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G-proteins in skeletal muscle

Evidence for a 40 kDa pertussis-toxin substrate in purified transverse tubules

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In muscle, it has been established that guanosine 5'-[γ-thio]triphosphate (GTP[S]), a non-hydrolysable GTP analogue, elicits a rise in tension in chemically skinned fibres, and that pretreatment with *Bordetella pertussis* toxin (PTX) decreases GTP[S]-induced tension development [Di Virgilio, Salviati, Pozzan & Volpe (1986) EMBO J. 5, 259–262]. In the present study, G-proteins were analysed by PTX-catalysed ADP-ribosylation and by immunoblotting experiments at cellular and subcellular levels. First, the nature of the G-proteins present in neural and aneural zones of rat diaphragm muscle was investigated. PTX, known to catalyse the ADP-ribosylation of the α subunit of several G-proteins, was used to detect G-proteins. Three sequential extractions (low-salt-soluble, detergent-soluble and high-salt-soluble) were performed, and PTX was found to label two substrates of 41 and 40 kDa only in the detergent-soluble fraction. The addition of pure βγ subunits of G-proteins to the low-salt-soluble extract did not provide a way to detect PTX-catalysed ADP-ribosylation of G-protein α subunits in this hydrophilic fraction. In neural as well as in aneural zones, the 39 kDa PTX substrate, very abundant in the nervous system ($G_o\alpha$), was not observed. We then studied the nature of the Gα subunits present in membranes from transverse tubules (T-tubules) purified from rabbit skeletal muscle. Only one 40 kDa PTX substrate was found in T-tubules, known to be the key element of excitation–contraction coupling. The presence of a G-protein in T-tubule membranes was further confirmed by the immunoreactivity detected with an anti-β-subunit antiserum. A 40 kDa protein was also detected in T-tubule membranes with an antiserum raised against a purified bovine brain $G_o\alpha$. The presence of two PTX substrates (41 and 40 kDa) in equal amounts in total muscle extracts, compared with only one (40 kDa) found in purified T-tubule membranes, suggests that this 40 kDa PTX substrate might be involved in excitation–contraction coupling.

INTRODUCTION

Guanine-nucleotide-binding proteins (G-proteins) are a family of regulatory proteins that transduce extracellular signals to specify intracellular effectors [1]. These proteins are heterotrimers composed of different α subunits and sharing common β and γ subunits [1]. The β and γ subunits are tightly associated and may serve to anchor the complex in the membrane [2]. The α subunits bind GTP, exhibit GTPase activity and are ADP-ribosylated by specific toxins such as *Bordetella pertusis* toxin (PTX) and cholera toxin. At least three Gα subunits were identified as substrates for PTX, the $G_o\alpha$ (39 kDa), the $G_i\alpha$ (41 kDa) and a new Gα of 40 kDa [3,4,5]. G_i (41 kDa) is believed to mediate adenylate cyclase inhibition [6], but the function of the two other PTX substrates is not yet clearly established.

In muscle, it has been recently demonstrated that GTP[S], a non-hydrolysable GTP analogue, elicits the development of isometric force in skinned muscle fibres, and that the threshold for such an effect is shifted to higher concentrations by preincubation with PTX [7]. These observations suggest that G-proteins are involved in the development of contraction in skeletal muscle, and that at least one of them is a substrate for ADP-ribosylation catalysed by PTX. A major step of the excitation–contraction coupling in skeletal muscle takes place at the level of sarcolemmal invaginations called transverse tubules (T-tubules), junctionally associated with sarcoplasmic reticulum. The aim of this study was to investigate in skeletal muscle and in T-tubules the nature and the subcellular localization of Gα subunits either by ADP-ribosylation with PTX or by cross-reactivity with antibodies raised against the $G_o\alpha$ subunit. In addition, the subcellular distribution of the Gβ subunit was investigated with specific anti-β-subunit antibodies. We report that at least two G-proteins were found in total muscle extract and one of them was present in purified T-tubule membranes.

EXPERIMENTAL

Preparations derived from muscles

In rat diaphragm muscle, the neuromuscular junctions are located in a central zone which is accurately detected under a dissecting microscope after acetylcholinesterase activity is made visible [8]. This central region was dissected: in this study, it is called neural zone (N). The two other distal regions containing the tendinous ends of the muscle fibres devoid of nerve endings were also removed by dissection: they constitute the aneural zones

Abbreviations used: G-proteins, guanine-nucleotide-binding proteins; GTP[S], guanosine 5'-[γ-thio]triphosphate; LSS, low-salt-soluble extract; DS, detergent-soluble extract; HSS, high-salt-soluble extract; PTX, *Bordetella pertussis* toxin; T-tubule membranes, transverse tubule membranes.

(AN). Crude membrane preparation of rat diaphragm muscle was obtained by homogenization and centrifugation as described previously for other tissues [9].

Rat diaphragm muscles were also subjected to three sequential extractions by a procedure adapted from that described by Bon & Massoulié [10] for the *Torpedo* electric organ. Thus, immediately after dissection, muscles were cut into small pieces and frozen in liquid N_2. The frozen material was crushed in a mortar to a powder. This powder was then successively extracted with 10 vol. (v/w) of three different buffers, all containing 20 mM-Tris/HCl, pH 7.4, 50 mM-NaCl, 10 mM-EGTA, bacitracin (0.1 mg/ml), aprotinin (25 kallikrein-inhibitory units/ml) and 10 mM-benzamidine. After resuspension in the above buffer (low salt), the powder was homogenized in a Teflon/glass Potter homogenizer (20 strokes) at 0 °C and centrifuged for 1 h at 100000 ***g***. The supernatant was the low-salt-soluble fraction (LSS). The pellet, subjected to another cycle of extraction/centrifugation in the buffer containing 1 % Triton X-100, yields the detergent-soluble fraction (DS). Finally, extraction of the second pellet in high-salt buffer (supplemented with 1 M-NaCl) gave the high-salt-soluble fraction (HSS).

T-tubule membranes were isolated from rabbit white skeletal muscle as previously described [11]. The quality of the T-tubule membrane preparation was assessed by binding of the dihydropyridine (+)[^{3}H]PN 200-110, which reached 60 pmol/mg of protein [12], and by the cholesterol content (0.8 μmol/mg of protein) [13].

Purification of G-proteins and production of antibodies

Purification of G-proteins from bovine brain membranes was performed by successive elution through DEAE-Sephacel (Pharmacia), AcA34 (LKB) and heptylamine–Sepharose columns. Pure $G_o\alpha$ subunit and enriched G_i and G_o fractions were obtained as described previously [8]. Polyclonal antibodies were raised in rabbits against purified $G_o\alpha$ and $G\beta\gamma$ subunits. The specificity of these antibodies has been established [8].

PTX-catalysed ADP-ribosylation

Crude extract, LSS, DS and HSS fractions and purified T-tubule membranes (20 μg of protein) or purified G_o and G_i proteins (0.25 μg of protein) were ADP-ribosylated with PTX (List Biological Laboratories, Campbell, CA, U.S.A.) as described previously [4].

SDS/polyacrylamide-gel electrophoresis and immunoblotting

Before electrophoresis, samples were treated with *N*-ethylmaleimide and then separated on a modified Laemmli 10 %-polyacrylamide gel as described previously [4].

Immunoblotting experiments with SDS/polyacrylamide gels were performed with rabbit polyclonal antibodies raised against either the β-subunit or the $G_o\alpha$ subunit purified from calf brain G-proteins as described previously [14].

RESULTS

When a crude preparation of membranes from rat diaphragm was incubated with [^{32}P]NAD^+ in presence of PTX, a very weakly radiolabelled band in the region of 40 kDa was detected on SDS/polyacrylamide-gel electrophoresis by autoradiography. With two distinct antibodies raised against the $G_o\alpha$ and $\beta\gamma$ subunits purified from bovine brain, no cross-reactivity was observed. It is likely that in such crude muscle preparations, mainly composed of contractile proteins, G-proteins represent only a small fraction of the material loaded on the gel and therefore cannot be easily detected.

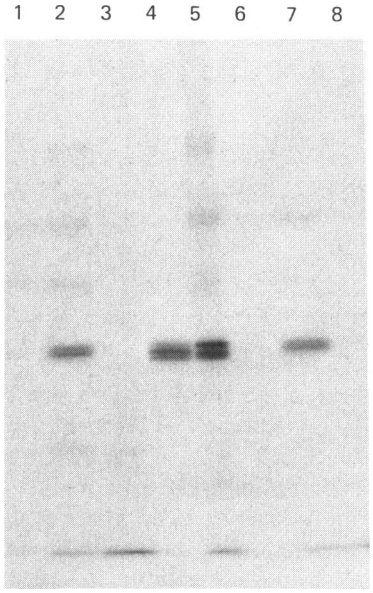

Fig. 1. Autoradiogram of PTX-catalysed ADP-ribosylated proteins with [^{32}P]NAD^+

Proteins loaded on a 10 %-polyacrylamide gel were (lanes): 1 and 6, LSS fractions; 2 and 7, DS fractions; 3 and 8, HSS fractions; 1, 2 and 3 were extracted from the neural part of rat diaphragm muscles, whereas 6, 7 and 8 were extracted from the aneural part of the same muscles; 4, chromaffin-cell granules (10 μg); 5, pre-synaptic plasma membranes from *Torpedo* electric organ (10 μg). For all muscle fractions 100 μg of protein were ADP-ribosylated by PTX and loaded.

We then analysed different fractions obtained from a sequential extraction of rat diaphragm. In this procedure, the LSS fraction (see the Experimental section) is known to contain the low-M_r soluble proteins, the DS fraction contains the membrane-bound components, and the HSS fraction is mainly composed of high-M_r proteins. In the particular case of skeletal muscle, the latter fraction mostly contains the myofibrillar proteins.

Fig. 1 shows PTX-catalysed ADP-ribosylation of such fractions prepared from either neural or aneural zones of the diaphragm. No ADP-ribosylation was observed in LSS and HSS fractions (lanes 1, 6 and 3, 8 respectively), but two radiolabelled bands of similar intensity were observed in the DS fractions (lanes 2 and 7). The electrophoretic mobility of the three Gα subunits (39, 40 and 41 kDa) [4] of chromaffin granules (Fig. 1, lane 4) and of the two α subunits (39 and 41 kDa) [14] of *Torpedo* electric-organ synaptosomes (Fig. 1, lane 5) compared with that of the two Gα substrates ADP-ribosylated by PTX in the DS fraction indicates that these two ADP-ribosylated substrates in muscle have molecular masses of 40 and 41 kDa (Fig. 1). No difference was detected between preparations obtained from neural or aneural zones.

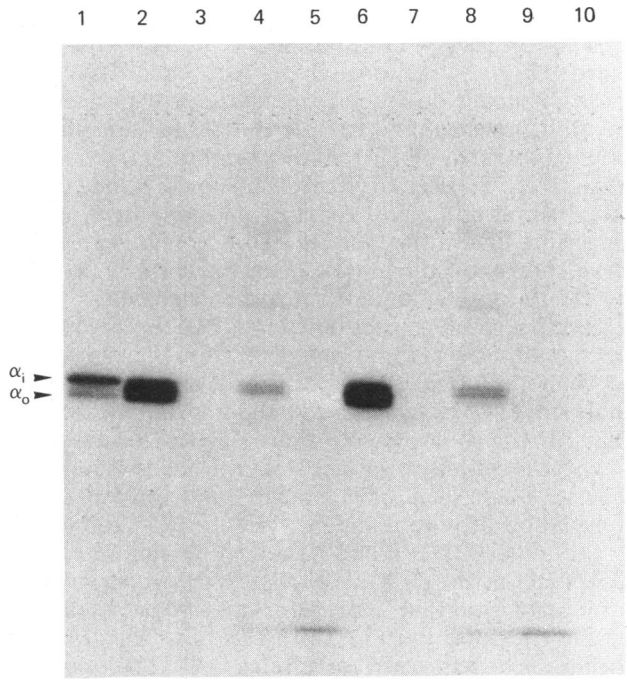

Fig. 2. Effect of addition of $\beta\gamma$ subunits on PTX-catalysed ADP-ribosylation of proteins in diaphragm-muscle extracts

Samples subjected to ADP-ribosylation were: a mixture of purified calf brain G_o and G_i proteins (lane 1), chromaffin granules (lanes 2 and 6), LSS, DS, HSS fractions (lanes 3, 4, 5 respectively) from the aneural part of rat diaphragm muscle, and LSS, DS, HSS fractions to which a pure $\beta\gamma$ fraction was added (lanes 7, 8, 9 respectively); $\beta\gamma$ fraction alone (lane 10). For muscle and chromaffin fractions 20 μg of protein were ADP-ribosylated with pertussis toxin and loaded on a 10%-polyacrylamide gel. Only 0.25 μg of ADP-ribosylated G_o, G_i and 0.5 μg of $\beta\gamma$ were loaded on the same gel.

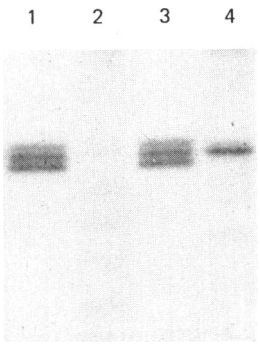

Fig. 3. Comparison of the PTX substrates present in T-tubule membranes with those of chromaffin granules

On an SDS/10%-polyacrylamide gel, the ADP-ribosylated proteins of chromaffin granules (lanes 1 and 3) are compared with those of purified T-tubule membranes in the absence (lane 2) or in the presence of PTX (lane 4). For each fraction 20 μg of protein was ADP-ribosylated with pertussis toxin.

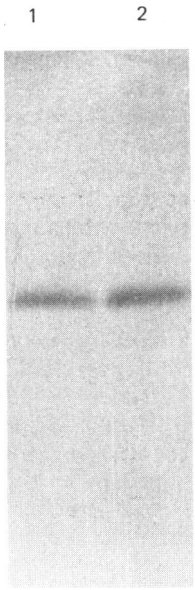

Fig. 4. Immunoblot analysis of β subunit present in T-tubule membranes purified from rabbit skeletal muscle

Samples loaded were (lane 1) T-tubule membranes (100 μg of protein) and (lane 2) 0.25 μg of a mixture of G_o and G_i proteins purified from calf brain. The samples were subjected to SDS/10%-polyacrylamide-gel electrophoresis and transferred on to nitrocellulose. The blot was probed with an antiserum raised against bovine brain β subunit. This antiserum was used at a 1:100 dilution. The antibody reaction was detected by indirect immunoperoxidase staining.

In the sequential extraction procedure used, it could be possible that some α subunits are solubilized in the LSS fraction, as can be observed for proteins loosely attached to the membrane (reviewed by Toutant & Massoulié [15]). If such a 'solubilization' of α subunits occurs in the LSS fraction without an equivalent solubilization of β and γ subunits, these α subunits should not be detected, since PTX-catalysed ADP-ribosylation requires the presence of β and γ subunits [16,17]. We therefore analysed the ADP-ribosylation of LSS, DS and HSS fractions to which a pure preparation of $\beta\gamma$ subunits purified from bovine brain was added (Fig. 2). In these conditions, when 0.5 μg of $\beta\gamma$ subunits was added to each sample, we did not detect any significant amount of α subunits in the LSS and HSS fractions (Fig. 2; lanes 3, 7 and 5, 9 respectively), whereas 0.5 μg of purified $\beta\gamma$ subunit was necessary and sufficient to ADP-ribosylate pure α_o subunit. Thus skeletal muscle possesses two PTX-dependent ADP-ribosylation substrates, of 41 and 40 kDa, both of which are associated with membrane fractions.

Since it has been reported that a G-protein might be involved in excitation–contraction coupling in rabbit skeletal muscle, we looked for the presence of G-proteins in a preparation of T-tubule membranes purified from rabbit skeletal muscle which offered the advantage of being well characterized by the use of several specific markers. In the two parts (rapid and slow) of the rabbit semi-membranosus muscle, as in rat diaphragm muscle, two PTX-dependent substrates were detected in the DS fraction in equivalent amounts. Fig. 3 shows the ADP-ribosylation of T-tubule membranes in comparison with those of chromaffin granules. A major labelled band was detected in T-tubule membranes (Fig. 3, lane 4), which

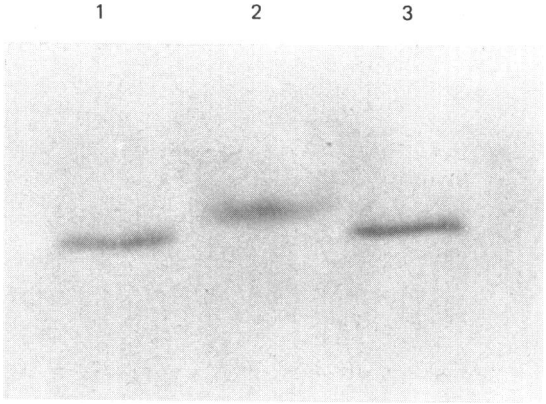

Fig. 5. $G_o\alpha$ immunoreactivity in purified T-tubule membranes

Proteins loaded were 0.25 μg of purified $G_o\alpha$ (lanes 1 and 3) and 100 μg of protein of purified T-tubule membranes (lane 2). The affinity-purified anti-$G_o\alpha$ antibodies were used at a 1:100 dilution. The antibody reaction was detected by indirect immunoperoxidase staining.

migrates in a manner similar to the 40 kDa component of chromaffin granules (Fig. 3, lanes 1 and 3). This ADP-ribosylation was shown to be toxin-dependent, since, in absence of PTX, no ADP-ribosylation occurred in T-tubule membranes (Fig. 3, lane 2). Interestingly, the 41 kDa PTX substrate, which was observed in the DS fraction of both rat diaphragm muscle and rabbit skeletal muscle, is hardly detectable in a subcellular fraction of T-tubule membranes isolated from rabbit skeletal muscle (Fig. 3).

To confirm the presence of a G-protein in T-tubule membranes, immunoblotting experiments were also performed with rabbit polyclonal antibodies raised against β subunits purified from calf brain G-proteins [8]. With this antiserum, a band of immunoreactivity was detected in T-tubule membranes (Fig. 4, lane 1), and the labelled band has the same electrophoretic mobility as the β subunits of G-proteins present in brain (Fig. 4, lane 2). This indicates that in muscle T-tubule membranes there is a G subunit immunologically related to the β subunit of brain G-proteins.

To identify further the G-protein detected in T-tubule membranes, we performed immunoblot analysis with polyclonal antibodies raised against a pure bovine brain $G_o\alpha$ with an apparent molecular mass of 39 kDa [8]. A cross-reactivity at 40–41 kDa was detected with this antiserum in purified T-tubule membranes (Fig. 5). With the same affinity-purified anti-$G_o\alpha$ antibodies, a similar 40–41 kDa polypeptide cross-reacted in adipocytes and in HL-60 cells, a leukaemic cell line of human origin (B. Rouot, J. Carrette, M. Lafontan, P. L. Tran, J. Bockaert & M. Toutant, unpublished work), but not in cells of neural origin [5]. Adipocytes and HL-60 cells, like skeletal muscle, do not have the $G_o\alpha$ protein, since they do not exhibit at 39 kDa either a PTX substrate or a cross-reactivity with the anti-$G_o\alpha$ antibodies.

DISCUSSION

Recently, it has been suggested that in skeletal muscle a GTP-binding protein is involved in excitation–contraction coupling [7]. In the present study, we have investigated the nature of PTX substrates present in muscular extracts and in purified T-tubule membranes.

Sequential extractions (LSS, DS and HSS) from aneural zones of rat diaphragm muscle provide a suitable way of demonstrating two polypeptides (41 and 40 kDa) ADP-ribosylated by PTX in the membrane fraction (DS extract). No 39 kDa PTX substrate could be detected in either neural or aneural zones of rat diaphragm muscle, suggesting that, if the $G_o\alpha$ (39 kDa) protein is present in nerve endings of the muscle neural zone, it is probably present in too low an amount to be detected.

Since one could expect that some α subunits might be functionally (or during homogenization) released from the membrane [18], we looked for the presence of PTX-catalysed ADP-ribosylation in the hydrophilic fraction LSS. It has been stated that PTX-catalysed ADP-ribosylation of pure α subunits of G-proteins requires the integrity of the heterotrimers $\alpha\beta\gamma$, and thus the ADP-ribosylation of pure α subunits requires the addition of $\beta\gamma$ subunits [16,17]. When we added $\beta\gamma$ subunits to the LSS fraction, no PTX-dependent ADP-ribosylation of α subunits could be detected. This indicates that no soluble PTX-sensitive Gα was present in skeletal-muscle extract, whereas two substrates (41 and 40 kDa) associated with membrane fractions can be found.

The component detected at 41 kDa by ADP-ribosylation was originally called G_i, since it was believed to transduce inhibition of adenylate cyclase [6]. It is now known that PTX not only affects adenylate cyclase inhibition but, depending on the system studied, also affects GTP-dependent activation of phospholipases of the C and/or A_2 type [19–23]. PTX also affects acetylcholine activation of heart, rectifying K^+ channels [24] and neurotransmitter inhibition of voltage-sensitive Ca^{2+} channels [25,26]. The multiplicity of the signal-transduction processes sensitive to blockade by PTX may be paralleled with the multiplicity of the cDNA encoding for G-proteins. Recently, in the human genome three non-allelic genes encoding $G_i\alpha$-type PTX substrates with molecular masses between 40 and 41 kDa and different from $G_o\alpha$ (39 kDa) were reported [27]. These results suggest that distinct G-protein activities probably reside on distinct molecules. However, it is not yet possible to relate a given PTX-sensitive biological function to a precise $G_i\alpha$ sequence.

In T-tubule membranes purified from rabbit skeletal muscle, only the 40 kDa PTX substrate was observed. Such a PTX substrate with an apparent molecular mass of 40 kDa has also been detected in other tissues, e.g. C_6 glioma cells, rat fat-cells, rabbit heart, neutrophils, chromaffin cells, astrocytes, neurons and adeno-hypophysis [28–31,4,5]. A 40 kDa species has also recently been purified from pig brain [32] and from neutrophils [33,34]. The presence of a G-protein in T-tubule membranes was further ascertained by the immunoreactivity detected with an anti-β-subunit antiserum. With polyclonal antibodies raised against pure $G_o\alpha$ subunit, a cross-reactivity was observed at 40 kDa in T-tubule membranes, which may be related to those observed in adipocytes and HL-60 cells (B. Rouot, J. Carrette, M. Lafontan, P. L. Tran, J. Bockaert & M. Toutant, unpublished work). In these cells, the phospholipase C stimulation by membrane-bound receptors is PTX-sensitive [19,20,30], differing from what is observed in brain tissues [35]. Previous studies have shown that our $G_o\alpha$ antiserum does not cross-react with

the 40 kDa PTX substrate of brain and chromaffin cells [4,5]. These immunological data suggest that the 40 kDa PTX substrate of brain and chromaffin cells is not identical with those of muscle, adipocytes and HL-60.

In summary, two PTX substrates were detected in total muscle extract, whereas only one was found in purified T-tubule membranes. This could suggest that, if a G-protein is involved in excitation–contraction coupling [7], it might be the 40 kDa protein detected in the T-tubule fraction. It remains to clarify the role of this G-protein, which might be linked either to voltage-dependent Ca^{2+} channels or to phospholipase C.

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