SKELETAL MUSCLE PURINE NUCLEOTIDE LEVELS IN NORMAL AND PHOSPHORYLASE KINASE DEFICIENT MICE

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1. Introduction

It has been known for many years that the glycogen phosphorylase *b* (EC 2.4.1.1) of skeletal muscle can be activated non-covalently by AMP or IMP as well as by covalent phosphorylation to phosphorylase *a* [1]. A physiological role for AMP or IMP mediated activation has not, however, been firmly established. Recent studies on the regulation of muscle glycogenolysis have emphasized the role of covalent phosphorylation [2,3].

The most convincing evidence for non-covalent activation of phosphorylase *b* in vivo came with the discovery of a strain of mice which lacks phosphorylase kinase (EC 2.7.1.38) in its skeletal muscle and so cannot form phosphorylase *a* [4]. These 'I-strain' mice were reported to break down muscle glycogen to lactate on exercise without forming detectable phosphorylase *a* [5] and this has been taken as evidence that non-covalent activation of phosphorylase *b* can be significant in vivo [2]. Gross and Mayer [6], however, have recently suggested that these mice may form significant phosphorylase *a*.

If phosphorylase-kinase deficient mice activate phosphorylase *b* non-covalently one would expect the muscle nucleotide or nucleotides concerned to be present at higher concentrations than in normal mice. The evidence for non-covalent activation would be strengthened if the hypothetical activator were to rise in concentration during exercise and if its concentration were comparable to its activation constant for phosphorylase *b*. In this study we examined muscle extracts from normal (SAS/4) and phosphorylase kinase deficient (ICR/IAn) mice and concluded that IMP is the most probable mediator of non-covalent phosphorylase activation.

2. Materials and methods

ICR/IAn mice were bred in our animal house from mice kindly supplied by Dr P. Cohen. They carry the phosphorylase kinase deficiency gene on the X-chromosome but are otherwise normal [7]. SAS/4 mice used as controls were from a long-term colony maintained in the same animal house. All mice were matched for age (ca. 8–12 months) and sex.

Trichloroacetic acid, methanol and ethanol were purchased from BDH Chemicals, Poole, England, Tris-base from Sigma Chemical Co., London, England and radioactive nucleotides and nucleosides from the Radiochemical Centre, Amersham, England.

The mice were killed by cervical dislocation either while normally active or after swimming for 15–30 min in warm water (ca. 30°C). The quadriceps muscles of both sides were clamped in liquid nitrogen cooled stainless steel tongs within about 30 s of death and the material between the jaws was powdered in liquid nitrogen, weighed into a pre-cooled homogeniser tube and then homogenised at 0°C in 10 vol of 10% aqueous trichloroacetic acid (w/v)–20% methanol (v/v) which contained 18.6 μCi/ml [^{14}C]Inosine. The homogenate was centrifuged at 14 000 × *g*. for 1 min and the supernatant freed of trichloroacetic acid and most of the methanol by extraction with wet ether. It was adjusted to pH 5–6 with Tris-base crystals and kept at −20°C until it could be analysed. These extracts were stable for at least three months.

Analysis was performed by high performance liquid chromatography as described by Perrett [8] and nucleotides were identified by comparison of their elution times with known standards and by co-chromatography. The radioactive inosine served as an internal standard since it was not retained on the column. Control experiments in which ^{14}C-labelled ATP, ADP and AMP were added at the start of the extraction established that no significant inter-conversion of nucleotides occurred and that recovery was better than 95%.

3. Results

ATP, ADP, AMP, IMP, NAD, GDP and GTP were identified in the chromatograms. The last three showed no variation between the three groups and their concentrations are not given. Table 1A shows the concentrations of ATP, ADP, AMP and IMP found in skeletal muscles of the two strains during normal activity. There was no significant difference between ADP or ATP levels but AMP was 23% higher and IMP was over twice as high in the phosphorylase kinase deficient mice. Both differences were statistically significant.

When mice from the two strains were made to swim for 15–30 min, there was no significant change in their AMP levels ($P < 0.25$). In fact, the ICR/IAn mice showed a slight fall (13%). IMP levels, on the other hand, rose in both strains by a factor of 2.3 (table 1B). There was considerable variation in IMP levels and the difference between the strains lies just outside the limit of statistical significance ($P < 0.1$) although the mean level in deficient mice was still twice as high as in normals. This may reflect a difference in the exertion induced by swimming in individual mice. The rise in IMP concentration during swimming was significant in both strains ($P < 0.05$) but ATP and ADP levels did not alter significantly during exercise and still showed no significant difference between the strains.

4. Discussion

Both AMP and IMP were found in muscles from the two strains at concentrations sufficient to activate phosphorylase *b*. Three criteria were suggested in the introduction for deciding whether an activator was physiologically important in deficient mice:

Table 1
Nucleotide levels[a] in skeletal muscle of normal and phosphorylase kinase deficient mice

A. Normally active mice

Nucleotide	Normal (SAS/4) (16)[b]	Deficient (ICR/IAn) (14)[b]	P[c]
AMP	48.9 ± 7.7	60.3 ± 5.3	< 0.05
IMP	202 ± 47	423 ± 106	< 0.05
ADP	731 ± 55	837 ± 75	< 0.25
ATP	5863 ± 411	5378 ± 412	< 0.25
Free AMP[d]	1.99 ± 0.22	3.41 ± 0.56	< 0.01

B. After 15-30 minutes forced exercise by swimming

Nucleotide	Normal (SAS/4) (12)[b]	Deficient (ICR/IAn) (11)[b]	P[c]
AMP	59.8 ± 13.1	52.2 ± 9.3	< 0.4
IMP	476 ± 144	958 ± 303	< 0.1
ADP	749 ± 46	713 ± 61	< 0.4
ATP	5147 ± 415	4642 ± 419	< 0.25
Free AMP[d]	2.77 ± 0.51	2.51 ± 0.29	< 0.4

[a] All concentrations in nmol/gram wet weight expressed as mean ± standard error of the mean.
[b] Number in each group.
[c] Statistical difference by Student's *t*-test.
[d] Calculated from the expression described in the text. All other values refer to total concentrations.

(i) It should be present at higher concentrations in deficient animals. This is true for AMP in resting mice (23% higher) but not after exercise. IMP is more than twice as concentrated in both groups.

(ii) It should rise in concentration after exercise. IMP doubled in both strains whereas AMP did not change significantly.

(iii) Its concentration should be comparable to its activation constant for phosphorylase *b*. This is a more complex question. The total intracellular concentrations of the two activators can be calculated if the intracellular water content is assumed to be 0.58 (the value for frog muscle [9]). Since IMP is not thought to bind tightly to any abundant intracellular protein, its total concentration, which varies from 0.35 mM in resting normal mice to 1.65 mM in exercising deficient mice, should approximate to its concentration in muscle water. The activation constant for IMP and phosphorylase *b* is 2 mM [10] so that up to 45% of the potential phosphorylase activity (71 μmol glucose/gram net weight/minute [6]) could be expressed during exercise – more than enough to supply the needs of glycolysis in deficient mice.

If all the muscle AMP were free in solution, it would also strongly activate phosphorylase *b* but it is thought to bind extensively to intracellular proteins [11]. The 'free' AMP concentration can be calculated if it assumed that ATP, ADP and AMP are maintained at equilibrium by adenylate kinase with an equilibrium constant of 0.42 [12]. ATP is so abundant that its concentration is probably not significantly depleted by binding to proteins whereas ADP is known to be about 90% bound [13,14].

$$AMP_f = \frac{(ADP/10)^2}{0.44 \times ATP} = 3.4–5.9\ \mu M$$

The free AMP content of each muscle was calculated on these assumptions for each set of results and the averaged values are given (in nanogrammes per gramme wet weight) in table 1. Deficient mice had a significantly higher free AMP content than normals but this difference was lost after exercise. There was no significant difference between free AMP concentrations before and after exercise in either strain and all the levels (3.4–5.9 μM) were well below the activation constant (20–100 μM [2]). IMP is thus a more likely activator than AMP if these assumptions are valid.

The enhanced rate of glycogenolysis during exercise cannot be accounted for by a fall in concentration of an inhibitor. ATP and ADP did not change concentration significantly while glucose 6-phosphate, the most potent inhibitor, rises markedly (Danforth and Lyon [5] and our own unpublished results). All these inhibitors will significantly reduce AMP induced activity and our unpublished results show that they also inhibit IMP-induced activity. It is therefore not possible to decide the precise degree of activation afforded by either activator but, on balance, IMP is more likely to be physiologically significant in phosphorylase kinase deficient mice.

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