Activation of AMP Aminohydrolase during Skeletal-Muscle Contraction

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AMP aminohydrolase activity is enhanced by 60% after 5 s tetanic stimulation of phosphorylase kinase-deficient mouse muscle and after 60 s tetanus in normal mice. During the recovery from tetanus the activity in the contralateral leg is similarly enhanced. The activation is stable to 1000-fold dilution and has a half-life of approx. 1 h.

The regulatory properties of AMP aminohydrolase (EC 3.5.4.6), the first enzyme in the purine nucleotide cycle (see Lowenstein, 1972), have recently been studied in detail, since deamination of AMP to IMP, catalysed by AMP aminohydrolase, is thought to stabilize the energy charge {i.e. the function $([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])$ } of contracting muscle (see Chapman & Atkinson, 1973; Coffee & Solano, 1977; Solano & Coffee, 1978; Sahlin *et al.*, 1978). AMP aminohydrolase activity is allosterically controlled by a number of effectors (Ronca-Testoni *et al.*, 1970; Lomax & Henderson, 1975) which are said to have the combined effect of activating it during a fall in energy charge (Coffee & Solano, 1977; Solano & Coffee, 1978).

We have suggested another role for this reaction: production of IMP to activate phosphorylase *b* (EC 2.4.1.1), particularly in phosphorylase kinase-deficient mice, whose skeletal muscles do not form phosphorylase *a* (Rahim *et al.*, 1976, 1978; Griffiths & Rahim, 1978). In the course of investigations on the 6-fold more rapid accumulation of IMP in working muscles of phosphorylase kinase-deficient mice (Griffiths & Rahim, 1978), we have found a novel activation mechanism for AMP aminohydrolase, one that is quite different from the allosteric mechanisms discussed above.

In this paper we report a 60% activation of AMP aminohydrolase that occurs within 5 s of stimulation in phosphorylase kinase-deficient muscle, but which attains the same value after 30 s tetanus in normal muscle. It survives 1000-fold dilution and is stable for several hours.

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Materials and Methods

Apart from AMP, which was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), all reagents were obtained from British Drug Houses, (Poole, Dorset, U.K.) and were Analytical Grade when obtainable. C3H and ICR/IAn (phosphorylase kinase-deficient) mice were obtained from colonies kept in our animal house and used at 6 months.

Mice were anaesthetized in two stages by injections of 0.015 and 0.005 ml of 0.5% sodium pentobarbitone/g body wt. with an interval of 15 min. After a further 30 min, the hamstrings-muscle group of one or both legs was exposed and the sciatic nerve of one leg exposed and crushed. Electrodes were applied distal to the crushed portion and a strain gauge was attached to the foot. After 10 min recovery time the nerve was stimulated by 25 pulses/s, 0.3 ms pulse duration at 40 V, which induced an isometric tetanus and a tension of up to 5 g.

After the predetermined period the stimulated muscle (and, in some experiments, the contralateral hamstrings group) was clamped in liquid-N₂-cooled tongs and the material between the jaws was then pulverized under liquid N₂ and homogenized in 10 vol. (ml/g) of 50 mM-imidazole/10 mM-mercapto-ethanol buffer, pH 6.5, containing either 200 mM- or 20 mM-KCl. The homogenate was left for 30 min at 0°C to permit solubilization of the enzyme and then diluted 1:50 with the same buffer. Control samples were obtained from anaesthetized unstimulated animals and extracted under identical conditions unless otherwise stated.

AMP aminohydrolase was assayed by addition of 0.5 ml of the diluted extract to 0.5 ml of 50 mM-AMP (both preincubated at 37°C) and incubation for 10 min at 37°C. The reaction was stopped by addition of 1 ml of 10% (w/v) trichloroacetic acid and the NH₃ formed was measured by the method of Chaney

Fig. 1. Activation of AMP aminohydrolase after tetanic stimulation of mouse hamstrings muscle

(a) Groups of six mice were anaesthetized and after 30 min the sciatic nerve of one leg was crushed. After 10 min it was stimulated tetanically, distal to the crushed section, at 25 Hz, with 0.3 ms 40 V pulses. The stimulated and contralateral hamstrings groups were then freeze-clamped, extracted in 10 vol. (v/w) of 50 mM-imidazole/200 mM-KCl/10 mM-mercaptoethanol buffer, pH 6.5, kept at 0°C for 30 min, diluted 1:50 with the same buffer, and 0.5 ml of this extract was assayed by addition of 0.5 ml of 50 mM-AMP and measurement of NH₃ formed at 37°C (see the text for full details). The activity in the stimulated hamstrings is expressed as a percentage increase over that in unstimulated muscles, $\pm$ S.E.M. The activities in the stimulated legs were significantly greater than those in the control legs in all cases. For ICR/IA-mouse muscles stimulated for 5, 10 and 30 s the *P* values and numbers of stimulated muscles were *P*<0.001 (*n*=6), *P*<0.02 (*n*=7) and *P*<0.001 (*n*=6). Analogous values for C3H-mouse muscles were *P*<0.05 (*n*=12), *P*<0.005 (*n*=12) and *P*<0.001 (*n*=12). Mice: $\square$, ICR/IA (phosphorylase kinase-deficient); $\blacksquare$, C3H (normal). (b) Hamstrings of ICR/IA mice were subjected to 30 s tetanic stimulation as in (a), but the muscles of both the stimulated ($\blacksquare$) and the contralateral unstimulated ($\square$) legs were freeze-clamped after 10–60 min recovery. All other conditions were as in (a). Results are expressed as mmol of NH₃/min per g, $\pm$ S.E.M. The numbers of muscles in the stimulated and unstimulated groups and the significance of the differences between them were: 0 min recovery, *n*=6, *P*<0.001; 5 min recovery, *n*=18, *P*<0.05; 30 min recovery, *n*=12, *P*<0.005; 60 min recovery, *n*=12, not significantly different. (c) Homogenates were prepared as in (a), from ($\blacksquare$) stimulated (30 s tetanus) and ($\square$) contralateral unstimulated legs of a single ICR/IA mouse at the times indicated. Results are expressed as mmol of NH₃/min per g, $\pm$ S.E.M. The shaded portion indicates the 30 min during which the enzyme was extracted at 0°C, as explained in the text.

& Marbach (1962). Activity is expressed as the mean of *n* observations $\pm$ S.E.M., in terms of mmol of NH₃/min per g wet wt.

Results

The activity of AMP aminohydrolase in muscles from 16 unstimulated ICR/IA mice was 0.37 ± 0.11 mmol/min per g, and in 14 unstimulated C3H mouse muscles it was 0.57 ± 0.05 mmol/min per g. The data from the stimulation experiments are shown in Fig. 1(a), expressed in terms of percentage increase in activity in the control groups of muscles. In both normal C3H mice and phosphorylase kinase-deficient ICR/IA mice there was approx. 60% activation compared with the activity in control, unstimulated muscles, but this activation occurred 6 times more rapidly in abnormal muscle.

Fig. 1(b) shows the variation in AMP aminohydrolase activity in the 60 min after a 30 s period of tetanus. Instead of reverting to the original activity, as expected, the activity in the stimulated leg was

maintained. The activity in the contralateral, unstimulated leg surprisingly rose to the same value after 60 min. No change in activity with time of anaesthesia was observed in muscles from unstimulated animals.

Fig. 1(c) shows the stability of the enhanced activity in an extract kept at 0°C, compared with an extract from a control leg under the same conditions. This is a typical result from a series of experiments. Both extracts lost activity over 5 h, but the enhanced activity was still observable. It seemed to be more labile, however, with a half-life of approx. 1 h.

Diluted muscle homogenates from ICR/IA mice were centrifuged for 15 min at 3300g at -4°C in order to ascertain the distribution of the enhanced activity between the particulate and soluble fractions. In eight tetanized muscles the activity of the whole homogenate was 0.598 ± 0.018 mmol of NH₃/min per g and the soluble fraction had an activity of 0.298 ± 0.009 mmol/min per g.

The six control-leg whole homogenates had an activity of 0.46 ± 0.006 mmol of NH₃/min per g and their supernatants had an activity of $0.163 \pm$

0.025 mmol/min per g. This implies that the enhanced activity in the stimulated legs was entirely confined to the soluble fraction of the homogenate.

No significant activation in AMP aminohydrolase activity was observed if the 30 min incubation at 0°C was omitted or if 20 mM-NaF was included in the extraction buffer (experiments with groups of eight ICR/1An mice at 0 s and 30 s stimulation).

Discussion

These effects are quite different from the adsorptive binding of effectors previously described (e.g. by Ronca-Testoni *et al.*, 1970), since the activation survives 1000-fold dilution and is then stable for several hours (Fig. 1c). Only if a low-molecular-weight effector were to bind with an extremely slow rate of dissociation would such data be explicable in terms of adsorptive binding, and the picture is more strongly reminiscent of a covalently mediated activation of a less-active AMP aminohydrolase *b* to a more-active AMP aminohydrolase *a*, similar to the phosphorylation of phosphorylase *b* to *a*, which occurs within less than 1 s of the onset of tetanus (Danforth *et al.*, 1962).

No abnormal property, other than an increased maximal velocity, was found in the *a* form of enzyme from the stimulated leg. Its K_m was not significantly changed and no difference was detectable on polyacrylamide-gel electrophoresis or by gradient elution from cellulose phosphate. It should be remembered, however, that the lifetime of the activation effect *in vitro* is similar to the time taken for these physical studies (see Fig. 1c).

The observation that tetanus-induced activation of AMP aminohydrolase affects only the soluble fraction of the muscle extract is of interest because the enzyme is thought to bind tightly to myosin *in vivo* (Byrnes & Suelter, 1965; Ashby & Frieden, 1977; Shiraki *et al.*, 1979a,b). Shiraki *et al.* (1979a) found that rat muscle AMP aminohydrolase required 0.3 M-KCl for complete extraction, and if this were true of mouse enzyme, the increased activity in the supernatant from stimulated muscle might be due to an enhanced susceptibility to extraction by the 0.2 M-KCl used in the present experiments. However, one would then expect the residual activity of the pellet to fall, whereas it stayed constant. Shiraki *et al.* (1979b) found that myosin binding caused activation of AMP aminohydrolase when the complex was suspended in 50 mM-Tris/HCl buffer, pH 7.0, but that no activity was found in the supernatant on centrifugation at 5000g for 10 min. Clearly this is different from the activation observed in the present paper. The simplest explanation of the present preliminary experiments would be that activation *in vivo* affects only a more-soluble fraction of the enzyme, but further studies on purified enzyme are clearly

necessary. There was no enhanced extraction of myosin (measured as ATPase activity at 25°C by using as substrate 1 mM-ATP in 10 mM-CaCl₂/0.6 M-KCl/20 mM-Tris/HCl, pH 7.0) from the stimulated muscles, so activation by myosin could not account for the enhanced AMP aminohydrolase activity.

Since the crushing of the sciatic nerve should have eliminated any transmission of nerve impulses to the contralateral leg, the activation of AMP aminohydrolase in that leg during the 60 min after tetanus is not easily explained in terms of the hypothetical mechanism that enhances activity in the stimulated leg. One possible explanation would involve the analogy with conversion of phosphorylase *b* into *a*, which is promoted both by muscle contraction (via Ca²⁺) and by hormone administration (via cyclic AMP). An attempt was therefore made to evoke the activation response by adrenaline administration (results not shown), but a dose sufficient to promote glycogenolysis had no effect on AMP aminohydrolase activity. This suggests that the activation is not mediated by cyclic AMP, and the failure to enhance activation by addition of 20 mM-NaF (an inhibitor of protein phosphatases) suggests that, if the enzyme is phosphorylated by a protein kinase, then there is no significant inactivation by a protein phosphatase. The kinase concerned could not, of course, be phosphorylase kinase, since this is absent from the muscle of ICR/1An mice (Cohen *et al.*, 1976; Daegelen-Proux *et al.*, 1978), but Varsanyi *et al.* (1978) have shown the presence of another Ca²⁺-activated protein kinase in these animals.

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