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Cellular distribution and biochemical characterization of G proteins in skeletal muscle: comparative location with voltage-dependent calcium channels

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GTP binding proteins have been proposed to play a role in excitation-contraction coupling. In a precedent study [Toutant *et al.*, (1988), *Biochem. J.*, 405-409], we determined that *Bordetella pertussis* toxin is able to catalyse ADP-ribosylation of two substrates in the detergent soluble fraction of total muscle extracts. Purified fractions of transverse tubule membranes (T-tubule membranes), a key element of the excitation-contraction coupling, were shown to exhibit a major ADP-ribosylated substrate at 40 kd and an immunoreactivity with antisera raised against purified bovine brain $G_o\alpha$ or $G\beta$. In the present study, we have investigated the cellular distribution of G protein subunits in comparison with that of the voltage-dependent Ca^{2+} channels by immunofluorescence on transverse and longitudinal sections of fast and slow muscles. With affinity-purified antibodies against $G\beta$ subunits, a fluorescent labelling underlined the myofibrils and sarcolemma, whereas a strong immunoreaction in a dotted pattern evoked the presence of the subunit in repetitive triadic structures. With anti-$G_o\alpha$ antibodies, the immunofluorescence was more clearly focussed on a dotted pattern and the co-location with the voltage-dependent Ca^{2+} channel immunoreactivity indicates that both proteins were located in very close subcellular structures. Immunoblot analysis and PTX ADP-ribosylation of the purified light sarcoplasmic reticulum (LSR), heavy sarcoplasmic reticulum (HSR) and T-tubule subcellular fractions indicate the discrete presence of G proteins in LSR, an unambiguous labelling of the HSR fraction, while T-tubule membranes clearly appear very rich in a G_o-like protein, confirming the observed preferential immunocytochemical distribution of G protein subunits. Such a distribution suggests that the G_o-like protein mostly represented in the T-tubule membranes might be involved in excitation-contraction coupling modulation, while G protein(s) present in non-negligible amounts in HSR might be involved in Ca^{2+} release.

Key words: Bordetella pertussis toxin/G protein/skeletal muscle/transverse tubule membrane

Introduction

Guanine nucleotide binding proteins (G proteins) are a family of regulatory proteins that transduce extracellular signals into cellular responses [for reviews see Birnbaumer *et al.* (1987); Bockaert *et al.* (1987) and Casey and Gilman (1988)]. Transducing G proteins are heterotrimers (α,β,γ) differing in their α subunits (from 39 to 52 kd). $\beta\gamma$ subunits were generally considered to be interchangeable among G proteins, although several different nucleotide sequences have been evidenced recently for each subunit (Gao *et al.*, 1987; Fukada *et al.*, 1989). In contrast, the distinctive α chains which contain the guanyl nucleotide binding site allow each G protein to interact specifically with its own effectors.

Some of the $G\alpha$ proteins can be specifically ADP-ribosylated by the toxin of *Bordetella pertussis* (PTX). In cells from neural origin, three substrates of PTX are detected by SDS-PAGE analysis at 39, 40 and 41 kd (Neer *et al.*, 1984; Toutant *et al.*, 1987). The ADP-ribosylated component(s) of 40-41 kd was originally called $G_i\alpha$, since it was reported to transduce the inhibition of adenylate cyclase (Katada and Ui, 1982). It is now known that PTX not only affects adenylate cyclase inhibition, but depending on the system studied, also affects (i) GTP-dependent activation of phospholipidases of the C and/or A_2 type (Bokoch and Gilman, 1984; Okajima and Ui, 1984; Cockcroft and Gomperts, 1985; Murayama and Ui, 1985; Burch *et al.*, 1986); (ii) acetylcholine activation of heart rectifying K^+ channels (Yatani *et al.*, 1987); and (iii) neurotransmitter inhibition of voltage-sensitive Ca^{2+} channels (Holz *et al.*, 1986; Hescheler *et al.*, 1987; Harris-Warrick *et al.*, 1988). The multiplicity of signal transduction processes sensitive to blockade by PTX may be paralleled with the diversity of α subunits. The recent finding of three non-allelic human genes encoding $G_i\alpha$ type PTX substrates with molecular masses between 40 and 41 kd and different from $G_o\alpha$ (39 kd) (Suki *et al.*, 1987), suggests that distinct G protein activities reside on distinct α molecules. However, it is still difficult to attribute a given PTX-sensitive biological function to a precise $G_i\alpha$ sequence.

In skeletal muscle, it has been reported that guanosine 5'-[γ-thio]triphosphate (GTP-γ-S), a non-hydrolysable GTP analogue, elicits the development of isometric force in skinned muscle fibres. The threshold for such an effect is shifted to higher concentrations by preincubating with PTX (Di Virgilio *et al.*, 1986). These observations suggest that PTX-sensitive G protein(s) are involved in the development of contraction in skeletal muscle. In agreement with this proposition, substrates for PTX were evidenced in different skeletal muscle preparations (Scherer *et al.*, 1987; Toutant *et al.*, 1988).

In our previous study, we demonstrated the presence of two PTX substrates in total muscle extracts (40 and 41 kd).

Purified fractions of transverse tubule membranes (T-tubule membranes), a key element in excitation–contraction coupling, essentially exhibit an ADP-ribosylated substrate of 40 kd. Immunoreactivity at 40 kd was also detected in T-tubule membranes with an antiserum raised against a purified bovine brain $G_o\alpha$, suggesting the presence in this fraction of a G_o-like protein. The presence of G protein(s) in T-tubule membranes was further confirmed by the detection of a 36 kd subunit by immunoblotting with an anti-β subunit antiserum (Toutant *et al.*, 1988; Villaz, *et al.*, 1990).

In the present study, we investigated the cellular distribution of two subunits of G protein in comparison with the location of the dihydropyridine (DHP)-sensitive voltage-dependent Ca^{2+} channels, by immunofluorescence using anti-Gβ and anti-$G_o\alpha$ antibodies. In parallel, immunoblot analysis was performed in three subcellular fractions: heavy and light sarcoplasmic reticulum (HSR and LSR) and T-tubule membranes. Our results indicate that the β subunit is not only located at the T-tubule level, but is also observed in HSR (the fraction of sarcoplasmic reticulum junctionally associated to T-tubules) and weakly in LSR, which does not contain the ryanodine-sensitive Ca^{2+} channels. Furthermore, the very similar distribution of fluorescent immunoreactivities to anti-$G_o\alpha$ and anti-Ca^{2+} channel antibodies reported here confirms that the T-tubules are rich in a G_o-like protein.

Results

Immunofluorescent location of G protein subunits in mammalian skeletal muscle

The subcellular location of the G protein subunits in the skeletal muscle was investigated with rabbit polyclonal anti-Gβ (Figure 1) and anti-$G_o\alpha$ (Figure 2) antibodies. These antibodies have already been shown to exhibit a positive immunoreactivity with blotted T-tubule membranes (Toutant *et al.*, 1988). They also recognized native G protein subunits, since they have provided a suitable way of determining the distribution of G proteins in various cell types of the central nervous system (Brabet *et al.*, 1988; Peraldi *et al.*, 1989; Gabrion *et al.*, 1989).

Our investigation was carried out on three types of striated muscles, the psoas, the semi-membranosus proprius and the diaphragm, classified as purely fast, purely slow or mixed fast and slow respectively. On muscle longitudinal sections, the fluorescent patterns observed with the G protein antibodies depend on both the muscle fibre type and on the section obliquity. Figure 1 illustrates the various patterns observed with the anti-β antibodies. On quasi-longitudinal sections of psoas (Figure 1A), diaphragm (Figure 1B) and semi-membranosus proprius (Figure 1C and 1D), the fluorescent pattern appeared regularly striated. This immunostaining seemed to be related to sarcoplasmic elements rather than to sarcomeric structures, as shown by the reactivity of

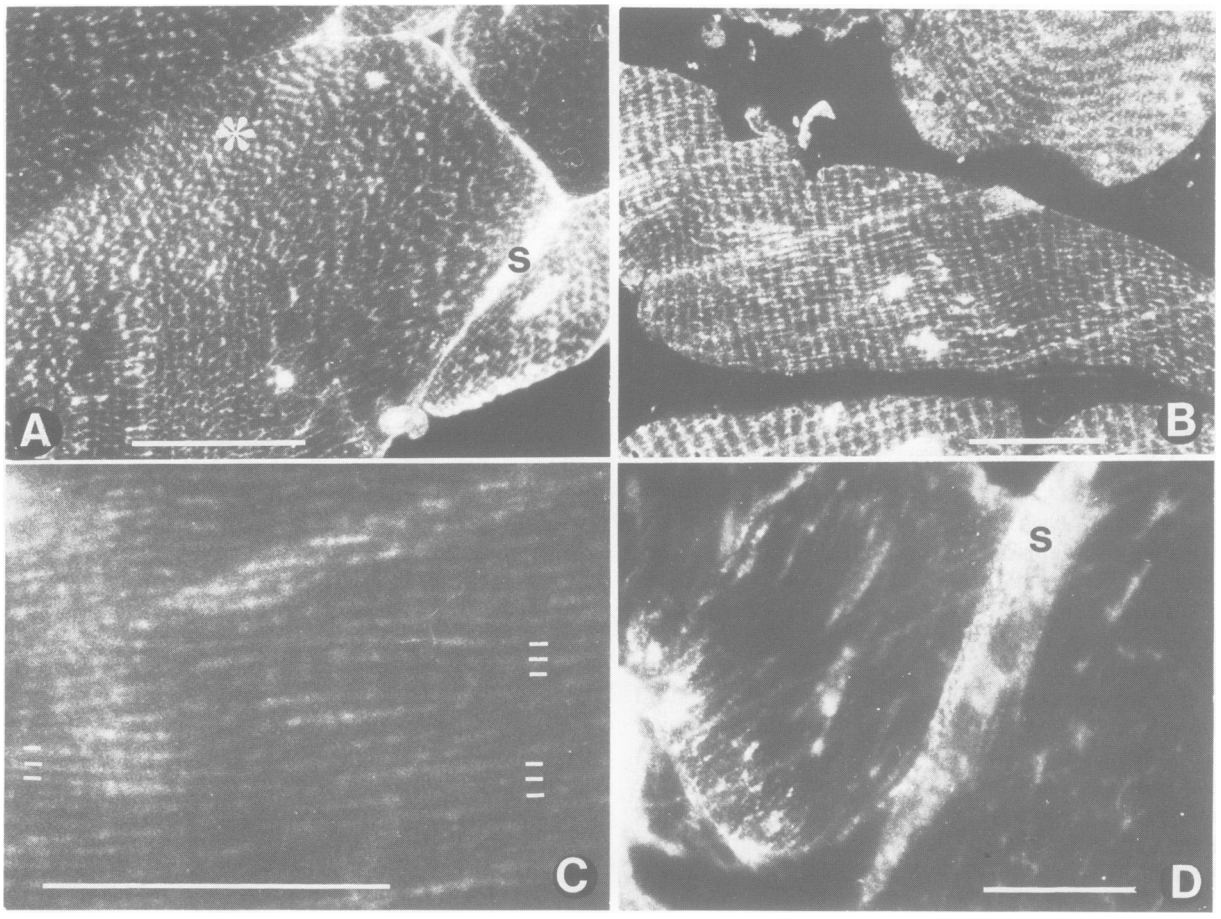

Fig. 1. Distribution of β subunit of G proteins in rabbit striated muscles. Longitudinal or quasi-longitudinal sections of: psoas (**A**), diaphragm (**B**), semi-membranosus proprius (**C** and **D**) were stained with a polyclonal antibody to Gβ. The observed patterns, i.e. dotted (*) in fast muscle cells (**A**) and striated (≡) in slow muscle cells (**C**), evoke a sarcoplasmic distribution. The localization of the G proteins in the subsarcolemmal cytoplasm exhibits differences when psoas (**A**) and semi-membranosus proprius (**D**) are compared. The sarcoplasm (S) enlarged in slow muscle fibres compared with fast muscle fibres also appears enriched in β subunit. Bars = 20 μm.

the subsarcolemmal cytoplasm (Figure 1A and D) and sarcoplasm between myofibrils (Figure 1C) and by antithetical labelling obtained with antibodies to sarcomeric components (not shown).

Figure 2 shows the labelling obtained on transversal (Figure 2A) and longitudinal (Figure 2B) sections of the diaphragm with the anti-α_o antibodies. The periphery of the cell appeared strongly labelled (Figure 2A) as seen with anti-Gβ antibodies (Figure 1A and D). The intracellular fluorescent staining detected in the transverse section of the diaphragm (Figure 2A) exhibits a typical honey-comb pattern in which the positive labelling circled the myofibrils. An obvious striated pattern evoking triadic structures is observed in longitudinal sections (Figure 2B).

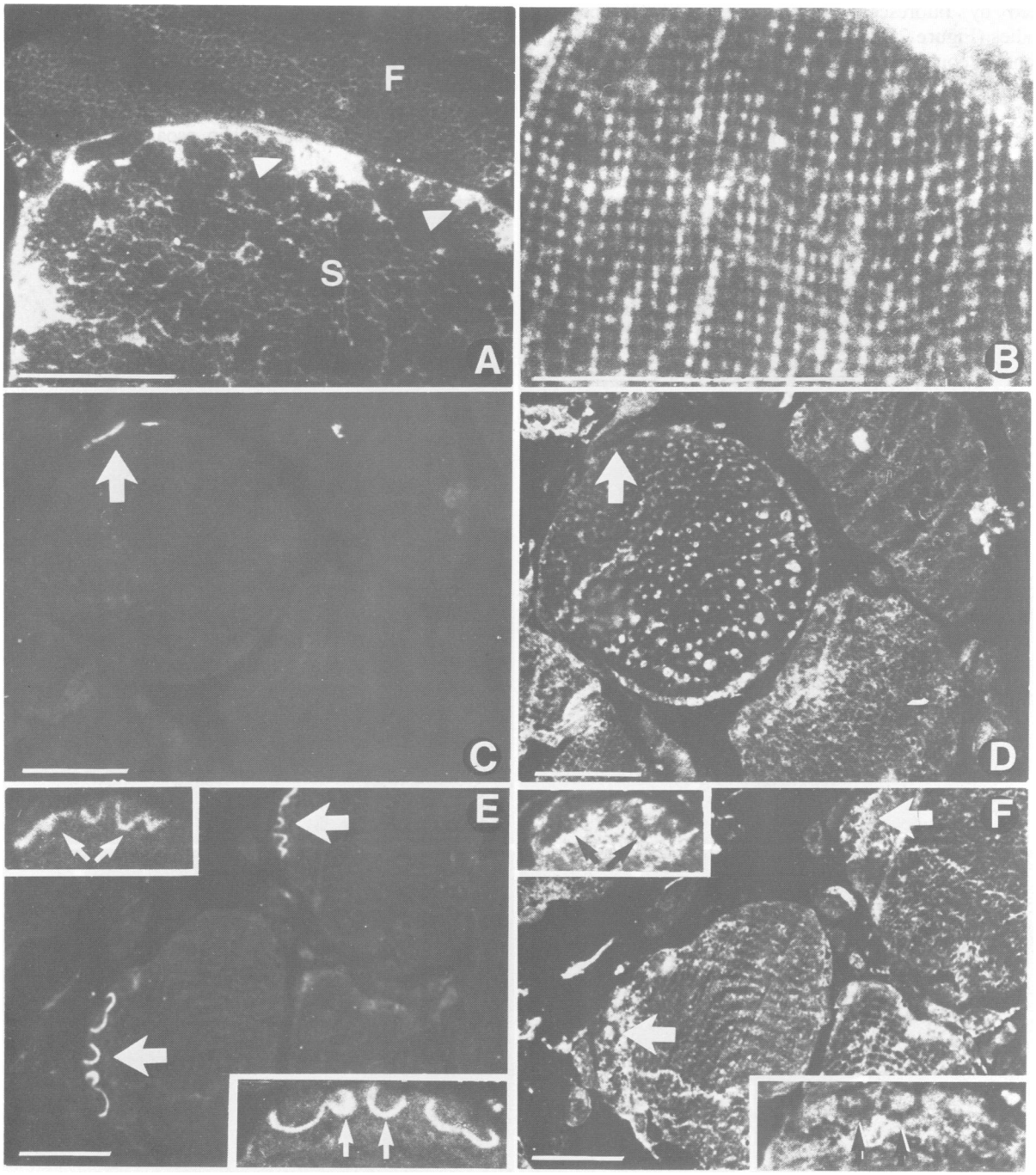

Fig. 2. Distribution of $G_o\alpha$-like subunits in rabbit diaphragm and comparison with the distribution of cholinergic receptors detected with rhodamine conjugated α-bungarotoxin. The $G_o\alpha$ immunoreactivity is evidenced in transverse sections (**A**) of slow (S) or fast (F) muscle cells, at the periphery of the cells, and weakly with a honey-comb distribution around the myofibrils. The subsarcolemmal cytoplasm (arrow heads) is intensively stained in slow muscle fibres. In longitudinal sections (**B**), the stronger reaction between myofibrils is observed as dotted lines, evoking triadic structures. Double labelling on the same diaphragm transversal section of slow (**C** and **D**) and fast (**E** and **F**) fibres was also carried out. Rhodamine-linked α-bungarotoxin fluorescence is shown in (**C**) and (**E**), while those corresponding to fluorescein labelled anti-rabbit antibodies are depicted in (**D**) and (**F**) respectively. The areas where neuromuscular synapses are shown by the presence of α-bungarotoxin binding sites (large arrows) are not specifically reactive with the antibodies to $G_o\alpha$, in slow as well as in fast muscle fibres. Synapses observed in (**E**) and (**F**) are also shown at a higher magnification in the corresponding insets. In these insets, small arrows point out corresponding areas which are positive with α-bungarotoxin and negative with antibodies to $G_o\alpha$. Bars = 20 μM.

Since the true $G_o\alpha$ subunit, highly concentrated in cells from the central nervous system is also present in the peripheral nervous system, we looked for a possible $G_o\alpha$-immunoreactivity at the neuromuscular junctions level. Thus, a double labelling on the same transverse section of the diaphragm was carried out using rhodamine-conjugated α-bungarotoxin for the labelling of the cholinergic receptor (Figure 2C and E) and an anti-$G_o\alpha$ antibody secondarily detected by fluorescein isothiocyanate (FITC)-labelled antibodies (Figure 2D and 2F). It appeared that no evident relationship could be noted between the distribution of the two fluorescent reactions. The absence of $G_o\alpha$ or $G_o\alpha$-like subunits in the post-synaptic neuromuscular junction indicated that in muscle, the G_o-like protein is not basically linked to the regulation of muscle contraction by nerve terminals.

Co-location of $G_o\alpha$ or $G\beta$ immunoreactivities and the DHP-sensitive voltage-dependent Ca^{2+} channels

The immunofluorescent staining with the anti-$G_o\alpha$ antibody indicated a location in sarcoplasmic regions containing T-tubules. In order to compare this pattern with that of a polypeptide component of the dihydropyridine (DHP)-sensitive voltage-dependent Ca^{2+} channel known to be specifically located at the T-tubule level, we used in double staining, a monoclonal antibody, selected to immunoprecipitate the dihydropyridine receptor (DHPR) (Vandaele *et al.*, 1987). We observed in psoas muscle, a clear co-

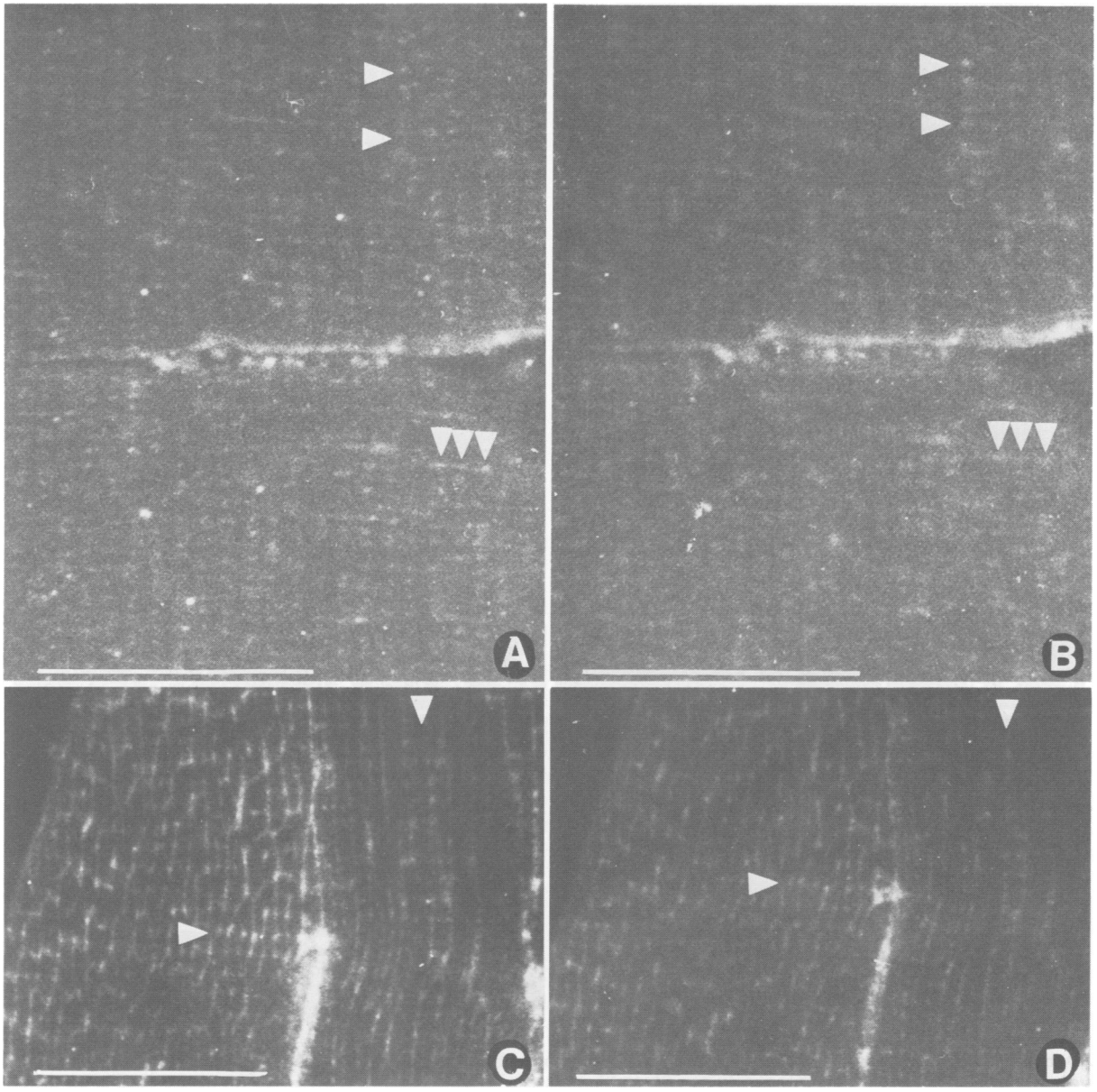

Fig. 3. Comparison of the distribution of $G_o\alpha$ and $G\beta$ with DHPR immunoreactivities on longitudinal sections of psoas muscle. Double labelling was performed on the same sections, (**A**) and (**B**) on one hand and (**C**) and (**D**) on the other. Labelling was performed as described in Materials and methods with anti-$G_o\alpha$ antibodies (**A**); anti-$G\beta$ antibodies (**C**); and the monoclonal antibody anti-DHPR (**B**) and (**D**). In both cases (**A** and **B**) and (**C** and **D**), the intense linear labelling corresponds to the subsarcolemmal cytoplasm of two adjacent cells. The arrowheads show the co-location of G proteins and DHP-sensitive voltage-dependent Ca^{2+} channels in sarcoplasm and, more specifically, in a punctuate pattern, evoking triadic structures. Bars = 20 μm.

location of Gβ with the DHPR (Figure 3C and D). It was interesting to note a similar distribution of the two immunoreactions, in sarcoplasmic elements, i.e. subsarcolemmal cytoplasm and sarcoplasm between myofibrils. Mainly with anti-$G_o\alpha$ antibodies, the reaction was focussed on a clear-cut dotted pattern (Figure 3A and B). As a perfect similarity could be observed between the two images, it seemed clear that both proteins were located in very close subcellular structures, which might be principally T-tubule membranes, where DHPR was previously evidenced (Fosset *et al.*, 1983).

Preferential distribution of G proteins and Ca^{2+} channels in T-tubule fractions

In a previous study, we reported that PTX was found to label two substrates of 41 and 40 kd in the detergent-soluble fraction of total muscle extracts and that the latter was present in purified T-tubule membranes. We then analysed not only T-tubule membranes, but also sarcoplasmic reticulum fractions (LSR and HSR). These fractions were incubated with [^{32}P]NAD in presence of PTX and subjected to electrophoresis on SDS−PAGE using modified Laemmli conditions (Toutant *et al.*, 1987). Figure 4 shows PTX-catalysed ADP-ribosylation of different subcellular fractions of rabbit skeletal muscle: LSR, HSR and T-tubules (Figure 4, lanes 2, 3 and 4 respectively), compared with those of chromaffin granules (Figure 4, lane 1). The electrophoretic mobility of the three Gα subunits (39, 40 and 41 kd) (Toutant *et al.*, 1987) of chromaffin granules (Figure 4, lane 1) indicates that the two ADP-ribosylated substrates in sarcoplasmic reticulum fractions correspond to masses of 40 and 41 kd.

Taking into account that the total amount of loaded proteins is the same for the three subcellular fractions of skeletal muscle, it appears that the intensity of the labelling of the two ADP-ribosylated bands is weaker in the LSR fraction than in HSR and that T-tubule membranes are clearly enriched in the 40 kd substrate, as compared to the other fractions.

It was then assessed whether the β subunit quantitatively followed the distribution of the PTX substrates in the same fractions. Immunoblotting experiments were performed with our anti-β antibodies. With this antiserum, a band of immunoreactivity was detected at 36 kd in the three studied subcellular fractions and the immunoreactive band appears more intensely labelled in T-tubules (Figure 5, lane 3) than in HSR fractions (Figure 5, lane 1). The LSR fraction (Figure 5, lane 2) displays a very weak immunoreactivity with this antiserum compared with the other fractions. Thus, in agreement with ADP-ribosylation experiments, immunoreactivity was stronger in T-tubule membranes than in sarcoplasmic reticulum.

The autoradiogram of an immunoblot of HSR, LSR and T-tubule membranes stained with a polyclonal anti-DHPR is shown in Figure 6. The results pointed out the presence of a very strong immunoreactivity in purified T-tubule membranes (Figure 6, lane 3), a weaker labelling was observed in HSR fractions (Figure 6, lane 1), whereas no reactivity was observed in LSR fractions (Figure 6, lane 2). These results ascertain the purity of the studied fractions that was already checked by the criteria of DHP binding. On the other hand, the comparison of Figures 5 and 6 indicates that T-tubule membranes are enriched in both Ca^{2+} channels and G proteins. These results are in good agreement with the immunocytochemical distribution of G protein subunits in muscle cells.

Discussion

In the present study, we showed the subcellular distribution of the G proteins in different types of rabbit muscles by immunofluorescence. The comparison of three muscles differing by their composition in fast and slow fibres, clearly indicates that the major Gβ and $G_o\alpha$ immunoreactivities

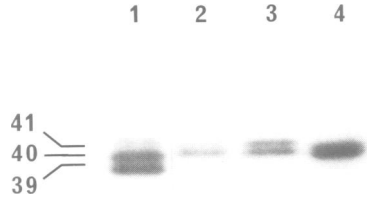

Fig. 4. Autoradiogram of PTX-catalysed ADP-ribosylated proteins with [^{32}P]NAD. Proteins loaded on a 10% polyacrylamide gel were: chromaffin cell granules (**lane 1**), LSR (**lane 2**), HSR (**lane 3**) and T-tubule membranes (**lane 4**). For each muscle fraction, 20 μg of protein were ADP-ribosylated with pertussis toxin and loaded. Only 10 μg of ADP-ribosylated chromaffin fractions were loaded on the same gel.

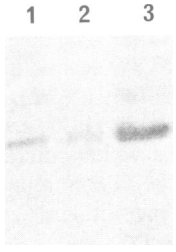

Fig. 5. Immunoblot analysis of β subunits present in muscle subcellular fractions purified from rabbit skeletal muscle. Samples loaded were: **lane 1**, HSR; **lane 2**, LSR; **lane 3**, T-tubule membranes. The samples were subjected to SDS/10% polyacrylamide gel electrophoresis and transferred onto nitrocellulose. The blot was probed with an antiserum against bovine brain β subunit. This antiserum was used at a 1:100 dilution. The antibody reaction was detected by indirect immunoperoxidase staining.

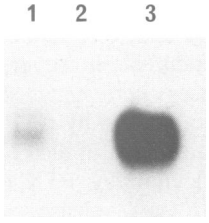

Fig. 6. Immunoblot assay of skeletal muscle subcellular fractions with an antiserum raised against DHPRs. Proteins loaded were: **lane 1**, HSR; **lane 2**, LSR; **lane 3**, T-tubule membranes. The samples were treated as in Figure 5 for electrophoresis and transfer to nitrocellulose. The blot was probed with antiserum raised against DHP sensitive Ca^{2+} channels and then treated with radioiodinated protein A. The antiserum was used at a 1:100 dilution (Barhanin *et al.*, 1987).

were regularly distributed as a dotted pattern evoking triadic structure repetition, while a weaker immunostaining was seen at the level of the sarcoplasmic reticulum surrounding the myofibrils. A labelling with both Gβ and $G_o\alpha$ antibodies also appears very intense, in subsarcolemmal cytoplasm, particularly in slow muscle fibres. This important subsarcolemmal labelling is probably not due to soluble G proteins, since none of them could have been identified in the fraction obtained after low salt extraction (Toutant *et al.*, 1988). In spite of the particular care we took, no specific immunoreaction could be observed with anti-$G_o\alpha$ antibodies at the synaptic membrane level of neuromuscular junctions evidenced with rhodamine labelled α-bungarotoxin. This result is in complete agreement with the absence of labelling noted at the level of neuro-neuronal synapses (Gabrion *et al.*, 1989). When compared with anti-Gβ immunoreactivities, the labelling observed with the affinity purified anti-$G_o\alpha$ antibodies appears more precisely concentrated in the triadic junction, suggesting the presence of a G_o-like protein in the structure where the major step of the excitation−contraction coupling arises. This interpretation is comforted by the comparison with the distribution of the immunofluorescent labelling obtained with an anti-DHPR monoclonal antibody.

We have also compared G-proteins in purified membranes of LSR, HSR and T-tubules, either after ADP-ribosylation in presence of PTX or immunoblotting. The data obtained indicate that the 40 kd PTX substrate is in greater amounts in T-tubule membranes than in HSR, whereas in LSR, the labelling is very poor. The HSR fraction exhibits an equal labelling at 40 and 41 kd, while T-tubule membranes mainly possess the 40 kd substrate. The quantitative distribution of the Gβ immunoreactivity seems to parallel that of the intensity of PTX labelling. Again, the non-negligible β immunoreactivity found in HSR seems to truly reflect the presence of G proteins which could be either sensitive or insensitive to PTX. The $G_o\alpha$ immunoreactivity which was observed in T-tubule membranes (Toutant *et al.*, 1988) is hardly detectable in HSR and LSR fractions (data not shown). Thus, the biochemical data are in good agreement with the immunocytochemical observations and argue for (i) the selective presence in T-tubules of the PTX-sensitive substrate at 40 kd, which appears to be recognized by the anti-$G_o\alpha$ antibodies (Toutant *et al.*, 1988); and (ii) the presence of Gβ subunits in HSR, together with at least 40 and 41 kd α subunits.

In the HSR, the presence of PTX-sensitive G proteins first reported by Scherer *et al.* (1987) raised the question of their possible function. In muscle skinned fibre experiments, GTP-γ-S was shown to be unable to induce Ca^{2+} release from the sarcoplasmic reticulum; however, Ca^{2+}- or caffeine-induced Ca^{2+} releases were enhanced by GTP-γ-S and these responses were blocked by GTP-β-S or PTX (Villaz *et al.*, 1990). Therefore, one of the G proteins we detected in sarcoplasmic reticulum by PTX or immunoreactivity might be involved in Ca^{2+} release.

In skeletal muscle, the presence of a protein recognized by antibodies raised against $G_o\alpha$, an α subunit selectively found in nervous tissues, is at a first glance surprising. However, Milligan *et al.* (1989) reported the presence of a novel form of a G_o in the rat myometrium. We have shown also that the positive immunoreactivity detected in adipocytes with our anti-$G_o\alpha$ antibody was in fact due to a polypeptide of higher mol. wt (Rouot *et al.*, 1989). In addition, Goldsmith *et al.* (1988) reported the purification and the characterization of another isoform of G_o in bovine brain based on analysis with specific antibodies and two-dimensional gel electrophoresis. These authors suggested that the two evidenced isoforms of G_o probably differ in post-translational modifications rather than in the primary sequence. We have also found that our anti-$G_o\alpha$ antibody recognizes two isoforms of $G_o\alpha$ in neuronal cells (Brabet *et al.*, 1990). All these data, together with those reported here, strongly suggest the existence in various tissues of specific G proteins, differing from the well-established G proteins either by their sequences or by post-translational modifications.

Regarding the putative function of the $G_o\alpha$-like polypeptide in T-tubules, it might be suggested that this protein could regulate the Ca^{2+} channel. Thus, in neuronal cells, the involvement of G proteins, particularly G_o in the control of Ca^{2+} channels has been proposed (Holz *et al.*, 1986; Hescheler *et al.*, 1987; Harris-Warrick *et al.*, 1988). As in neuronal cells, the functional reconstitution of Ca^{2+} channel activity involving partially purified Ca^{2+} channels from isolated rabbit skeletal muscle and bovine brain protein G_o, indicates that G_o might regulate Ca^{2+} channel current (Horne *et al.*, 1988). More evidence for the control of Ca^{2+} channels by G proteins has been provided in a recent report by Yatani *et al.* (1988), who demonstrated that exogenously added GTP-γ-S-activated human $G_s\alpha$ subunit, stimulates T-tubule channels in a manner that is independent of phosphorylation by cAMP dependent protein kinase. Another possibility is that the G proteins found in T-tubule membranes might be linked to phospholipase C (Volpe *et al.*, 1985; Vergara *et al.*, 1985). However, current evidence does not favour the view that IP_3 is the activator of contraction, although it may play a role in modulating or modifying contraction (Caswell and Brandt, 1989). Thus, the role of the G proteins present in T-tubules remains to be definitely established. Another more immediate approach would consist of assessing more precisely the nature of the sequence of the $G_o\alpha$-like subunit in T-tubules as well as that of the 41 kd in HSR.

Materials and methods

Membrane preparation

Rabbit white skeletal muscle subcellular fractions (T-tubules, light and heavy sarcoplasmic reticulum) were isolated as described by Horgan and Kuypers (1987). A crude microsomal fraction was centrifuged on continuous sucrose density gradients (0.7−1.4 M sucrose) buffered with 20 mM Tris−maleate, pH 7.0. Material sedimenting at 1.2 M sucrose was further purified by centrifugation on ion free gradients. In this study, the binding of dihydropyridine (+) [^{3}H]PN 200 −110 reached 75 pmol/mg of protein in T-tubule membrane preparations, 0.8 pmol/mg of protein in LSR fractions and 3.7 pmol/mg of protein in the HSR membrane preparations.

Preparation and affinity purification of antibodies

Polyclonal antibodies were raised in rabbit against purified $G_o\alpha$ or $G\beta\gamma$ subunits and the specificity of these antibodies has been previously established (Homburger *et al.*, 1987). The affinity purification of antibodies against $G_o\alpha$ has previously been described (Brabet *et al.*, 1988; Gabrion *et al.*, 1989). Purification of anti-βγ antibodies was carried out similarly using a CH-Sepharose gel (Pharmacia) onto which pure mixture of G_o/G_i were immobilized. The anti-DHPR monoclonal antibody has been characterized by its ability to immunoprecipitate a polypeptide component of the voltage-dependent Ca^{2+} channel (Vandaele *et al.*, 1987). Polyclonal guinea pig antibodies raised against the 140 kd subunit of the voltage dependent Ca^{2+} channel were obtained and characterized as described (Barhanin *et al.*, 1987).

Immunocytochemistry

Thin frozen sections (0.1–0.2 μM) of psoas, semi-membranosus proprius and diaphragm muscles from rabbit were obtained according to the method described by Tokuyasu (1980). Briefly, small pieces of muscles were fixed in 3% formaldehyde in phosphate buffered saline (PBS) infused in 2 M sucrose and frozen in liquid nitrogen. Thin sections were prepared at −80°C with a Reichert Ultracut microtome with a FC4 cryo-attachment and treated for immunofluorescent staining. They were first incubated overnight in a moisture chamber with antibodies diluted in PBS containing 0.2% gelatin and 2% normal goat serum. After thorough washing in PBS, specimens were stained 45 min with rhodamine- or fluorescein-conjugated goat anti-rabbit IgG (Nordic Immunologicals, Belgium; 1:50 in PBS). Double stainings were performed by incubating the sections (i) in a mixture of anti-$G_o\alpha$ antibodies and a rhodamine-labelled α-bungarotoxin or (ii) in a mixture of two antibodies, the anti-Ca^{2+} channel monoclonal and anti-$G_o\alpha$ or Gβ-polyclonal antibodies which were raised in different species (mouse and rabbit respectively). After extensive washing, a mixture of two secondary antibodies directed to rabbit immunoglobulins (heavy and light) [IgG (H + L)] and mouse Ig (H + L), both obtained in a third species (i.e. goat) and labelled with FITC or trimethyl rhodamine isothiocyanate (TRITC) respectively, was used in the double immunostaining. Controls were made by (i) omitting the first specific antibody, (ii) by replacing the antibodies to G protein subunits with anti-actin, anti-myosin or anti-tropomyosin antibodies to sarcomeric components or (iii) by using antibodies preincubated in the presence of purified G proteins (1 h, 30°C).

Sections were examined with a Zeiss epifluorescence microscope equipped with interferential filters to detect FITC and TRITC fluorescences and with planapochromat objectives (×40 and ×63). Photographs were taken on Kodak Tmax Professional films (400 ASA), exposed and developed to 1600 ASA with Ilford Microphen developer.

PTX catalysed ADP-ribosylation

Purified T-tubule membranes, LSR and HSR fractions (20 μg of protein for each fraction) were ADP-ribosylated with PTX (List Biological Laboratories, Campbell, CA, USA) as previously described (Toutant *et al.*, 1987, 1988).

SDS–PAGE

Prior to electrophoresis, samples were treated with *N*-ethylmaleimide and then separated on a modified Laemmli 10% polyacrylamide gel as previously described (Toutant *et al.*, 1987).

Immunoblotting experiments with SDS–polyacrylamide gels were performed with rabbit polyclonal antibodies raised against either the βγ subunit purified from calf brain G proteins, or the voltage-dependent Ca^{2+} channel (Toutant *et al.*, 1987; Barhanin *et al.*, 1987).

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