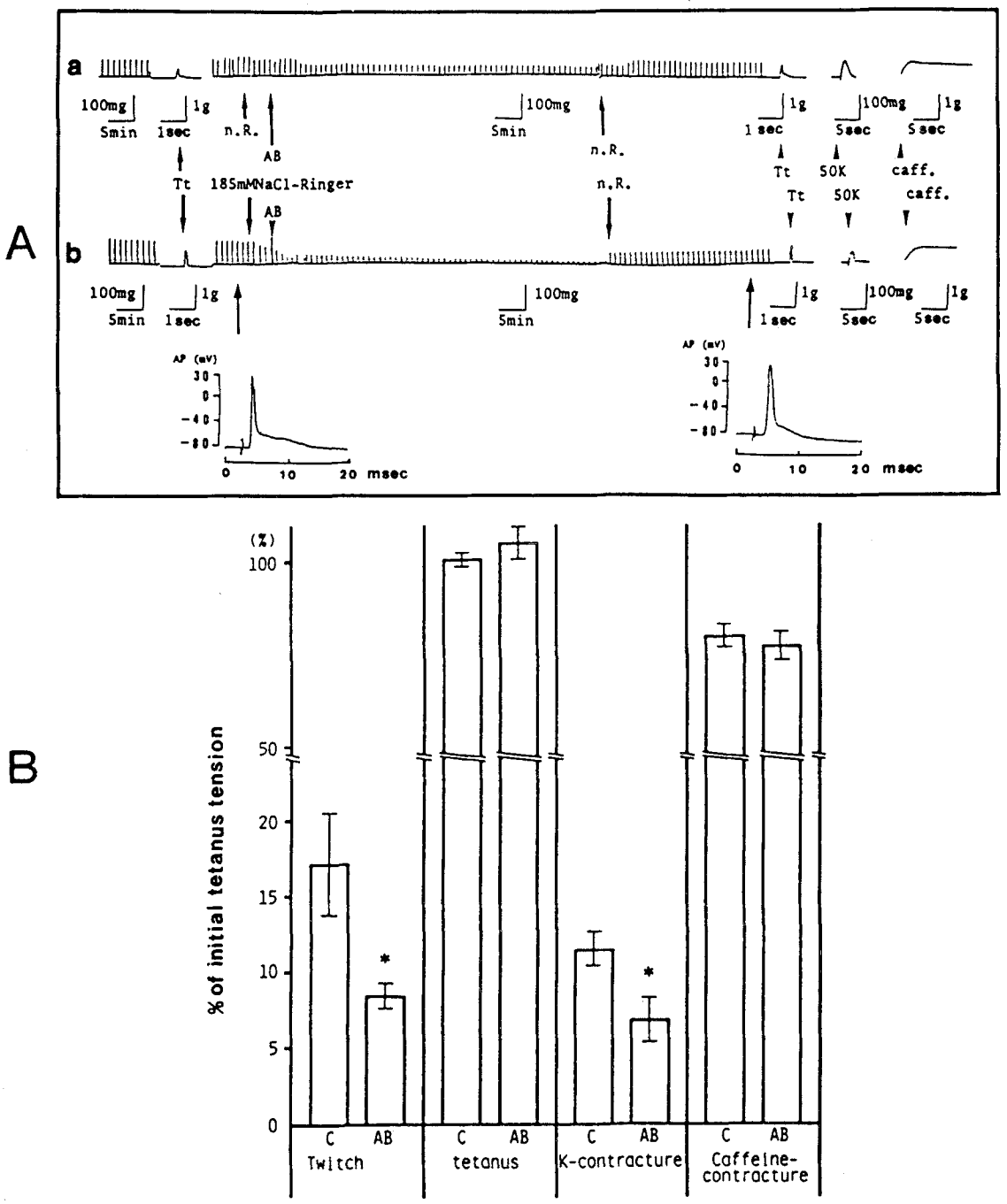


Figure 4. Effect of monoclonal antibody (AB) against the 31.5 kDa protein on E-C coupling of single fibres of frog skeletal muscle. *A* An observation of electrical and mechanical behaviors in single fibres. Resting and action potentials were measured at two time-points, before and after AB-administration, as indicated by the arrows; at n.R., Ringer was substituted. *B* Histograms to show summarized results on peak tensions of contractile responses in A-b. In each mechanogram, the initial tetanus tension, the tension when full muscle equilibrium is reached in normal Ringer before conditioning, is taken as 100; the contractile force of any time points and applied conditions in each mechanogram is expressed as a percentage of this; comparison between AB- and uncoupled AB-conditioning (i.e., C) was in terms of percent of contractile tension at corresponding time points and conditions; vertical bars indicate $\pm$SE of the mean (*$p < 0.05$), which was obtained from more than 5 observations.

pared from TTM-JSR inhibits E-C coupling in living muscle cells, showing that this protein plays a key functional role in E-C coupling, and that it exists in the plasma membrane of the T-tubules and is in contact with the extracellular fluid space. 2) This protein possesses a saccharide moiety (supported by its isolation by means of Con A affinity chromatography (fig. 3C) and by the inhibition of E-C coupling due to Con A[13]). This saccharide constitutes at least a part of the active site, which is accessible extracellularly. The protein has a mol.wt of 31.5 kDa (SDS-PAGE method).

The question arises whether this 31.5 kDa protein is related to the dihydropyridine (DHP) receptor complex. The γ subunit of the DHP complex has a mol.wt of 30–32 kDa, and it has carbohydrate moieties[16, 18]. Numerous recent publications[19–22] have proved that the DHP receptor complex, especially its α_1 subunit, is the voltage sensor. However, our protein is probably different from the known subunits of the DHP receptor complex, because our protein has a high affinity for Con A. It can be obtained by Con A-affinity chromatography from the void fraction from a wheat germ agglutinin (WGA)-sepharose column (detailed results will be given elsewhere), which efficiently removes the DHP receptor protein[16]. Furthermore, the 30–32 kDa protein is not found in DHP receptor preparations from frog skeletal muscle[23], which was our source. It follows, then, that there exist two kinds of voltage sensors on the plasma membrane of skeletal muscle cells; one is Con A- and the other is WGA-sensitive. The functional relationship between them remains to be clarified. The ryanodine receptor protein has basic subunits with a molecular weight of over 300 kDa[24, 25], and these are, therefore, probably not identical with our protein.

The extracellular active site for E-C coupling of the protein binds PGO, and is inhibited by it[12, 14]. The essential structure of the extracellular active site for E-C coupling is likely to contain arginyl residues of the protein, or a similar set-structure consisting of two amino-radicals or two similar radicals. If such radicals are dissociated electrically and carry positive charges under physiological conditions, one of them should move mechanically depending on the value of membrane potential. The protein thus behaves as an electrometer so as to measure membrane potential. Therefore, we suggest that the new protein should be called 'electrometrin'.

We have recently found evidence from physiomorphological studies that a small, but distinct, mechanical movement of a Con A-sensitive microstructure evoked by membrane potential changes exists at localized sites of the extracellular surface of the T-tubular membrane opposite the feet[12, 14]. This movement is abolished under the condition where PGO uncouples E-C[12]. The AB against 'electrometrin' is bound to sites on the T-tubular membrane opposite the feet[26]. It is, thus, very probable that a mechanical change in the PGO- and Con A-sensitive protein 'electrometrin' is the first step in E-C coupling. Since the available knowledge is as yet scanty, the entire scheme for E-C coupling cannot yet be described; however, it is likely that the mechanical movement described above is transmitted to the terminal cisternae by the mediation of the 'electrometrin' molecule. Our preliminary observations[27, 28] have shown that the protein consists of a head and a tail. The former is capable of moving and the latter is both long enough to span the triadic-junctional gap and thin enough to occupy the foot-core[22].

Recently, there have been many reports[20, 29–31] that intramembrane charge movement couples depolarization of the TTM with release of Ca^{2+} from the JSR. Etter (Ref. 30 and personal communication) carried out studies which were initiated by our suggestion that the weakened action of PGO evokes a selective inhibition of E-C coupling in frog twitch muscle fibres. PGO was found to inhibit charge 1, which has often been pointed out to have a close relation to E-C coupling, much more (25–60%) than charge 2 (2–12%). These studies on charge movement are considered to measure electrically the mechanical movement of the mechanism discussed above.

Although the precise roles of the protein 'electrometrin' in the E-C coupling process are as yet unclear, there can be no doubt that the protein is a key substance for E-C coupling.

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