Protein synthesis in isolated rabbit forelimb muscles

The possible role of metabolites of arachidonic acid in the response to intermittent stretching

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(Received 4 February 1983/Accepted 28 March 1983)

1. Protein synthesis was measured in isolated intact rabbit muscles by the incorporation of [^3H]phenylalanine added at a high concentration (2.5 mM) to the incubation medium. Intermittent mechanical stretching substantially increased the rate of protein synthesis relative to that in control muscles incubated under a constant tension. 2. Indomethacin and meclofenamic acid, inhibitors of the enzyme cyclo-oxygenase, which converts free arachidonic acid into the prostaglandins, prostacyclins and thromboxanes, decreased the rate of protein synthesis in intermittently stretched muscles, but had no effect on synthesis rates in the unstimulated controls. 3. Arachidonic acid at concentrations of 0.2 and 1.0 μM gave a highly significant increase in the rate of protein synthesis in muscles incubated under a constant tension. The ability of arachidonic acid to increase protein-synthesis rates was abolished by the addition of indomethacin. 4. Activation of protein synthesis by intermittent stretching persisted for 10–20 min after the stretch stimulation had ceased. Indomethacin, added either during the initial incubation with intermittent stretching or during the subsequent period when protein synthesis was measured after stimulation had ceased, decreased protein-synthesis rates. This decrease was similar whether indomethacin was present during the initial, final or entire incubation period. 5. In experiments analogous with those in (4) above, when Ca^{2+} was withheld and EGTA added for the entire incubation, rates of protein synthesis were again decreased. The rates of protein synthesis observed when Ca^{2+} was present during either an initial stimulation phase or a final, unstimulated, measurement phase were similar, and were intermediate between control rates and those in muscles incubated without Ca^{2+} for the whole experiment. 6. Two prostaglandins, $\text{F}_{2\alpha}$ (2.8 μM) and A_1 (28 μM), increased rates of protein synthesis in unstimulated muscles, but prostaglandins E_2 and D_2 and the leukotrienes C_4 and D_4 failed to do so. 7. It is concluded that the stretch-stimulated increase in protein synthesis may be caused by activation of membrane phospholipases, release of arachidonic acid and a consequent increase in prostaglandin synthesis.

Mechanical forces affect cell and tissue growth, differentiation and morphogenesis (reviewed by Rodan, 1981). The responses of skeletal muscle to exercise or to inactivity, leading to changes in protein accretion, are well documented (Laurent *et al.*, 1978; Goldspink, 1978), but the mechanisms involved in the accretion of protein by mechanically stimulated tissue remain poorly understood.

Intermittent mechanical stretching of isolated rabbit forelimb muscles *in vitro* increased the tissues' rate of protein synthesis, compared with controls maintained at constant tension (Palmer *et al.*,

1981a). However, changes in amino acid transport, in the supply of O_2 or in the availability of energy were not found to be major factors in determining this response. Furthermore, Palmer *et al.* (1981b) found no support for the view, suggested by experiments with rat diaphragm (Reeds *et al.*, 1980), that the increase in protein synthesis might be mediated by electrical changes in the muscle plasma membrane induced by intermittent stretching. Thus the neurotoxins tetrodotoxin and α -bungarotoxin, which respectively block fast Na^+ channels and acetylcholine receptors, failed to block the increase in protein synthesis in stretched isolated rabbit forelimb muscles. At the same time, an underlying mechanism of humoral rather than electrical character was clearly suggested by the finding that

Abbreviations used: PG, prostaglandin; LT, leukotriene.

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indomethacin added *in vitro* blocked the stretch-stimulated increase in muscle protein synthesis (Palmer *et al.*, 1981b). Indomethacin, a cyclo-oxygenase inhibitor, blocks the conversion of arachidonic acid, via the cyclo-oxygenase pathway, into prostaglandins, endoperoxides and thromboxanes. In the present paper we describe further evidence for the involvement of metabolites of arachidonic acid in the stretch-induced stimulation of protein synthesis in muscle. Part of this work has appeared as a preliminary communication (Smith *et al.*, 1983).

Experimental

Materials

L-[2,6-³H]Phenylalanine (50 Ci/mol) was purchased from Amersham International (Amersham, Bucks., U.K.).

Arachidonic acid, indomethacin, succinylcholine and the prostaglandins PGA₁ and PGD₂ were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). PGF_{2α} (as the tromethamine salt) was purchased from Upjohn Ltd. (Crawley, Sussex, U.K.). PGE₂ was kindly given by Mr. Andrew Coulson, Upjohn Ltd. The leukotrienes D₄ and C₄ (potassium salts) were kindly given by Dr. J. Rokasch, Merck Frosst (Montreal, Canada). Calcium ionophore A23187 was purchased from Boehringer (London) Ltd. (Lewes, E. Sussex, U.K.). EGTA was purchased from British Drug Houses (Poole, Dorset, U.K.). Meclofenamic acid was kindly given by Dr. T. G. Goodburn, Warner-Lambert/Parke-Davis (Pontypool, Gwent, Wales, U.K.). All other chemicals, including those used in the preparation of the incubation medium, were purchased from Gibco Europe (Paisley, Renfrewshire, Scotland, U.K.), Sigma Chemical Co. or British Drug Houses.

Animals and tissues

New Zealand White rabbits weighing 600–1300 g and which had been allowed free access to food and water were the source of the muscles. Three pairs of forelimb muscles were used, extensor carpi ulnaris, extensor quinti digitorum proprius and extensor quarti digitorum proprius. The muscles were 2–3 cm long, 2–3 mm in diameter and weighed 40–90 mg. Extensor quinti digitorum proprius, the largest of the three muscles, was only of a suitable size in smaller animals weighing up to 850 g.

Incubation procedure

The apparatus and procedures used in all the incubations were described previously (Palmer *et al.*, 1981a). Muscles were incubated either under a constant tension of 10 g or with intermittent mechanical stretching equivalent to an 8 g increase in weight for 100 ms every 3 s. In each experiment the

different muscles used were represented equally in each condition of incubation. The medium used was based on that of Trowell (1959), but was without insulin and contained both essential and non-essential amino acids in concentrations similar to those in rabbit blood (Reeds *et al.*, 1980); the concentration of phenylalanine in the basic medium was 200 μM. The medium was sterilized by filtration before use, and during the incubation was continuously bubbled with O₂/CO₂ (19:1) to maintain a pH of 7.4. All incubations were performed at 37 ± 0.1 °C in 3 ml of medium. Muscles were preincubated for 30 or 60 min in unlabelled medium before protein synthesis was measured; the length of the preincubation period is indicated in the Tables. PGF_{2α}, LTC₄ and LTD₄ were added to the medium, as supplied, in aqueous solution. The other prostaglandins and the inhibitors (indomethacin and meclofenamic acid) were dissolved in ethanol and added to the medium at the start of the incubation. Up to 10 μl of the ethanol solution was added to each incubation vessel; an equal amount of ethanol was added to the controls. Arachidonic acid was supplied as a solution in toluene; portions were evaporated at room temperature under a stream of O₂-free N₂ and redissolved in ethanol. In the Ca²⁺-free medium, CaCl₂ was replaced with NaCl, to give the same osmolarity as the control medium (300 mosmol/kg). EGTA was added to the Ca²⁺-free medium to give a concentration of 50 μM.

Protein synthesis was measured during a period of 10–30 min in the presence of a high concentration of phenylalanine (Garlick *et al.*, 1980). The labelled medium contained 2.5 mM-phenylalanine with a nominal specific radioactivity of 1500 d.p.m./nmol. This was prepared by the addition of 0.2 ml of a stock solution of phenylalanine in 0.9% NaCl to 20 ml of the basic medium. At the end of the incubation, muscles were removed from the labelled medium and immediately washed (2–3 s) in ice-cold 0.9% NaCl, blotted dry and stored at –20 °C.

Muscles were homogenized in 3–4 ml of 0.5 M-HClO₄ and the homogenate was centrifuged at 2500 g for 5 min. The pellet was washed once with 0.5 M-HClO₄ (3 ml), twice with ethanol (5 ml) and once with diethyl ether (5 ml), dried and hydrolysed for 24 h in 5.6 M-HCl at 105 °C. After drying under reduced pressure, the hydrolysate was redissolved in 0.2 M-sodium citrate buffer, pH 2.2.

Phenylalanine in hydrolysates and medium was measured by ion-exchange chromatography on a column (24 cm × 0.9 cm) of LA49 resin (Locarte Co., London W.12, U.K.) by elution with 0.2 M-sodium citrate buffer, pH 4.27, at 50 °C on a Locarte automatic amino acid analyser.

Radioactivity was measured in a Beckman LS45 liquid-scintillation counter by using Lumax/xylene (1:1, v/v) scintillation fluid (L.K.B., Croydon,

Surrey, U.K.). The counting conditions were identical for all samples and the efficiency of counting was 33.8%.

Protein synthesis (k_s , days⁻¹) was calculated as

$$k_s = \frac{S_B}{S_M} \times \frac{1440}{t}$$

where S_B = the specific radioactivity of protein-bound phenylalanine (d.p.m./nmol), S_M = the specific radioactivity of the phenylalanine in the medium (d.p.m./nmol) and t = incubation time (min).

Results

Equilibration of homogenate pool of phenylalanine and rate of incorporation of labelled phenylalanine into muscle proteins

The concentration of phenylalanine in the incubation medium, 2.5 mM, was considerably higher than the normal intracellular (homogenate) concentration of 90–100 nmol/g fresh wt. of muscle. Under these incubation conditions the intracellular concentration of phenylalanine increased very rapidly. In preliminary experiments muscles were incubated in labelled phenylalanine (2.5 mM) and removed from the medium after 1, 2, 5, 10, 20, 30 or 60 min of incubation. The specific radioactivity of the homogenate pool of free phenylalanine attained a value 81% of that of the medium after 1 min incubation, and this rose to 96% after 2 min and maintained a value of 96–106% of that of the medium at all subsequent times measured. In the same experiments the mean specific radioactivity of protein-bound phenylalanine (which differed between experiments by less than 20%) was 0.27 d.p.m./nmol after 10 min incubation, rising to 0.62, 0.91 and 1.93 d.p.m./nmol after 20, 30 and 60 min respectively. These values correspond to rates of protein synthesis of 2.59, 2.93, 2.91 and 3.08%/day at the four times noted, indicating a linear rate of synthesis over the period.

Stretch-induced stimulation of protein synthesis in succinylcholine-treated muscle

To confirm our previous conclusions (Palmer *et al.*, 1981b), obtained with specific neurotoxins, that membrane charge effects are unlikely to be critically involved in the stretch-stimulation of muscle protein synthesis, we measured rates of protein synthesis in muscles depolarized by succinylcholine. Table 1 shows that when isolated muscle preparations are incubated in the presence of 20 μ M-succinylcholine there was no change in rate of protein synthesis by the control muscle and no significant decrease in the stimulation of the protein synthesis associated with intermittent stretching.

Dependence of stretch-induced stimulation on metabolites of the arachidonic acid cyclo-oxygenase pathway

The arachidonic acid cyclo-oxygenase inhibitors indomethacin (50 μ M) or meclofenamic acid (50 or 100 μ M) were added to the media in which muscles were incubated, either under constant tension or with intermittent stretching. Table 2 shows that both indomethacin and meclofenamic acid significantly decreased the rate of protein synthesis in the stretched muscle ($P < 0.05$ for each inhibitor), but neither compound influenced the basal rate of protein synthesis in the unstimulated muscle.

The effect of meclofenamate, at concentrations below 50 μ M, on protein synthesis in intermittently stretched muscles was examined in three experiments; 20 μ M-meclofenamate decreased this rate in the stimulated muscles by less than 10%.

Stimulation of muscle protein synthesis by arachidonate metabolites

The potential of eicosanoids of the arachidonic acid cyclo-oxygenase pathways to function as activators of muscle protein synthesis, and thus as potential mediators of stretch-stimulation, was examined in muscles incubated, without stretching, with

Table 1. *Effect of succinylcholine on protein synthesis in muscles incubated under a constant tension or with intermittent mechanical stretching*

Muscles were incubated with intermittent stretching or under a constant tension for 60 min in unlabelled medium and then for 30 min in fresh medium containing 2.5 mM-phenylalanine (sp. radioactivity 1500 d.p.m./nmol). Succinylcholine (20 μ M, added from a stock solution in 0.9% NaCl) was present throughout the 90 min of incubation. The effect of succinylcholine was not significant in either treatment.

	Constant tension		Mechanical stretching	
	Control	+ Succinylcholine	Control	+ Succinylcholine
Protein-synthesis rate (%/day) ($n = 4$)	2.01	2.18	3.39	3.47
Mean difference $\pm$ S.E.M.		0.18 $\pm$ 0.09		0.07 $\pm$ 0.13

Table 2. *Effects of indomethacin and meclofenamate on the rate of protein synthesis in isolated muscles*

Muscles were incubated for 60 min in unlabelled medium and for 30 min in labelled medium as described previously. Indomethacin or meclofenamic acid was added from stock solutions in ethanol; a total of 10 μ l was added to each incubation vessel (3 ml of medium), or 10 μ l of ethanol was added to the controls. Indomethacin or meclofenamic acid was present in the medium during both the initial 60 min and the final 30 min of incubation. Results are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses: *significantly different by paired *t* test from corresponding intermittently stretched control ($P < 0.05$).

Treatment	Rate of protein synthesis (%/day)	
	Constant tension	Intermittent stretching
Control	2.47 $\pm$ 0.33 (8)	5.21 $\pm$ 0.77 (12)
+ Indomethacin (50 μ M)	2.85 $\pm$ 0.50 (8)	3.01 $\pm$ 0.40 (12)*
Control	2.19 $\pm$ 0.64 (4)	4.12 $\pm$ 0.99 (4)
+ Meclofenamate (50 μ M)	1.91 $\pm$ 0.37 (4)	2.66 $\pm$ 0.53 (4)*
+ Meclofenamate (100 μ M)		1.99 $\pm$ 0.37 (4)*

arachidonic acid in the presence and absence of indomethacin. In a preliminary report (Smith *et al.*, 1983), arachidonic acid was found to mimic stretch-stimulation in muscles maintained without intermittent stretching. Table 3 summarizes the effect of two concentrations of arachidonic acid (0.2 and 1.0 μ M), alone and with indomethacin, on the rate of protein synthesis in muscles held under constant tension. Arachidonic acid alone, at concentrations of 0.2 or 1.0 μ M, gave a significant increase in the rate of protein synthesis; the two concentrations gave a similar increase in rate. The addition of 50 μ M-indomethacin to muscles incubated in the presence of arachidonic acid caused a significant decrease in protein synthesis when compared with the effect of arachidonic acid alone, and the inhibitory effect of indomethacin was not dependent on the concentration of arachidonic acid at the values used.

The effect of indomethacin on muscle protein synthesis measured in the presence of 1 μ M-arachidonic acid was examined at lower concentrations of inhibitor; 20 μ M-indomethacin decreased the rate of protein synthesis by 30%, i.e. by a similar proportion to that obtained with 50 μ M-indomethacin, but lower concentrations of 10 μ M- and 5 μ M-indomethacin were without effect.

In experiments with mechanically stimulated muscle, the increase in rate of protein synthesis associated with stretching was not further augmen-

Table 3. *Effect of arachidonic acid alone and arachidonic acid + indomethacin on the rate of protein synthesis in muscles maintained under a constant tension*

Muscles were incubated under a constant tension for 60 min in unlabelled medium and for 30 min in labelled medium; 50 μ M-indomethacin or 0.2–1.0 μ M-arachidonic acid or both arachidonic acid and indomethacin were added for the entire 90 min. Results are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. By two-way analysis of variance: * not significantly different from treatment 1; † significantly different from treatment 1 ($P < 0.001$), but not significantly different from each other; ‡ significantly different from treatments 3 and 5, but not significantly different from each other.

Treatment no.	Addition	Protein-synthesis rate (%/day)
1	None	2.50 $\pm$ 0.15 (16)
2	50 μ M-Indomethacin	2.88 $\pm$ 0.33 (6)*
3	0.2 μ M-Arachidonic acid	3.46 $\pm$ 0.26 (6)†
4	0.2 μ M-Arachidonic acid + indomethacin	2.58 $\pm$ 0.32 (4)‡
5	1 μ M-Arachidonic acid	3.67 $\pm$ 0.21 (9)†
6	1 μ M-Arachidonic acid + indomethacin	2.73 $\pm$ 0.29 (5)‡

ted by the addition of arachidonic acid to the incubation medium (results not shown).

Residual activation of protein synthesis after withdrawing mechanical stimulus

If eicosanoid(s), released by intermittent stretching, underlie the activation of muscle protein synthesis, this activation may be expected to persist, albeit briefly, depending on the stability of the factor, after the mechanical stimulus is withdrawn ('residual activation'). Previously, in incubations lasting 5 h we failed to demonstrate residual activation (Palmer *et al.*, 1981a), but the ability of the technique used in the previous studies to measure short-term changes in protein synthesis was severely limited. Indeed, one reason for adopting our present technique was to enable us to measure protein synthesis over periods of less than 15 min. When the period of mechanical stimulation was shortened to 30 min and protein synthesis was subsequently measured over 10 min intervals, the activation of muscle protein synthesis persisted for between 10 and 20 min after stretching ceased (Fig. 1).

Table 4 shows that the addition of indomethacin to this test system, in which mechanical stimulation of the muscle and measurement of [3 H]phenylalanine incorporation were performed consecutively, decreased the rate of protein synthesis to an extent similar to that shown in Table 2. The extent of this decrease was, moreover, essentially the same whether indomethacin was present for the entire

Fig. 1. *Residual activation of protein synthesis after withdrawing the mechanical stimulus*

Muscles were incubated (a) under a constant tension or (b) with intermittent stretching for 30 min and then under a constant tension for 10–40 min. The rate of protein synthesis was measured over a 10 min period, commencing when intermittent stretching ceased, or 10, 20 or 30 min later. The rate of protein synthesis in the control muscles, incubated under a constant tension throughout was also measured over a 10 min period after 30 min incubation and was $2.66 \pm 0.4\%$ /day (broken line). In all experiments the measurement of the rate of protein synthesis was commenced by the replacement of the initial unlabelled medium with a medium containing [³H]-phenylalanine (sp. radioactivity 1500 d.p.m./nmol). Results are means $\pm$ S.E.M.

Ca²⁺ requirement in the stretch-induced stimulation of muscle protein synthesis

Ca²⁺, as the calmodulin complex, is an essential cofactor in the phospholipase A₂-mediated release of arachidonic acid from phosphatidylcholine, a rate-limiting step in eicosanoid synthesis (Zenser *et al.*, 1980; Irvine, 1982). In general accord with the idea that the stretch-stimulation of muscle protein synthesis is mediated by eicosanoids, there was a significant decrease in the effect when Ca²⁺ was omitted and EGTA was added to the incubation medium (Table 5), confirming the work of Palmer *et al.* (1981b). We attempted to define further the requirement for Ca²⁺ by conducting the muscle stimulation and the measurement of phenylalanine incorporation as two separate consecutive steps, and, as in the previous section, examined the Ca²⁺ requirement of each step in relation to the overall stretch-induced stimulation of protein synthesis. Table 5 shows that the effect of incubating muscles in Ca²⁺-free medium containing EGTA, during either the mechanical stimulation or the phenylalanine-incorporation stages, was similar. The mean rate of protein synthesis of each series in which Ca²⁺ was present for part of the incubation was lower than that of controls in which Ca²⁺ was present during both steps, but higher than the rate of protein synthesis obtained when Ca²⁺ was withheld for the whole experiment. Thus the two small effects obtained by withholding Ca²⁺ at each consecutive step appeared to be additive.

The Ca²⁺ ionophore A23187 may stimulate the influx of extracellular Ca²⁺ as well as liberating intracellular Ca²⁺ stores (Irvine, 1982). It was added to the muscle incubation medium in an attempt to raise the concentration of intracellular Ca²⁺, and to examine the consequences of this change on rates of protein synthesis. Table 6 shows that when muscles were incubated, either at constant tension or with intermittent stretching, there was no increase in rate of muscle protein synthesis associated with the addition to the medium, which contained 2 mM-Ca²⁺, of ionophore A23187 at concentrations of 25 and 2.5 μ M. In all experiments the mean rates of protein synthesis were slightly decreased by these concentrations of A23187, which may reflect a toxic action (Reed & Lardy, 1972; Schanne *et al.*, 1979).

Effect of prostaglandins and leukotrienes on rate of protein synthesis in muscles incubated without mechanical stimulation

In conflict with the above evidence for the involvement of prostanoids in the stretch-stimulation of muscle protein synthesis is the earlier failure (Smith *et al.*, 1983) to simulate this effect by incubating the muscle with prostaglandins *in vitro*, without mechanical stimulation. On re-examination of the action of prostaglandins at higher concen-

incubation, for only the first part, comprising incubation with mechanical stimulation, or for only the final incubation, in which the amino acid incorporation was measured without stimulation. This response to indomethacin added only after mechanical stimulation has ceased is consistent with the view that stretching the muscle promotes the release of arachidonic acid, which is retained by the tissue, and subsequently converted via the cyclo-oxygenase pathway into prostanoids.

Table 4. *Effect of indomethacin added for different periods of the incubation on the rate of protein synthesis in isolated muscles*

Muscles were incubated with intermittent stretching for 30 min in radioisotope-free medium; 50 μ M-indomethacin was present in the medium of two of the four muscles. Simultaneously, after 30 min: (a) intermittent stretching was stopped; (b) the unlabelled medium was removed, the muscles were washed with three changes of normal medium and then incubated in medium containing [3 H]phenylalanine; (c) one pair of muscles was changed from medium without indomethacin to medium + indomethacin or from medium + indomethacin to medium without indomethacin. The incubation was continued subsequently for 15 min under constant tension. Results are means $\pm$ S.E.M. for five experiments. Significantly different from treatment 1 by paired *t* test: **P* < 0.005; †*P* < 0.01; ‡*P* < 0.05.

Treatment no.	Treatment	Time of incubation (min)	Rate of protein synthesis (%/day)
1	Intermittent stretching	30	
	Constant tension	15	5.09 $\pm$ 0.65
2	Intermittent stretching + indomethacin	30	
	Constant tension + indomethacin	15	3.39 $\pm$ 0.50*
3	Intermittent stretching + indomethacin	30	
	Constant tension	15	3.06 $\pm$ 0.34‡
4	Intermittent stretching	30	
	Constant tension + indomethacin	15	3.23 $\pm$ 0.47†

Table 5. *Effect of withholding Ca²⁺ for different periods of the incubation on the rate of protein synthesis in isolated muscles*

Muscles were incubated under a constant tension or with intermittent stretching for 30 min in normal medium containing 2 mM-Ca²⁺, or Ca²⁺-free medium containing 50 μ M-EGTA. Simultaneously, after 30 min: (a) intermittent stretching was stopped; (b) unlabelled medium was replaced by medium containing [3 H]phenylalanine; and (c) pairs of intermittently stretched muscles were changed from normal to Ca²⁺-free medium (treatment 5) or from Ca²⁺-free to normal medium (treatment 6). Protein synthesis was measured in all muscles for a subsequent period of 10 min, under constant tension. Results are means $\pm$ S.E.M. for six experiments. By paired *t* test: * significantly different from treatment 1 (*P* < 0.01); † significantly different from treatment 3 (*P* < 0.05); ‡ not significantly different from treatment 3.

Treatment no.	Treatment	Time of incubation (min)	Ca ²⁺ status	Rate of protein synthesis (%/day)
1	Constant tension	30	+Ca ²⁺	
	Constant tension	10	+Ca ²⁺	2.14 $\pm$ 0.30
2	Constant tension	30	-Ca ²⁺ + EGTA	
	Constant tension	10	-Ca ²⁺ + EGTA	1.85 $\pm$ 0.30
3	Intermittent stretching	30	+Ca ²⁺	
	Constant tension	10	+Ca ²⁺	3.87 $\pm$ 0.41*
4	Intermittent stretching	30	-Ca ²⁺ + EGTA	
	Constant tension	10	-Ca ²⁺ + EGTA	2.73 $\pm$ 0.31†
5	Intermittent stretching	30	+Ca ²⁺	
	Constant tension	10	-Ca ²⁺ + EGTA	3.21 $\pm$ 0.45‡
6	Intermittent stretching	30	-Ca ²⁺ + EGTA	
	Constant tension	10	+Ca ²⁺	3.24 $\pm$ 0.55‡

trations (28 and 2.8 μ M) than those used previously, PGF_{2 α} stimulated muscle protein synthesis at each concentration, and PGA₁ was similarly active at the higher concentration only; PGE₂ and PGD₂ gave no significant stimulation. The leukotrienes LTC₄ and LTD₄ did not influence the rate of muscle protein synthesis (Table 7).

Discussion

While the present work was in progress, Rodemann & Goldberg (1982) reported that PGF_{2 α}

and PGE₂ influence the rate of turnover of muscle protein *in vitro*. Their findings, together with the present evidence, support the hypothesis (Palmer *et al.*, 1981b) that the stretch-induced stimulation of protein synthesis in this muscle preparation is mediated by a change in intracellular prostaglandin concentrations. Evidence that prostaglandins are released during skeletal-muscular work has been reported by several groups (e.g. Herbaczynska-Cedro *et al.*, 1976; Young & Spinks, 1980), and indomethacin and meclofenamate, two compounds of different structure but with similar actions on the

Table 6. *Effect of ionophore A23187 on the rate of protein synthesis in muscles incubated under constant tension or with intermittent stretching*

Muscles were incubated for 60 min in unlabelled medium and for 30 min in labelled medium. Ionophore A23187 was present throughout the incubation. Results are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. By paired *t* test the effect of A23187 was not significant in any treatment.

Addition	Rate of protein synthesis (%/day)	
	Constant tension	Intermittent stretch
None (8)	2.93 $\pm$ 0.38	3.96 $\pm$ 0.49
25 μ M-A23187 (4)	2.82 $\pm$ 0.90	3.12 $\pm$ 0.55
2.5 μ M-A23187 (4)	2.24 $\pm$ 0.57	3.11 $\pm$ 0.91

Table 7. *Effect of prostaglandins and leukotrienes on the rate of protein synthesis in muscles incubated under a constant tension*

Muscles were incubated under a constant tension for 60 min in unlabelled medium and for 30 min in labelled medium. The eicosanoids were present in the concentrations stated throughout the 90 min incubation period. Results are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. Significantly different from corresponding control by paired *t* test: **P* < 0.05; †*P* < 0.02.

Eicosanoid added	Concn. (μ M)	Rate of protein synthesis (%/day)
None		2.38 $\pm$ 0.22 (12)
PGA ₁	2.8	2.98 $\pm$ 0.37 (5)
	17	3.77 $\pm$ 0.58 (5)†
PGD ₂	2.8	2.84 $\pm$ 0.68 (5)
	14	2.79 $\pm$ 0.53 (5)
PGE ₂	2.8	2.82 $\pm$ 0.35 (5)
	17	2.82 $\pm$ 0.56 (5)
PGF _{2α}	2.8	3.03 $\pm$ 0.59 (5)*
	22	3.24 $\pm$ 0.42 (5)*
LTD ₄	0.3	2.27 $\pm$ 0.43 (3)
	1.1	2.40 $\pm$ 0.45 (4)
	2.2	2.16 $\pm$ 0.37 (4)
LTC ₄	1.4	2.53 (2)
	2.8	2.41 (2)

cyclo-oxygenases, block the stretch-induced stimulation of protein synthesis. This observation was in striking contrast with the consistently negative outcome of attempts to invoke, as mechanisms of stretch stimulation, either ameliorative effects of tissue energy availability (Palmer *et al.*, 1981a) or membrane charge effects (Palmer *et al.*, 1981a). Additional evidence that changes in the membrane potential induced by intermittent stretching, as discussed by Katz (1950), do not underlie the stimulation of muscle protein synthesis is provided by the results with succinylcholine.

Indomethacin and meclofenamate are potent inhibitors of the conversion of free arachidonic acid into prostaglandins, prostacyclins and thromboxanes (Flower, 1974), but, because we have used unskinned preparations, we have had to add high (> 20 μ M) concentrations of the two inhibitors. It is possible that they may have acted by inhibiting other enzymes, including phospholipase A₂ (Franson *et al.*, 1980), and their action in decreasing the stretch-induced stimulation of protein synthesis may not be attributed solely to a blockade of cyclo-oxygenase. However, three observations lead to the conclusion that the effects of these compounds relate specifically to a product of cyclo-oxygenase rather than to a general blockade of phospholipase A₂ activity. First, the two inhibitors share similar activities as inhibitors of cyclo-oxygenase (Flower, 1974) and of stretch-stimulated protein synthesis (Table 2), but may differ considerably in their activity towards phospholipase A₂ (Franson *et al.*, 1980; Kaplan-Harris & Elsbach, 1980). Second, unlike PGF_{2 α} and PGA₁, neither LTC₄ nor LTD₄ (whose synthesis would be affected by phospholipase A₂ inhibition) stimulated protein synthesis. Third, the stimulation of protein synthesis by added arachidonic acid was blocked by indomethacin.

Despite the conclusion that indomethacin and meclofenamate are acting via an inhibition of cyclo-oxygenase activity, we would propose that the primary effect of mechanical activity on protein synthesis is a stimulation of arachidonic acid release from membrane-bound phospholipids. Thus the addition of arachidonic acid at concentrations greater than 0.1 μ M increased the rate of protein synthesis in muscles held at a constant tension, but it has been our consistent observation that the addition of arachidonic acid to muscles subjected to intermittent stretching has no further stimulatory effect of protein synthesis. Rodemann & Goldberg (1982) have also reported an activation of protein synthesis in unstretched rat soleus muscles by 5 μ M-arachidonic acid, although surprisingly they were unable to observe an effect in extensor digitorum longus muscles.

According to this view of the link between mechanical activity and protein synthesis *in vitro*, the persistence of an activation of protein synthesis in intermittently stretched muscle, observed after the stimulus is withdrawn (Fig. 1), is most simply explained by the assumption that free arachidonate is released by stretching, is retained intracellularly and continues to be metabolized to the prostaglandins. The data in Table 4 are consistent with this, and suggest that the persistence of the stimulation of protein synthesis is not due to the continued presence of prostaglandins synthesized during the period of intermittent stretching. Nevertheless, the effect on protein synthesis of a period of mechanical

stimulation does fall progressively after the stimulus is withdrawn, and this may reflect the re-esterification of free arachidonic acid into tissue phospholipids, including phosphatidylinositol (Irvine, 1982).

In glycerinated skeletal- and heart-muscle preparations, Brenner (1979) reported a stretch-induced release of Ca^{2+} from the sarcoplasmic-reticulum vesicles. A similar release of Ca^{2+} in our intermittently stretched muscle preparations might therefore offer a simple explanation for the stimulation of eicosanoid synthesis, via a Ca^{2+} -activated phospholipase. However, in sequential incubations (Table 5) from which Ca^{2+} was absent but in which $50\mu\text{M}$ -EGTA was present during the stretching phase, there was only a small non-significant impairment in the stimulation of protein synthesis. From the earlier sequential incubations in the presence of Ca^{2+} (Table 4), the release of free arachidonic acid during the stretch phase is inferred from the effects of indomethacin. The apparently low Ca^{2+} requirement for this stretch-stimulated release, and the consequent stimulation of protein synthesis thus indicated, is clearly not consistent with a central regulatory role for Ca^{2+} in the activation. However, work with intact platelets suggests that EGTA does not penetrate to the locus of phospholipase A_2 (Pickett *et al.*, 1976; Van den Bosch, 1980); consequently, the unexpectedly small decrease in stimulation obtained when Ca^{2+} was withheld during the stretching phase may simply reflect a low availability of EGTA at the phospholipase locus in our unskinned muscle preparation. A small decrease in stretch-induced stimulation of protein synthesis was found when Ca^{2+} was omitted and EGTA added during the phenylalanine incorporation phase. This effect may reflect, among other things, a Ca^{2+} requirement at the $\text{PGF}_{2\alpha}$ -binding sites in muscle, as found by Rao (1975) at the high-affinity $\text{PGF}_{2\alpha}$ -binding sites in corpora lutea.

The failure of ionophore A23187 to increase the rate of protein synthesis in rabbit forelimb muscle held at a constant tension (Table 6) is in contrast with the report by Kameyama & Etlinger (1979) (of a significant stimulation of protein synthesis in rat soleus muscles incubated with A23187 plus Ca^{2+}), but is, however, in agreement with work by Rodemann *et al.* (1982), in which the compound failed to stimulate protein synthesis in either rat soleus or extensor digitorum longus muscles. Those authors did nevertheless obtain a large increase in $\text{PGF}_{2\alpha}$ production in rat diaphragm incubated with A23187. In their earlier work (Rodemann & Goldberg, 1982) and in our present study, $\text{PGF}_{2\alpha}$ was found to stimulate muscle protein synthesis, and Rodemann *et al.* (1982) concluded that some other consequences of the A23187 treatment prevented this effect of the prostaglandin. Such consequences

might include cell killing, triggered by A23187 plus Ca^{2+} (Shier & DuBourdieu, 1982), and this could underlie the inhibitory effects suggested by the data in Table 6. Thus the general lack of support, offered by the present experiments, for the idea of a Ca^{2+} -controlled initiation of the stretch-stimulation effect in muscle may well reflect the shortcomings of the chemical tools employed.

Although the phospholipases are mostly Ca^{2+} -dependent, not all are Ca^{2+} -controlled (Irvine, 1982), and possible alternative or perhaps additional mechanisms of phospholipase activation in the stretched muscle preparation must be considered. Among the mechanisms for membrane-bound phospholipase A_2 regulation, reviewed by Van den Bosch (1980), is the proposal that the activation of phospholipases might occur by changes in the structural arrangement of phospholipids in plasma membranes (Vogt, 1978). There is increasing evidence [reviewed by Chapman *et al.* (1979) and Sandermann (1978)] that membrane phospholipids can influence the activity of membrane-bound enzymes considerably. The possibility that plasma-membrane structural changes induced by intermittent stretching of the muscle may activate phospholipase and initiate the chain of events inferred from the present work is therefore one that deserves serious consideration.

We are grateful to Mr. I. McDonald for the statistical analysis, and to Dr. E. J. Harris and Dr. R. F. Irvine for helpful discussions.

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