Masses of inositol phosphates in resting and tetanically stimulated vertebrate skeletal muscles

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The masses of inositol phosphates have been determined in isolated skeletal muscles from *Xenopus laevis* (sartorius, tibialis anterior and iliofibularis) and rat (gastrocnemius and soleus) which were quick-frozen in the resting state and at different stages of an isometric (*Xenopus*) or isotonic (rat) tetanus. The isomeric spectrum of inositol phosphates detected was similar to that in other tissues and cell types. The total sarcoplasmic concentrations of the isomers $\text{Ins}(1,4,5,6)\text{P}_4/\text{Ins}(3,4,5,6)\text{P}_4$ (0.2–0.9 μM), $\text{Ins}(1,3,4,6)\text{P}_4$ (not detectable), $\text{Ins}(1,3,4,5,6)\text{P}_5$ (about 1 μM) and InsP_6 (3.2–4.6 μM) were lower than in other cell types. Variations in these concentrations were due to the muscle type rather than to the donor species. The putative second messenger $\text{Ins}(1,4,5)\text{P}_3$, as well as its dephosphorylation product $\text{Ins}(1,4)\text{P}_2$, were present at surprisingly high total myoplasmic resting concentrations, ranging from 1.2 to 2.5 μM and 3.5 to 6.9 μM respectively. Upon tetanic stimulation these two inositol phosphates in particular exhibited significantly increased total sarcoplasmic concentrations, up to 4.2 μM and 11.3 μM respectively, with a time scale of seconds. From the initial rate of increase in the total sarcoplasmic concentrations of $\text{Ins}(1,4,5)\text{P}_3$ and its rapidly formed metabolic products, a minimal phosphoinositidase C (PIC) activity in tetanically activated *Xenopus* skeletal muscle of about 1.7–2.6 $\mu\text{M/s}$ can be estimated. This PIC activity observed *in vivo* seems to be far too low to account for a functional role for $\text{Ins}(1,4,5)\text{P}_3$ as a chemical transmitter in the fast excitation–contraction coupling (ECC) process in skeletal muscle. The presence of $\text{Ins}(1,3,4,5)\text{P}_4$ in all muscle types is indicative of a Ca^{2+} -activated $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activity. The rapid transient increases in $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3)\text{P}_2$ in isometrically contracting *Xenopus* muscles suggest that corresponding $\text{Ins}(1,3,4,5)\text{P}_4$ phosphatases are operating in skeletal muscle as well. In all muscles investigated except rat soleus, the fructose 1,6-bisphosphate [$\text{Fru}(1,6)\text{P}_2$] concentration increased substantially during a tetanus, up to about 2 mM. This increase is correlated with a simultaneous decrease in phosphocreatine, whereas the energy charge of the muscles was essentially unaffected by the applied tetani. The time course of the rise in $\text{Fru}(1,6)\text{P}_2$ was used to model changes in the free concentrations of high-affinity aldolase-binding inositol phosphates during the course of a tetanus. These calculations demonstrate that the free concentration of $\text{Ins}(1,4,5)\text{P}_3$ and other aldolase-bound inositol phosphates can increase much faster and to a larger extent than the corresponding total concentrations as a result of their competitive displacement from aldolase-binding sites by the rapidly rising concentration of $\text{Fru}(1,6)\text{P}_2$.

INTRODUCTION

Skeletal muscle fibres are equipped with the biochemical machinery of the phosphoinositide-linked signal-generating pathway, consisting of phosphatidylinositol kinase, phosphatidylinositol 4-phosphate kinase and phosphatidylinositol 4,5-bisphosphate-specific phosphoinositidase C (PIC) [1,2]. The activities of these enzymes are comparable with those found in other cell types in which a functional role for the phosphoinositide-linked signalling system in intracellular Ca^{2+} mobilization has been established (for review, see [3]). In contrast, in skeletal muscle neither the initiating signal for the generation of $\text{Ins}(1,4,5)\text{P}_3$ nor the physiological function of this second messenger are unequivocally known. There is some experimental evidence that skeletal muscle depolarization leads to a transient increase in $\text{Ins}(1,4,5)\text{P}_3$ turnover, as measured by means of radioisotope techniques [4,5]. On the other hand, the generation of $\text{Ins}(1,4,5)\text{P}_3$ by PIC appears to be Ca^{2+} -dependent [6], which is not in agreement with a direct activation of PIC by membrane depolarization. $\text{Ins}(1,4,5)\text{P}_3$ has been described as the initiator of Ca^{2+} release from the sarcoplasmic reticulum (SR) during excitation–contraction coupling (ECC); however, the available data are controversial (for review, see [7]). The Ca^{2+} release observed after a rapid increase in sarcoplasmic $\text{Ins}(1,4,5)\text{P}_3$ [8] is much

slower than that observed under voltage clamp conditions [9]. The functional Ca^{2+} -release channel of the SR, i.e. the ryanodine receptor complex [10] (which has been expressed in Chinese hamster ovary cells) is not activated by $\text{Ins}(1,4,5)\text{P}_3$, which also argues against $\text{Ins}(1,4,5)\text{P}_3$ being the major physiological messenger of ECC in skeletal muscle [11].

Another line of evidence indicates the existence of a functional role for $\text{Ins}(1,4,5)\text{P}_3$ as a modulator of different cellular processes occurring on a time scale of seconds. $\text{Ins}(1,4,5)\text{P}_3$ and other inositol phosphates are potent competitive inhibitors of the glycolytic enzyme aldolase A [12]. We have shown that a fraction of the sarcoplasmic aldolase that is bound to cellular structures such as myofilaments and triads is released upon binding of inositol phosphates and fructose 1,6-bisphosphate [$\text{Fru}(1,6)\text{P}_2$] to the enzyme [13]. This repartitioning of aldolase might also be responsible for the reported $\text{Ins}(1,4,5)\text{P}_3$ -induced enhancement of the Ca^{2+} sensitivity of the contractile mechanism [14]. In order to evaluate concentration changes in inositol phosphates and $\text{Fru}(1,6)\text{P}_2$ in skeletal muscle at different states of activity, mass determinations of these compounds rather than radiolabelling experiments are necessary. We have used the novel h.p.l.c./metal-dye detection method [15] in order to quantify inositol phosphate isomers in vertebrate skeletal muscles under resting conditions and after various periods of tetanic stimulation. This investi-

Abbreviations used: 2,3-BPG, 2,3-bisphosphoglycerate; $\text{Fru}(1,6)\text{P}_2$, fructose 1,6-bisphosphate; P_3i , trimetaphosphate; CrP, phosphocreatine; PIC, phosphoinositidase C; PFK, phosphofructokinase; SR, sarcoplasmic reticulum; ECC, excitation–contraction coupling.

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gation provides the first comprehensive quantitative spectrum of inositol phosphate metabolite masses and their changes, on a time scale of seconds, in a variety of skeletal muscles. The data are interpreted in terms of potential functional roles for inositol phosphates and a likely coupling between activation of glycolysis and increases in free inositol phosphates in glycolytic or mixed-type muscles.

MATERIALS AND METHODS

Muscle preparation and stimulation

Skeletal muscles of frog [*Xenopus laevis*: sartorius (fast type), iliofibularis (predominantly fast type) and tibialis anterior (mixed type)] and rat [gastrocnemius (mixed type) and soleus (slow type)] were used in the experiments. The animals, obtained from commercial breeding stations, were killed by cervical dislocation and the specified intact muscles were simultaneously dissected in pairs by two experimenters. The preparations were completed between 10 and 15 min after the animals were killed. Before the experiments the isolated muscles were incubated pairwise in oxygenated physiological solutions (*Xenopus*: NaCl 115 mM, KCl 2.5 mM, NaH₂PO₄ 0.85 mM, Na₂HPO₄ 2.15 mM, CaCl₂ 1.8 mM, glucose 5 mM, pH 7.0; rat: NaCl 140 mM, KCl 5 mM, CaCl₂ 2 mM, MgCl₂ 1 mM, Hepes 10 mM, glucose 5 mM, pH 7.1) for 15–45 min at room temperature. Muscles were used in pairs, one for stimulation and the contralateral homologue as a control, making sure that the times of experimental treatment were matched for each pair. The *Xenopus* muscles were mounted between two clamps at their approximate length *in situ* and stimulated under isometric conditions. Tension development in the muscles was measured by a force-displacement transducer (Grass FT03C) connected to one of the clamps. The transducer output signal was fed into a bridge circuit and the bridge output was recorded by a pen recorder. Two extracellular stimulus electrodes attached to a flexible holder were applied to a mounted muscle by a micro manipulator. The muscle-electrode set-up was immersed in an oxygenated bathing solution kept at room temperature. After 5 min, the solution chamber was lowered and the moist muscle was stimulated. Prior to tetanic stimulation the adequate stimulus voltage was determined by single pulses of 5 ms duration interrupted by resting intervals of 30 s. A stimulus voltage of twice the value giving maximum twitch contraction and a stimulus frequency of 30 Hz were used in the experiments. In the 'out of bath' position for stimulation, the muscle was accessible to a freeze-clamping device (effective clamp width 1.5 cm) that was cooled in liquid nitrogen and brought into position just before the beginning of tetanic stimulation. At different times after the onset of stimulation the muscles were quick-frozen by operating this device manually. Since the flexible electrode holder was automatically removed by the closing clamp, the time between stopping stimulation and starting freezing was of the order of milliseconds. Freezing of a preparation itself was estimated to take less than 0.2 s. The freeze-clamped material was immediately dipped into liquid nitrogen and stored in precooled polypropylene vials at -70°C until further processing. Contralateral control muscles were treated likewise, except for the electrical stimulation. The experimental procedure for rat muscles was essentially the same as for *Xenopus* muscles, except that the rat muscles were allowed to exert unloaded shortening during tetanic stimulation, i.e. to operate isotonically.

Mass analyses of inositol phosphates and other phosphocompounds

Quick-frozen muscle specimens were weighed in tightly capped

preweighed tubes. Specimen weights were between 80 and 350 mg wet wt. The muscles were put into a Teflon capsule containing a single steel ball of 5 mm diameter, both precooled in liquid nitrogen. The capsule was mounted into a Braun Dismembrator vibrating device, which after 5 s of operation gave a fine frozen muscle powder. The powder was instantaneously suspended in 2 ml of ice-cold 0.8 M-HClO₄ containing 50 mM-acetic acid and homogenized for 10 s with a small Ultra-Turrax. The pellet obtained after 3 min of centrifugation (5000 g) in a cooled bench-top centrifuge was extracted once more by the same procedure. To a number of samples 1 nmol of Ins-(1,2,3,4,5)P₅ was added prior to extraction as an internal standard. EDTA (4 μmol /100 mg wet wt.) and NaF (5 μmol) were added from concentrated stock solutions [15]. The subsequent steps of sample processing were as described in [15]. Samples in HClO₄ were kept at 0 $^{\circ}\text{C}$ and were reneutralized within 20 min. In a number of samples, 5% of the neutralized extract was removed before freeze-drying for measurement of phosphocreatine (CrP) enzymically [16] and of Fru(1,6)P₂, PP_i, ATP, ADP and AMP by h.p.l.c. analysis. Fru(1,6)P₂ was analysed as described below, whereas the nucleotides were determined from their u.v. absorbances recorded before post-column derivatization. After freeze-drying, the extracts were dissolved in 0.7 ml of water and submitted to three charcoal treatments with each time, a 20% (w/v) suspension of acid-treated Norit A in 0.1 M-NaCl (20 μl /100 mg wet wt.) [15]. The charcoal pellets were subsequently washed with 0.2 ml of 0.1 M-NaCl per sample. Two charcoal treatments, with even larger volumes of the charcoal suspension, did not remove all the ATP present. No solid-phase extraction was performed. Samples were dissolved in 2.2 ml of 2.5 mM-sodium acetate and between 40 and 90% of this volume was injected for h.p.l.c. analysis, with metal-dye detection, of inositol phosphates and 2,3-bisphosphoglycerate (2,3-BPG) [15]. A 0.5 cm $\times$ 20 cm column of Mono Q (Pharmacia) with a 0.5 cm $\times$ 5 cm guard column (Mono Q 5/5, Pharmacia) was employed with the acidic elution system (long column elution system I) described in [15]. The remaining fractions of the samples were employed for control analyses as described below, and between 1 and 5% of each sample was separately analysed for Fru(1,6)P₂, PP_i, and for higher concentrations of the compound trimetaphosphate (P₃i) (see the Results section). Fru(1,6)P₂ and PP_i were separated from inositol bisphosphates and other hexose bisphosphates by the above-specified h.p.l.c. elution system. The short HClO₄ extraction times and the omission of the harsh solid-phase extraction [15] ensured that these compounds, and the other acid-labile phosphocompounds that were identified (see the Results section), were not degraded until analysis. The average volume trapped in the KClO₄ pellets was determined in a number of samples by weighing these pellets wet and then after having dried them in an oven.

Data evaluation

All peaks of phosphorylated compounds of interest were quantified by applying non-linear numeric calibration techniques [15]. Calibration was carried out by injecting a series of standard mixtures containing all substances of interest in defined amounts (between 0.05 and 8 nmol). Data were corrected for the trapped volumes in the KClO₄ pellets. Statistical evaluation of the data was performed by Student's *t* test. Differences between treated muscles and contralateral controls were tested by Student's *t* test for paired samples. All controls and all muscles stimulated for the same periods of time were also tested by the *t* test for unpaired samples. The energy charge of *Xenopus* sartorius and tibialis anterior muscles was calculated from the measured concentrations of ATP, ADP and AMP, as: $([\text{ATP}] + [\text{ADP}]/2)/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$.

Analytical controls

Fractions of processed samples from larger muscle specimens (> 250 mg) were submitted either to a limited acid treatment to remove acid-labile compounds (see the Results section) and to assess the presence of inositol phosphates undergoing acid migration of their phosphate groups, or to treatment with alkaline phosphatase. For the acid treatment, samples were brought to 1 M with trichloroacetic acid and boiled for periods of either 7 or 14 min. After five ether extractions they were analysed as normal. For alkaline phosphatase treatment, samples of up to 1 ml were diluted to 3 ml with water, the pH was adjusted to 9.0 with Tris/base, and MgCl_2 and alkaline phosphatase (molecular biology grade) were added to final concentrations of 5 mM and 0.2 or 2 units/ml respectively. After 16 h at room temperature, the enzyme was precipitated by adding trichloroacetic acid to 1 M and the supernatants were ether-extracted five times and analysed as normal.

Chemicals

Chemicals and reference compounds used for sample processing and analysis were as described in [15]. All other chemicals were at least of analytical grade.

RESULTS

The total masses of all separated isomers of inositol bis-, tris-, tetrakis- and pentakis-phosphate, and those of InsP_6 , 2,3-BPG, $\text{Fru}(1,6)\text{P}_2$ and PP_i were analysed in intact skeletal muscles in the resting state and in muscles tetanically stimulated under isometric conditions (*Xenopus*) or under unloaded shortening (i.e. close to isotonic) conditions (rat). [Since all isomer analyses were not enantiomer-specific, the exclusive use of the abbreviations $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ for the corresponding pairs of D- and L-enantiomorphs does not necessarily imply that all of the corresponding isomers can be attributed to the D-enantiomers according to the relaxation of the I.U.P.A.C. rules. For other isomers, the two possible enantiomorphs, abbreviated in their D-form according to this relaxation of the rules, are both given in the text and are separated by a slash.] In *Xenopus sartorius* and tibialis anterior the concentrations of ATP, ADP, AMP and CrP were also determined (see the Materials and methods section) in order to monitor the energy metabolic state. Fig. 1 shows the protocol of stimulation and freeze-clamping of *Xenopus* tibialis anterior muscles as an example of the general experimental procedure. After lowering the chamber containing the oxygenated physiological solution, tetanic stimulation of a muscle was started. The activated muscles were quick-frozen at about 2 s, 10 s and 30 s after the onset of electrical stimulation (Fig. 1). Only the muscle material held between the jaws of the freeze-clamp was subjected to analysis by h.p.l.c. with metal-dye detection.

Figs. 2(a) and 2(b) show h.p.l.c. chromatograms of extracts from resting rat gastrocnemius and soleus respectively, whereas in Figs. 2(c) and 2(d) analyses from resting and tetanically activated *Xenopus sartorius* and tibialis anterior respectively are presented. The chromatograms reveal the spectrum of inositol phosphates (starting with InsP_2 isomers up to InsP_6), as well as $\text{Fru}(1,6)\text{P}_2$ (eluting after the group of inositol bisphosphates), PP_i , 2,3-BPG and some unidentified phosphocompounds (designated XP_3 , XP_4 , YP_4 and ZP_4 due to their apparent phosphate content). Since the compounds $\text{Ins}(1,4,5)\text{P}_3$ and XP_3 were eluted close together (see Fig. 2), evaluation of the two individual peak areas was done by hand. A subset of samples treated with acid before analysis (see the Materials and methods section), i.e. containing only the acid-stable inositol phosphates, was used to

Fig. 1. Force development after tetanic stimulation and freeze-clamping of *X. laevis* tibialis anterior muscle

At the time marked by 0 the bathing solution was lowered and at time point S direct electrical stimulation of a muscle was started. Freeze-clamping of a muscle F was carried out at 2 s (a), 10 s (b) and 30 s (c) after the onset of tetanic stimulation.

optimize this evaluation (see Fig. 3). XP_4 was eluted almost precisely at the position of $\text{Ins}(1,2,4,6)\text{P}_4$, YP_4 close to the position of $\text{Ins}(1,2,4,5)\text{P}_4$, and ZP_4 about 0.4 min after YP_4 . Acid treatment revealed that all of these additional phosphocompounds are highly acid-labile substances, each containing at least one pyrophosphate- or polyphosphate-like group (Fig. 3). Peak P_3 was tentatively assigned to trimetaphosphate because of its coelution with commercial trimetaphosphate. It has also been found in avian erythrocyte extracts, where it was isolated and demonstrated by n.m.r. to contain only phosphate (T. Radenberg & G. W. Mayr, unpublished work). Since upon acid hydrolysis two closely spaced peaks in the sugar bisphosphate region (Fig. 3) but none in the InsP_3 region increased, the basic (more acid-stable) structural moieties of the compounds XP_3 , XP_4 , YP_4 and ZP_4 appear to be sugar bisphosphates. The disappearance of all four compounds upon treatment with alkaline phosphatase (results not shown) indicated that each compound contains at least one phosphomonoester. The most likely structures therefore are sugar phosphate di(poly)- and tri(poly)-phosphates (PP-X-P and PPP-X-P). XP_3 was not identical to phosphoribosyl pyrophosphate. The acid treatment controls revealed that practically all of the material eluting at the positions of $\text{Ins}(1,2,4,6)\text{P}_4$ and $\text{Ins}(1,2,4,5)\text{P}_4$ was assignable to the acid-labile compounds XP_4 and YP_4 .

In *Xenopus sartorius* and tibialis anterior muscles we realized that, although the absolute concentrations of the compounds PP_i , P_3 , XP_3 , XP_4 and YP_4 were quite different (varying from about $0.5 \mu\text{mol/g}$ for PP_i to below 1 nmol/g for the compounds XP_4 , YP_4 and ZP_4 ; see Fig. 2), their rapid changes during contraction all paralleled the decreases in the concentration of CrP (see Fig. 5). In contrast, the decrease in ATP concentration (results not shown) and in the energy charge (see below) were much less pronounced. When the reneutralized muscle extracts were analysed for these compounds without a preceding lyophilization (carried out with portions of the samples from the same muscles; see the Materials and methods section), most of these compounds were absent, except for a small amount of PP_i . This indicates that all of the 'energy-rich' products were artificially produced by chemical *trans*-phosphorylation reactions between CrP and phosphate or sugar phosphates during or after lyophilization. Since this lyophilization is essential for decreasing the extract volume prior to charcoal treatment (see the Materials and methods section), a nearly complete hydrolysis of CrP before lyophilization was found to adequately prevent these reactions. Such removal of CrP is easily brought about by incubating the HClO_4 extracts for 30 min at room temperature before reneutralization.

Fig. 2. H.p.l.c. with metal-dye detection analysis of charcoal-treated skeletal muscle extracts from rat and *Xenopus*

Quick-frozen muscles were processed as described in the Materials and methods section. (a) Extract from 267 mg wet wt. of resting rat gastrocnemius. The upper trace is the metal-dye detection signal at 546 nm; the lower trace is the absorbance at 260 nm (same sensitivity as indicated for 546 nm), recorded prior to post-column derivatization [15]. (b) Extract from 205 mg wet wt. of resting rat soleus. (c) Extracts from 138 and 130 mg wet wt. respectively of resting (continuous line) and tetanically stimulated (broken line) *Xenopus* sartorius. (d) Extracts from 147 and 154 mg wet wt. respectively of resting (continuous line) and tetanically stimulated (broken line) *Xenopus* tibialis anterior. Tetanic stimulation in (c) and (d) was for 30 s. Letters on all chromatograms denote: a, ATP; b, Ins(1,3) P_2 ; c, Ins(1,4) P_2 ; d, GTP; e, Fru(1,6) P_2 ; f, UDP; g, PP $_i$; h, 2,3-BPG; i, UTP; j, Ins(1,3,4) P_3 ; k, Ins(1,4,5) P_3 ; l, XP_3 ; m, P_3 ; n, XP_4 ; o, YP_4 and ZP_4 (close together); p, Ins(1,3,4,5) P_4 ; q, Ins(1,4,5,6) P_4 /Ins(3,4,5,6) P_4 ; r, Ins(1,2,4,5,6) P_5 /Ins(2,3,4,5,6) P_5 ; s, Ins(1,3,4,5,6) P_5 ; t, Ins P_6 .

Nucleoside polyphosphates were almost completely removed before mass analysis (see the Materials and methods section and Fig. 2a), and interference of remaining small amounts with inositol phosphates was ruled out by routine u.v. monitoring prior to post-column derivatization. Because of the high concentrations of phosphate and sugar monophosphates in the extracts and the fluoride which was added to each sample as a stabilizer [15], no analysis of inositol monophosphates was performed.

Four of the true inositol phosphate peaks, namely those assigned to Ins(1,3) P_2 , Ins(1,4) P_2 , Ins(1,3,5) P_3 and Ins(1,4,5,6) P_4 , could not be unequivocally attributed to one isomer or pair of enantiomorphs. From standard analyses we know that Ins(1,5) P_2 , Ins(3,4) P_2 , Ins(2,4,6) P_3 and Ins(1,2,3,4,6) P_5 respectively are eluted at the same positions. The presence of large amounts of Ins(3,4) P_2 is unlikely, since its metabolic precursor, Ins(1,3,4) P_3 , is found in low concentrations in all muscle types under all conditions. The

Fig. 3. Acid treatment of extract from resting *Xenopus* tibialis anterior muscle

Extract corresponding to 900 mg wet wt. was pooled from eight samples and divided into three equal parts, one of which was left as a control (upper trace); the other two were treated with acid as described in the Materials and methods section for 7 min (middle trace) or 14 min (lower trace). Letters on peaks have the same meaning as in Fig. 2. Additional letters denote: u, Ins(3,4,5) P_3 ; v, Ins(1,2,4,5) P_4 ; w, Ins(2,3,4,5) P_4 ; x, Ins(2,4,5,6) P_4 . These extra peaks arise during acid treatment by acid migration of phosphate groups occurring in inositol phosphates with the 1-phosphomonoester group. Ins(2,4,5) P_3 is generated from Ins(1,4,5) P_3 together with Ins(3,4,5) P_3 (u) by phosphate migration. Its elution just before that of Ins(1,4,5) P_3 causes only a broadening of peak k. Peaks v and w are generated from p, peak x from q and peak r from s. Note the rapid disappearance of the acid-labile compounds marked e, g, l, m, n and o (cf. Fig. 2).

presence of high amounts of the other known dephosphorylation product of Ins(1,3,4) P_3 , i.e. Ins(1,3) P_2 , in some of the muscles (see below) is intriguing in this context. Therefore we cannot rule out at present the possibility that some of the Ins P_2 eluting at the position of Ins(1,3) P_2 is a different isomer, e.g. Ins(1,5) P_2 . Neither the presence of Ins(1,3,5) P_3 nor that of Ins(2,4,6) P_3 has been reported previously in animal cells. Since upon acid treatment in both cases compounds eluting just ahead of Ins(1,3,4) P_3 were generated, the true isomeric nature of this novel, less abundant, Ins P_3 isomer (below 0.2 μM) remains unclear. The presence of significant amounts of Ins(1,2,3,4,6) P_5 in the peak assigned to Ins(1,4,5,6) P_4 /Ins(3,4,5,6) P_4 could be ruled out by the rapid disappearance of this peak upon alkaline phosphatase treatment [Ins(1,2,3,4,6) P_5 is highly resistant to this treatment], and by the rapid formation of the same percentage of Ins(2,4,5,6) P_4 upon acid treatment (see Fig. 3) as is formed from pure Ins(1,4,5,6) P_4 [17]. Ins(1,2,3,4,6) P_5 does not undergo phosphate migration. The detection limits for inositol phosphates were about 10 pmol for isomers of Ins P_3 , and 5–10 pmol for Ins P_4 , Ins P_5 and Ins P_6 .

2,3-BPG, which is a completely acid-stable compound, was

found at resting concentrations of 17.3 ± 0.8 , 20.3 ± 1.2 and 21.2 ± 1.3 nmol/g wet wt. in *Xenopus* sartorius, tibialis and iliofibularis respectively, and of 52.0 ± 2.9 and 20.2 ± 0.6 nmol/g wet wt. in rat gastrocnemius and soleus respectively. These concentrations did not change significantly during tetanic contraction, and they are about an order of magnitude higher than those reported in perfused muscle [18]. Assuming a concentration of 3800 nmol of 2,3-BPG/g in blood [18], these masses might be mainly attributed to 0.5–1.5% contamination of the muscle tissues with blood.

Table 1 shows the masses (per g muscle wet wt.) of all assigned inositol phosphate isomers identified in concentrations above the detection limit, and the concentrations of Fru(1,6) P_2 . Data were accumulated from 13–17 control muscles for *Xenopus* sartorius, tibialis anterior and iliofibularis. Corresponding data after stimulation were from 2–7 muscles for each tetanus duration. In rats, data from 23 and 11 analyses of control gastrocnemius and soleus muscles respectively, and from 2–12 analyses after tetanic stimulation, were accumulated. Pairwise statistical evaluation of data from matched control and stimulated muscles (results not shown) did not reveal any more significant differences between these groups of muscles than those obtained by unpaired *t* test analysis. The data in Table 1 illustrate that in all muscles investigated the resting concentrations of Ins(1,4) P_2 , Ins(1,4,5) P_3 , Ins(1,3,4,5,6) P_5 and Ins P_6 did not vary very much. When corrected for the myoplasmic space of muscle by assuming a value of 0.58 ml/g muscle wet wt. [19], the concentrations are 3.5–6.9 μM , about 2.3 μM , about 1 μM , and 3.2–4.6 μM respectively for these isomers. However, in rat soleus the concentrations of Ins(1,4,5) P_3 and Ins P_6 were markedly lower (1.2 and 3.2 μM respectively). The second Ins P_2 isomer or group of Ins P_2 isomers, which was tentatively assigned to Ins(1,3) P_2 (see above), as well as Ins(1,3,4,5) P_4 and Ins(1,4,5,6) P_4 /Ins(3,4,5,6) P_4 showed greater variations in their resting levels between the different muscle types. Their sarcoplasmic concentrations ranged from 0.5 to 3.2 μM , 0.2 to 1.0 μM and 0.2 to 0.9 μM respectively. Ins(1,3,4,6) P_4 and its acid migration product Ins(1,2,4,6) P_4 were always below the detection limit in control samples subjected to limited acid treatment in order to eliminate the interfering compounds XP_4 , YP_4 and ZP_4 . This large compilation of inositol phosphate masses in resting muscles indicates no marked differences between species, but rather variations between muscle types. Surprisingly, the putative second messenger Ins(1,4,5) P_3 is always present above micromolar resting concentrations.

Tetanic stimulation of the skeletal muscles led to significant changes in the masses of some of the inositol phosphates. The time courses of the derived total sarcoplasmic concentrations of Ins(1,4,5) P_3 , Ins(1,4) P_2 , Ins(1,3,4,5) P_4 , Ins(1,3,4) P_3 and Ins(1,3) P_2 in *Xenopus* sartorius and tibialis as well as in rat gastrocnemius are depicted in Fig. 4. The total Ins(1,4) P_2 concentration approximately doubled within 10 s of the isometric tetanus in *Xenopus* sartorius and tibialis (Figs. 4a and 4b). The concentration of the Ins P_2 isomer tentatively assigned as Ins(1,3) P_2 showed a marked but transient increase in *Xenopus* sartorius, and a kinetically similar but much smaller transient in *Xenopus* tibialis anterior. In the former muscle, the rise in concentration from about 1.5 to 4.1 μM was the fastest change of any of the inositol phosphate concentrations measured. In the isotonically contracting rat muscles only a transient rise in Ins(1,4) P_2 , to above 11 μM , was observed in the gastrocnemius (Fig. 4c). In all muscles of the fast and mixed type, Ins(1,4,5) P_3 increased significantly during the tetanus with similar kinetics (Fig. 4), but in the slow rat soleus (Table 1) the Ins(1,4,5) P_3 concentration remained unchanged. Ins(1,3,4) P_3 exhibited transient increases within a few seconds in the isometrically contracting *Xenopus* muscles (up to 0.65 μM), but remained essentially unchanged in the isotonically con-

Table 1. Masses of inositol phosphates and Fru(1,6) P_2 in isolated skeletal muscles of *Xenopus laevis* and rat

Masses are given in nmol/g wet wt., and results are means $\pm$ S.E.M. for the numbers of individual muscles analysed (given in parentheses). Significant differences in stimulated muscles compared with controls (tested by Student's *t* test for $n > 2$ and by the *U* test for $n = 2$) are indicated by: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.005$, § $P < 0.001$. In cases of pairs of enantiomorphs, the isomer name (omitting the D or L prefix) following the IUPAC rule of lowest locants is given.

(a) *Xenopus sartorius*

Compound	Control	2 s tetanus	10 s tetanus	30 s tetanus
Ins(1,3) P_2	0.87 $\pm$ 0.17 (12)	2.42 $\pm$ 0.71 (3)†	0.75 $\pm$ 0.71 (2)	0.69 $\pm$ 0.39 (3)
Ins(1,4) P_2	2.36 $\pm$ 0.17 (15)	2.83 $\pm$ 0.49 (4)	3.50 $\pm$ 0.56 (4)	3.68 $\pm$ 0.55 (5)*
Fru(1,6) P_2	195 $\pm$ 7 (4)¶	1026 $\pm$ 287 (6)§	1168 $\pm$ 116 (5)§	1032 $\pm$ 80 (7)§
Ins(2,4,6) P_3 /Ins(1,3,5) P_3	0.05 $\pm$ 0.01 (12)	0.03 $\pm$ 0.03 (3)	0.03 $\pm$ 0.02 (2)	0.04 $\pm$ 0.04 (3)
Ins(1,3,4) P_3	0.07 $\pm$ 0.02 (12)	0.18 $\pm$ 0.02 (2)	0.10 $\pm$ 0.10 (2)	0.12 $\pm$ 0.08 (3)
Ins(1,4,5) P_3	1.21 $\pm$ 0.08 (17)	1.27 $\pm$ 0.26 (3)	1.81 $\pm$ 0.10 (5)†	1.92 $\pm$ 0.22 (5)†
Ins(1,3,4,5) P_4	0.12 $\pm$ 0.03 (14)	0.19 $\pm$ 0.04 (3)	0.14 $\pm$ 0.02 (3)	0.13 $\pm$ 0.07 (5)
Ins(1,2,5,6) P_4	0.05 $\pm$ 0.02 (12)	0.13 $\pm$ 0.01 (2)	< 0.02 (2)	< 0.02 (3)
Ins(1,4,5,6) P_4	0.17 $\pm$ 0.03 (12)	0.12 $\pm$ 0.04 (3)	0.11 $\pm$ 0.08 (2)	0.23 $\pm$ 0.14 (3)
Ins(1,2,4,5,6) P_5	0.14 $\pm$ 0.03 (12)	0.25 $\pm$ 0.12 (3)	0.11 $\pm$ 0.08 (2)	0.17 $\pm$ 0.15 (3)
Ins(1,3,4,5,6) P_5	0.59 $\pm$ 0.05 (12)	0.25 $\pm$ 0.11 (3)†	0.47 $\pm$ 0.11 (2)	0.70 $\pm$ 0.22 (3)
Ins P_6	2.44 $\pm$ 0.14 (12)	2.52 $\pm$ 0.53 (3)	3.08 $\pm$ 0.17 (2)	3.01 $\pm$ 0.74 (3)

(b) *Xenopus tibialis anterior*

Compound	Control	2 s tetanus	10 s tetanus	30 s tetanus
Ins(1,3) P_2	0.28 $\pm$ 0.05 (11)	0.39 $\pm$ 0.15 (5)	0.35 $\pm$ 0.11 (2)	0.36 $\pm$ 0.28 (2)
Ins(1,4) P_2	2.03 $\pm$ 0.17 (11)	2.18 $\pm$ 0.25 (7)	3.89 $\pm$ 0.73 (4)†	4.08 $\pm$ 0.30 (5)†
Fru(1,6) P_2	183 $\pm$ 14 (4)¶	386 $\pm$ 72 (9)†	886 $\pm$ 172 (6)†	1027 $\pm$ 103 (7)†
Ins(2,4,6) P_3 /Ins(1,3,5) P_3	0.13 $\pm$ 0.03 (11)	0.14 $\pm$ 0.05 (5)	0.12 $\pm$ 0.10 (2)	0.04 $\pm$ 0.04 (2)
Ins(1,3,4) P_3	0.18 $\pm$ 0.04 (11)	0.38 $\pm$ 0.19 (5)	0.12 $\pm$ 0.04 (2)	0.22 $\pm$ 0.08 (2)
Ins(1,4,5) P_3	1.25 $\pm$ 0.08 (15)	1.46 $\pm$ 0.11 (7)	1.75 $\pm$ 0.21 (4)*	1.83 $\pm$ 0.09 (6)†
Ins(1,3,4,5) P_4	0.19 $\pm$ 0.06 (13)	0.30 $\pm$ 0.10 (7)	0.12 $\pm$ 0.02 (3)	0.05 $\pm$ 0.02 (5)*
Ins(1,2,5,6) P_4	0.03 $\pm$ 0.02 (11)	0.03 $\pm$ 0.03 (3)	< 0.02 (2)	< 0.02 (2)
Ins(1,4,5,6) P_4	0.12 $\pm$ 0.02 (11)	0.06 $\pm$ 0.03 (4)	0.11 $\pm$ 0.03 (2)	0.07 $\pm$ 0.06 (2)
Ins(1,2,4,5,6) P_5	0.24 $\pm$ 0.08 (11)	0.19 $\pm$ 0.05 (5)	0.30 $\pm$ 0.24 (2)	0.22 $\pm$ 0.21 (2)
Ins(1,3,4,5,6) P_5	0.52 $\pm$ 0.09 (11)	0.63 $\pm$ 0.16 (5)	0.70 $\pm$ 0.24 (2)	0.34 $\pm$ 0.25 (2)
Ins P_6	2.49 $\pm$ 0.12 (11)	2.44 $\pm$ 0.41 (5)	2.01 $\pm$ 0.59 (2)	1.96 $\pm$ 0.57 (2)

(c) *Xenopus iliofibularis*

Compound	Control	20 s tetanus
Ins(1,3) P_2	0.79 $\pm$ 0.21 (12)	0.63 $\pm$ 0.34 (2)
Ins(1,4) P_2	2.69 $\pm$ 0.25 (12)	5.60 $\pm$ 0.51 (2)
Fru(1,6) P_2	525 $\pm$ 37 (10)	1030 $\pm$ 444 (2)
Ins(2,4,6) P_3 /Ins(1,3,5) P_3	0.10 $\pm$ 0.01 (12)	0.09 $\pm$ 0.04 (2)
Ins(1,3,4) P_3	0.13 $\pm$ 0.03 (12)	0.13 $\pm$ 0.13 (2)
Ins(1,4,5) P_3	1.46 $\pm$ 0.06 (14)	2.47 $\pm$ 0.04 (2)
Ins(1,3,4,5) P_4^b	0.12 $\pm$ 0.05 (12)	0.15 $\pm$ 0.07 (2)
Ins(1,2,5,6) P_4	0.03 $\pm$ 0.02 (12)	< 0.02 (2)
Ins(1,4,5,6) P_4	0.12 $\pm$ 0.03 (12)	0.13 $\pm$ 0.08 (2)
Ins(1,2,4,5,6) P_5	0.18 $\pm$ 0.06 (12)	0.29 $\pm$ 0.23 (2)
Ins(1,3,4,5,6) P_5	0.65 $\pm$ 0.08 (12)	0.71 $\pm$ 0.55 (2)
Ins P_6	2.91 $\pm$ 0.21 (12)	1.83 $\pm$ 0.25 (2)

(d) Rat gastrocnemius

Compound	Control	10 s tetanus	20 s tetanus	30 s tetanus
Ins(1,3) P_2	1.85 $\pm$ 0.14 (12)	1.69 (1)	1.41 $\pm$ 0.33 (6)	1.41 $\pm$ 0.31 (4)
Ins(1,4) P_2	4.07 $\pm$ 0.16 (12)	6.65 (1)	4.51 $\pm$ 0.83 (6)	4.47 $\pm$ 0.82 (4)
Fru(1,6) P_2	684 $\pm$ 71 (12)	1722 $\pm$ 76 (2)	1452 $\pm$ 202 (6)§	1528 $\pm$ 61 (4)§
Ins(2,4,6) P_3 /Ins(1,3,5) P_3	n.d.	n.d.	n.d.	n.d.
Ins(1,3,4) P_3	0.13 $\pm$ 0.04 (15)	0.17 $\pm$ 0.02 (2)	0.24 $\pm$ 0.15 (7)	0.20 $\pm$ 0.09 (5)
Ins(1,4,5) P_3	1.48 $\pm$ 0.07 (23)	2.02 $\pm$ 0.05 (3)*	2.12 $\pm$ 0.04 (11)§	2.18 $\pm$ 0.08 (8)§
Ins(1,3,4,5) P_4	0.59 $\pm$ 0.04 (23)	0.66 $\pm$ 0.06 (3)	0.50 $\pm$ 0.05 (12)	0.58 $\pm$ 0.04 (8)
Ins(1,2,5,6) P_4	0.06 $\pm$ 0.01 (12)	0.05 $\pm$ 0.05 (2)	0.04 $\pm$ 0.03 (6)	0.04 $\pm$ 0.03 (4)
Ins(1,4,5,6) P_4	0.24 $\pm$ 0.02 (23)	0.23 $\pm$ 0.05 (3)	0.17 $\pm$ 0.03 (11)*	0.18 $\pm$ 0.03 (8)
Ins(1,2,4,5,6) P_5	0.15 $\pm$ 0.02 (20)	0.14 $\pm$ 0.06 (3)	0.08 $\pm$ 0.02 (12)†	0.11 $\pm$ 0.02 (8)
Ins(1,3,4,5,6) P_5	0.48 $\pm$ 0.03 (23)	0.48 $\pm$ 0.05 (3)	0.43 $\pm$ 0.03 (12)	0.47 $\pm$ 0.04 (8)
Ins P_6	3.03 $\pm$ 0.19 (23)	3.07 $\pm$ 0.27 (3)	2.81 $\pm$ 0.16 (12)	3.35 $\pm$ 0.33 (8)

Table 1 (cont.)

(e) Rat soleus

Compound	Control	10 s tetanus	20 s tetanus	30 s tetanus
Ins(1,3) P_2	1.33 ± 0.13 (9)	1.22 ± 0.30 (2)	1.37 ± 0.11 (6)	1.34 ± 0.21 (4)
Ins(1,4) P_2	3.29 ± 0.20 (7)	2.74 ± 0.37 (2)	3.93 ± 0.34 (6)	3.14 ± 0.25 (4)
Fru(1,6) P_2	60.2 ± 2.2 (10)	60.7 ± 2.3 (2)	55.7 ± 3.9 (6)	55.1 ± 7.3 (4)
Ins(2,4,6) P_3 /Ins(1,3,5) P_3	< 0.03 (10)	< 0.03 (2)	< 0.03 (6)	< 0.03 (4)
Ins(1,3,4) P_3	0.13 ± 0.03 (10)	0.07 ± 0.03 (2)	0.10 ± 0.01 (6)	0.10 ± 0.02 (4)
Ins(1,4,5) P_3	0.69 ± 0.04 (9)	0.58 ± 0.02 (2)	0.71 ± 0.08 (6)	0.74 ± 0.11 (4)
Ins(1,3,4,5) P_4	0.17 ± 0.02 (11)	0.14 ± 0.03 (2)	0.16 ± 0.06 (4)	0.13 ± 0.02 (3)
Ins(1,2,5,6) P_4	< 0.03 (10)	< 0.03 (2)	< 0.03 (6)	< 0.03 (4)
Ins(1,4,5,6) P_4	0.50 ± 0.02 (9)	0.38 ± 0.14 (2)	0.46 ± 0.04 (6)	0.48 ± 0.02 (4)
Ins(1,2,4,5,6) P_5	< 0.02 (10)	< 0.02 (2)	< 0.02 (6)	< 0.02 (4)
Ins(1,3,4,5,6) P_5	0.65 ± 0.02 (11)	0.58 ± 0.07 (2)	0.52 ± 0.05 (6)†	0.46 ± 0.05 (4)§
Ins P_6	1.85 ± 0.14 (9)	1.53 ± 0.58 (2)	1.54 ± 0.16 (6)	1.75 ± 0.13 (4)

|| Only the average of a group of muscles with lowest resting values is given (see text).

Fig. 4. Time courses of the total sarcoplasmic inositol phosphate concentrations in tetanically stimulated *Xenopus* and rat muscles

Micromolar sarcoplasmic concentrations were obtained by multiplying the masses in nmol/g wet wt. by a factor of 1.7 [19]. (a) *Xenopus* sartorius, (b) *Xenopus* tibialis anterior (c) rat gastrocnemius. ●, Ins(1,4,5) P_3 ; ○, Ins(1,4) P_2 ; □, Ins(1,3) P_2 ; ■, Ins(1,3,4) P_3 ; △, Ins(1,3,4,5) P_4 . Data are represented as means ± S.E.M.

Fig. 5. Changes of the total sarcoplasmic concentrations of Fru(1,6) P_2 (○, ●) and CrP (□, ■) during tetanic stimulation in *Xenopus* muscles

Concentration in sartorius (●, ■, ▲) and tibialis anterior (○, □, △) muscles are shown. Sarcoplasmic concentrations were derived as described. Data are means ± S.E.M. The single triangles represent average Fru(1,6) P_2 concentrations in corresponding metabolically stressed resting muscles (see the text).

tracting rat muscles (Fig. 4 and Table 1). The rapid concomitant increase in Ins(1,3) P_2 in the former muscles might identify this isomer as a likely dephosphorylation product of Ins(1,3,4) P_3 . Ins(1,3,4,5) P_4 , the likely precursor of the latter inositol phosphates, in fact shows very similar transient increases in the *Xenopus* muscles (Figs. 4a and 4b), but not in rat muscles (Fig. 4c, Table 1).

The other isomers detected, Ins(2,4,6) P_3 /Ins(1,3,5) P_3 , Ins(1,4,5,6) P_4 /Ins(3,4,5,6) P_4 , Ins(2,3,4,5) P_4 /Ins(1,2,5,6) P_4 , Ins(1,3,4,5,6) P_5 , Ins(1,2,4,5,6) P_5 /Ins(2,3,4,5,6) P_5 and Ins P_6 , did not change significantly or decreased during the course of a tetanus (Table 1). They do not appear to be involved in the essential regulation of muscle function in the observed time domain. Their functional roles in other animals cells are largely unknown [20].

Several observations indicate interference between inositol phosphates and the key glycolytic compound Fru(1,6) P_2 via the high-affinity inositol phosphate binding protein aldolase [12,13] and the glycolytic key enzyme phosphofructokinase (PFK) [21], as well as interference of this and other bisphosphorylated glycolytic intermediates with Ins(1,4,5) P_3 5-phosphomonoesterase [22]. Therefore we have also measured the masses of Fru(1,6) P_2 and, as indicators of the energy state of the muscles, those of ATP, ADP, AMP (results not shown) and CrP (Fig. 5) under resting and tetanically activated conditions. In the resting

Fig. 6. Influence of the binding of Fru(1,6) P_2 to aldolase on the free concentrations of Ins(1,4,5) P_3 and Ins(1,4) P_2 .

The time courses of the changes of these compounds were modelled for *Xenopus sartorius* (a–c) and *tibialis anterior* (d–f). The total sarcoplasmic concentrations derived as described [19] were approximated by single exponentials of the type $y(t) = y_0(1 - e^{-\alpha t}) + C$, yielding the time courses of Fru(1,6) P_2 , Ins(1,4,5) P_3 and Ins(1,4) P_2 (broken lines). The free sarcoplasmic concentrations (continuous lines) were computed by solving the following system of conservation equations using the Nelder–Meade simplex algorithm:

$$\begin{aligned} \text{FBP}_{\text{tot.}}(t) &= \text{FBP}_{\text{free}}(t) + \text{FBP}_{\text{free}}(t) \cdot K_{\text{FBP}} \cdot \text{ALD}/N(t) \\ \text{IP3}_{\text{tot.}}(t) &= \text{IP3}_{\text{free}}(t) + \text{IP3}_{\text{free}}(t) \cdot K_{\text{IP3}} \cdot \text{ALD}/N(t) \\ \text{IP2}_{\text{tot.}}(t) &= \text{IP2}_{\text{free}}(t) + \text{IP2}_{\text{free}}(t) \cdot K_{\text{IP2}} \cdot \text{ALD}/N(t) \end{aligned}$$

where $N(t)$ is given by:

$$N(t) = 1 + \text{FBP}_{\text{free}}(t) \cdot K_{\text{FBP}} + \text{IP3}_{\text{free}}(t) \cdot K_{\text{IP3}} + \text{IP2}_{\text{free}}(t) \cdot K_{\text{IP2}}$$

FBP_{tot.}(t), IP3_{tot.}(t) and IP2_{tot.}(t) represent the total sarcoplasmic concentrations of Fru(1,6) P_2 , Ins(1,4,5) P_3 and Ins(1,4) P_2 respectively at a given time t , and FBP_{free}(t), IP3_{free}(t) and IP2_{free}(t) are the corresponding free sarcoplasmic concentrations. Values of $K_{\text{FBP}} = 10^5 \text{ M}^{-1}$, $K_{\text{IP3}} = 1.7 \times 10^5 \text{ M}^{-1}$ and $K_{\text{IP2}} = 2.1 \times 10^4 \text{ M}^{-1}$, which are the apparent affinity constants of aldolase for Fru(1,6) P_2 , Ins(1,4,5) P_3 and Ins(1,4) P_2 respectively, were taken from [12]. For the total concentration of aldolase sites in skeletal muscle (ALD) a value of 810 μM was used [37,38].

fast and mixed-type muscles, the average mass of Fru(1,6) P_2 was significantly higher than in rat soleus (Table 1). It very likely varies with the metabolic state of the muscles prior to analysis. Muscles which took more time to dissect, which were re-oxygenated more briefly or which were subjected to single twitches prior to tetanic stimulation showed higher Fru(1,6) P_2 concentrations. The single triangles in Fig. 5 indicate the elevated mean resting Fru(1,6) P_2 concentrations in these muscles (sartorius 459 nmol/g wet wt., tibialis anterior 367 nmol/g wet wt.) as compared with muscles prepared quickly (within 5 min), well-oxygenated (more than 30 min) and untwitched prior to tetanic stimulation (Table 1). It is noteworthy that the total concentrations of inositol phosphates did not vary significantly between these subgroups. During tetanic contraction the mass of Fru(1,6) P_2 increased significantly in the former muscles but remained unchanged in rat soleus. Both the rise in Fru(1,6) P_2 and the

decrease in CrP were half-maximal within 2 s in *Xenopus sartorius* and about 6 s in *Xenopus tibialis anterior* (Fig. 5). However, the energy charges of these muscles calculated from the concentrations of ATP, ADP and AMP determined essentially did not decrease during these periods (sartorius: 0.88 at rest, 0.86 at 2 s, 0.83 at 10 s, 0.84 at 30 s; tibialis anterior: 0.90 at rest, 0.89 at 2 s, 0.87 at 10 s, 0.87 at 30 s).

DISCUSSION

Although killing the animals might have led to mass changes of inositol phosphates due to hormonal stress, the inositol phosphate concentrations in non-stimulated muscles are assumed to represent resting values *in vivo*. The spectrum of inositol phosphate isomers found in resting skeletal muscles of *Xenopus* and rat (Table 1) shows some differences from the spectrum of these substances in non-stimulated cultured cells ([17,20,33] G. W. Mayr, unpublished work). The apparent absence of Ins(1,3,4,6) P_4 in all muscles investigated, the high levels of the putative isomer Ins(1,3) P_2 in several muscle types, and the low concentrations of Ins(1,4,5,6) P_4 /Ins(3,4,5,6) P_4 , Ins(1,3,4,5,6) P_5 and Ins P_6 are the most prominent differences from cultured or isolated cells [20,23] and parenchymal tissues (G. W. Mayr, unpublished work). In contrast, the resting values of Ins(1,4,5) P_3 and Ins(1,4) P_2 were unexpectedly high, ranging from about 1.2 to 2.5 μM and 3.5 to 6.9 μM respectively. Upon tetanic stimulation, the latter two inositol phosphates exhibited significant increases in concentration in all muscles investigated except for the rat soleus (Fig. 4 and Table 1) within about 10 s. This shows clearly that PIC of fast and mixed-type skeletal muscle is activated during tetanic stimulation. A recent paper by Lagos & Vergara [24] reached the same conclusion from analysis of radiolabel in the phosphoinositides. Upon tetanic stimulation (50 Hz) of sartorii and semitendinosi from the bullfrog (*Rana catesbeiana*) prelabelled for 2 h with [^3H]inositol or [^{32}P]P_i, the authors observed a substantial decrease in radiolabel in PtdIns P_2 and in PtdIns P after 2 and 8 s of tetanus. Although a putative voltage sensor of the T-tubule membrane is involved in the process of ECC[25], at present there is no evidence for activation of PIC by this protein or any other receptor-coupling mechanism. Activation of PIC in isolated triads by Ca^{2+} [6] and a recent finding of a Ca^{2+} -activated PIC in bovine rod outer segments [26] argue for this activation mechanism of PIC in muscle, although the alternative, i.e. activation by increased substrate access, cannot be ruled out. It has been shown that Ca^{2+} influx through the T-tubular membrane is not necessary for initiation of Ca^{2+} release from the SR [27]. Consequently, a Ca^{2+} activation of skeletal muscle PIC would imply that this process does not play an essential role in the key mechanism of ECC. The recent report of an opening of T-tubular dihydropyridine-sensitive Ca^{2+} channels by Ins(1,4,5) P_3 [28] would in fact point to a downstream effect of increasing Ins(1,4,5) P_3 in skeletal muscle. An activation of PIC by increased substrate access would require a preceding mechanism deprotecting unaccessible PtdIns(4,5) P_2 , i.e. it would also be a secondary event.

The sum of the initial rates of increase of Ins(1,4) P_2 , Ins(1,4,5) P_3 , and, if rising, of Ins(1,3,4,5) P_4 , Ins(1,3,4) P_3 and Ins(1,3) P_2 , can be used to estimate the PIC activity of tetanically stimulated skeletal muscle. In *Xenopus sartorius* and *tibialis anterior* these isomers increased at maximal rates of about 2.6 and 1.7 $\mu\text{M/s}$ (i.e. 92 and 61 nmol/min per g of muscle) respectively. These rates represent PIC activities *in vivo* for PtdIns(4,5) P_2 hydrolysis when the enzyme releases no Ins(1,4) P_2 from PtdIns4 P or exhibits equal activities for both substrates. These activities are 3–5-fold higher than the PIC activity of rabbit skeletal muscle microsomes *in vitro* (25 nmol/min per mg of microsomal protein

[6], which corresponds to about 20 nmol/min per g of muscle). From the initial rates of increase of $\text{Ins}(1,4)P_2$, an estimate of the minimal activity of $\text{Ins}(1,4,5)P_3$ 5-phosphatase *in vivo* can be made, assuming that no $\text{Ins}(1,4)P_2$ is released from $\text{PtdIns}4P$. The derived activities in *Xenopus* sartorius and tibialis anterior of about 0.5 $\mu\text{M/s}$ (i.e. 18 nmol/min per g of muscle) are in good agreement with the activities determined in isolated triads [29] and T-tubule membranes [30,31]. According to these rate comparisons, our experimental time resolution appears to cope with the maximal rates of inositol phosphate release and metabolism in skeletal muscle. If assuming that all $\text{Ins}P_2$, $\text{Ins}P_3$ and $\text{Ins}P_4$ isomers increasing during tetanic stimulation are originally derived from $\text{PtdIns}(4,5)P_2$ present prior to stimulation, the mass of this precursor should at least equal or, since it is likely that $\text{Ins}P_1$ also increases, in fact exceed the sum of these mass increases. In fact, the mass determined for $\text{PtdIns}P_2$ in bullfrog muscles is 28 ± 11 nmol/g wet wt. [24], i.e. about three times the sum of these increases.

A recent abstract has reported significant transient increases in $\text{Ins}(1,4,5)P_3$ during repetitive single twitches (1.5 Hz) in rat diaphragm [32]. These preliminary data might explain our continuous rises in $\text{Ins}(1,4,5)P_3$ during tetanic stimulation (30 Hz) as fused $\text{Ins}(1,4,5)P_3$ transients. $\text{Ins}(1,4,5)P_3$ in skeletal muscle has been suggested controversially in several reports [5,7,8,11,31] to function as a chemical transmitter of ECC, inducing Ca^{2+} release from the SR by binding to a putative receptor. In fact, the skeletal muscle ryanodine receptor, which appears to be the Ca^{2+} -release channel of the SR [10], shows some sequence similarity to the brain $\text{Ins}(1,4,5)P_3$ receptor [33], but does not show high-affinity binding of $\text{Ins}(1,4,5)P_3$ [34,35]. Despite this inconsistency and despite the lack of direct coupling of PIC to membrane depolarization (above) we have tested our data for compatibility with such a model. The net rate of production of $\text{Ins}(1,4,5)P_3$ should then correlate with a lag of about 2 ms between T-tubule depolarization and SR Ca^{2+} release [36]. Provided that the K_d *in vivo* of a putative receptor in the junctional gap is of the order of 0.1 μM [35], this concentration at least should be attained within the lag time. A minimal PIC activity of 50 $\mu\text{M/s}$ would then be required in the junctional gap, which is about 19–29 times the overall PIC activity estimated for *Xenopus* sartorius and tibialis anterior (see above). Thus, even if the entire PIC activity of skeletal muscle were concentrated in the junctional face of the T-tubules, it would be unlikely to suffice for ECC by such a mechanism [i.e. $\text{Ins}(1,4,5)P_3$ production]. Furthermore, the fact that bound aldolase (50 pmol of aldolase tetramer/mg of triad protein [13]) will function as an $\text{Ins}(1,4,5)P_3$ -buffering protein in the triadic gap (see below) makes it even more unlikely that $\text{Ins}(1,4,5)P_3$ functions as such a chemical transmitter.

The relatively low resting values of $\text{Ins}(1,3,4,5)P_4$ despite the high concentrations of $\text{Ins}(1,4,5)P_3$ indicate a low Ca^{2+} -independent $\text{Ins}(1,4,5)P_3$ 3-kinase activity. Since only small transient increases in $\text{Ins}(1,3,4,5)P_4$ occur during isometric tetani in *Xenopus* muscles, with subsequent decreases, one is tempted to make a similar assumption also for the Ca^{2+} -activated activity. However, the rapid appearance of the dephosphorylation products $\text{Ins}(1,3,4)P_3$ and $\text{Ins}(1,3)P_2$ in these muscles argues for a certain Ca^{2+} -activation of this enzyme which may be obscured by rapid dephosphorylation of its product $\text{Ins}(1,3,4,5)P_4$, i.e. by rapid turnover. Such rapid dephosphorylation may be due to increased phosphatase susceptibility (discussed below) and might explain why the dephosphorylation products increase only transiently.

Due to the reported binding of inositol phosphates to aldolase, an abundant enzyme in muscle [12,13], the determined concentrations of these compounds (Fig. 4) will not reflect their free sarcoplasmic concentrations. By competitive binding to aldolase,

the increase in $\text{Fru}(1,6)P_2$ during contraction (Fig. 5) will dramatically influence the free concentrations of $\text{Ins}(1,4,5)P_3$ and other inositol phosphates that bind to aldolase [12]. This is exemplified in Fig. 6 for *Xenopus* sartorius and tibialis anterior. In the resting state the binding capacity of aldolase for inositol phosphates (one binding site per subunit [12]) will decrease the free sarcoplasmic $\text{Ins}(1,4,5)P_3$ concentration to about 24 nM, a concentration insufficient to activate $\text{Ins}P_3$ -sensitive Ca^{2+} channels *in vivo* [3]. Due to the marked increase in $\text{Fru}(1,6)P_2$ concentration during a tetanus, the increase relative to resting concentrations will be much larger for free sarcoplasmic $\text{Ins}(1,4,5)P_3$ than for total $\text{Ins}(1,4,5)P_3$ (compare the broken and continuous lines in each panel of Fig. 6). As a result, in *Xenopus* sartorius the 1.7-fold increase in total $\text{Ins}(1,4,5)P_3$ will lead to a 60-fold increase in free $\text{Ins}(1,4,5)P_3$. This phenomenon will be also valid for $\text{Ins}(1,4)P_2$ (Fig. 6) and other inositol phosphates binding to aldolase [12,13], even if their total concentrations are unchanged or decreasing. Under conditions where the $\text{Fru}(1,6)P_2$ concentration rises very quickly, as is the case in the purely glycolytic *Xenopus* sartorius (Fig. 5, Figs. 6a–6c), the maximum rates of increase of the free inositol phosphate concentrations will also be markedly higher than those of the total concentrations. In this muscle, half-times for the free inositol phosphates are thus 10-fold smaller (less than 1 s) than those for the total inositol phosphates. They will be even smaller than those reported for agonist-stimulated increases in $\text{Ins}(1,4,5)P_3$ in most cells [3].

In oxidative muscles such as the rat soleus, where $\text{Fru}(1,6)P_2$ does not increase during contraction (see Table 1), the phenomenon described above will not occur. However, in fast and mixed-type muscles a coupling of inositol phosphate changes to changes in glycolytic activity, i.e. in energy metabolism, will take place. Although apparently not fast enough to cope with the requirements of ECC (see above), these free inositol phosphate changes might be suitable for modulation of processes in tetanically contracting muscle in a time domain of seconds, i.e. long before significant changes in energy charge occur. A similar coupling might occur in other cells with high glycolytic capacity, e.g. erythrocytes. Another aspect of this interplay in muscle is that each activation of glycolysis by muscle contraction should increase the susceptibility of inositol phosphates to phosphomonoesterases by mass action. The decrease during tetanic contraction (see the Results section) in the concentrations of a number of inositol phosphates also known to bind to aldolase [12,13] would agree with this proposal. The observed inhibition of the $\text{Ins}(1,4,5)P_3$ 5-phosphatase activity by $\text{Fru}(1,6)P_2$ [22] might counteract this effect to a certain extent in the second messenger $\text{Ins}(1,4,5)P_3$.

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