CORRELATION BETWEEN CONTRACTION AND PHOSPHORYLATION OF THE INHIBITORY SUBUNIT OF TROPONIN IN PERFUSED RAT HEART

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1. Introduction

The inhibitory subunit of troponin (troponin-I) from rabbit skeletal muscle has been shown to be phosphorylated by both phosphorylase kinase and cyclic 3′, 5′ AMP dependent protein kinase [1–3]. Both kinases also phosphorylate the 37 000 dalton component (troponin-T) [3–5], and whole troponin from cardiac muscle [6]. A hypothesis has been proposed that in vivo phosphorylation of troponin could modify its properties so as to allow an increase in the contractility of muscle [2,5]. The phosphorylation could be initiated by hormonal stimulation of the muscle, in particular by catecholamines. This may be of special importance in cardiac muscle, where both contractility and cyclic 3′, 5′ AMP are increased by adrenaline [7,8].

However, before this hypothesis is acceptable as a mechanism for enhancing contractility, several criteria have to be met [9]. In particular, it has to be shown that phosphorylation occurs in vivo under conditions where contractility is increased. This paper presents results which show that when perfused rat heart is stimulated by 10^{-6} M adrenaline there is a time dependent increase in phosphorylation of troponin-I which coincides with the increase in force of contraction. A direct correlation between contraction and phosphorylation is also seen in experiments where the concentration of adrenaline is varied from 3×10^{-8} M to 3×10^{-6} M.

2. Methods

2.1. *Heart perfusions*

Hearts from male Wistar rats (300–400 g) were perfused by the Langendorff technique with bicarbonate buffered medium [10] containing 11 mM glucose but with the P_i decreased to 0.234 mM. The P_i was lowered to increase the specific radioactivity of the medium during perfusions with ^{32}P. This modification did not affect the rate of $^{32}P_i$ exchange across the cell membrane, or the total intracellular P_i during the perfusions.

After 5 min of perfusion by drip-through with non-radioactive medium the hearts were switched to a small recirculating system [11] and perfused for 15 min with medium containing 10 μCi $^{32}P_i$/ml. The perfusion pressure was maintained at 6.0 kPa throughout the perfusions. Experiments showed that equilibration of $^{32}P_i$ was slow across the cell membrane, and after 15 min the specific radioactivity of the intracellular P_i was only 4–5% of the medium. Following the recirculation period the hearts were given a 1 min perfusion by drip-through with non-radioactive medium and the drip-through perfusion was then continued for up to 25 sec with medium containing adrenaline. The contractile force was measured with a UF-1 force-displacement transducer and an MX-2 recorder (Devices Instruments Ltd., Welwyn Garden City, UK). At the end of the perfusions the hearts were quick-frozen [12], and powdered under liquid N_2 in a mortar and pestle.

2.2. *ATP concentration and specific radioactivity*

Samples of frozen heart powder (0.3–0.5 g) were extracted in 5% (w:v) $HClO_4$, and the extracts neutralised with $KHCO_3$. The ATP concentration in the extracts was measured [13], and found in control perfusions to be 16–20 μmole/g dry heart. ATP concentrations below 16 μmole/g were considered an indication of anoxia, and the results discarded. This occurred

with less than 5% of the adrenaline stimulated hearts.

The specific radioactivity of [γ-^{32}P] ATP in heart extracts was measured by a new, simple, method. The extract was incubated with phosphorylase *b* and phosphorylase kinase, and the radioactivity incorporated into phosphorylase measured. Under conditions in which there was an excess molarity of ATP over phosphorylase the amount of ^{32}P incorporated into a fixed amount of phosphorylase was dependent only on the specific radioactivity of the γ-phosphate of ATP. An incubation under similar conditions was made using [γ-^{32}P] ATP of known specific radioactivity, and that of the unknown ATP calculated using this result.

Samples of heart extract (150 μl) were incubated at 30°C with 150 μl of 5 mg/ml phosphorylase *b* in 100 mM Tris–Cl, pH 8.0, 5 mM $MgCl_2$, 15 mM 2-mercaptoethanol, and 1 μg phosphorylase kinase prepared as described by Cohen [14]). These conditions gave a 3–6-fold excess of ATP over phosphorylase *b*. The incubations were sampled at 0, 20, 40 and 60 min after addition of phosphorylase kinase, and 35 μl aliquots spotted onto small squares of Whatman No. 1 filter paper and dropped into cold 10% (w:v) trichloracetic acid. The papers were repeatedly washed and ^{32}P bound to the papers measured in a liquid scintillation spectrometer [15]. The incorporation of ^{32}P reached a plateau between 20 and 40 min, and an average of the 40 and 60 min values was used in subsequent calculations. Comparison of the method with one using ion-exchange chromatography and enzymic conversion [16] showed the new method to be superior in terms of reproducibility, speed and simplicity, while giving identical results.

2.3. *Isolation of troponin-I*

Troponin-I was isolated using the troponin-C affinity chromatography method of Syska et al. [17]. Rabbit skeletal muscle troponin-C was prepared [18,19] and coupled to Sepharose 4B [20]. A sample of frozen heart powder (0.5–0.8 g) was homogenised in 10 ml of 8 M urea, 75 mM Tris–Cl, pH 8.0, 1 mM $CaCl_2$, 15 mM 2-mercaptoethanol, and after centrifugation and dialysis against the same buffer, the solution was applied to a 3–4 ml column of troponin-C Sepharose. The column was extensively washed, and then washed with 20 ml of the same buffer plus 0.4 M KCl. The troponin-I was eluted with 7ml of the original buffer containing 10 mM ethanedeoxybis (ethylamine) tetra-acetate (EGTA), the eluate dialysed to remove urea, and ^{32}P in the whole sample measured using Čerenkov radiation. Protein in the sample was estimated by the method of Lowry et al. [21]. The yield of troponin-I was approximately 0.3 mg/g wet heart and was independent of the amount of phosphate present in the protein. Analysis of the samples by polyacrylamide–sodium dodecyl sulphate electrophoresis [22] showed the troponin-I to be at least 97% pure, and the ^{32}P localised in the troponin-I band. A molecular weight of 29 000 was obtained from the electrophoresis, which was identical to that reported for rabbit cardiac troponin-I [17].

For the analysis of total phosphate in troponin-I the protein was precipitated, washed and digested in 2.5 N NaOH at 100°C for 20 min [23]. After neutralisation, P_i was measured with ammonium molybdate/malachite green [24].

2.4. *Calculation of results*

The ^{32}P per mole troponin-I was calculated from the mol. wt above, and the [γ-^{32}P] ATP specific radioactivity used to calculate the moles of ^{32}P per mole troponin-I. This calculation assumes that the ^{32}P donor is ATP, which will be true if either phosphorylase kinase or a protein kinase catalyses the reaction. Also it is assumed that the ATP specific radioactivity does not change during the period of phosphorylation. As is shown below, phosphorylation occurred with 25 sec of adrenaline administration, during which time no significant changes in ATP specific radioactivity were detected.

3. Results and discussion

Fig.1 shows the time course of change in force of contraction and phosphorylation of troponin-I following administration of 10^{-6} M adrenaline to perfused rat heart. After a delay of 5 sec the contraction gradually increased reaching a maximum after 20 sec. The amount of ^{32}P in troponin-I followed an identical time course, increasing from a control value of 0.2 mole ^{32}P/mole troponin-I to a maximum of 1.2 mole/mole.

Fig.2 shows the changes which occurred in contraction and troponin-I phosphorylation after 20 sec of perfusion with different concentrations of adrenaline. The contraction increased with increasing adrenaline concentrations from 3×10^{-8} M to 10^{-6} M, but at

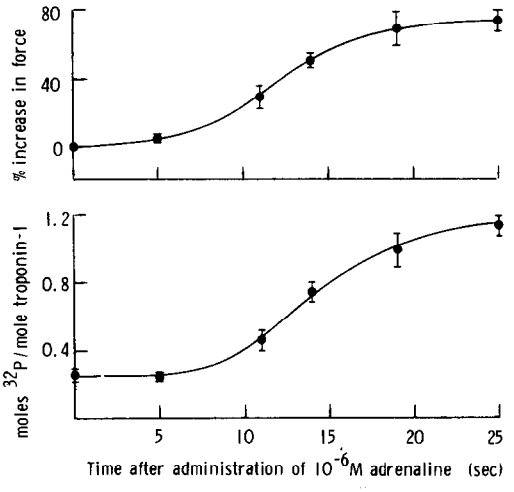

Fig.1. Time course of changes in force of contraction (upper panel) and phosphorylation of troponin-I (lower panel) following administration of 10^{-6} M adrenaline. Hearts were perfused with $^{32}P_i$ as described in Methods, and then perfused by drip-through during a control period. The hearts were freeze clamped [12] at the times shown following continuous perfusion with adrenaline. The increase in force of contraction is expressed as a percentage of that during the control period. Each point is the mean of three hearts, and the vertical bars are 2 S.E.M.

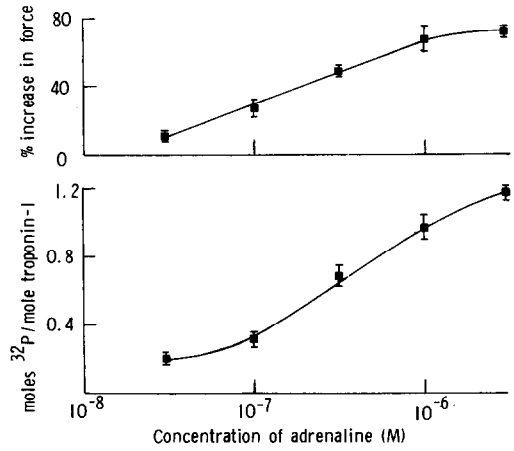

Fig.2. Dose-response curve of force of contraction (upper panel) and phosphorylation of troponin-I (lower panel) at various adrenaline concentrations. The hearts were perfused for 20 sec with the stated concentration of adrenaline before being freeze-clamped. Other details are given in fig.1 or in the text. Each point is the mean of three hearts, and the vertical bars are 2 S.E.M.

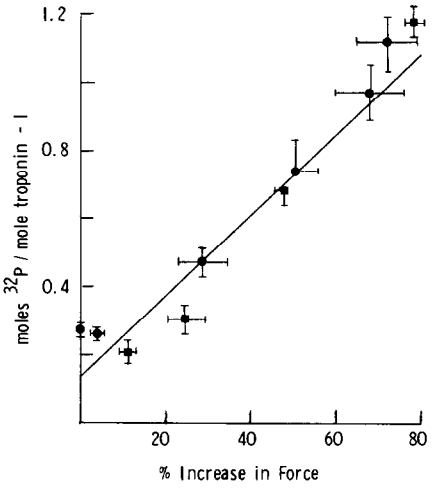

Fig.3. Correlation between heart contraction, and phosphorylation of troponin-I. The data are taken from figs. 1 (•) and 2 (▪). The line was calculated by linear regression. (Correlation coefficient = 0.93).

higher concentrations the response did not increase. The phosphorylation of troponin-I also followed a similar dose-response curve.

Fig.3 is a replot of the data figs.1 and 2, and shows the correlation between increase in contraction and troponin-I phosphorylation. The correlation coefficient, 0.93, was highly significant ($P<0.001$; Student's t test).

It has been reported that from 1–2 mole phosphate/mole troponon-I were incorporated after incubation of isolated troponin-I with protein kinase [25,26]. Since the maximum ^{32}P incorporated in the perfused hearts was 1.2 mole/mole, the troponin-I samples from the experiments of fig.2 were analysed for total phosphate to investigate the possibility that additional non-radioactive phosphate might be present. The troponin-I from the three hearts in each group were pooled, and total phosphate measured. The ratio of ^{32}P to total phosphate was found to be 0.83 ± 0.13 (mean ± S. E. M., 5 values), which was not significantly different from unity ($P>0.05$, Student's t test). It appears therefore that the phosphate in troponin-I measured as ^{32}P represents the total phosphate content.

The results of this paper show that a close correlation exists between increases in contraction and phosphorylation of troponin-I in rat heart in response to adrenaline. This suggests that changes in the phospho-

rylation state could be responsible for the changes in contraction which occur following adrenaline administration. However, more evidence that phosphorylation changes the properties of troponin-I is required to substantiate this hypothesis [5]. The concentrations of cyclic 3′, 5′ AMP and activated phosphorylase kinase, on administration of adrenaline to perfused rat heart, reach their maxima before the maximum increase in contractility [27,28]. Phosphorylase *a* reaches a maximum after this [27]. The phosphorylation of troponin-I is therefore intermediate in time between activation of phosphorylase kinase and production of phosphorylase *a*. A simple precursor–product analysis suggests that heart troponin-I may be phosphorylated by phosphorylase kinase rather than by a cyclic 3′, 5′ AMP dependent protein kinase. However, problems of availability of troponin-I to the kinases, and their translocation into the myofibrils do not allow a more detailed analysis. Also, nonactivated phosphorylase kinase can phosphorylate troponin-I [2], and other factors such as free Ca^{2+} may be of importance.

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