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Phosphoinositides in membranes that build up the triads of rabbit skeletal muscle

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Abstract

The total membrane concentrations of PtdIns, PtdIns4P, and PtdIns(4,5)P_2 contribute to the functional capacity of the Ins(1,4,5)P_3 signalling system which is operating in skeletal muscle but the function of which is still unknown. Total amounts of these phosphoinositides have been determined in purified membranes of transverse tubules (TT) and terminal cisternae (TC) of the sarcoplasmic reticulum (SR) of rabbit skeletal muscle. PtdIns and PtdIns4P have been detected in both membrane systems whereas PtdIns(4,5)P_2 (290 μmol/mol phospholipid) is confined only to TT. A much greater pool of PtdIns(4,5)P_2 seems, however, to be located in the sarcolemma away from the triadic junction.

Key words: Phosphoinositide; PtdIns(4,5)P_2; Phospholipase C, Inositol 1,4,5-trisphosphate; Triad; Skeletal muscle

1. Introduction

The concept that inositol 1,4,5-triphosphate (Ins(1,4,5)P_3) acts as an intracellular chemical transmitter in the fast excitation–contraction coupling process of skeletal muscle has been put forward by several groups [1–3]. There are, however, severe objections to this idea which have been worked out in detail by [4]. A second concept favours a messenger action of Ins(1,4,5)P_3 on a larger time scale aiming at the excitation–contraction coupling mechanism and/or the energy metabolism of the skeletal muscle fibre [5]. A key event of the Ins(1,4,5)P_3-signalling pathway is the cleavage of Ins(1,4,5)P_3 from phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) by phospholipase C (PLC) [6] that has to fit in quite different time frames depending on which of the concepts is realised in skeletal muscle. It is well established that the activity of skeletal muscle PLC is strongly regulated by myoplasmic Ca^{2+} in a concentration range that is reached during muscle activation [7,8]. Up to now it is unknown whether this Ca^{2+}-sensitivity, which is common to all known PLC isoforms [9], represents the primary event for PLC-activation or whether a different membrane borne process switches on the enzyme Ca^{2+}-independently. A putative assignment of the activation mechanism to one of the established classes (G protein activated for PLC-β; receptor tyrosine kinase activated for PLC-γ [6,9]) is not possible because a PLC isoform of skeletal muscle has not yet been identified by protein or cDNA sequencing. There is, however, evidence that two PLC, i.e. a cytosolic and a membrane associated form, coexist in skeletal muscle [7,8] and that the myoplasmic enzyme might be different from the known PLC families [10]. The membrane associated enzyme is localised mainly at the TT [7,8]. Membranes of the SR seem either to be devoid of this enzyme [7] or contain marginal PLC activity [8]. Compared to the knowledge accumulated on skeletal muscle PLC only little is known about membrane localisation and content of its substrate, PtdIns(4,5)P_2. In the present work we have studied in detail the total concentration and localisation of phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P), and PtdIns(4,5)P_2 in TT membranes and in membranes of the TC of the SR which come in close apposition at the level of triads (for review see [11]). The data provide information on the capacity and limitations of the Ins(1,4,5)P_3 signalling system in skeletal muscle focused on the phosphoinositides in the different membrane systems. Preliminary results of the work have been presented in abstract form [12].

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Abbreviations: GroPIns, glycerophosphoinositol; GroPIns4P, glycerophosphoinositol 4-phosphate; GroPIns(4,5)P_2, glycerophosphoinositol 4,5-bisphosphate; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; 4-methylumbelliferyl phosphate, MUM; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PLC, PtdIns(4,5)P_2-directed phospholipase C; SR, sarcoplasmic reticulum; TC, terminal cisternae; TT, transverse tubules.

2. Materials and methods

2.1. Preparation of purified triads, TT, and TC

Skeletal muscle microsomes and triads were isolated from rabbit back muscle according to [13]. The pellet resulting from the final centrifugation at 100,000 × *g* was homogenised in 250 mM sucrose, 3 mM histidine, pH 7.3, and centrifuged at 70,000 × *g* overnight on a linear sucrose density gradient (15–50% w/w sucrose). The gradient was fractionated and the fractions (1.5 ml) were assayed for dihydropyridine binding according to [14] using 0.5 nM [^{3}H]PN200/110 and for ryanodine binding according to [15] employing 1.5 nM [^{3}H]ryanodine. The distribution of the SR Ca^{2+} transport ATPase activity was monitored

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by measuring the dephosphorylation of 4-methylumbelliferyl phosphate (MUM) in the fractions according to [16]. The phospholipid content of the fractions was determined by combining phospholipid extraction [17] and determination of organic phosphorus [18]. Aliquots of the fractions (20 μl) were mixed with 500 μl chloroform/methanol (4:1, v/v), 33 μl 2.4 N HCl, and 30 μl H_2O and stirred vigorously for 1 min. After brief centrifugation, the organic phases were transferred to phosphate-free glass tubes and their phospholipid phosphorus contents were determined as described below. Protein was determined with the bicinchoninic acid method [19] using bovine serum albumin as standard. Certain gradient fractions which contained purified triads (see section 3) were pooled and subjected to French press treatment according to [20]. The pressure treated purified triads were separated by isopycnic sucrose density centrifugation and fractionated as described above. The distribution of protein and phospholipid and likewise of ryanodine binding and PN200/110 binding in the gradients was determined as described before. Fractions that contained only one defined membrane type (i.e. purified TT or purified TC, see section 3) were pooled.

2.2. Extraction and deacylation of phospholipids

Characterised membrane fractions were subjected to a two-step lipid extraction procedure according to [17]. Purified triads (5–20 mg) purified TT (1–7 mg), and purified TC (1–7 mg), respectively, were extracted with 5 volumes of chloroform/methanol (4:1, v/v) and 0.67 volumes of 2.4 N HCl. After vortexing for 1 min and brief centrifugation, the lower organic phases were carefully removed and transferred to other glass tubes. These samples were further extracted with one volume of 2.4 N HCl/methanol (1:1, v/v) and the organic phases were again transferred quantitatively to new glass vials. The organic phases were evaporated to dryness in a speedvac and the dried lipid extracts were redissolved in 200 μl chloroform. The recovery of the lipid extraction procedure was determined by adding [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 (both 1.5×10^4 Bq/ml), respectively, to the purified membranes and measuring the radioactivity regained in the final chloroform phase by scintillation counting. For both phosphoinositides, the obtained recoveries were in the range of 90 to 96% of the initially employed radioactivity. Analysis of these chloroform phases by one-dimensional high-performance thin-layer chromatography, HPTLC (see below), revealed a single spot in both cases, indicating that no significant dephosphorylation of phosphoinositides occurs during the lipid extraction procedure. An aliquot of the chloroform phases obtained from the purified membranes was used for the determination of PtdIns by HPTLC (see below). The remains of these lipid extracts were deacylated according to the method of [21]. The chloroform phase was mixed with 1 ml monomethylamine reagent (25%, v/v) and incubated in a sealed glass tube at 53°C for 40 min. Thereafter, 500 μl ice-cold *n*-propanol was added and the sample was evaporated to dryness in a speedvac. The resulting pellet was redissolved in a mixture of 1 ml H_2O and 1.2 ml *n*-butanol/petrolether/ethyl formate (20:4:1, by volume). The aqueous phase, which contains the glycerophosphoinositol-derivatives of phosphoinositides and other phospholipids, was quantitatively transferred to another tube and dried in a speedvac. It was redissolved in HPLC sample buffer (2.5 mM sodium acetate, 1 mM NaF, pH 5.0) for quantitative determination of glycerophosphoinositol phosphates by high-performance liquid chromatography (HPLC) with metal-dye detection (see below).

Deacylation of reference compounds, [^{3}H]PtdIns4P respectively [^{3}H]PtdIns(4,5)P_2, according to the described protocol revealed a deacylation efficiency of 85–93% of the originally employed phosphoinositides. Moreover, deacylation of [^{3}H]PtdIns4P produced only [^{3}H]GroPIns4P and deacylation of [^{3}H]PtdIns(4,5)P_2 yielded only [^{3}H]GroPIns(4,5)P_2 indicating that no dephosphorylation of phosphoinositides occurred during the procedure.

2.3. Analysis of glycerophosphoinositol phosphates

The glycerophosphoinositol phosphates resulting from PtdIns4P and PtdIns(4,5)P_2 after deacylation of purified membranes were analysed by HPLC with metal-dye detection as described previously [5]. A 0.5×20 cm Mono Q column (Pharmacia, Uppsala, Sweden) with a 0.5×1 cm guard column (Mono Q) was employed with a neutral elution system (weak eluant: 10 mM triethanolamine.HCl, pH 8.0; strong eluant: 1 M KCl, 10 mM triethanolamine.HCL, pH 8.0) recommended for the quantitative determination of phosphoinositide deacylation products [22]. Separated glycerophosphoinositol derivatives were identified and quantified with the aid of calibration runs with the standard compounds GroPIns, GroPIns4P and GroPIns(4,5)P_2.

2.4. Determination of PtdIns by HPTLC

The PtdIns content of purified triads, TT and TC, respectively, was determined by two-dimensional HPTLC employing three solvent systems as described by [23]. An aliquot (4 μl) of the final organic phase resulting from the lipid extraction of purified membranes (see above) was spotted on a silica gel 60 HPTLC plate. After solvent development, lipid spots were visualised by exposing the plates to iodine vapour. In parallel experiments the PtdIns spot was labelled by spiking the purified membranes with [^{3}H]PtdIns (9×10^5 Bq/ml) prior to the lipid extraction procedure described above. It was identified on the plates by autoradiography before lipid staining and appeared, in agreement with [23], well separated from the major phospholipids of the purified membranes, i.e. phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine, as well as from phosphatidic acid. For quantification of PtdIns in an identified spot, the corresponding silica gel was scraped out and transferred to a phosphate-free glass tube for phospholipid phosphorus determination as described below. The method was calibrated by analysing different amounts of PtdIns spotted on the HPTLC plates. The same standards were also directly applied to tubes and analysed. The phosphorus determinations with the scraped out silica gel yielded values which were in the order of 95% of those obtained with the latter method.

2.5. One-dimensional HPTLC of [^{3}H]phosphoinositides

Degradation of ^{3}H-labelled phosphoinositides during extraction of phospholipids and preparation of skeletal muscle microsomes, respectively, was followed by one-dimensional HPTLC on silica gel 60 HPTLC plates which were previously sprayed with potassium oxalate (pH 7.0, 1% v/v in methanol/H_2O (2:3, v/v)) and dried overnight at 70°C. Aliquots (2–5 μl) of [^{3}H]phosphoinositide-labelled phospholipid extracts of purified membranes (see above) and of phospholipid extracts of raw muscle homogenates which were initially injected with [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 (both 1.5×10^4 Bq/ml), respectively, were spotted on the plates. Chloroform/acetone/methanol/100% acetic acid/H_2O (40:15:13:12:8, by volume) was used as solvent system. After solvent development, ^{3}H-labelled phosphoinositides were visualized by autoradiography and the autoradiograms were quantified with the aid of Whole Band Analyzer software on a Bio Image system (Millipore, Ann Arbor, MI, USA).

2.6. Determination of phospholipid phosphorus

The phospholipid phosphorus content of chloroform/methanol extracts of sucrose gradient fractions as well as of purified triads, TT, and TC, respectively, was determined by incineration of the extracts [18] followed by phosphorus determination according to [24]. The extracts were evaporated to dryness by centrifugation in a speedvac. The dried lipid extracts were incinerated by addition of 100 μl 10 M H_2SO_4 and incubation at 180°C for 1 h. Thereafter, 100 μl H_2O_2 (33% v/v, phosphate-free) were added and the tubes were again incubated for 1 h at 180°C. After cooling down, 50 μl H_2O were added and the total volume of fluid was determined in each tube. The phosphate content of the incinerated material was then determined in aliquots (10 μl) with a spectrophotometric method [24] using microtiter plates. The phospholipid phosphorus determinations were calibrated by means of phosphate standards treated in the same way as the samples.

2.7. Materials

[9,21-^{3}H(N)]ryanodine and (+)-[5-methyl-^{3}H]-PN200/110 were from NEN DuPont de Nemours (Bad Homburg, Germany). L-3-Phosphatidyl[2-^{3}H]inositol 4-monophosphate and L-3-phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate were from Amersham (Braunschweig, Germany). L-3-phosphatidyl [2–3H]inositol was from BioTrends (St. Louis, MO, USA). Glycerol 3-phosphate, 4-methylumbelliferyl phosphate, GroPIns, GroPIns4P, and GroPIns(4,5)P_2 were from Boehringer (Mannheim, Germany). Silica gel 60 HPTLC plates (10×10 cm) were from Merck (Darmstadt, Germany). PtdIns, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidic acid were from Sigma (St. Louis, MO, USA). Monomethylamine, petrolether and triethanolamine were from Fluka (Buchs, Switzerland). HCl and *n*-propanol were from Baker (Deventer, The Netherlands). Ethyl

formate and *n*-butanol were from Riedel de Haen (Seelze, Germany). Chemicals and reference compounds used for HPLC analysis were as described by [22]. All other chemicals were at least of analytical grade.

3. Results

Separation of rabbit skeletal muscle microsomes by centrifugation on a linear sucrose gradient (Fig. 1a,b) leads to a protein distribution as shown in Fig. 1a. Part of this protein is due to proteins incorporated in or associated with membrane structures as can be deduced from the concomitant appearance of phospholipids (Fig. 1a). The distribution of sarcolemmal fragments derived from the surface membrane or the T-tubular network was followed by high affinity binding of [^{3}H]PN200/110 to L-type Ca^{2+} channels (Fig. 1b). Fragments of the SR containing the junctional membrane of TC were identified on the gradient by high affinity [^{3}H]ryanodine binding to the SR Ca^{2+} release channel (Fig. 1b). In addition, the dephosphorylation of MUM by the Ca^{2+} transport ATPase of the SR [16] was measured in order to identify fractions containing extra-junctional SR parts (Fig. 1b). In agreement with other studies [7,13,20], the fractions under the MUM phosphatase activity peak at 30% (w/w) sucrose can be assigned to fragments of the longitudinal SR because of only minor binding of PN200/110 and ryanodine. The fractions around 40% (w/w) sucrose can be identified as triads [7,13,14]. Those of them which showed substantial binding of PN200/110 and ryanodine as well as significant MUM phosphatase activity (Fig. 1b, filled symbols) were pooled and called *purified triads*. French press treatment of purified triads leads to a clear separation of TC from T-tubular fragments on a linear sucrose gradient (Fig. 1c) indicating a breakage of the triadic superstructure [20]. Fractions which showed significant ryanodine binding but practically no binding of PN200/110 (Fig. 1c, filled circles) were pooled and denoted as *purified TC*. Those which showed PN200/110 binding at the absence of ryanodine binding (Fig. 1c, filled squares) were pooled and designated as *purified TT*. Each of these two pools contained virtually only one of the two membrane types existing in skeletal muscle triads. They were, together with purified triads, subjected to phospholipid extraction and phosphoinositide analysis.

In phospholipid extracts of purified triads, GroPIns4P and GroPIns(4,5)P_2 can be determined and quantified by HPLC with metal-dye detection [5,22] after deacylation [21] of the extracts (Fig. 2). These glycerophosphoinositol phosphates report the total amounts of PtdIns4P and PtdIns(4,5)P_2, respectively, in purified triads. The determined mean values ± S.E. were 2.2 ± 0.1 nmol PtdIns4P and 0.3 ± 0.2 nmol PtdIns(4,5)P_2 per mg triadic protein. The much smaller peak of GroPIns4P compared to GroPIns(4,5)P_2 reflects the much lower sensitivity of the detection method for the former substance [22]. The major phospholipids of cell membranes, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine, as well as phosphatidic acid do not lead, after processing, to products interfering with the detection of GroPIns4P or GroPIns(4,5)P_2 (Fig. 2 e–h). Their deacylation products, glycerophosphocholine, glycerophosphoserine, glycerophosphoethanolamine, and glycerol 3-phosphate, respectively, together with GroPIns (Fig. 2d) which is the deacylation product of PtdIns, contribute to the dominant peak detected in purified triads (Fig. 2a, assigned with ×). Because of this lack of separation of GroPIns from the other deacylation products of biological membranes and because of the relatively low sensitivity of the detection method for this compound (Fig. 2d), the content of PtdIns in the purified skeletal muscle membranes was determined by quantitative HPTLC (see below).

GroPIns(4,5)P_2 was readily detectable and quantifiable in deacylated phospholipid extracts of purified TT (Fig. 3a) whereas it was not detectable in those of purified TC (Fig. 3c). On the contrary, GroPIns4P could be identified and quantified in both triadic membrane types. The determined glycerophosphoinositol phosphates correspond to total amounts (mean value ± S.E.) of 22 ± 4 nmol PtdIns4P and 1.1 ± 0.2 nmol PtdIns(4,5)P_2 per mg protein in TT and to 12 ± 3 nmol PtdIns4P per mg protein in TC. We have observed that storage of purified TT at −70°C prior to phospholipid extraction led to reduced total amounts ofGroPIns(4,5)P_2 compared to immediately processed purified TT. For that reason, only data obtained from purified membranes processed immediately after preparation were considered. Storage of deacylation products of extracted phospholipids in HPLC buffer at −70°C did, however, not lead to significant decomposition of glycerophosphoinositol phosphates.

The total content of PtdIns, which is commonly the most abundant phosphoinositide in tissues, was determined directly by HPTLC of phospholipid extracts of purified skeletal muscle membranes (Fig. 4). The spot corresponding to PtdIns separated well from those of the other phospholipids of these membrane structures. Quantification of PtdIns by phospholipid phosphorus determination revealed total amounts (mean value ± S.E.) of 11 ± 4 nmol, 141 ± 18 nmol, and 292 ± 27 nmol per mg of protein in purified triads, TT, and TC, respectively.

Table 1 compiles the total amounts of PtdIns, PtdIns4P, and PtdIns(4,5)P_2 determined in purified triads, TT, and TC. The tabulated values have been corrected by the mean yield coefficients of the experimental procedures that were combined in order to analyse the purified membranes (see legend of Table 1). The obtained data were related to the mass of protein in the purified membrane structures that were subjected to phosphoinositide analysis as well as to the phospholipid

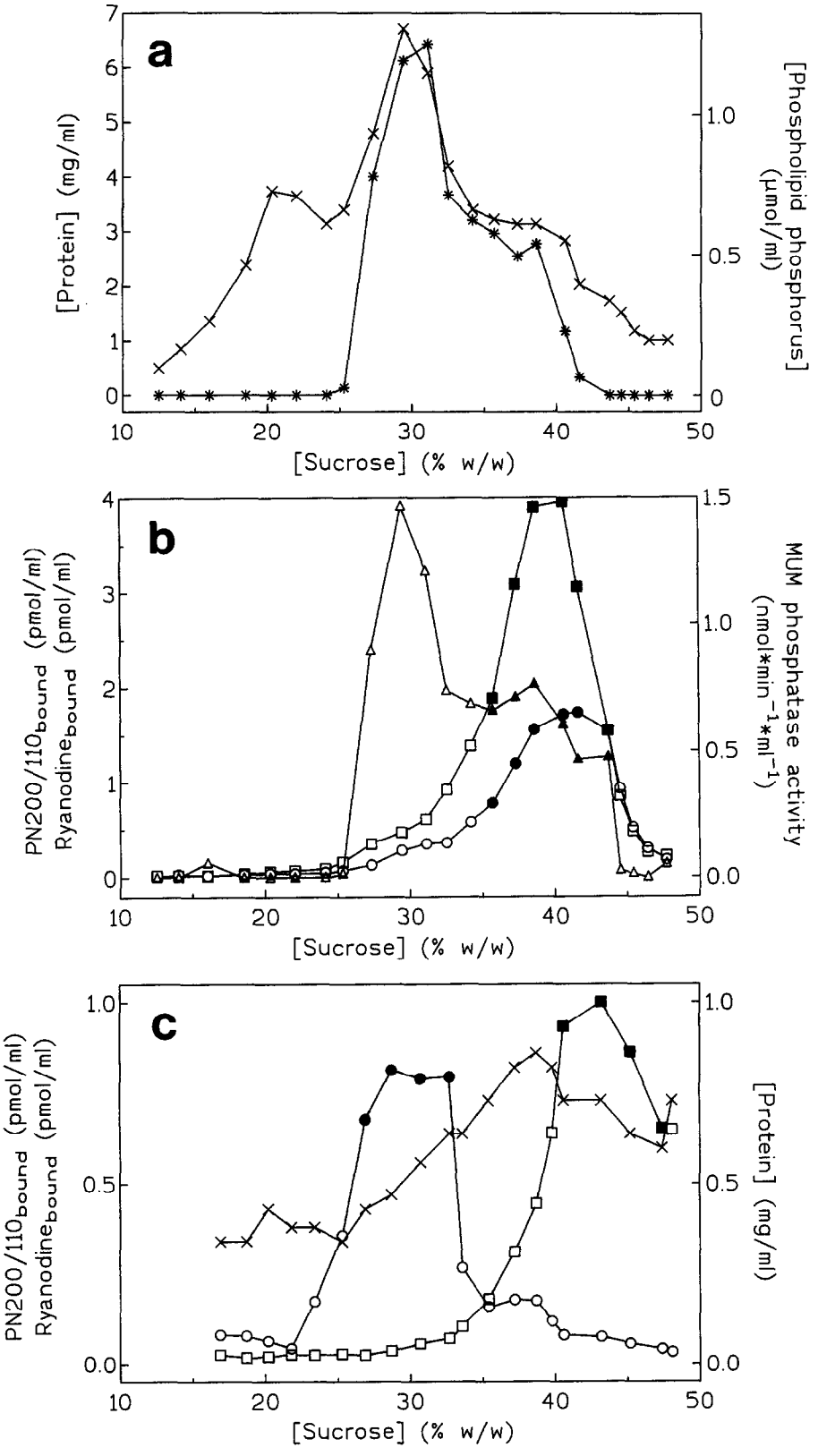

Fig. 1. Separation, identification and purification of triads and triadic membranes prior to phospholipid extraction and analysis. (a, b) Isopycnic sucrose density centrifugation of rabbit skeletal muscle microsomes on a linear sucrose gradient. The gradient fractions were scanned for protein concentration (a, ×), concentration of phospholipid-derived phosphorus (a, *), ryanodine binding (b, □), PN200/110 binding (b, ○), and MUM phosphatase activity (b, △). The filled symbols in b denote those fractions which, according to the actual measurement (corresponding open symbols in b), contain *purified triads* (see text). (c) Separation of pressure cell treated purified triads by centrifugation on a linear sucrose gradient. The gradient fractions were scanned for protein concentration (×), ryanodine binding (□), and PN200/110 binding (○). Fractions containing *purified TT* and *purified TC* are assigned (●) and (■), respectively (see text).

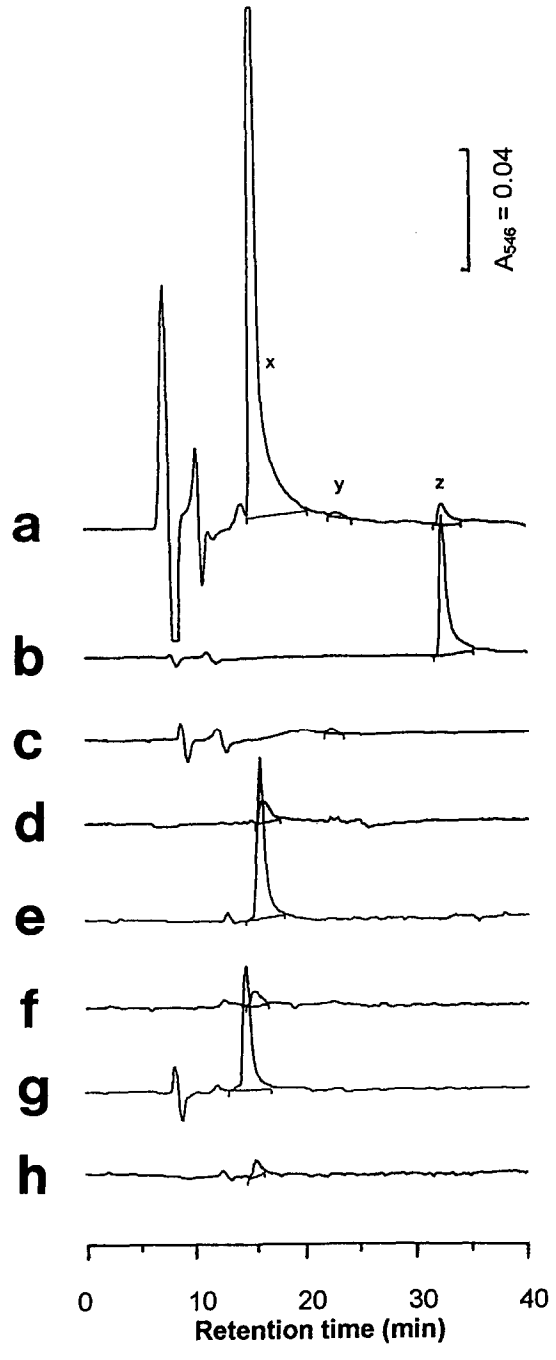

Fig. 2. Identification of GroPIns4P and GroPIns(4,5)P_2 in deacylated phospholipid extracts of purified triads by HPLC with metal-dye detection. Shown are baseline-subtracted chromatograms of: a, processed purified triads (5.7 mg); b, GroPIns(4,5)P_2 (3.3 nmol); c, GroPIns4P (16 nmol); d, GroPIns (110 nmol); e, glycerol 3-phosphate (8.3 nmol); f, processed phosphatidylcholine (450 nmol); g, processed phosphatidylserine (50 nmol); h, processed phosphatidylethanolamine (50 nmol). The peaks assigned y and z in purified triads correspond to 24 nmol GroPIns4P and 0.8 nmol GroPIns(4,5)P_2, respectively.

content of these membranes approximated by the phospholipid-derived phosphorus. In purified triads and TT, the phosphoinositides separate to about the same degree into PtdIns (80–86%), PtdIns4P (14–18%), and PtdIns(4,5)P_2 (0.7–2.2%). It is obvious that the PtdIns(4,5)P_2 detected in purified triads originates solely from TT fragments assembled in this complex superstructure. In the purified membrane of TC, only PtdIns and PtdIns4P are present. Here, they make up about 19% of the total phospholipids and separate into 96% PtdIns and 4% PtdIns4P.

The question whether degradation during membrane preparation could have had affected the obtained data

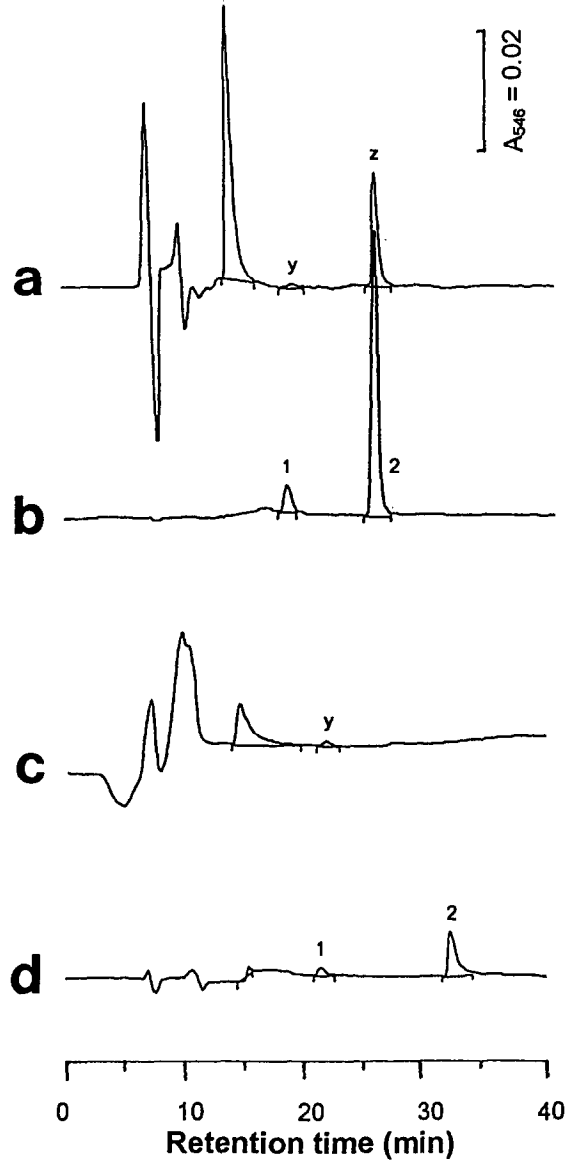

Fig. 3. Analysis of deacylated phospholipid extracts of purified TT and TC by HPLC with metal-dye detection. Shown are baseline-subtracted chromatograms of: a, processed purified TT (2 mg); b, mixture of GroPIns4P (40 nmol, peak 1) and GroPIns(4,5)P_2 (5.8 nmol, peak 2); c, processed purified TC (1 mg); d, mixture of GroPIns4P (16 nmol, peak 1) and GroPIns(4,5)P_2 (1.2 nmol, peak 2). In purified TT, the peaks assigned y and z correspond to 26 nmol GroPIns4P and 1.5 nmol GroPIns(4,5)P_2 (compared a and b). In purified TC, the peak y corresponds to 10 nmol GroPIns4P and GroPIns(4,5)P_2 is not detectable (compare c and d). The elution protocol used in a and b was slightly different from that used in c and d.

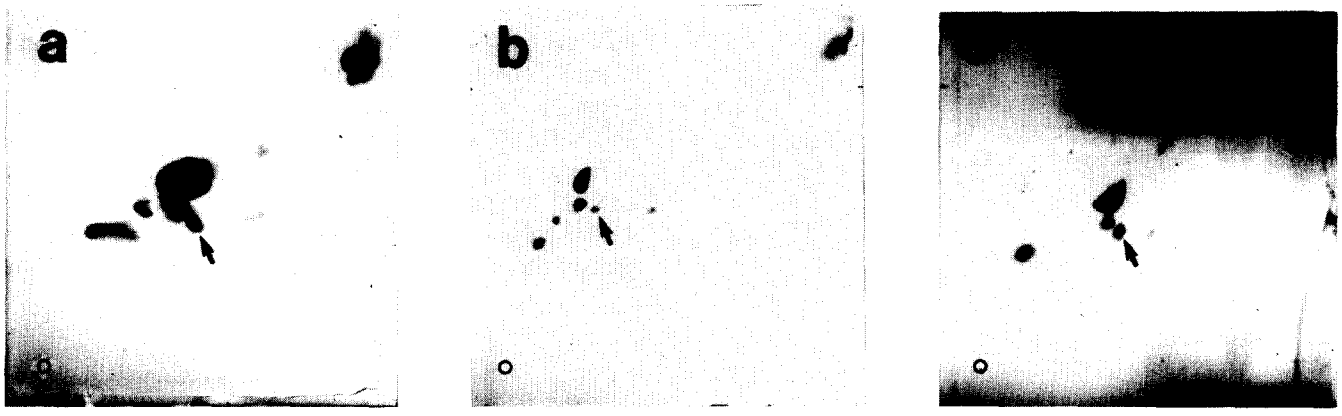

Fig. 4. Determination of PtdIns in purified triads, TT and TC by HPTLC. The chloroform/methanol extracts (4 μl) of processed purified triads (a, 420 μg), TT (b, 60 μg), and TC (c, 140 μg), respectively, were applied on the plates at the spots marked by circles. After solvent development the phospholipids were visualized by exposition to iodine vapour. The arrow on each plate indicates the PtdIns spot. The amount of PtdIns in these spots obtained by phospholipid phosphorus determination (see section 2) were 9 nmol (a), 19 nmol (b), and 57 nmol (c), respectively. Vertical, 1st dimension; horizontal, 2nd dimension.

was investigated in the following way. [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2, respectively, was added to aliquots of the raw muscle homogenate and these samples were incubated under conditions comparable to the corresponding preparative steps. After lipid extraction of the raw homogenate samples, the radioactivity appearing in the organic phase was assayed by scintillation counting and HPTLC (see section 2). The mean recovery of [^{3}H]inositol-containing compounds stemming from [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 was 71.9% and 62.6%, respectively, which would correspond to a phosphodiesteratic cleavage of the initially employed phosphoinositides in the raw muscle homogenate by 28.1% and 37.4%, respectively. According to HPTLC analysis of these lipid extracts, the compounds originating from [^{3}H]PtdIns4P separated into 91.4% [^{3}H]PtdIns4P and 8.6% [^{3}H]PtdIns and those stemming from [^{3}H]PtdIns(4,5)P_2 into 87.4% [^{3}H]PtdIns(4,5)P_2, 3.5% [^{3}H]PtdIns4P, and 9.1% [^{3}H]PtdIns. Net formation of [^{3}H]PtdIns(4,5)P_2 from [^{3}H]PtdIns4P was not found. These data suggest that about 8.6% of the initially employed [^{3}H]PtdIns4P and about 12.6% of the initially introduced [^{3}H]PtdIns(4,5)P_2 were degraded by phosphomonoesteratic cleavage in the raw muscle homogenate.

Recently, Ins(1,4,5)P_3 has been detected in triads of rabbit skeletal muscle using a radioreceptor assay [25]. For that reason, we have analysed the supernatant of skeletal muscle microsomes after denaturation with trichloroacetic acid by HPLC with dye-detection as described previously [5]. Total amounts of about 5 pmol Ins(1,4,5)P_3/mg microsomal protein were detected in freshly prepared microsomes which is much less than the values reported by [25].

4. Discussion

The general composition of the major phospholipids in skeletal muscle SR membranes is fairly well known [26–28]. There is, however, only very limited knowledge about the contents of phosphoinositides in the different membrane systems of skeletal muscle especially with re-

Table 1
Phosphoinositide content of different rabbit skeletal muscle membranes determined by non-radioactive mass determinations[a]

	PtdIns[b]		PtdIns4P[c]		PtdIns(4,5)P_2[c]	
	(mol/mg protein)	(mol/mol phospholipid)	(mol/mg protein)	(mol/mol phospholipid)	(mol/mg protein)	(mol/mol phospholipid)
Purified triads	$1.2 \pm 0.4 \times 10^{-8}$	$2.1 \pm 0.6 \times 10^{-4}$ (4)	$2.7 \pm 0.1 \times 10^{-9}$	$4.6 \pm 0.2 \times 10^{-5}$ (3)	$3.4 \pm 2.5 \times 10^{-10}$	$5.8 \pm 4.3 \times 10^{-6}$ (4)
Purified TT	$1.6 \pm 0.2 \times 10^{-7}$	$36 \pm 4 \times 10^{-3}$ (3)	$2.6 \pm 0.5 \times 10^{-8}$	$5.8 \pm 1.2 \times 10^{-3}$ (5)	$1.3 \pm 0.3 \times 10^{-9}$	$2.9 \pm 0.7 \times 10^{-4}$ (9)
Purified TC	$3.3 \pm 0.3 \times 10^{-7}$	$181 \pm 17 \times 10^{-3}$ (3)	$1.4 \pm 0.4 \times 10^{-8}$	$7.5 \pm 2.4 \times 10^{-3}$ (5)	not detectable	not detectable

[a]The values represent mean values (± S.E.M.) of the number of determinations given in parenthesis.
[b,c]The tabulated amounts, N, were derived from the measured total amounts, M, according to $N = M/(p \times q)$, where $p = 0.93^{b,c}$ represents the mean recovery of phosphoinositides with the lipid extraction procedure, $q = 0.95^{b}$ is the yield coefficient of the phospholipid phosphorus determinations in HPTLC spots and $q = 0.89^{c}$, respectively, is the mean yield coefficient of phosphoinositides with the phospholipid deacylation procedure (see section 2).

spect to PtdIns4P and PtdIns(4,5)P_2 [26–30]. Apart from skeletal muscle, differential determinations of total mass levels of the polyphosphoinositides in subcellular membranes are generally missing [31]. Our data give a first report on the total amounts of phosphoinositides in previously characterised subcellular membrane systems which are of fundamental importance to muscle function [11]. The results indicate that the phosphoinositide composition of TT membranes is significantly different from that of TC membranes. Triadic TT contain all the phosphoinositides which establish the complete membrane localised branche of the Ins(1,4,5)P_3 signalling system. In constrast, TC of the SR are devoid of PtdIns(4,5)P_2 and thus lack the capacity of Ins(1,4,5)P_3 generation. A localisation of PtdIns(4,5)P_2 restricted to T-tubular membranes has previously been shown for frog skeletal muscle my means of radioisotope techniques [28] which essentially do not report total mass levels. The distribution of phosphoinositides in TT and TC membranes, respectively, is in agreement with the targeting of enzymes that are involved in the conversion of these molecules. PtdIns 4-kinase, which catalyses the phosphorylation of PtdIns to PtdIns4P, has been found in longitudinal SR, TC and TT [7,32]. In contrast, formation of PtdIns(4,5)P_2 from PtdIns4P catalysed by PtdIns4P 5-kinase has been detected only in TT [7,32,33]. The presence of PtdIns(4,5)P_2 exclusively in TT could thus be due to a specific targeting of PtdIns4P 5-kinase to TT membranes. The same seems to apply to a membrane associated form of PLC which has been detected in preparations of TT [7,8] but not in TC membranes [7] and only to a little extend in SR membranes [8].

In purified TT, phosphoinositides represent about 4.2% of the total phospholipids. This relative content as well as the partition of the phosphoinositides into 85.5% PtdIns, 13.8% PtdIns4P, and 0.7% PtdIns(4,5)P_2 is in accordance with data obtained from various tissues and might be regarded as typical for membranes involved in the generation of Ins(1,4,5)P_3. The total amount of PtdIns relative to the total phospholipid content of purified TT obtained in this study is similar to values reported previously for this membrane of rabbit skeletal muscle [29,30]. However, the total phospholipid content determined by us is about 3-times higher than the corresponding values given in these studies [29,30]. This could be due to our elaborate procedure for the selection of purified membranes which might have led to a higher ratio of phospholipid to protein. The total membrane concentration of about 290 μmol PtdIns(4,5)P_2/mol phospholipid phosphorus determined in purified TT is nearly 5-times the value given in a review [34]. If a surface area of 60–70 Å^2 obtained for lecithins in a model bilayer [35] is assumed for the phospholipids of the TT membrane, a packing density of about 1.5×10^6 phospholipids/μm^2 would result. The determined total PtdIns(4,5)P_2 content of TT would then correspond to about 435 molecules of PtdIns(4,5)P_2/μm^2 TT membrane. In view of the chemical transmitter concept of Ins(1,4,5)P_3 in excitation-contraction coupling [34] this would mean, that the total concentration of the precursor is in the order of the concentration of the putative receptor in mammalian skeletal muscle, the junctional feet of the TC membrane (estimated at 340–390/μm^2 of total TT surface of red and white leg muscle of guinea pig [36]).

The relative phosphoinositide content of the intracellular TC membrane was significantly higher than that of the sarcolemmal TT membrane. The total amount of PtdIns relative to the total phospholipid content of purified TC is about twice as high as values obtained with different SR preparations of rabbit skeletal muscle [26,27,29,30]. We have shown previously that PtdIns is tightly associated with the Ca^{2+} transport ATPase of the SR and that phosphorylation of this PtdIns activates the Ca^{2+} transport mechanism [37]. Part of the relatively high PtdIns content could be due to this association for Ca^{2+} transport ATPase is detectable in these membranes (see Fig. 1b).

In chicken skeletal muscle α-actinin represents a high capacity binding site for PtdIns(4,5)P_2 [38]. The purified membranes we have investigated contained no α-actinin as revealed by immunoblotting with commercially available monoclonal antibodies (not shown). The group of Hidalgo has recently reported, that surprisingly high amounts of Ins(1,4,5)P_3 in the range of 0.3–0.4 nmol/mg protein are present in triads of frog and rabbit skeletal muscle [25]. We have determined much lower mass levels of about 5 pmol Ins(1,4,5)P_3/mg microsomal protein which corresponds to about 6.7 pmol/mg triadic protein. This quantity comes to only 2% of the PtdIns(4,5)P_2 content of purified triads (Table 1).

The data given in Table 1 could represent lower limits of total mass levels of phosphoinositides in purified skeletal muscle membranes. If the degradation estimated with reference compounds in raw muscle homogenate would affect in the same manner the phosphoinositides endogenously present in the skeletal muscle membranes, all values given in Table 1 should be multiplied by 1.4–1.6 in order to compensate the diesteratic cleavage. The amounts of PtdIns4P and PtdIns(4,5)P_2 should further be multiplied by 1.1 in order to correct the estimated monoesteratic cleavage.

Mass determinations of PtdIns(4,5)P_2 carried out with skeletal muscle microsomes revealed total amounts of 17–43 nmol PtdIns(4,5)P_2/mg microsomal protein which correspond to 22–56 nmol PtdIns(4,5)P_2/g muscle mass. A very similar value of 28 ± 11 nmol PtdIns(4,5)P_2/g wet weight has been obtained with a radioisotope method for frog skeletal muscle [28]. With the aid of the yield coefficients for purified triads (0.97 mg triadic protein/g muscle mass) and purified TT (0.31 mg TT protein/g muscle mass) the total amount of PtdIns(4,5)P_2 given in

Table 1 can be extrapolated from the triadic junction to the whole cell. The cellular PtdIns(4,5)P_2 content estimated in this way is about 0.3–0.4 nmol/g muscle mass which is only about 0.5–1.8% of the total amount of PtdIns(4,5)P_2 determined with microsomes. These results clearly show that overall determinations of phosphoinositides in whole muscle do not clarify the situation in special cellular compartments. They further suggest that the major PtdIns(4,5)P_2 pool of the sarcolemma is located either in the surface membrane or in the TT away from the triadic junction. During tetanic activation of skeletal muscle, the total myoplasmic content of inositol 1,4-biphosphate and Ins(1,4,5)P_3 increases by about 1.2 nmol/g muscle mass on a time scale of seconds [5]. This increase would comprise about 2–5% of the estimated total mass level of PtdIns(4,5)P_2 in skeletal muscle and could hardly be supplied by the PtdIns(4,5)P_2 pool of triadic TT alone.

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