PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Inositol Trisphosphate, Calcium and Muscle Contraction [and Discussion]

A. P. Somlyo, J. W. Walker, Y. E. Goldman, D. R. Trentham, S. Kobayashi, T. Kitazawa, A. V. Somlyo and I. C. H. Smith

Phil. Trans. R. Soc. Lond. B 1988 **320**, 399-414
doi: 10.1098/rstb.1988.0084

Phil. Trans. R. Soc. Lond. B **320**, 399–414 (1988)
Printed in Great Britain

Inositol trisphosphate, calcium and muscle contraction

By A. P. Somlyo[1], J. W. Walker[2], Y. E. Goldman[1], D. R. Trentham[2], F.R.S., S. Kobayashi[1], T. Kitazawa[1] and A. V. Somlyo[1]

[1] *Pennsylvania Muscle Institute and Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania* 19104–6083, *U.S.A.*

[2] *National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.*

The identity of organelles storing intracellular calcium and the role of Ins(1,4,5)P_3 in muscle have been explored with, respectively, electron probe X-ray microanalysis (EPMA) and laser photolysis of 'caged' compounds. The participation of G-protein(s) in the release of intracellular Ca^{2+} was determined in saponin-permeabilized smooth muscle.

The sarcoplasmic reticulum (SR) is identified as the major source of activator Ca^{2+} in both smooth and striated muscle; similar (EPMA) studies suggest that the endoplasmic reticulum is the major Ca^{2+} storage site in non-muscle cells. In none of the cell types did mitochondria play a significant, physiological role in the regulation of cytoplasmic Ca^{2+}.

The latency of guinea pig portal vein smooth muscle contraction following photolytic release of phenylephrine, an α_1-agonist, is 1.5 ± 0.26 s at 20 °C and 0.6 ± 0.18 s at 30 °C; the latency of contraction after photolytic release of Ins(1,4,5)P_3 from caged Ins(1,4,5)P_3 is 0.5 ± 0.12 s at 20 °C. The long latency of α_1-adrenergic Ca^{2+} release and its temperature dependence are consistent with a process mediated by G-protein-coupled activation of phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) hydrolysis.

GTPγS, a non-hydrolysable analogue of GTP, causes Ca^{2+} release and contraction in permeabilized smooth muscle. Ins(1,4,5)P_3 has an additive effect during the late, but not the early, phase of GTPγS action, and GTPγS can cause Ca^{2+} release and contraction of permeabilized smooth muscles refractory to Ins(1,4,5)P_3. These results suggest that activation of G protein(s) can release Ca^{2+} by, at least, two G-protein-regulated mechanisms: one mediated by Ins(1,4,5)P_3 and the other Ins(1,4,5)P_3-independent.

The low Ins(1,4,5)P_3 5-phosphatase activity and the slow time-course (seconds) of the contractile response to Ins(1,4,5)P_3 released with laser flash photolysis from caged Ins(1,4,5)P_3 in frog skeletal muscle suggest that Ins(1,4,5)P_3 is unlikely to be the physiological messenger of excitation–contraction coupling of striated muscle. In contrast, in smooth muscle the high Ins(1,4,5)P_3-5-phosphatase activity and the rate of force development after photolytic release of Ins(1,4,5)P_3 are compatible with a physiological role of Ins(1,4,5)P_3 as a messenger of pharmacomechanical coupling.

Introduction

Muscle contraction can be triggered by electromechanical coupling initiated by depolarization of the surface membrane leading to an increase in cytoplasmic free Ca^{2+}, as well as by 'pharmacomechanical coupling' (Somlyo & Somlyo 1968), a mechanism that is not regulated by the surface membrane potential changes. Both electromechanical and pharmacomechanical coupling operate primarily through the modulation of cytoplasmic free Ca^{2+}, although Ca^{2+}-independent regulation may also play a role, particularly in cardiac and in smooth muscles (see, for example, Nishikawa *et al.* 1984; Pfitzer *et al.* 1985).

Cytoplasmic Ca^{2+} can be increased by Ca^{2+} influx through voltage- or ligand-gated (Somlyo & Somlyo 1971; Benham & Tsien 1987) channels, as well as through the electromechanical or pharmacomechanical release of Ca^{2+} from an intracellular storage site (table 1). Consequently, the mechanisms of release and the structural identity of the intracellular organelles responsible for the storage and release of Ca^{2+} have been central questions of excitation–contraction coupling.

Table 1. Excitation–contraction coupling

(1) electromechanical coupling: voltage-controlled
  (*a*) release of intracellular, stored Ca^{2+}
  (*b*) Ca^{2+}-influx through voltage-gated channels

(2) pharmacomechanical coupling: voltage-independent
  (*a*) Ca^{2+} release by messenger: Ins(1,4,5)P_3
  (*b*) Ca^{2+} influx through ligand-gated channels
  (*c*) other (e.g. inhibition through phosphorylation of myosin light chain kinase by kinase A)

The primary role of sarcoplasmic and endoplasmic reticulum in Ca^{2+} regulation

The primary role of the sarcoplasmic reticulum (SR) in the regulation of Ca^{2+} in skeletal muscle has been generally accepted (Martonosi 1984; Luttgau & Stephenson 1986; Endo 1985), and recent studies also indicate that the SR has a similarly dominant role in mammalian cardiac (Fabiato 1985) and in smooth muscle (see below and A. P. Somlyo (1985)). The regions of the SR (terminal cisternae or junctional SR) serving as the major store of releasable intracellular Ca^{2+} are separated from the surface membrane by a 12–18 nm gap, traversed by irregularly periodic electron densities; 'feet' or 'bridging structures' (Franzini-Armstrong 1986; A. V. Somlyo 1979). The question then is: what is the message that is transmitted from the surface membrane to the SR, causing it to release Ca^{2+}?

Functional studies have shown that the amount of intracellular Ca^{2+} stored is sufficient in both striated (see, for example, Armstrong *et al.* 1972) and in smooth muscle (Bond *et al.* 1984*a*) to activate a maximal contraction. The rise in cytoplasmic Ca^{2+} after excitation has been characterized with Ca^{2+}-sensitive indicators (e.g. for striated muscle see Blinks (1986); Baylor & Hollingworth (1988); Maylie *et al.* (1987); cardiac muscle: Fabiato (1985); smooth muscle: DeFeo & Morgan (1985); Fay *et al.* (1979); Himpens & Somlyo (1988); Rembold & Murphy (1986)), and the SR has been identified, with electron probe X-ray microanalysis of rapidly frozen muscles (see, for example, A. V. Somlyo *et al.* 1981, 1985*b*; Bond *et al.* 1984*a*; Kowarski *et al.* 1985), as the principal source of released Ca^{2+}.

The terminal cisternae (TC) of frog skeletal muscle contain approximately 120 mmol Ca kg^{-1} dry mass, 60% of which is released during a 1.2 s tetanus (A. V. Somlyo *et al.* 1981, 1985*b*). The large quantity of Ca^{2+} released is consistent with the high concentration (0.35 mM) of the soluble cytoplasmic calcium binding protein, parvalbumin, in frog muscle (Gosselin-Rey & Gerday 1977), in addition to the regulatory protein, troponin. The Ca^{2+} content is significantly lower (about 10–50 mmol kg^{-1} dry SR) in the SR of cardiac (Wheeler-Clark & Tormey 1987; Jorgensen *et al.* 1988) and smooth muscles (Bond *et al.* 1984*a*, Kowarski *et al.* 1985) that do not contain parvalbumin.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

In smooth muscle, Ca^{2+} can be released from the SR both in normally polarized (Kowarski *et al.* 1985) and in depolarized (Bond *et al.* 1984*a*; Himpens & Somlyo 1988) preparations, in the presence and in the absence of extracellular Ca^{2+}, providing direct evidence for the suggestion (A. P. Somlyo *et al.* 1971; Devine *et al.* 1972) that the release of Ca^{2+} from the SR is a major mechanism of pharmacomechanical coupling. These and other experiments in the literature (reviewed in A. P. Somlyo (1985)) indicate that intracellularly stored Ca^{2+} is sufficient to activate smooth muscle contraction, but do not answer the question whether, under physiological conditions (in the presence of normal extracellular Ca^{2+}), the influx of extracellular Ca^{2+} makes a major contribution to activator Ca^{2+}. The amount of Ca^{2+} current carried by an action potential is insufficient to supply the total Ca^{2+} required to activate contraction (Johansson & Somlyo 1980; Bond *et al.* 1984*b*). Contraction of many, though not all (see, for example, Bozler 1969; Devine *et al.* 1972) smooth muscles is abolished in Ca^{2+}-free solution, and total cellular calcium significantly increases during maintained depolarization (see, for example, Bond *et al.* 1984*b*). These findings are frequently considered as evidence of a major role of influx in contractile activation (Van Breemen *et al.* 1986; Ratz & Murphy 1987). Nevertheless, Ca^{2+}-free solutions could also inhibit contraction through the loss of calcium from the SR or through uncoupling of excitation from contraction. For example, in view of the role of *myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3, see below) in pharmacomechanical coupling in smooth muscle, inhibition of phospholipase C activity could lead to the uncoupling of agonist-induced contractions. The activity of phospholipase C is reduced at low free Ca^{2+} (less than 100 nM) levels (Sasaguri *et al.* 1985; Mallows & Bolton 1987; Rapoport 1987; Roth 1987) that are in the range of free cytoplasmic Ca^{2+} in some smooth muscle cells placed in Ca-free solutions (Himpens & Somlyo 1988), although Ins(1,4,5)P_3 production may persist in the presence of Ca^{2+} entry blockers that inhibit contraction (Best *et al.* 1985).

Mitochondria, as is now generally agreed (A. P. Somlyo *et al.* 1987; Carafoli 1987), do not play a significant role in the physiological regulation of cytoplasmic Ca^{2+}, although under pathological conditions they are capable of massive and reversible calcium accumulation (Broderick & Somlyo 1987). This may be a protective mechanism preventing cell death or, alternatively, mitochondria may become initial sites of cellular calcification. The endogenous calcium content of mitochondria in normal, intact cells, muscle and nonmuscle, is low (approximately 0.5–3 nmol mg^{-1} mitochondrial protein), and is not measurably increased even during a tetanus in skeletal muscle (A. V. Somlyo *et al.* 1981), prolonged (30 min) contracture of smooth muscle (A. P. Somlyo *et al.* 1979; Bond *et al.* 1984*b*) or hormonal stimulation of liver (Bond *et al.* 1987). Mitochondrial Ca^{2+} increases when cytoplasmic Ca^{2+} rises to abnormally high levels in injured cells or, even in frog striated muscle, under the influence of caffeine (Yoshioka & Somlyo 1984). Obviously, such mitochondrial calcium accumulation in injured or caffeine treated (skeletal or cardiac) muscle does not constitute evidence of a physiological role of mitochondria in Ca^{2+} regulation.

The SR is developmentally related to the endoplasmic reticulum (ER) found in all eukaryotic cells. Therefore, it seems appropriate to note that, like the SR in muscle, the ER appears to be the primary organelle regulating cytoplasmic Ca^{2+} in non-muscle cells (for review see A. P. Somlyo (1984)). It is not surprising, therefore, that a messenger that releases Ca^{2+} from the ER (Streb *et al.* 1983; Berridge & Irvine 1984) has similar effects on the SR of at least some types of muscle (e.g. smooth muscle).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Smooth muscle: the role of Ins(1,4,5)P_3, G-protein mediated Ca^{2+} release and Ins(1,4,5)P_3 5-phosphatase

The major physiological role of Ins(1,4,5)P_3 as the mediator of pharmacomechanical Ca^{2+} release in smooth muscle is supported by a continuously growing body of evidence, and is, by now, the best understood mechanism of excitation–contraction coupling. Stimulation of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) hydrolysis and/or other evidence of Ins(1,4,5)P_3 production in smooth muscle (reviewed in Abdel-Latif 1986) has been demonstrated in response to cholinergic (Abdel-Latif *et al.* 1987; Baron *et al.* 1984; Duncan *et al.* 1987; Mallows & Bolton 1987; Takuwa *et al.* 1986; Ueno *et al.* 1987) and α_1-adrenergic agents (Berta *et al.* 1986; Fox *et al.* 1985; Rapoport 1987), histamine, substance P (Mallows & Bolton 1987), serotonin (Berta *et al.* 1986), ATP (Phaneuf *et al.* 1987), angiotensin II and vasopressin (Nabika *et al.* 1985). The rate of agonist-stimulated hydrolysis of PtdIns(4,5)P_2 is rapid: an increase in Ins(1,4,5)P_3 has been demonstrated as early as 1 s after stimulus (Duncan *et al.* 1987).

Ins(1,4,5)P_3 can activate contraction (see, for example, A. V. Somlyo *et al.* 1985*a*; Bitar *et al.* 1986; Walker *et al.* 1987) of smooth muscle by releasing Ca^{2+} from an intracellular, non-mitochondrial store (see, for example, Suematsu *et al.* 1984; A. V. Somlyo *et al.* 1985*a*; Iino 1987; Saida & Van Breemen 1987; Smith *et al.* 1985). Because α_1-adrenergic stimulation causes Ins(1,4,5)P_3 production and also releases Ca^{2+} from the SR (Bond *et al.* 1984*a*; Kowarski *et al.* 1985), we conclude that the SR is the major source of Ins(1,4,5)P_3-releasable Ca^{2+}.

G-*protein mediated* Ca^{2+} *release:* Ins(1,4,5)P_3-*dependent and independent*

G protein(s) couple receptors to PtdIns(4,5)P_2 hydrolysis in non-muscle cells (Litosch & Fain 1986) and, probably, also in smooth muscle: the non-hydrolysable analogues of GTP, GTPγS (Sasaguri *et al.* 1985; Fulle *et al.* 1987) and Gpp(NH)p (Roth 1987) can activate PtdIns(4,5)P_2 hydrolysis, release Ca^{2+} (figure 1) and trigger contraction in (permeabilized) smooth muscle (figures 2 and 3) (Kobayashi *et al.* 1988). The threshold for GTPγS action is about 0.3 μM, with the maximal contractile effect obtained at 30 μM. Because the effects of GTPγS are competitively blocked by GDPβS and also produced by Gpp(NH)p, it is probable that they are mediated by a G protein, and do not involve either nucleotide hydrolysis or thiophosphorylation. The contractile effect of fluoride (1–10 mM) on permeabilized smooth muscle (Kobayashi *et al.* 1988), provides further evidence of G protein(s) coupled to the Ca^{2+} release, as fluoride (in the presence of contaminating Al) interacts with G-proteins (see, for example, Higashijima *et al.* 1987) to activate PtdIns(4,5)P_2 hydrolysis (see, for example, Blackmore *et al.* 1985).

A novel, and possibly important, finding is that GTPγS can also release Ca^{2+} and induce contraction in smooth muscles that have become refractory to the action of Ins(1,4,5)P_3 (figure 2). Therefore, the G-protein-coupled processes must include other mechanism(s) in addition to stimulation of Ins(1,4,5)P_3 production and Ins(1,4,5)P_3-induced Ca^{2+} release. Ins(1,4,5)P_3 has little or no further additive effect when added during the initial peak of GTPγS-induced contraction, in permeabilized (pulmonary artery) smooth muscle, in contrast to its additive effect during the later, sustained phase of GTPγS-induced contraction (figure 3) (Kobayashi *et al.* 1988). This demonstration of Ins(1,4,5)P_3-independent GTPγS-induced Ca^{2+} release suggests that the release of Ca^{2+} by GTP analogues in skeletal muscle (Di Virgilio *et al.* 1986), cannot be taken as *prima facie* evidence of a mechanism mediated by Ins(1,4,5)P_3.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

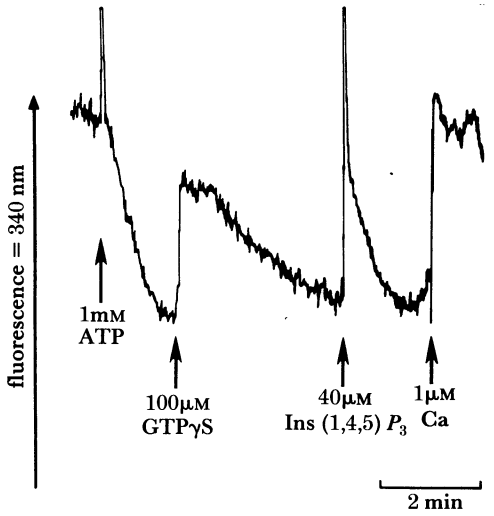

FIGURE 1. The effects of ATP (1 mM), GTPγS (100 μM), and Ins(1,4,5)P_3 (40 μM) on the fluorescence signal of fura 2 in the bath medium of saponin permeabilized rabbit main pulmonary artery. The compounds were applied by rapid manual addition. The application of 1 mM ATP to the permeabilized MPA resulted in a decrease in the fluorescence signal, indicating the uptake of Ca^{2+} by the strip. (The sharp upward deflection during addition of ATP is a bubble artefact.) Both GTPγS and Ins(1,4,5)P_3 caused a rapid increase in the fluorescence signal, indicating Ca^{2+} release from the strip. For the relative Ca^{2+} calibration, a known amount of $CaCl_2$ was added to the medium.

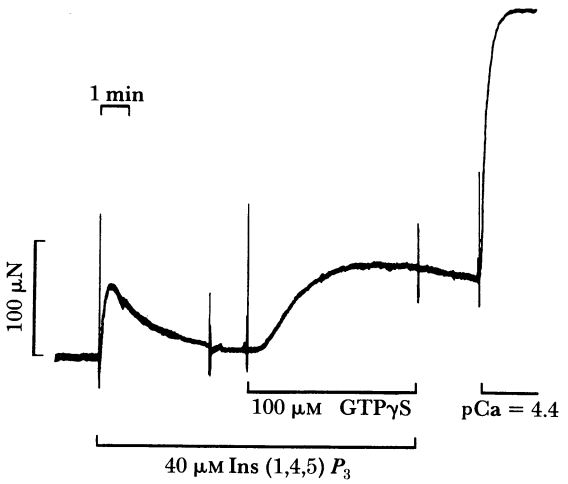

FIGURE 2. Ins(1,4,5)P_3 and GTPγS-induced contractions in saponin permeabilized rabbit main pulmonary artery smooth muscle. After the transient contraction induced by the first application of 40 μM Ins(1,4,5)P_3, the subsequent application of 'fresh' Ins(1,4,5)P_3 had no effect. However, 100 μM GTPγS induced tonic contraction in the continuous presence of Ins(1,4,5)P_3 (the unlabelled, sharp deflections are due to solution change with solutions containing Ins(1,4,5)P_3 or GTPγS or both as indicated by the horizontal bars. Note that the lack of response to Ins(1,4,5)P_3 added in the wash indicates that the decline of the initial Ins(1,4,5)P_3-induced contraction was not due solely to hydrolysis of Ins(1,4,5)P_3).

Kinetics of contractions induced by photolysis of caged phenylephrine and caged Ins(1,4,5)P_3

A physiological role of PtdIns(4,5)P_2 hydrolysis and Ins(1,4,5)P_3-induced Ca^{2+} release in excitation–contraction coupling requires their rates to be sufficiently fast for the activation of contraction. It is also of interest whether, in smooth muscle, these steps contribute to the long latency between activation and contraction (Fay 1977; Kamm & Stull 1986; Yagi *et al.* 1987; A. V. Somlyo *et al.* 1988). The introduction of photolabile, biologically inert precursors (caged

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

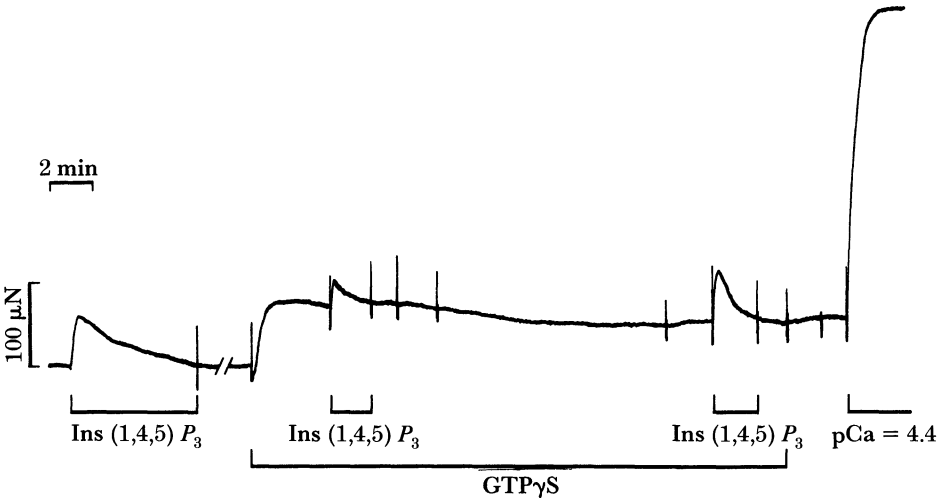

FIGURE 3. The effects of repetitive applications of 40 μM Ins(1,4,5)P_3 in, respectively, the absence and the presence of 100 μM GTPγS. The first contraction shows the effect of Ins(1,4,5)P_3 alone. Subsequently, when 40 μM Ins(1,4,5)P_3 was applied within 5 min after the application and in the presence of 100 μM GTPγS, the contractile response to Ins(1,4,5)P_3 was markedly reduced. However, during the late, sustained contraction induced by GTPγS, the effects of Ins(1,4,5)P_3 and GTPγS were approximately additive. The addition of Ca at the end of the experiment shows the maximal force developed by the muscle strip. The unlabelled, sharp deflections are due to solution changes as described in the legend to figure 2.

compounds) that can be activated through photolysis with a near-UV laser (Kaplan *et al.* 1978; reviewed in Gurney & Lester 1987) has made it possible to resolve rapid kinetics of cellular events without the limitations imposed by diffusion. Phenylephrine is an α_1-adrenergic agent that stimulates inositol phosphate production in vascular smooth muscle (Berta *et al.* 1986). We have used caged phenylephrine (Walker & Trentham 1988) and caged Ins(1,4,5)P_3 (Walker *et al.* 1987) to determine the kinetics of, respectively, α_1-adrenergic or Ins(1,4,5)P_3-induced activation of contraction.

Photolysis of caged phenylephrine caused contraction of intact (guinea pig portal vein) smooth muscle (figure 4), whether it was polarized or depolarized by high K^+ solution. These contractions could be blocked by the α-adrenergic blocking agent, phentolamine. The latency between photolysis and the onset of contraction was long: 1.5 ± 0.26 s and 0.6 ± 0.18 s at 20 and 30 °C, respectively. The latencies and their temperature dependence were similar following 'phenylephrine-jumps' with extremely high concentration (100 μM) of the agonist, confirming that the long latencies following photolysis of caged phenylephrine were not due to slow dark reactions (Walker & Trentham 1988) or laser-induced damage of the surface membrane. The temperature dependence ($Q_{10} = 2.7$) of the latency of phenylephrine action, and the significantly shorter latency of Ins(1,4,5)P_3-induced contractions (figure 4 and see below), are compatible with a significant portion of the latency being related to phospholipase C action and, possibly (Kohl & Hofmann 1987), G-protein coupling. The latency of agonist-induced changes in membrane potential (Purves 1974; Bolton 1976) may be due to the same or similar processes, but the relation between phospholipase C action and changes in smooth muscle plasma membrane permeability is yet to be determined.

The release of Ins(1,4,5)P_3 from caged Ins(1,4,5)P_3, by photolysis, caused contraction (figure 4) of permeabilized smooth muscle at rates comparable to those observed in the intact tissue (Walker *et al.* 1987), with a latency of 0.5 ± 0.12 s (at 20 °C, in the presence of 5 μM

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

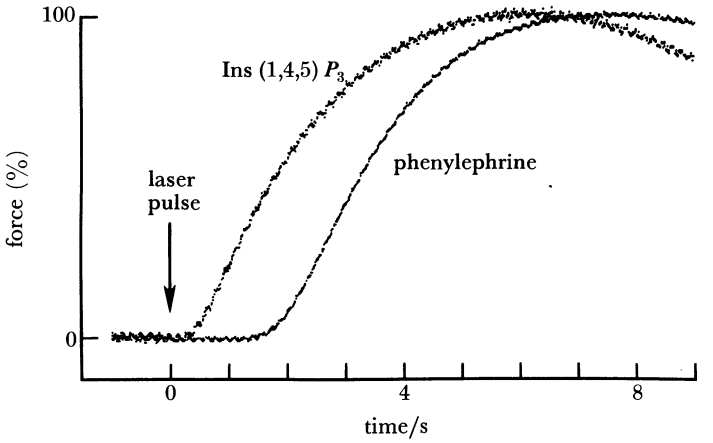

FIGURE 4. Two force transients recorded after photolysis of caged Ins(1,4,5)P_3 in a permeabilized muscle strip, and caged phenylephrine in an intact muscle strip of guinea-pig portal vein at 20 °C. A 50 ns laser pulse at 347 nm is indicated by the arrow. The peak force and $t_{\frac{1}{2}}$ to peak force for Ins(1,4,5)P_3 and phenylephrine were 177 μN, 1.4 s and 205 μN, 1.4 s, respectively. The lag-phase preceding force development was 0.4 s for Ins(1,4,5)P_3 and 1.8 s for phenylephrine. The intact strip used for the caged phenylephrine experiment had been treated with 6-hydroxydopamine for 20 min to produce adrenergic denervation (Aprigliano & Hermsmeyer 1976). This experiment was done in the presence of 143 mM potassium to depolarize the cell membrane and 50 μM caged phenylephrine. The Ins(1,4,5)P_3 response was obtained in a muscle strip permeabilized with 50 μg ml^{-1} saponin for 15 min, calcium loaded for 5 min at pCa 6.6 with 1 mM EGTA, followed by a 2 min wash in 0 calcium containing 1 mM EGTA solution, and subsequent incubation with 10 μM caged Ins(1,4,5)P_3 (esterified on the P^5 position) in a solution containing 0.1 mM EGTA and 90 μM calmodulin for 3 min before the laser flash. Approximately 10% Ins(1,4,5)P_3 and phenylephrine were released from the caged precursors.

calmodulin). This is significantly shorter than the latency (1.5 s), following photolysis of caged phenylephrine. The timing of these events is consistent with a sequence in which receptor activation is followed, with some delay, by Ins(1,4,5)P_3 production and Ca^{2+} release.

Ins(1,4,5)P_3 5-*phosphatase*

The presence of well-developed pathways for the metabolic inactivation of Ins(1,4,5)P_3 (Walker *et al.* 1987) as well as for its production (Sasaguri *et al.* 1985), provide further evidence for its physiological role as a messenger in smooth muscle. At 200 μM substrate the Ins(1,4,5)P_3 5-phosphatase activity of permeabilized rabbit main pulmonary artery hydrolysed Ins(1,4,5)P_3 at approximately 12 μM s^{-1}, which corresponds to 720 ± 252 nmol min^{-1} g^{-1} tissue (table 2). A similar level of activity (430 ± 195 nmol min^{-1} g^{-1}), 70% of which was associated with the particulate fractions, was found in homogenates of main pulmonary artery (table 2). This enzyme activity specifically hydrolysed the 5-phosphate residue of Ins(1,4,5)P_3 and displayed many of the properties characteristic of Ins(1,4,5)P_3 5-phosphatases from non-muscle tissues (Downes *et al.* 1982), including a Mg^{2+} requirement for full activity, a relatively high K_m for Ins(1,4,5)P_3 (20 μM) and inhibition by 2,3- diphosphoglycerate. Taken together, the measurements of hydrolysis rates and K_m indicate that a pulse of Ins(1,4,5)P_3 would be hydrolysed within main pulmonary artery in an approximately exponential decay at a rate of 0.6 s^{-1} (see table 2). This rate is fast enough to accommodate the relaxation rate of main pulmonary artery following release of 1 μM from caged Ins(1,4,5)P_3 (estimated to be 0.05 s^{-1} from data as in figure 1*a* in Walker *et al.* (1987)).

Metabolism of Ins(1,4,5)P_3 in saponin-skinned muscle preparations appeared to yield only

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 2. Ins(1,4,5)P_3-5-PHOSPHATASE ACTIVITY IN SKINNED FIBRES AND HOMOGENATES

	skinned fibres†		homogenates‡		
	total activity / nmol g^{-1} min^{-1}	V_{max}§/μM s^{-1}	total activity / nmol g^{-1} min^{-1}	K_m/μM	Q_{10}
main pulmonary artery	720±252‖ ($n=6$)	12±4 ($n=6$)	430±195 ($n=3$)	21	1.8
frog semitendinosus	24±6 ($n=4$)	0.4±0.1 ($n=4$)	72±26 ($n=2$)	19	1.8

† Main pulmonary artery strips or frog semitendinosus fibres were skinned by 15 min exposure to 50 μg ml^{-1} saponin, followed by three washes to remove saponin. Alternatively, frog fibres were also skinned by mechanically removing the sarcolemma. Incubations were done in 45 μl troughs containing 200 μM [^{3}H]Ins(1,4,5)P_3 (2.25 Ci mol^{-1} ($= 8.325 \times 10^{10}$ Bq mol^{-1})) to obtain maximum hydrolysis rates at 22 °C; inositol phosphates were analysed by HPLC (for experimental details see Walker *et al.* (1987)). Tissue mass was calculated from fibre dimensions assuming a density of 1 (average dimensions (mass): main pulmonary artery, 2.8 mm × 0.36 mm × 0.12 mm (0.12 mg); frog, 3.5 mm × 0.25 mm diameter (0.17 mg)). The errors due to uncertainty of fibre dimensions are estimated to be about ±30%.

‡ Homogenization with a glass–PTFE homogenizer was done in the incubation buffer of the following composition: 100 mM K^+ PIPES pH 7.1, 5.5 mM ATP, 7.9 mM $MgCl_2$, 9.5 mM creatine phosphate, 50 U ml^{-1} creatine kinase and 15 mM K^+ 1,6-diaminohexane-N,N,N′,N′-tetraacetate and containing 10 μM leupeptin and 0.1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis. Enzyme activity was measured in the same buffer without the protease inhibitors and with 1 μM $CaCl_2$ and 5 μM calmodulin. Activities were calculated from initial rates measured under conditions such that the hydrolysis of Ins(1,4,5)P_3 was linear with time; typically 5–200 μM [^{3}H]Ins(1,4,5)P_3 was incubated with 0.02 mg protein for 1–5 min at 22 °C. Differential centrifugation of homogenates resulted in three fractions containing activity, the distribution of which was similar in the main pulmonary artery and in frog fibres. The low-speed pellet (600 *g* for 5 min) contained 30%, the high speed pellet (85000 *g* for 2 h) 40%, and the high-speed supernatant 30% of the total activity. Q_{10} values were derived from measurements at 8 °C, 22 °C and 30 °C. First-order rate constants quoted in the text represent V_{max}/K_m which is valid for concentrations of Ins(1,4,5)$P_3 < K_m$.

§ V_{max} is defined as the maximum hydrolysis rate of Ins(1,4,5)P_3 within the fibre volume. Activities in main pulmonary artery represent lower limit estimates for V_{max}, as the hydrolysis rates did not show saturation at 200 μM Ins(1,4,5)P_3, indicating that diffusion of Ins(1,4,5)P_3 into fibres is partly rate-limiting. Saturation was observed at 200 μM Ins(1,4,5)P_3 in mechanically skinned frog fibres.

‖ Values given are means±s.d.

inositol 1,4-bisphosphate (Ins(1,4)P_2). However, enzymes were released during homogenization that further hydrolysed Ins(1,4)P_2 to inositol 1(or 4)-monophosphate and inositol, and were sensitive to inhibition by 5 mM LiCl as it is typical for Ins(1,4)P_2 and Ins(1 or 4)P_1 phosphatases (Storey *et al.* 1984). Another pathway for Ins(1,4,5)P_3 metabolism (and probably Ins(1,4,5)P_3 inactivation) consists of a Ca^{2+}-calmodulin sensitive Ins(1,4,5)P_3 3-kinase that converts Ins(1,4,5)P_3 to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) (Irvine *et al.* 1986) that is subsequently degraded to inositol 1,3,4-trisphosphate (Ins(1,3,4)P_3). Although we did not detect Ins(1,3,4,5)P_4 or Ins(1,3,4)P_3 formation in permeabilized main pulmonary artery in the presence of 5 μM calmodulin, nor was Ins(1,4,5)P_3 3-kinase activity found in homogenates, evidence for the existence of this pathway was found in other smooth muscles (Rossier *et al.* 1987; Yamaguchi *et al.* 1987).

SKELETAL AND CARDIAC MUSCLE: ELECTROMECHANICAL COUPLING AND THE QUESTION OF Ins(1,4,5)P_3 ACTION

Contraction of most vertebrate skeletal and mammalian cardiac muscles is activated by action potentials that release Ca^{2+} from the terminal cisternae (TC) or junctional SR. The two dominant mechanisms proposed for transmission of the message, to release Ca^{2+}, from the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

surface membrane to the SR are, respectively, a conformational change related to movement of electrical charge (Chandler *et al.* 1975) and the production of a chemical messenger (see, for example, Luttgau & Stephenson 1986; Martonosi 1984; A. P. Somlyo 1985; Caille *et al.* 1985). An earlier hypothesis, that ionic currents can directly invade and depolarize the SR membrane, is no longer considered plausible, as no evidence of such ion movements could be found by electron probe X-ray microanalysis of tetanized muscles (Kitazawa *et al.* 1984). The role of a chemical messenger is supported by the delay between T-tubule depolarization and Ca^{2+}-release (Zhu *et al.* 1986) and by the temperature sensitivity of this delay (Miledi *et al.* 1982), although it must be noted that charge movement is also temperature sensitive. The $Q_{10} = 2.0$ for the time to peak of Q_γ, the charge component implicated in excitation–contraction coupling (C. S. Hui, personal communication). A diffusible substances(s) that causes oscillatory Ca^{2+} release from the SR (Kumbaraci & Nastuk 1982; Herrmann 1986) is generated under the influence of caffeine and this, or a similar chemical messenger, may mediate electromechanical coupling.

Several laboratories have reported contraction of skinned skeletal muscle on exposure to Ins(1,4,5)P_3 (Volpe *et al.* 1985, 1986; Donaldson *et al.* 1987; Nosek *et al.* 1986; Vergara *et al.* 1987), whereas others found no effect of Ins(1,4,5)P_3 on skinned muscle (Lea *et al.* 1986) or isolated SR preparations (Palade 1987; Mikos & Snow 1987; Sherer & Ferguson 1985; but see Volpe *et al.* 1985). However, some of the experimental manipulations used to inhibit Ins(1,4,5)P_3-5-phosphatase activity and so enhance Ins(1,4,5)P_3 action in frog fibres or to show the specificity of Ins(1,4,5)P_3 action are non-specific and unphysiological. Cadmium (Cd), used as an inhibitor of Ins(1,4,5)P_3-5-phosphatase (Vergara *et al.* 1987), can release Ca^{2+} from fragmented SR (Abramson *et al.* 1983) and, likewise, neomycin, an inhibitor of phospholipase C (Downes & Michell 1981; Van Rooijen & Agranoff 1985) used to block Ins(1,4,5)P_3-mediated effects (Vergara *et al.* 1987), also non-specifically inhibits SR Ca^{2+}-channels (Palade 1987). Moreover, careful, comparative studies of intact and skinned frog, mouse and guinea-pig twitch-muscle failed to show any effect of Ins(1,4,5)P_3 injected into intact fibres, although contractions were evoked in skinned muscles (Hannon *et al.* 1988; Blinks *et al.* 1987). These authors concluded (but see Donaldson *et al.* 1987) that the Ins(1,4,5)P_3-induced Ca^{2+} release observed by others was due to its effects on the membrane potential of sealed off T-tubules detached by the skinning procedure. Similarly, the effects of Ins(1,4,5)P_3 on cardiac Ca^{2+} release and contraction are minimal or very slow or both, perhaps sufficient to modulate the force of contraction, but not indicative of a primary role in cardiac excitation–contraction coupling (Hirata *et al.* 1984; Fabiato 1986; Nosek *et al.* 1986). It is difficult to evaluate some of the reported effects of Ins(1,4,5)P_3 on striated muscles, because the obligatory use of weakly Ca^{2+}-buffered solutions, to detect force induced by Ca^{2+} release, is conducive to the development of spontaneous contractile oscillations. Such oscillatory Ca^{2+}-induced Ca^{2+} release occurs, in the absence of Ins(1,4,5)P_3, from the SR in cardiac muscle (Chiesi *et al.* 1981) and even in frog skeletal muscle (Endo *et al.* 1970). In cardiac (Otani *et al.* 1986) and, perhaps, skeletal (Vergara *et al.* 1987) muscle, Ins(1,4,5)P_3 production is increased by α-adrenergic or muscarinic agonists and electrical stimulation, but these increases seem to bear little or no direct relation to excitation–contraction coupling (Otani *et al.* 1986). The likelihood of Ins(1,4,5)P_3 being the major chemical messenger of electromechanical coupling is also negated by the fact that high potassium-induced depolarization of smooth muscle causes no increase in PtdIns(4,5)P_2 turnover (Baron *et al.* 1985; but see Jafferji & Michell 1976), whereas in skeletal

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

muscle phosphatidylinositol breakdown continues for a much longer period (3 h) than the contractile response to depolarization (Novotny *et al.* 1983). The physiological significance of kinases that phosphorylate phosphatidylinositol to phosphatidylinositol 4,5-bisphosphate in T-tubules isolated from frog muscle is uncertain, because of the low PtdIns(4,5)P_2 content of T-tubules (Hidalgo *et al.* 1986).

The low activity of the degradative enzyme, Ins(1,4,5)P_3 5-phosphatase in frog striated muscle (Walker *et al.* 1987) (table 2) also negates the hypothesis that Ins(1,4,5)P_3 is the chemical messenger of excitation–contraction coupling, because Ca^{2+} release is terminated within milliseconds (Maylie *et al.* 1987; Baylor & Hollingworth 1988). Maylie *et al.* (1987) estimate that for a mechanism in which removal of the messenger is directly linked to termination of Ca^{2+} release, its inactivation rate would have to be in the range of 200–300 s^{-1}, based on the duration of Ca^{2+} signals in frog fibres. Even taking into account the 3-fold higher Ins(1,4,5)P_3 5-phosphatase activity of homogenates compared with skinned fibres (table 2) (P. Volpe, personal communication), this activity (0.06 s^{-1} at 22 °C) appears to be too low by several orders of magnitude in frog muscle, especially at 4–7 °C (Q_{10} = 1.8), for the termination of Ca^{2+} release during a twitch. Rapid inactivation is also unlikely to occur via the phosphorylation or dephosphorylation pathway involving Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3, because formation of these inositol phosphates from Ins(1,4,5)P_3 could not be detected either in skinned fibres or in muscle homogenates. Because of the low Ins(1,4,5)P_3 5-phosphatase activity of frog muscle, it is unlikely that, as has been suggested (Vergara *et al.* 1987), the high concentrations of Ins(1,4,5)P_3 required to induce contraction in this preparation and the slowness of the contractile response are due to rapid hydrolysis of Ins(1,4,5)P_3.

To determine whether slow diffusion, for whatever other reason, could account for the modest and inconsistent effects of Ins(1,4,5)P_3 on skeletal muscle, we have determined the effects of Ins(1,4,5)P_3 released from caged Ins(1,4,5)P_3 with laser flash photolysis (Walker *et al.* 1987). Even under these conditions, much higher concentrations of caged Ins(1,4,5)P_3 were required to trigger contraction in striated than in smooth muscle, and the contractile response to Ins(1,4,5)P_3 released within milliseconds was orders of magnitude slower than a normal twitch of frog muscle (figure 2 in Walker *et al.* (1987) and figure 5 in Somlyo *et al.* (1987)). These results argue against Ins(1,4,5)P_3 being the major physiological messenger of excitation–contraction coupling in striated muscle, although they do not rule out the real possibility of chemical transmission by some other messengers.

Supported by HL15835 to Pennsylvania Muscle Institute and the MRC (D. R. T.). We thank Mrs M. Tokito for the illustrations and Mrs B. Tyrcha for preparation of manuscript.

References

Abdel-Latif, A. A. 1986 Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmac. Rev.* **38**, 227–272.

Abdel-Latif, A. A., Howe, P. H. & Akhtar, R. A. 1987 Muscarinic-receptor induced myo-inositol trisphosphate accumulation, myosin light chain phosphorylation and contraction in the rabbit iris sphincter smooth muscle, In *Mechanisms of signal transduction by hormones and growth factors* (ed. M. C. Cabot & W. L. McKeehan), pp. 119–132. New York: Alan R. Liss.

Abramson, J. J., Trimm, J. L., Weden, L. & Salama, G. 1983 Heavy metals induce rapid calcium release from sarcoplasmic reticulum vesicles isolated from skeletal muscle. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1526–1530.

Aprigliano, O. & Hermsmeyer, K. 1976 *In vitro* denervation of the portal vein and caudal artery of the rat. *J. Pharmac. exp. Ther.* **198**, 578–587.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Armstrong, C. M., Bezanilla, F. M. & Horowicz, P. 1972 Twitches in the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid. *Biochim. biophys. Acta* **267**, 605–608.

Baron, C. B., Cunningham, M., Strauss, J. F. III & Coburn, R. F. 1984 Pharmacomechanical coupling in smooth muscle may involve phosphatidylinositol metabolism. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6899–6903.

Baylor, S. M. & Hollingworth, S. 1988 Fura2 Ca^{2+} transients in frog skeletal muscle fibres. *J. Physiol., Lond.* (In the press.)

Benham, C. D. & Tsien, R. W. 1987 A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature, Lond.* **328**, 275–278.

Berridge, M. J. & Irvine, R. F. 1984 Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature, Lond.* **312**, 315–321.

Berta, P., Seguin, J., Vidal, N., Haiech, J., Mathieu, M.-N. & Chevillard, C. 1986 Influence of $[Ca^{2+}]_e$ on 5-HT_2- and α_1-induced arterial contraction and phosphoinositide metabolism. *Eur. J. Pharmac.* **132**, 253–257.

Best, L., Brooks, K. J. & Bolton, T. B. 1985 Relationship between stimulated inositol lipid hydrolysis and contractility in guinea-pig visceral longitudinal smooth muscle. *Biochem. Pharmac.* **34**, 2297–2301.

Bitar, K. N., Bradford, P. G., Putney, J. W. Jr & Makhlouf, G. M. 1986 Stoichiometry of contraction and Ca^{2+} mobilization by inositol 1,4,5-trisphosphate in isolated gastric smooth muscle cells. *J. biol. Chem.* **261**, 16591–16596.

Blackmore, P. F., Bocckino, S. B., Waynick, L. E. & Exton, J. H. 1985 Role of a guanine nucleotide-binding regulatory protein in the hydrolysis of hepatocyte phosphatidylinositol 4,5-bisphosphate by calcium-mobilizing hormones and the control of cell calcium. *J. biol. Chem.* **260**, 14477–14483.

Blinks, J. R. 1986 Intracellular $[Ca^{2+}]$ measurements. In *The heart and cardiovascular system* (ed. H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katc & H. E. Mortan), pp. 671–701. New York: Raven Press.

Blinks, J. R., Cai, Y.-D. & Lee, N. K. M. 1987 Inositol 1,4,5-trisphosphate causes calcium release in frog skeletal muscle only when transverse tubules have been interrupted. *J. Physiol., Lond.* **394**, 23*P*.

Bolton, T. B. 1976 On the latency and form of the membrane responses of smooth muscle to the iontophoretic application of acetylcholine or carbachol. *Proc. R. Soc. Lond.* B **194**, 99–119.

Bond, M., Kitazawa, T., Somlyo, A. P. & Somlyo, A. V. 1984*a* Release and recycling of calcium by the sarcoplasmic reticulum in guinea pig portal vein smooth muscle. *J. Physiol., Lond.* **355**, 677–695.

Bond, M., Shuman, H., Somlyo, A. P. & Somlyo, A. V. 1984*b* Total cytoplasmic calcium in relaxed and maximally contracted rabbit portal vein smooth muscle. *J. Physiol., Lond.* **357**, 185–201.

Bond, M., Vadasz, G., Somlyo, A. V. & Somlyo, A. P. 1987 Subcellular calcium and magnesium mobilization in rat liver stimulated *in vitro* with vasopressin and glucagon. *J. biol. Chem.* **262**, 15630–15637.

Bozler, E. 1969 Role of calcium in initiation of activity of smooth muscle. *Am. J. Physiol.* **216**, 671.

Broderick, R. & Somlyo, A. P. 1987 Calcium and magnesium transport by *in situ* mitochondria: electron probe analysis of vascular smooth muscle. *Circ. Res.* **61**, 523–531.

Caille, J., Ildefonse, M. & Rougier, O. 1985 Excitation–contraction coupling in skeletal muscle. *Prog. Biophys molec. Biol.* **46**, 185–239.

Carafoli, E. 1987 Intracellular calcium homeostasis. *A. Rev. Biochem.* **56**, 395–433.

Chandler, W. K., Schneider, M. F., Rakowski, R. F. & Adrian, R. H. 1975 Charge movements in skeletal muscle. *Phil. Trans. R. Soc. Lond.* B**270**, 501–505.

Chiesi, M., Ho, M. M., Inesi, G., Somlyo, A. V. & Somlyo, A. P. 1981 Primary role of sarcoplasmic reticulum in phasic contractile activation of cardiac myocytes with shunted myolemma. *J. Cell Biol.* **91**, 728–742.

DeFeo, T. T. & Morgan, K. G. 1985 Calcium-force relationships as detected with aequorin in two different vascular smooth muscles of the ferret. *J. Physiol., Lond.* **369**, 269–282.

Devine, C. E., Somlyo, A. V. & Somlyo, A. P. 1972 Sarcoplasmic reticulum and excitation–contraction coupling in mammalian smooth muscle. *J. Cell Biol.* **52**, 690–718.

Di Virgilio, F., Salviati, G., Pozzan, T. & Volpe, P. 1986 Is a guanine nucleotide-binding protein involved in excitation–contraction coupling in skeletal muscle? *EMBO J.* **5**, 259–262.

Donaldson, S. K., Goldberg, N. D., Walseth, T. F. & Huetteman, D. A. 1987 Inositol trisphosphate stimulates calcium release from peeled skeletal muscle fibers. *Biochim. biophys. Acta* **927**, 92–99.

Downes, C. P. & Michell, R. H. 1981 The polyphosphoinositide phosphodiesterase of erythrocyte membranes. *Biochem. J.* **198**, 133–140.

Downes, C. P., Mussat, M. C. & Michell, R. H. 1982 The inositol trisphosphate phosphomonoesterase of the human erythrocyte membrane. *Biochem. J.* **203**, 169–177.

Duncan, R. A., Krzanowski, J. J. Jr, Davis, J. S., Polson, J. B., Coffey, R. G., Shimoda, T. & Szentivanyi, A. 1987 Polyphosphoinositide metabolism in canine tracheal smooth muscle (CTSM) in response to a cholinergic stimulus. *Biochem. Pharmac.* **36**, 307–310.

Endo, M. 1985 Calcium release from sarcoplasmic reticulum. In *Current topics in membranes and transport* (ed. F. Bronner), vol. 25, pp. 181–230. New York: Academic Press.

Endo, M., Tanaka, M. & Ogawa, Y. 1970 Calcium induced release of calcium from the sarcoplasmic reticulum of skinned muscle fibers. *Nature, Lond.* **227**, 34–36.

Fabiato, A. 1985 Calcium-induced release of calcium from the sarcoplasmic reticulum. *J. gen. Physiol.* **85**, 189–320.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Fabiato, A. 1986 Inositol (1,4,5)-trisphosphate-induced release of Ca^{2+} from the sarcoplasmic reticulum of skinned cardiac cells. *Biophys. J.* **49**, 190a.

Fay, F. S. 1977 Isometric contractile properties of single isolated smooth muscle cells. *Nature, Lond.* **265**, 553–556.

Fay, F. S., Shlevin, H. H., Granger, W. C. & Taylor, S. R. 1979 Aequorin luminescence during activation of single isolated smooth muscle cells. *Nature, Lond.* **280**, 506–508.

Fox, A. W., Abel, P. W. & Minneman, K. P. 1985 Activation of α_1-adrenoceptors increases [^{3}H]inositol metabolism in rat vas deferens and caudal artery. *Eur. J. Pharmac.* **116**, 145–152.

Franzini-Armstrong, C. 1986 The sarcoplasmic reticulum and the transverse tubules. In *Myology* (ed. A. G. Engel & B. Q. Banker), pp. 125–154. New York: McGraw-Hill.

Fulle, H.-J., Hoer, D., Lache, W., Rosenthal, W., Schultz, G. & Oberdisse, E. 1987 *In vitro* synthesis of ^{32}P-labelled phosphatidylinositol 4,5-bisphosphate and its hydrolysis by smooth muscle membrane-bound phospholipase C. *Biochem. biophys. Res. Commun.* **145**, 673–679.

Gosselin-Rey, C. & Gerday, C. 1977 Parvalbumins from frog skeletal muscle (*Rana temporaria* L.); isolation and characterization; structural modifications associated with calcium binding. *Biochim. biophys. Acta* **492**, 53–63.

Gurney, A. M. & Lester, H. A. 1987 Light-flash physiology with synthetic photosensitive compounds. *Physiol. Rev.* **67**, 583–617.

Hannon, J. D., Lee, N. K. M. & Blinks, J. R. 1988 Calcium release by inositol trisphosphate in amphibian and mammalian skeletal muscle is an artifact of cell disruption, and probably results from depolarization of sealed-off T-tubules., *Biophys. J.* **53**, 607a.

Herrmann, A. 1986 Caffeine-induced sarcomeric oscillations in skeletal muscle fibres of the frog. *J. Physiol., Lond.* **378**, 106P.

Hidalgo, C., Carrasco, M. A., Magendzo, K. & Jaimovich, E. 1986 Phosphorylation of phosphatidylinositol by transverse tubule vesicles and its possible role in excitation–contraction coupling. *FEBS Lett.* **202**, 69–73.

Higashijima, T., Ferguson, K. M., Sternweis, P. C., Ross, E. M., Smigel, M. D. & Gilman, A. G. 1987 The effect of activating ligands on the intrinsic fluorescence of guanine nucleotide-binding regulatory proteins. *J. biol. Chem.* **262**, 752–756.

Himpens, B. & Somlyo, A. P. 1988 Free calcium and force transients during depolarization and pharmaco-mechanical coupling in guinea pig smooth muscle. *J. Physiol., Lond.* **395**, 507–530.

Hirata, M., Suematsu, E., Hashimoto, T., Hamachi, T. & Koga, T. 1984 Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate. *Biochem. J.* **223**, 229–236.

Iino, M. 1987 Calcium dependent inositol trisphosphate-induced calcium release in the guinea-pig taenia caeci. *Biochem. biophys. Res. Commun.* **142**, 47–52.

Irvine, R. F., Lecher, A. J., Heslop, J. P. & Berridge, M. J. 1986 The inositol tris/tetrakisphosphate pathway – demonstration of Ins(1,4,5)P_3 3-kinase activity in animal tissues. *Nature, Lond.* **320**, 631–634.

Jafferji, S. S. & Michell, R. H. 1976 Investigation of the relationship between cell-surface calcium-ion gating and phosphatidylinositol turnover by comparison of the effects of elevated extracellular potassium ion concentration on ileum smooth muscle and pancreas. *Biochem. J.* **160**, 397–399.

Johansson, B. & Somlyo, A. P. 1980 Electrophysiology and excitation–contraction coupling. In *The handbook of physiology: the cardiovascular system* (ed. D. F. Bohr, A. P. Somlyo & H. V. Sparks), vol. 2, pp. 301–324. Bethesda, Maryland: American Physiological Society.

Jorgensen, A. O., Broderick, R., Somlyo, A. P. & Somlyo, A. V. 1988 Two structurally distinct calcium storage sites in rat cardiac sarcoplasmic reticulum. *Biophys. J.* **53**, 437a.

Kamm, K. E. & Stull, J. T. 1986 Activation of smooth muscle contraction: relation between myosin phosphorylation and stiffness. *Science, Wash.* **232**, 80–82.

Kaplan, J., Forbush, B. III & Hoffman, J. F. 1978 Rapid photolytic release of adenosine 5′-trisphosphate from a protected analogue: utilization by the Na:K pump of human red blood cell ghosts. *Biochemistry, Wash.* **17**, 1929–1935.

Kitazawa, T., Somlyo, A. P. & Somlyo, A. V. 1984 The effects of valinomycin on ion movements across the sarcoplasmic reticulum in frog muscle. *J. Physiol., Lond.* **350**, 253–268.

Kobayashi, S., Somlyo, A. P. & Somlyo, A. V. 1988 Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3)-dependent and independent calcium release by guanine nucleotides in vascular smooth muscle. *FASEB J.* **2**, A330.

Kohl, B. & Hofmann, K. P. 1987 Temperature dependence of G-protein activation in photoreceptor membranes. *Biophys. J.* **52**, 271–277.

Kowarski, D., Shuman, H., Somlyo, A. P. & Somlyo, A. V. 1985 Calcium release by norepinephrine from central sarcoplasmic reticulum in rabbit main pulmonary artery smooth muscle. *J. Physiol., Lond.* **366**, 153–175.

Kumbaraci, N. M. & Nastuk, W. L. 1982 Action of caffeine in excitation–contraction coupling of frog skeletal muscle fibres. *J. Physiol., Lond.* **325**, 195–211.

Lea, T. J., Griffiths, P. J., Tregear, R. T. & Ashley, C. C. 1986 An examination of the ability of inositol 1,4,5-trisphosphate to induce calcium release and tension development in skinned skeletal muscle fibres of frog and crustacea. *FEBS Lett.* **207**, 153–161.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Litosch, I. & Fain, J. N. 1986 Regulation of phosphoinositide breakdown by guanine nucleotides. *Life Sci.* **39**, 187–194.

Luttgau, H. C. & Stephenson, G. D. 1986 Ion movements in skeletal muscle in relation to the activation of contraction. In *Physiology of membrane disorders* (ed. T. E. Andreoli, J. F. Hoffman, D. D. Fanestil & S. G. Schultz), pp. 449–468. New York: Plenum.

Mallows, R. S. E. & Bolton, T. B. 1987 Relationship between stimulated phosphatidic acid production and inositol lipid hydrolysis in intestinal longitudinal smooth muscle from guinea pig. *Biochem. J.* **244**, 763–768.

Martonosi, A. N. 1984 Mechanisms of Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle. *Physiol. Rev.* **64**, 1240–1320.

Maylie, J., Irvine, M., Sizto, N. L. & Chandler, W. K. 1987 Calcium signals recorded from cut frog twitch fibers containing antipyrylazo III. *J. gen. Physiol.* **89**, 83–143.

Miledi, R., Parker, I. & Zhu, P. H. 1982 Calcium transients evoked by action potentials in frog twitch muscle fibres. *J. Physiol., Lond.* **333**, 655–679.

Mikos, G. J. & Snow, T. R. 1987 Failure of inositol 1,4,5-trisphosphate to elicit or potentiate Ca^{2+}-release from isolated skeletal muscle sarcoplasmic reticulum. *Biochim. biophys. Acta* **927**, 256–260.

Nabika, T., Velletri, P. A., Lovenberg, W. & Beaven, M. A. 1985 Increase in cytosolic calcium and phosphoinositide metabolism induced by angiotensin II and [Arg]vasopressin in vascular smooth muscle cells. *J. biol. Chem.* **260**, 4661–4670.

Nishikawa, M., de Lanerolle, P., Lincoln, T. M. & Adelstein, R. S. 1984 Phosphorylation of mammalian myosin light chain kinases by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. *J. biol. Chem.* **259**, 8429–8436.

Nosek, T. M. N., Williams, M. F., Zeigler, S. T. & Godt, R. E. 1986 Inositol trisphosphate enhances calcium release in skinned cardiac and skeletal muscle. *Am. J. Physiol.* **250**, C807–C811.

Novotny, I., Saleh, F. & Novotna, R. 1983 K^+ depolarization and phospholipid metabolism in frog sartorius muscle. *Gen. Physiol. Biophys.* **2**, 329–337.

Otani, H., Otani, H. & Das, D. K. 1986 Evidence that phosphoinositide response is mediated by α_1-adrenoceptor stimulation, but not linked with excitation–contraction coupling in cardiac muscle. *Biochem. biophys. Res. Commun.* **136**, 863–869.

Palade, P. 1987 Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum. *J. biol. Chem.* **262**, 6149–6154.

Pfitzer, G., Ruegg, J. C., Zimmer, M. & Hofmann, F. 1985 Relaxation of skinned coronary arteries depends on the relative concentrations of Ca^{2+}, calmodulin and active cAMP-dependent protein kinase. *Pflügers Arch. Eur. J. Physiol.* **405**, 70–76.

Phaneuf, S., Berta, P., Casanova, J. & Cavadore, J.-C. 1987 ATP stimulates inositol phosphates accumulation and calcium mobilization in a primary culture of rat aortic myocytes. *Biochem. biophys. Res. Commun.* **143**, 454–460.

Purves, R. D. 1974 Muscarinic excitation: a microelectrophoretic study on cultured smooth muscle cells. *Br. J. Pharmac.* **52**, 77–86.

Rapoport, R. M. 1987 Effects of norepinephrine on contraction and hydrolysis of phosphatidylinositols in rat aorta. *J. Pharmacol. exp. Therap.* **242**, 188–194.

Ratz, P. H. & Murphy, R. A. 1987 Contributions of intracellular and extracellular Ca^{2+} pools to activation of myosin phosphorylation and stress in swine carotid media. *Circ. Res.* **60**, 410–421.

Rembold, C. M. & Murphy, R. A. 1986 Myoplasmic calcium, myosin phosphorylation, and regulation of the crossbridge cycle in swine arterial smooth muscle. *Circ. Res.* **58**, 803–815.

Rossier, M. F., Capponi, A. M. & Vallotton, M. B. 1987 Metabolism of inositol 1,4,5-trisphosphate in permeabilized rat aortic smooth-muscle cells. Dependence on calcium concentration. *Biochem. J.* **245**, 305–307.

Roth, B. L. 1987 Modulation of phosphatidylinositol-4,5-bisphosphate hydrolysis in rat aorta by guanine nucleotides, calcium and magnesium. *Life Sci.* **41**, 629–634.

Saida, K. & van Breemen, C. 1987 GTP requirements for inositol-1,4,5-trisphosphate-induced Ca^{2+} release from sarcoplasmic reticulum in smooth muscle. *Biochem. biophys. Res. Commun.* **144**, 1313–1316.

Sasaguri, T., Hirata, M. & Kuriyama, H. 1985 Dependence on Ca^{2+} of the activities of phosphatidylinositol 4,5-bisphosphate phosphodiesterase and inositol 1,4,5-trisphosphate phosphatase in smooth muscles of the porcine coronary artery. *Biochem. J.* **231**, 497–503.

Sherer, N. H. & Ferguson, J. E. 1985 Inositol 1,4,5-trisphosphate is not effective in releasing calcium from skeletal sarcoplasmic reticulum microsomes. *Biochem. biophys. Res. Commun.* **128**, 1064–1070.

Smith, J. B., Smith, L. & Higgins, B. L. 1985 Temperature and nucleotide dependence of calcium release by *myo*-inositol 1,4,5-trisphosphate in cultured vascular smooth muscle cells. *J. biol. Chem.* **260**, 14413–14416.

Somlyo, A. P. 1984 Cellular site of calcium regulation. *Nature, Lond.* **308**, 516–517.

Somlyo, A. P. 1985 Excitation–contraction coupling and the ultrastructure of smooth muscle. *Circ. Res.* **57**, 497–507.

Somlyo, A. P. 1985 Excitation–contraction coupling. The messenger across the gap. *Nature, Lond.* **316**, 298–299.

Somlyo, A. P., Devine, C. E., Somlyo, A. V. & North, S. R. 1971 Sarcoplasmic reticulum and the temperature-dependent contraction of smooth muscle in calcium-free solutions. *J. Cell Biol.* **51**, 722–741.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Somlyo, A. P. & Somlyo, A. V. 1971 Electrophysiological correlates of the inequality of maximal vascular smooth muscle contractions elicited by drugs. In *Physiology and pharmacology of vascular neuroeffector systems* (ed. J. A. Bevan, R. F. Furchgott, R. A. Maxwell and A. P. Somlyo), pp. 216–228. Basle: S. Karger.

Somlyo, A. P., Somlyo, A. V., Bond, M., Broderick, R., Goldman, Y. E., Shuman, H., Walker, J. W. & Trentham, D. R. 1987 Calcium and magnesium movements in cells and the role of inositol trisphosphate in muscle. In *Cell calcium and the control of membrane transport* (ed. D. C. Eaton & L. J. Mandel), vol. 42, pp. 77–92. New York: Rockefeller University Press.

Somlyo, A. P., Somlyo, A. V. & Shuman, H. 1979 Electron probe analysis of vascular smooth muscle: composition of mitochondria, nuclei and cytoplasm. *J. Cell Biol.* **81**, 316–335.

Somlyo, A. V. 1979 Bridging structures spanning the junctional gap at the triad of skeletal muscle. *J. Cell Biol.* **80**, 743–750.

Somlyo, A. V., Bond, M., Somlyo, A. P. & Scarpa, A. 1985*a* Inositol-trisphosphate ($Ins P_3$) induced calcium release and contraction in vascular smooth muscle. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5231–5235.

Somlyo, A. V., Goldman, Y. E., Fujimori, T., Bond, M., Trentham, D. R. & Somlyo, A. P. 1988 Crossbridge kinetics, cooperativity and negatively strained crossbridges in vertebrate smooth muscle: a laser flash photolysis study. *J. gen. Physiol.* **91**, 165–192.

Somlyo, A. V., Gonzalez-Serratos, H., Shuman, H., McClellan, G. & Somlyo, A. P. 1981 Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: an electron probe study. *J. Cell Biol.* **90**, 577–594.

Somlyo, A. V., McClellan, G., Gonzalez-Serratos, H. & Somlyo, A. P. 1985*b* Electron probe X-ray microanalysis of post tetanic Ca and Mg movements across the sarcoplasmic reticulum *in situ*. *J. biol. Chem.* **260**, 6801–6807.

Somlyo, A. V. & Somlyo, A. P. 1968 Electromechanical and pharmacomechanical coupling in vascular smooth muscle. *J. Pharmacol. exp. Therap.* **159**, 129–145.

Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. 1984 Stepwise enzymatic dephosphorylation of inositol 1,4,5-trisphosphate to inositol in liver. *Nature, Lond.* **312**, 374–376.

Streb, H., Irvine, R. F., Berridge, M. J. & Schultz, I. 1983 Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature, Lond.* **306**, 67–69.

Suematsu, E., Hirata, M., Hashimoto, T. & Kuriyama, H. 1984 Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. *Biochem. biophys. Res. Commun.* **120**, 481–485.

Takuwa, Y., Takuwa, N. & Rasmussen, H. 1986 Carbachol induces a rapid and sustained hydrolysis of polyphosphoinositide in bovine tracheal smooth muscle. Measurements of the mass of polyphosphoinositides, 1,2-diacylglycerol, and phosphatidic acid. *J. biol. Chem.* **261**, 14670–14675.

Ueno, H., Sumimoto, K., Hasimoto, T., Hirata, M. & Kuriyama, H. 1987 Effects of procaine on pharmacomechanical coupling mechanisms activated by acetylcholine in smooth muscle cells of porcine coronary artery. *Circ. Res.* **60**, 356–366.

Van Breemen, C., Cauvin, C., Johns, A., Leijten, P. & Yamamoto, H. 1986 Ca^{2+} regulation of vascular smooth muscle. *Fedn Proc. Fedn Am. Socs exp. Biol.* **45**, 2746–2751.

Van Rooijem, L. A. A. & Agranoff, B. W. 1985 Inhibition of polyphosphoinositide phosphodiesterase by aminoglycoside antibiotics. *Neurochem. Res.* **10**, 1019–1024.

Vergara, J., Asotra, K. & Delay, M. 1987 A chemical link in excitation–concentration coupling in skeletal muscle. In *Cell calcium and the control of membrane transport* (ed. D. C. Eaton & L. J. Mandel), vol. 42, pp. 133–152. New York: Rockefeller University Press.

Volpe, P., Di Virgilio, F., Pozzan, T. & Salviati, G. 1986 Role of inositol-1,4,5-trisphosphate in excitation–contraction-coupling in skeletal muscle. *FEBS Lett.* **197**, 1–4.

Volpe, P., Salviati, G., Di Virgilio, F. & Pozzan, T. 1985 Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. *Nature, Lond.* **316**, 347–349.

Walker, J. W., Somlyo, A. V., Goldman, Y. E., Somlyo, A. P. & Trentham, D. R. 1987 Kinetics of smooth and skeletal muscle activation by laser pulse photolysis of caged inositol 1,4,5-trisphosphate. *Nature, Lond.* **327**, 249–252.

Walker, J. W. & Trentham, D. R. 1988 Caged phenylephrine: synthesis and photochemical properties. *Biophys. J.* **53**, 5962.

Wheeler-Clark, E. S. & Tormey, J. McD. 1987 Electron probe X-ray microanalysis of sarcolemma and junctional sarcoplasmic reticulum in rabbit papillary muscles: low sodium-induced calcium alterations. *Circ. Res.* **60**, 246–250.

Yagi, S., Becker, B. L. & Fay, F. S. 1987 Measurement of force and cystolic calcium in single smooth muscle cells following electrical stimulation. *Biophys. J.* **51**, 101a.

Yamaguchi, K., Hirata, M. & Kuriyama, H. 1987 Calmodulin activates inositol 1,4,5-trisphosphate 3-kinase activity in pig aortic smooth muscle. *Biochem. J.* **244**, 787–791.

Yoshioka, T. & Somlyo, A. P. 1984 The calcium and magnesium contents and volume of the terminal cisternae in caffeine-treated skeletal muscle. *J. Cell Biol.* **99**, 558–568.

Zhu, P. H., Parker, I. & Miledi, R. 1986 Minimal latency of calcium release in frog twitch muscle fibres. *Proc. R. Soc. Lond.* B **229**, 39–46.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Note added in proof (25 *April* 1988)

In more recent studies we have found that, in rabbit pulmonary artery vascular smooth muscle, heparin inhibited the InsP_3-induced, but not the late phase ('I'-component) of GTPγS-induced contraction (Kobayashi *et al.* 1988). This component was also not inhibited by procaine, an inhibitor of Ca^{2+}-induced Ca^{2+} release. These finding also support the existence of an InsP_3-independent component of G-protein-mediated Ca^{2+} release in smooth muscle and, furthermore, indicate that this component is also not due to Ca^{2+}-induced Ca^{2+} release.

Reference

Kobayashi, S., Somlyo, A. V. & Somlyo, A. P. 1988 Heparin inhibits the inositol 1,4,5-trisphosphate-dependent, but not the independent, calcium release induced by guanine nucleotide in vascular smooth muscle. *Biochem. biophys. Res. Commun.* **153**, 625–631.

Discussion

I. C. H. Smith (*Department of Physiology, King's College London, U.K.*). Professor Somlyo has described a latency for the adrenoreceptor-mediated mechanical response, measured by using caged phenylephrine, of around 0.5 s at 30 °C. Has this been corrected for the delay in photolysis of the cage which I understand to have a similar time constant? In vas deferens smooth muscle the latency attributable to α_1-adrenoreceptor-activated responses can be less than 0.1 s (Amobi & Smith 1987) at 35 °C.

Reference

Amobi, N. I. B. & Smith, I. C. H. 1987 Adrenergic and 'non-adrenergic' contributions to the two-component tetanus in the rat vas deferens. *Eur. J. Pharmac.* **135**, 173–182.

A. P. Somlyo. The latencies of force development we measured in the portal vein were 1.5 s at 20 °C and 0.6 s at 30 °C. The contractions were blocked by phentolamine and were independent of the state of membrane polarization, indicating pure pharmacomechanical coupling. At 21 °C, the very long latency is not due to the dark reaction rate, estimated as approximately 3 s^{-1} in preliminary experiments (Walker & Trentham 1988). It might be recalled that in a first-order reaction the delay before reaching some critical threshold concentration is determined by the initial concentration of reactant, as well as by the rate constant. In our experiment at 20 °C, the concentration of phenylephrine released within 100 ms after the laser pulse, from 50 μM caged phenylephrine, would have been well above the value required to initiate contraction. The dark reaction rate at 30 °C has not been measured yet, but in the case of caged ATP the Q_{10} is 2.3. If the dark reactions of caged phenylephrine have a similar temperature dependence, then those reactions would also not account for the bulk of the latency at 30 °C.

The responses Dr Smith measured in vas deferens at 36.5–37 °C were not purely α_1-mediated pharmacomechanical coupling, because they were not fully blocked by an α_1 antagonist. The short latencies Dr Smith mentions might have been the result of electromechanical (rather than pharmacomechanical) coupling of the propagated action potentials evoked by (possibly non-α_1-adrenergic) neural stimuli. Those familiar with the literature of smooth muscle will also recognize that the latency of depolarization following neural stimulation is shorter in vas

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

deferens (Burnstock & Holman 1961; Kuriyama 1963) than in some other smooth muscles (Bolton 1976; Purves 1974). The latency of vascular smooth muscle contraction following neural stimulation of the rabbit main pulmonary artery at 39 °C is 1 s (Bevan & Verity 1966).

References

Bevan, J. A. & Verity, M. A. 1966 *J. Pharmac. exp. Therapy* **152**, 221–230.
Burnstock, G. & Holman, M. E. 1961 *J. Physiol., Lond.* **155**, 115–133.
Kuriyama, H. 1963 *J. Physiol., Lond.* **169**, 223–228.
Walker, J. W. & Trentham, D. R. 1988 *Biophys. J.* **35**, 569a.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES