RAPID FORMATION OF SPERMINE IN SKELETAL MUSCLE DURING TETANIC STIMULATION

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1. Introduction

It has been known for some years that the activation by AMP or IMP of muscle glycogen phosphorylase *b* (EC 2.4.1.1) is enhanced in the presence of the di-, tri- and tetra-amines, putrescine, spermidine and spermine [1]. There was no evidence that this activation had a physiological role in glycogenolysis although polyamines are thought to participate in control of such cellular functions as cell growth, division and differentiation [2], membrane stability and permeability [3] and activity of membrane-bound enzymes [4].

If polyamines were to have a role in the control of glycogenolysis, one would expect it to be manifested most strongly in the muscle of phosphorylase kinase (EC 2.7.1.38) deficient mice. These muscles perform glycogenolysis during exercise without converting phosphorylase *b* to phosphorylase *a* [5,6], apparently by rapid synthesis of IMP [7] (and probably of AMP [8]) which non-covalently activates the phosphorylase *b*. Since these mice depend on the activation of phosphorylase *b* by IMP and AMP, and since this nucleotide-induced activation is markedly enhanced in the presence of polyamines, synthesis of polyamines during exercise would be a logical adaptation of phosphorylase kinase deficiency.

Here, we show that the concentration of spermine rises 3-fold within 30 s of onset of tetanus in phosphorylase kinase-deficient muscle and that a statistically significant, though less clearcut rise occurs in normal, tetanised muscle. We also show that spermine extracts from muscle enhance the activation of rabbit muscle phosphorylase *b* at sub-optimal concentrations of AMP and raise V_{max} in the presence of IMP.

2. Materials and methods

Apart from cellulose-phosphate cation exchanger, Borax, 2,4-dinitrofluorobenzene, spermine and triethanolamine hydrochloride which were purchased from Sigma Chemical Co. (Poole, Dorset), all reagents were obtained from British Drug Houses (Poole, Dorset) and were analytical grade when obtainable. Phosphorylase kinase-deficient ICR/IAn mice and control C57B1 mice were obtained from colonies kept in our animal house and used at 6–8 weeks.

The mouse hamstring muscles were tetanically stimulated and freeze-clamped as in [7]. The biopsies were extracted with 10% trichloroacetic acid–20% methanol in water and the trichloroacetic acid and methanol were removed as in [9]. Spermine was assayed as in [10] except that a cellulose-phosphate cation-exchange column (16.0 × 1.0 cm) was used for the preliminary extractions. It gave good separation of standard polyamine mixtures. The colour yields obtained from muscle extracts were compared with those obtained from standard solutions of spermine in 10% trichloroacetic acid pre-treated as in [9] and passed through the column. Phosphorylase *b* was assayed as in [11].

The extract of spermine used to stimulate purified rabbit phosphorylase *b* was prepared (using the above column) from the unstimulated, freeze-clamped, muscles of 40 ICR/IAn mice. The spermine-containing eluents were lyophilised to concentrate them and redissolved in water. Aliquots of 50 μl were added to 1 ml phosphorylase *b* substrate (16 mM glucose-1-phosphate, 1% (w/v) glycogen, 50 mM triethanolamine, 100 mM KCl, pH 6.8) giving a final spermine dilution of 1:5 compared with the muscle cytosol (see below).

3. Results and discussion

The extract of muscle spermine caused a marked rise in the maximal velocity of the phosphorylase *b* catalysed reaction in the presence of saturating IMP but had little effect on the concentration of IMP required to give half-maximal activity (fig.1A). Its effect on AMP-induced activity was to markedly reduce the AMP concentration required for half-maximal activity with only a small rise in V_{max}. There was a slight tendency for high AMP concentrations to inhibit the enzyme (fig.1B).

Not all the spermine extracted from the muscles by the trichloroacetic acid–methanol mixture would have been free in solution in the muscle water. Spermine and other polyamines are extensively bound to nucleic acids, membranes and other sites within the cell [12] and all this bound material would have been liberated by the extraction procedure. The dilution of spermine extract in the phosphorylase *b* assays described in fig.1 would correspond to 80% sequestration of spermine in the intact muscle. In this context it should be remembered that other polyamines such as putrescine and spermidine are present in muscle [12, 13] and that they, too, enhance the nucleotide-induced activation [1]. Also, as indicated in [12], there are other intracellular cations, some present at much higher concentration than spermine, which will compete for the non-specific binding sites. These considerations suggest that polyamines, even in relaxed ICR/IAn muscle, could have a role in the catalysis of glycogenolysis.

Fig.2 shows that the total concentration of spermine in the muscle of phosphorylase kinase-deficient ICR/IAn mice was 5-fold lower than in normal muscle ($p < 0.001$). Thus the spermine concentration in normal muscle cytosol could have been sufficient to induce the activation of phosphorylase *b* seen in fig.1 even if 95% of the total spermine had been sequestered elsewhere in the cell. Fig.2 shows that after 30 s tetanus there was a significant, 3-fold rise ($p = 0.0009$) in

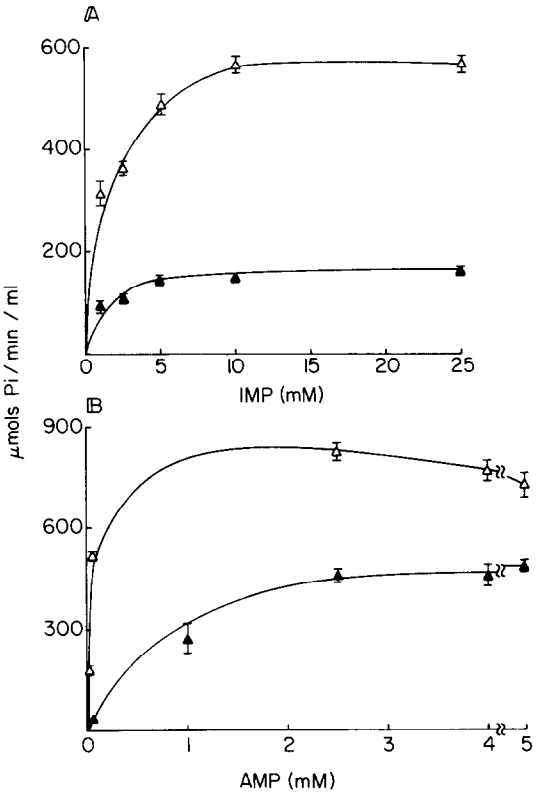

Fig.1. (A) Stimulation of IMP-induced activity of rabbit muscle phosphorylase *b* by spermine extracted from mouse muscles. The spermine extracts were prepared from unstimulated muscles of ICR/IAn mice and diluted into phosphorylase *b* substrate (see text). The final spermine concentration was 1/5-th of that which would have been found in muscle cytosol if all the extracted spermine had been free in solution in vivo. Each data point is the mean ± SEM of 4 assays. (B) Stimulation of AMP-induced activity of rabbit muscle phosphorylase *b* by spermine extracted from mouse muscle (conditions as in (A)).

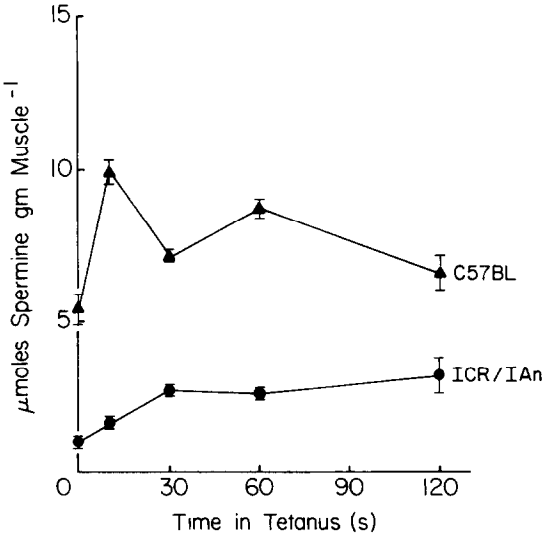

Fig.2. Formation of spermine during isometric tetanus of (▲) C57B1 and (●) ICR/IAn hamstring muscles. Muscles were stimulated with 0.3 ms pulses at 25 Hz. Each data point is the mean ± SEM of 6 obs.

the spermine content of ICR/IAn muscle and this was maintained at 60 s ($p = 0.0006$) and 120 s ($p = 0.01$). The rise in the spermine content of normal C57Bl muscle after the induction of tetanus was statistically significant at 10 s and 60 s ($p = 0.0006$ and 0.002, respectively) but not at 30 s and 120 s. Taken as a whole, the samples from normal muscles after 10–120 s tetanus showed a 40% higher spermine content than resting muscle and this difference was statistically significant ($p = 0.009$). During prolonged muscle work there is a decrease in concentration of ATP and a consequent release of Mg^{2+} from its ATP chelate. By competing for the non-specific cation binding sites this Mg^{2+} would tend to elevate the concentration of spermine in the cytosol.

These data, taken with those in fig.1, suggest that the rise in spermine content of ICR/IAn muscles during tetanus would enhance their rate of AMP or IMP induced glycogenolysis. The physiological significance of the rise in spermine content during tetanus of C57 mouse muscle is less clear since there is no strong evidence that AMP or IMP induce significant phosphorylase *b* activity in normal muscles, at least during brief tetani.

However, it has been reported that polyamines inhibit muscle phosphorylase phosphatase [14] and activate muscle glycogen synthetase phosphatase [15]. This might be the major physiological role in elevated polyamine concentration in normal working muscle.

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