Early events in the response of fast skeletal muscle to chronic low-frequency stimulation

Polyamine biosynthesis and protein phosphorylation

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The concentrations of putrescine, spermidine and spermine and the activities of ornithine decarboxylase (ODC) and *S*-adenosyl-L-methionine decarboxylase (SAM-D) were investigated in fast muscle subjected to chronic low-frequency electrical stimulation. Both ODC and SAM-D activities increased markedly between 18 and 48 h of stimulation. Changes in enzyme activities were followed by phasic elevations in the concentrations of putrescine, spermidine and spermine. Peak levels were reached first by putrescine at 3–4 days, followed by spermidine at about 9 days and then by spermine at about 11 days. A possible relationship was sought between these events and changes produced *in vitro* in the phosphorylation pattern of cytoplasmic proteins and the total activity of cyclic AMP-dependent protein kinase. However, during the early stages of stimulation, no prominent changes were seen either in the phosphorylation pattern or in the activity of cyclic AMP-dependent protein kinase. These characteristics changed significantly at a later stage (by 12 days of stimulation) and became indistinguishable from those of slow muscle by 3 to 4 weeks of stimulation.

When a fast skeletal muscle in a mammal is subjected to a continuous low-frequency pattern of electrical stimulation over a period of weeks, it acquires, through an orderly sequence of changes, the physiological, biochemical and ultrastructural characteristics of a slow muscle (Salmons & Henriksson, 1981; Eisenberg & Salmons, 1981). This transformation has two striking features. First, it is readily elicited in the muscles of an adult animal. This suggests that the changes reflect an inherent plasticity that enables a skeletal muscle to respond adaptively to changing functional demands. Secondly, the transformation involves changes not merely in the amount but also in the types of protein synthesized by the muscle. This has been shown in relation to the complement of myosin light chains (Sréter *et al.*, 1973, 1974; Salmons & Sréter, 1976; Pette *et al.*, 1976), myosin heavy chains (Sréter *et al.*, 1974, 1975) and proteins of the sarcoplasmic reticulum (Ramirez & Pette, 1974; Pette & Heilmann, 1977; Heilmann & Pette, 1979). Evidence

from gel electrophoresis performed on extracts from single muscle fibres (Pette & Schnez, 1977) and from immunocytochemical studies using antibodies specific for fast- or slow-muscle myosin (Rubinstein *et al.*, 1978) has established that changes in the synthesis of myosin take place within individual fibres. It would seem that the plasticity of adult skeletal muscle derives at least in part from an ability to switch from the synthesis of one isoform of a protein to another. As part of a study designed to gain insight into the regulatory mechanisms underlying these phenomena, we have been investigating a possible involvement of polyamines in the early stages of the response to chronic stimulation.

Polyamines are low-molecular-weight polycationic substances that occur widely in biological material, and are associated particularly with tissues that have a high biosynthetic activity. Their precise function in protein synthesis is still a matter for debate, since there is evidence to support a potential role in almost every step from transcription to translation (Cohen, 1971). The biogenesis of the polyamines putrescine, spermidine and spermine involves two decarboxylating enzymes, ODC and SAM-D, both of which have exceptionally short half-lives (approx. 11 min for ODC, 60 min for SAM-D) and which show a striking inducibility in

Abbreviations used: ODC, ornithine decarboxylase; SAM-D, *S*-adenosyl-L-methionine decarboxylase.

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response to stimuli that enhance protein synthesis (Bachrach, 1973; Tabor & Tabor, 1976; Russell & Durie, 1978; Jänne *et al.*, 1978).

Protein phosphorylation serves an important function in cell regulation (for reviews, see Jungmann & Russell, 1977; Nimmo & Cohen, 1977; Greengard, 1978; Weller, 1979). Russell *et al.* (1976) have attempted to link such phosphorylation events to the induction of ODC. In their proposed scheme, cyclic AMP is involved in the induction of ODC by activating one or more cyclic AMP-dependent protein kinases in the cytoplasm. This is followed by translocation of the active catalytic subunit to nuclear acceptor sites, where it is responsible for phosphorylation of nuclear acidic proteins (Russell *et al.*, 1976; Jungmann & Russell, 1977). In this way, the DNA sequence encoding ODC is released from repression.

In the present study, we have examined the levels of putrescine, spermidine and spermine and the activities of ODC and SAM-D in fast skeletal muscles subjected to chronic electrical stimulation. In addition, we have examined protein phosphorylation during the induction of ODC and during the later stages of muscle transformation. This has been achieved by investigating: (a) changes in the phosphorylation patterns of the cytoplasmic proteins; (b) the total cytoplasmic activity of cyclic AMP-dependent protein kinase; and (c) the DEAE-cellulose chromatographic pattern of cyclic AMP-dependent protein kinase activity, which reveals the relative proportions of type I and type II enzymic activity.

Materials and methods

Materials

DL-[1-¹⁴C]Ornithine (sp. radioactivity 59 Ci/mol), S-adenosyl-L-[1-¹⁴C]methionine (sp. radioactivity 61 Ci/mol) and the triethylammonium salt of adenosine 5-[γ -³²P]triphosphate (sp. radioactivity 2000 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. DE-52 DEAE-cellulose was purchased from Whatman, Springfield Mill, Maidstone, Kent, U.K. NSD-1055 (4-bromo-3-hydroxybenzoyloxyamine dihydrogen phosphate) was obtained from Smith and Nephew Research, Gilston Park, Harlow, Essex, U.K. Soluene was obtained from Packard Instruments, Caversham, Berks., U.K. Protein assays were performed with the use of a commercial kit (Bio-Rad Laboratories, Watford, Herts., U.K.).

All other chemicals were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Stimulation

Adult New Zealand White rabbits of either sex with body weights between 3 and 5 kg were

anaesthetized with an intravenous injection of pentobarbitone sodium (30 mg/kg body wt.) 30–40 min after pre-anaesthetic medication with atropine sulphate (50 μ g/kg body wt.) and chlorpromazine hydrochloride (Largactil; 25 mg/kg body wt.) administered subcutaneously. In some cases diethyl ether was used briefly as a supplementary anaesthetic. Under strictly aseptic conditions a miniature electronic stimulator, based on that described by Salmons (1967), was implanted intraperitoneally and the two flexible leads were conveyed subcutaneously to the left hind limb, where they were fixed near to the common peroneal nerve. The right limb served as an unoperated control. For short-term stimulations the implant incorporated a subcutaneous switch, which could be operated remotely by means of a magnet a week or more after implantation. In this way the effects of stimulation could be dissociated from the consequences of anaesthesia and surgery.

Stimulation was carried out for periods ranging from 6 h to 24 weeks. In terminal procedures the animals were anaesthetized and both the tibialis anterior muscles and the right soleus muscle were excised and used for the biochemical assays described below.

Determination of endogenous concentrations of polyamines

Muscle samples (400 mg), frozen to the temperature of liquid N₂, were ground and transferred to a glass pestle and vessel for homogenization in water (1 ml). Trichloroacetic acid (0.5 ml; 20%, w/v) was then added. The samples were vortex-mixed and left on ice for 30 min. The precipitates were spun down at 800 g for 10 min at 4°C. The supernatants were used for polyamine analysis and the precipitate for protein determination. Polyamines were analysed in a Locarte amino acid analyser incorporating a cation-exchange chromatography column from which the polyamines were eluted with stepwise increases in pH and ionic strength. They were visually detected by reaction with ninhydrin, and the colour formation was measured at two wavelengths and two pathlengths by a series of colorimeters. Outputs were plotted automatically on a three-channel point-plot recorder. Individual polyamines were identified on the basis of their retention time, being eluted in the order: putrescine, spermidine, spermine.

Assay of ODC activity

Muscles were excised, rinsed in ice-cold 0.9% NaCl, blotted, weighed and immediately homogenized with an Ilado X10/20 Disperser in 5 vol. of cold 0.05 M-sodium phosphate/potassium phosphate buffer, pH 7.2, containing 1 mM-dithiothreitol, 20 μ M-pyridoxal phosphate and 0.1 mM-EDTA. The

20000g supernatant was used to assay enzyme activity. The latter was determined using the liberation of $^{14}\text{CO}_2$ from DL-[1- ^{14}C]ornithine as described previously (Fuller *et al.*, 1977). It was necessary to use DL-[1- ^{14}C]ornithine of high specific radioactivity (59 Ci/mol) because the control levels of ODC activity in fast and slow muscle were very low. Additional experiments (results not shown) with DL-[1- ^{14}C]ornithine of lower specific radioactivity (4 Ci/mol) were carried out to check that changes in ODC activity after stimulation were not a consequence of a change in the levels of endogenous ornithine. Values were corrected by reference to a blank containing heat-inactivated enzyme.

Assay of SAM-D activity

The 20000g supernatant prepared for the determination of ODC activity was also used to assay the activity of SAM-D. The latter was determined by measuring the liberation of $^{14}\text{CO}_2$ from *S*-adenosyl-L-[1- ^{14}C]methionine (sp. radioactivity 5 Ci/mol) with 2.5 mM-putrescine as substrate, as described previously (Fuller *et al.*, 1977). All values were corrected by comparison with blank values obtained as in the assay for ODC activity.

Assay for total cyclic AMP-dependent protein kinase activity

Fresh muscle samples were homogenized in 5 vol. of cold 5 mM-Tris/HCl buffer, pH 7.5, containing 2 mM-EDTA and 3 mM-NaF. Samples were centrifuged at 20000g at 4°C for 20 min and the supernatant was used to determine enzyme activity. The assay was based on the methods described by Haddox *et al.* (1979) and Gill & Walton (1979). Protein kinase activity was determined in a total volume of 90 μl containing 30 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 6.8, 10 mM-magnesium acetate, 5 mM-NaF, 1 mM-aminophylline, 0.25 mM-EGTA, 2 mM-dithiothreitol, 100 μg of H2B histone, 0.5 μCi of [γ - ^{32}P]ATP and 0.1 mM-ATP, in the presence and in the absence of 10 μM -cyclic AMP. The reaction was initiated by the addition of 20 μl of supernatant that had been diluted 35–50-fold in homogenizing buffer. After incubating for 2 min at 30°C, 10 μl of a solution containing 0.1 μmol of ATP was added to the reaction mixture and 75 μl of the total were immediately transferred by pipette on to a numbered paper filter disc (Whatman 3MM; 2.4 cm), which was supported above a styrofoam board with a pin. The discs with their pins were then washed by transferring to a dish of ice-cold 10% trichloroacetic acid (approx. 10 ml/disc) containing 5 mM-ATP, where they were allowed to stand for 30 min. Washing was repeated for three additional 15-min periods in 5% trichloroacetic acid containing 10 mM- NaH_2PO_4 ; the second of these was carried out at 70–90°C, the others at 0–5°C. After the tri-

chloroacetic acid washes, the filters were placed in 95% ethanol and agitated for approx. 5 min. After removing the pins, the discs were dried with the aid of a hair dryer and placed in plastic vials containing 8 ml of xylene-based scintillant. Assays were performed in triplicate and values were corrected by reference to a blank containing homogenizing buffer instead of enzyme.

Column chromatography

The cyclic AMP-dependent protein kinases I and II were separated by the method of Haddox *et al.* (1979). DEAE-cellulose (10 g) was equilibrated with 50 mM-Tris/HCl (100 ml), pH 7.5, containing 10 mM-EDTA and 15 mM-NaF, after which a column (0.7 cm $\times$ 10 cm) was prepared. Just before use, the column was washed with 5 mM-Tris/HCl, pH 7.5, containing 2 mM-EDTA, 3 mM-NaF and 1 mM-dithiothreitol until the pH and the ionic strength of the eluate were the same as that of the buffer. The samples to be applied were prepared in the same way as those used for assaying total protein kinase activity except that 1 mM-dithiothreitol was included in the homogenizing buffer. The 20000g supernatant was diluted ten times. Approx. 3 mg of protein was applied to the column at a rate of 25 ml/hour. Columns were then washed with homogenizing buffer, at a rate of 33 ml/h, until no protein was present in the eluate. The kinases were then eluted from the column with a linear gradient of 0–0.3 M-KCl. Fractions (1 ml) were collected and 50 μl portions were assayed for protein kinase activity. The assay was performed in the same way as that described for total protein kinase activity in the supernatant, except that the reaction was started by the addition of 40 μl of the assay mixture containing the [γ - ^{32}P]ATP. Reaction was allowed to proceed for 8 min and terminated in the same manner as described for total protein kinase activity. To assess recovery, enzyme activity was also determined in a 20 μl portion of the 20000g supernatant diluted 35-fold. Between 41 and 64% of this supernatant activity was recovered after chromatography. The determination of recovery included two correction factors, obtained from Haddox *et al.* (1979), to compensate for salt inhibition of types I and II protein kinase activity in the eluted peaks.

Phosphorylation of cytoplasmic proteins in vitro

For the analysis of the phosphorylation patterns of cytoplasmic proteins, fresh muscle samples were homogenized in 5 vol. of cold 0.32 M-sucrose. Homogenates were then centrifuged at 2000g at 4°C for 10 min and the supernatants were re-centrifuged at 105000g at 4°C for 60 min. A portion of the resulting supernatant containing 150–350 μg of protein was incubated in a cocktail incorporating 40 mM-Mes (4-morpholine-ethanesulphonic acid),

pH 6.5, 10 mM-MgCl₂, 2.5 mM-aminophylline, 2.5 μ Ci of [³²P]ATP and 1 μ M-ATP in a total volume of 100 μ l as described by Malkinson (1977). The reaction was allowed to proceed for 4 min at 30°C in the presence and absence of cyclic AMP (10 μ M). The reaction was terminated with 50 μ l of disruption mixture consisting of 8% sodium dodecyl sulphate, 22.4% mercaptoethanol, 0.2 M-Tris/HCl, pH 7.0, 12% sucrose and two drops of Bromophenol Blue. After heating the resulting mixture to 80°C for 15 min and then cooling to room temperature, the proteins were analysed using a discontinuous gel system based on that described by Harper *et al.* (1979). The phosphorylation patterns were visually detected by autoradiography of the dried and flattened gel.

Results

Polyamine levels in chronically stimulated fast muscle

The concentrations of putrescine, spermidine and spermine showed marked phasic elevations in response to stimulation. The putrescine concentration increased sharply, reached a peak level 3–4 days after the onset of stimulation, and then declined rapidly (Fig. 1). Spermidine levels showed a similar rise and after 4 days of stimulation had increased 10-fold. This level persisted for about 2 weeks before falling to levels typical of control slow muscle (Fig. 2). Changes in spermine concentrations, although still significant, were less marked than those of the other two polyamines (Fig. 3). High levels were sustained over a period from 1 to 3 weeks after the

onset of stimulation. The concentration then declined to values typical of control slow muscle. Essentially similar changes were seen when the polyamine concentrations were expressed per mg of protein (results not shown).

Figs. 1–3 also provide some indication of a moderate rise in the levels of putrescine, spermidine and spermine preceding the main phasic increases. This was present at the earliest times studied (12 h).

Changes in polyamine levels were also evaluated in terms of the spermidine/spermine ratio. This ratio has been found to be high in tissue actively engaged in biosynthesis, and to decrease with age in a variety of tissues, including skeletal muscle (Jänne *et al.*, 1964; Takyi *et al.*, 1977). In the present study the

Fig. 1. Changes in putrescine levels in stimulated fast muscle

Each point represents a single determination. In this, and in the following Figures, the stippled bands marked F and S represent the 95% confidence limits of control values obtained from fast and slow muscles, respectively, in the right (unoperated) legs of treated animals. Control putrescine values (mean $\pm$ s.e.m.) were 4.0 ± 0.4 ($n = 15$) and 16 ± 3 ($n = 13$) nmol/g wet wt. for fast and slow muscles respectively.

Fig. 2. Changes in spermidine levels in stimulated fast muscle

Each point represents a single determination. Control spermidine values (mean $\pm$ s.e.m.) were 21 ± 2 ($n = 15$) and 75 ± 8 ($n = 13$) nmol/g wet wt. for fast and slow muscles respectively.

Fig. 3. Changes in spermine levels in stimulated fast muscle

Each point represents a single determination. Control spermine values (mean $\pm$ s.e.m.) were 115 ± 6 ($n = 15$) and 169 ± 13 ($n = 13$) nmol/g wet wt. for fast and slow muscles respectively.

spermidine/spermine ratio rose to a peak value some five times that in the control fast muscle after 4 days of stimulation (Fig. 4). Thereafter the ratio declined very slowly to control slow-muscle values.

Fig. 4. Changes in the spermidine/spermine ratio in stimulated fast muscle

Each point represents a single determination. Control values (mean $\pm$ S.E.M.) were 0.18 ± 0.01 ($n = 15$) and 0.45 ± 0.03 ($n = 13$) for fast and slow muscles respectively.

Fig. 5. Changes in ODC activity in stimulated fast muscle

The activity was determined in duplicate with $[1-^{14}\text{C}]$ ornithine (sp. radioactivity 59 mCi/mol) as substrate. Control values for ODC activity (means $\pm$ S.E.M.) were 46 ± 18 ($n = 19$) and 33 ± 8 ($n = 18$) d.p.m./h per 20mg of tissue for fast and slow muscles respectively. Additional experiments were carried out with DL- $[1-^{14}\text{C}]$ ornithine of lower specific radioactivity (4 mCi/mol) to determine whether the increased counts were a consequence of enhanced ODC activity rather than of reduced levels of endogenous ornithine. These experiments confirmed the enhancement of ODC activity during the peak, although at this specific activity control levels of ODC were below the detection limit of the assay (results not shown).

Activities of ODC and SAM-D

ODC activity was low in both fast and slow types of skeletal muscle. However, marked elevations in ODC activity were seen between 18 and 48h after the onset of stimulation (Fig. 5).

The changes in ODC activity were accompanied by changes in the activity of SAM-D. During the first 20h of stimulation levels of SAM-D activity appeared to be depressed below those normally found in fast muscle. Thereafter SAM-D activity increased rapidly to almost double the normal level, reaching a maximum approx. 28h after the commencement of stimulation. The activity then declined, and by 1 week had reached values lower than those for control fast muscle, but within the range of those for control slow muscle (Fig. 6).

Cytoplasmic proteins and their phosphorylation

Control fast and slow muscles had different cytoplasmic protein profiles (Fig. 7). The difference was pronounced in the region of mol.wt. 32000–56000. During short-term stimulation of fast muscle, the protein pattern did not undergo any major changes (results not shown). However, by 3 weeks of stimulation the pattern had started to resemble that of slow muscle. By 8 weeks of stimulation, the pattern of the stimulated fast muscle was very similar to that of slow muscle.

The phosphorylation patterns of cytoplasmic proteins of fast and slow muscle showed marked differences (Fig. 8). Particularly noteworthy was a band corresponding to mol.wt. 54000, which was seen only in slow muscle and had the same mobility as the regulatory subunit of type II cyclic AMP-

Fig. 6. Changes in SAM-D activity in stimulated fast muscle

The activity was determined in duplicate in the presence of 2.5 mM-putrescine, with *S*-adenosyl-L- $[1-^{14}\text{C}]$ methionine (sp. radioactivity 5 mCi/mol) as substrate. Control values for SAM-D (means $\pm$ S.E.M.) were 3.01 ± 0.13 ($n = 19$) and 0.69 ± 0.10 ($n = 18$) nmol/30min per g of tissue for fast and slow muscles respectively.

Fig. 7. Densitometric tracings of the protein-staining pattern obtained from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of cytosol from control and stimulated muscles

Samples containing 19 μg of protein were electrophoresed in a 10% acrylamide slab gel. Protein bands were stained with Coomassie Brilliant Blue R. The molecular weights of the major protein bands were determined by means of the standard curve obtained using the following standards: haemoglobin, 15 000; myoglobin, 17 200; trypsin inhibitor, 21 000; trypsin, 23 300; aldolase, 40 000; bovine serum albumin, 66 000; phosphorylase α , 95 000; β -galactosidase, 120 000; myosin, 190 000. In addition, a mixture of proteins ('Dalton mixture'; Sigma Chemical Co.) was used. This contained β -lactoglobulin (mol.wt. 18 400), trypsinogen (24 000), ovalbumin (45 000) and bovine serum albumin (66 000).

dependent protein kinase (Liu & Greengard, 1976). This was confirmed by examining the phosphorylation pattern of cytoplasmic proteins of rabbit heart muscle (Figs. 8d and 8e), a tissue that has been shown to contain significant amounts of protein kinase II (Corbin & Keely, 1977). In the early stages of stimulation, only minor changes occurred in the phosphorylation pattern of fast muscle. By 13 days of stimulation the pattern of phosphorylation of cytoplasmic proteins showed some change towards that of slow muscle (results not shown). By 20 days of stimulation the pattern showed some of the features of slow muscle, including the presence of a faint band at mol.wt. 54 000, whose phosphorylation was stimulated by cyclic AMP (Figs. 8f and 8g).

Fig. 8. Autoradiographs showing the incorporation of ^{32}P into cytoplasmic proteins

Phosphorylations were carried out by incubating portions of the 105 000g supernatant with [γ - ^{32}P]ATP. Samples containing 50 μg of protein were electrophoresed in a 10% acrylamide slab gel. (a), Fast muscle incubated in the presence of cyclic AMP; (b) and (c), slow muscle incubated in the presence and absence respectively of cyclic AMP; (d) and (e), cardiac muscle incubated in the presence and absence respectively of cyclic AMP; (f) and (g), fast muscle after 20 days stimulation, incubated in the presence and absence respectively of cyclic AMP.

Activity of cyclic AMP-dependent protein kinase

Results are summarized in Fig. 9. Slow muscle exhibited a significantly higher activity of cyclic AMP-dependent protein kinase than fast muscle. On short-term stimulation, there was no significant change in the activity of cyclic AMP-dependent protein kinase. By 12 days of stimulation, the enzymic activity had increased significantly, and it reached the level characteristic of control slow muscle by 4 weeks of stimulation.

The activity of protein kinase in the absence of cyclic AMP was very low and no significant changes were observed to result from stimulation.

Chromatographic separation of type I and type II cyclic AMP-dependent protein kinase

Fig. 10 illustrates the results of separating chromatographically the type I and type II cyclic AMP-dependent protein kinase activities for control fast (Fig. 10a) and slow (Fig. 10b) muscle. Fast muscle exhibited mainly type I cyclic AMP-dependent protein kinase activity; very little type II

Fig. 9. Changes in the cyclic AMP-dependent protein kinase activity in stimulated fast muscle

The activity was determined in triplicate by measuring the incorporation of ^{32}P into histones/min per mg of protein in the presence of cyclic AMP (●) and in the absence of cyclic AMP (○). The shaded bands marked F and S represent the 95% confidence limits of control values obtained for fast and slow muscles respectively in the presence of cyclic AMP. Mean values $\pm$ S.E.M. were 0.21 ± 0.01 nmol ($n = 10$) for fast and 0.63 ± 0.04 nmol ($n = 10$) for slow muscle.

activity was present. Slow muscle, however, displayed both types of activity, and in addition the level of type I activity was much higher than in control fast muscle. The chromatographic pattern of fast muscle showed no change after 17h of stimulation (Fig. 10c). Changes in this pattern did take place by 12 days of stimulation, and these were characterized by an increase in type I activity and a smaller increase in type II activity (Fig. 10d). After 4 weeks of stimulation, fast muscle acquired a chromatographic pattern similar to that of slow muscle (Fig. 10e).

Discussion

The concentration of polyamines differed in control fast and slow muscles of the rabbit, an observation that may reflect the known higher level of synthetic activity in the slow muscle (Dreyfus, 1967; Goldberg, 1967). Such differences in polyamine concentrations have also been found in fast and slow muscles of the rat (C. Matri, S. Salmons & G. H. Thomas, unpublished work) and the mouse (Kremzner *et al.*, 1978). In the long term, low-frequency stimulation of fast muscle established

polyamine levels typical of slow muscle. In this respect the results were concordant with the transformation of fibre type, which has already been described in terms of other physiological, histochemical, biochemical and ultrastructural criteria. Of greater significance, however, were the marked changes in the first week after the onset of stimulation. The polyamines exhibited a succession of phasic increases, peak concentrations being reached for putrescine, spermidine and spermine in the same sequence as they appear in the biosynthetic pathway. These changes were preceded by changes in enzymic activities. ODC activity showed a sharp increase within 24h of the onset of stimulation and this was accompanied by a smaller, although still highly significant, rise in SAM-D activity.

In addition there was some evidence of small increases in the concentrations of putrescine, spermidine and spermine as early as 12h after the onset of stimulation. These changes were too early to have been the result of the increases in enzymic activities. They may have been brought about by a stimulation-induced release from conjugated forms already present within the cells.

The early occurrence, magnitude and transient nature of the phasic changes in polyamine biosynthesis are strikingly similar to the patterns of response observed in many tissues after trophic stimulation (for review, see Russell & Durie, 1978). During many of these experiments, hyperplasia is a major element in the response of the tissue. It is therefore important to note that this is not the case in the transformation of muscle type by chronic stimulation, which takes place without any alteration in the total number of fibres present in the muscle (Pette *et al.*, 1976; Salmons & Henriksson, 1981). Similar transient increases in polyamine concentration have been observed in skeletal muscles of the mouse and rabbit as a result of denervation (Kremzner *et al.*, 1978). However, in view of the increase in the satellite cell population, which is a known consequence of denervation (Ontell, 1974), such changes cannot be dissociated entirely from those that would be expected to accompany mitotic division. The same considerations apply to the study of denervation hypertrophy in rat diaphragm by Hopkins & Manchester (1981), who observed phasic increases in polyamine levels and ODC and SAM-D activities with an early time course similar to that reported here.

Although the stimulation model is not complicated by hyperplasia, there remain at least two aspects of the response that may bear a relationship to the observed changes: (a) the qualitative change, referred to above, in the protein complement of the muscle during type transformation; (b) a generalized increase in both protein synthesis and protein

Fig. 10. DEAE-cellulose elution profiles of cyclic AMP-dependent protein kinase

Muscle samples were homogenized and supernatant fractions (3 mg of protein) were subjected to chromatography as described in the Materials and methods section. Fractions (1 ml) were collected and 50 μ l portions were assayed for protein kinase activity in the presence of cyclic AMP (●). Activities have not been corrected for salt inhibition. [KCl] was measured in each fraction and expressed in mol/litre (○). (a) Control fast muscle; (b) control slow muscle; (c), (d) and (e), fast muscles stimulated for 17 h, 12 days and 4 weeks respectively.

degradation during the first few weeks of stimulation, for which there is now preliminary evidence (B. McGurk & S. Salmons, unpublished work). At this stage we cannot point to a direct association between enhanced levels of polyamine biosynthesis and either of these processes.

In view of interest in the possible link between ODC activation and protein kinase activity in many biological systems (Russell *et al.*, 1976), we have examined changes in kinase activity in stimulated muscle. The experiments used provided information on the total enzyme activity and also, by chromatographic separation, on the proportions of

type I and type II kinase activities contributing to that total. In terms of both total activity and chromatographic pattern, fast and slow muscles were markedly different. Total activity was higher in slow muscle than in fast, partly because of higher type I activity and partly because of a significant contribution of type II activity in slow muscle. The latter finding, which contrasted with the minimal presence of type II activity in fast muscle, was consistent with our observations of phosphorylation of the 54 000-mol.wt. phosphoprotein *in vitro* in the present investigation, and agrees with the work of Burchell *et al.* (1976).

During the early stages of stimulation of fast muscle there were no significant changes in total protein kinase activity, whether in the presence or absence of cyclic AMP, or in the proportions of type I and type II activity. At a later stage, considerably after the peak of ODC activity had subsided, changes in activity were observed, consisting of an increase in type I activity, followed by an increase in type II activity. By 4 weeks, levels of activity had been attained that resembled those of slow muscle. These observations were in agreement with the autoradiographs of Fig. 8, for by 20 days the phosphorylation patterns of stimulated fast muscle were similar to those of control slow muscle in every respect, including the appearance of the band at mol.wt. 54 000 presumed to be cyclic AMP-dependent protein kinase II.

The changes in protein kinase activities appeared, therefore, to be part of the general process of type transformation of the stimulated muscle, reflecting the higher level of biosynthetic activity in slow muscle. This long-term change in the level of cytoplasmic protein kinase activity should be distinguished from the activation of existing protein kinase which, if it is an initiating event in ODC induction, would have to occur within 18 h of the onset of stimulation. According to the model proposed by Russell *et al.* (1976), activation would be followed by nuclear translocation of the catalytic subunit, phosphorylation of chromatin-associated proteins and release of template activity for the synthesis of messenger RNA coding for ODC. In view of the magnitude of the ODC response reported here, these other processes assume additional interest as possible intermediate stages in the response of skeletal muscle to electrical stimulation.

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