cGMP-dependent protein kinase decreases calcium sensitivity of skinned cardiac fibers

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Chemically skinned (Lubrol WX) cardiac muscle fibres produce half-maximum isometric tension at pCa 6.18 (pH 6.7) in presence of MgATP (10 mM). After addition of cGMP (5 μM) and cGMP-depenent protein kinase (0.1 μM), the pCa required for half-maximum activation is 5.96, while maximum tension is not affected. Similar shifts in the tension/pCa-relationship have been observed after incubation of skinned cardiac muscle fibres with cAMP of catalytic subunit of the cAMP-dependent protein kinase. The shift in the Ca^{2+}-sensitivity is associated with an increased incorporation of radioactivity into a M_r 28000 band (presumably troponin-I) and a M_r 145000 band.

cGMP-dependent protein kinase *Skinned cardiac muscle* *Troponin-I phosphorylation*

1. INTRODUCTION

In cardiac muscle the positive inotropic effect of β-adrenergic stimulation is mediated by cAMP-dependent phosphorylation of myofibrillar, sarcoplasmic and sarcolemmal proteins [1,2]. In contrast to cAMP, the physiological significance of cGMP as a regulator of cardiac contractility is unclear. Originally it was suggested that cGMP mediates the negative inotropic effect of acetylcholine [3]. This suggestion has been questioned [4] since changes in cardiac contractility have been dissociated from changes in cGMP levels [5,6]. Immunocytochemistry of cardiac muscle indicated that protein bound cGMP is associated with myofibrils [7] suggesting the presence of a specific cGMP receptor in that area. In agreement with this localization it was found that an increase in cGMP was associated with increased dephosphorylation of cardiac troponin in vivo [8] and increased calcium sensitivity of tension development in hyperpermeable cardiac fibers [9]. This raised the possibility that cGMP stimulates directly or indirectly a protein phosphatase. The latter possibility was considered to be more likely since only a cGMP-dependent protein kinase (cGMP-kinase) has been identified in and purified from cardiac muscle extract [10]. In contrast to this consideration, in vitro incubation of cGMP-dependent protein kinase with cardiac troponin did not result in dephosphorylation but in phosphorylation of the inhibitory subunit of troponin (TN-I) [11,12]. Phosphorylation of troponin by cAMP-dependent protein kinase (cAMP-kinase) [13,14] reduces the calcium sensitivity of tension development [15,16] presumably by increasing the rate of dissociation of calcium from troponin [17]. It has been assumed that dephosphorylation of troponin is associated with an increase in calcium sensitivity of tension development in this preparation [9,15]. Therefore, determination of the calcium sensitivity of such fibers before and after addition of cGMP-kinase might give a clue whether this enzyme is involved in the phosphorylation or dephosphorylation of myofibrillar proteins. Such experiments show that addition of cGMP-kinase to skinned cardiac fibers decreases the calcium sensitivity of tension development.

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2. METHODS

Chemically skinned muscle fibers were prepared from the subendocardial layer of the right ventricle and from the coronary arteries of porcine hearts, and from muscular strips of porcine trachea as in [16,18]. Conditions for measuring isometric tension development in the presence of various [Ca^{2+}] were as in [16]. In some experiments ATP was replaced by [γ-^{32}P]ATP in the absence of an ATP regenerating system. In these experiments reactions were stopped by the addition of 50% trichloroacetic acid to fibers still attached to the force transducer. Denatured proteins were separated on SDS-gradient gels (6–15%), and radioactivity associated with individual bands was detected by autoradiography as in [19]. Homogeneous preparations of cGMP-kinase [10], catalytic subunit of cAMP-kinase [20], and calmodulin [21] were obtained from bovine lung, bovine heart and porcine brain.

3. RESULTS AND DISCUSSION

Chemically skinned cardiac muscle fibers were relaxed in an ATP solution containing <0.01 μM Ca^{2+} and were maximally contracted at 20 μM Ca^{2+}. Reproducible contraction/relaxation cycles were obtained for ≥2 h (fig. 1). Preincubation of fibers with low concentrations of cGMP-kinase (0.1 μM), cGMP (5 μM) and isobutylmethylxanthine (IBMX) (0.1 mM) at low [Ca^{2+}] caused an inhibition of the subsequent tension development elicited by submaximal [Ca^{2+}] (fig. 1). cGMP-kinase induced also relaxation if added after fibers had been contracted submaximally. The relaxing effect of cGMP-kinase persisted after changing buffers. cGMP (5 μM) alone or cGMP-kinase in the absence of cGMP did not affect tension development. cGMP (5 μM) added in the presence of IBMX (0.1 mM) minimally relaxed skinned fibers contracted at a pCa of 6.08.

Incubation of fibers with cGMP-kinase, cGMP and IBMX caused a reproducible rightward shift of the force/pCa-relationship without affecting the maximal tension developed (fig. 2). In the absence and presence of cGMP-kinase halfmaximal tension occurred at a pCa of 6.18 ± 0.05 and 5.96 ± 0.04, respectively. Statistical evaluation showed that this 2-fold shift in calcium sensitivity was highly significant ($p < 0.001$). No significant change in the Hill coefficient was observed suggesting that cGMP-kinase did not alter the cooperativity of the force/pCa-relationship. Similar small shifts in tension/pCa-relationships have been observed after incubation of skinned cardiac fibers with cAMP [15,16] or with the catalytic subunit of the cAMP-kinase [26]. The potential significance of the observed rightward shift is further supported by biochemical experiments which showed that phosphorylation of cardiac TN-I increased the [Ca^{2+}] required for inducing a 50% change in the fluorescence signal of 2-(4′-iodoacetamidoanilino)naphthalene-6-sulfonic acid modified TN-C by 0.27 pCa units [17], a value almost identical to that obtained here. Therefore, the most likely explanation of the experiments shown was, that

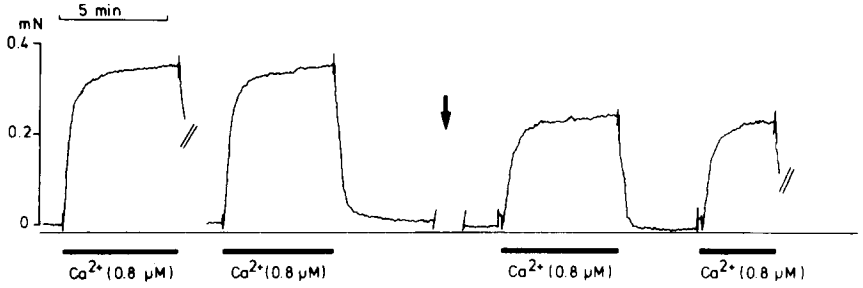

Fig. 1. Isometric tension of a skinned cardiac muscle fiber was recorded after raising [Ca^{2+}] from 0.01–0.8 μM in the presence of IBMX (0.1 mM), Mg·ATP (10 mM), and an ATP regenerating system. After the second contraction (↓), cGMP (5 μM) and cGMP-kinase (0.1 μM) were added in the presence of 0.01 μM Ca^{2+}. The fiber was incubated for 20 min before contraction was initiated by changing the bathing medium to one containing 0.8 μM Ca^{2+} but no cGMP and cGMP-kinase.

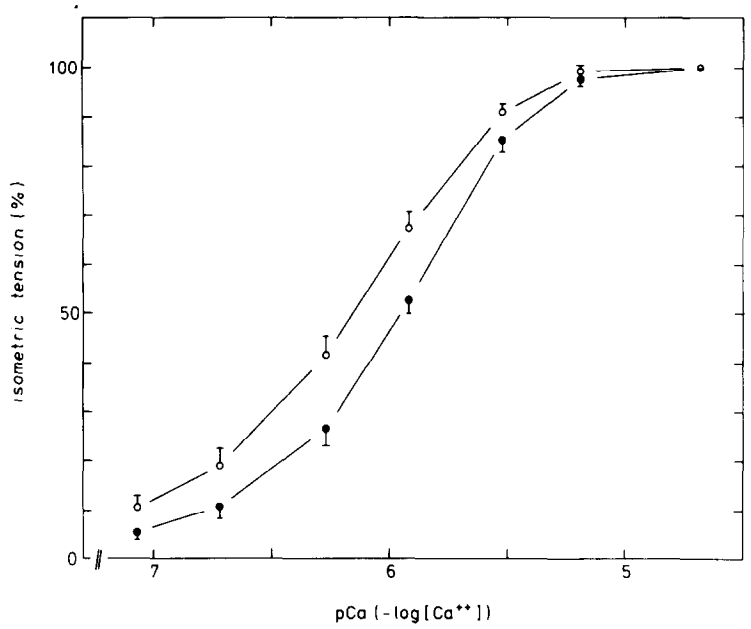

Fig. 2. cGMP-kinase shifts the force/pCa-relationship to the right. Fibers were first contracted by increasing the [Ca^{2+}] cumulatively in the absence of cGMP-kinase (○), then after incubation of the relaxed fibers in the presence of cGMP-kinase (●). Values are $\bar{x} \pm$ SEM for 6 different fibers. In individual fibers maximal tension developed was not changed by addition of cGMP-kinase. For further details see legend to fig. 1.

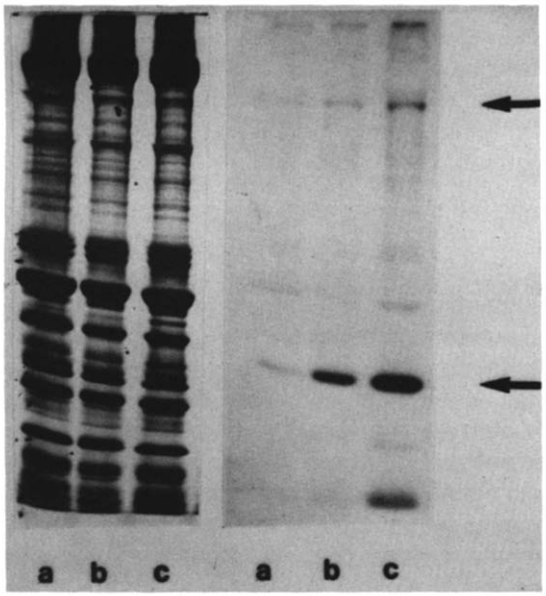

Fig. 3. Coomassie blue staining of SDS–polyacrylamide gels (6–15% acrylamide) (left) and the corresponding autoradiographs (right) of individual skinned cardiac fibers incubated in the presence of [γ-^{32}P]ATP·Mg, IBMX (0.1 mM) and: (a) 0.8 μM Ca^{2+}; (b) 0.8 μM Ca^{2+} and 5 μM cGMP; or (c) 0.8 μM Ca^{2+}, 5 μM cGMP and 0.1 μM cGMP-kinase. Radioactivity is associated mainly with a M_r 28000 and 145000 band (→).

cGMP-kinase phosphorylated TN-I during the incubation and induced thereby the rightward shift in the force/pCa-relationship. This explanation is supported by experiments which showed that radioactivity associated with a M_r 28000 band (corresponding to that of purified cardiac TN-I) increased dramatically in the presence of cGMP-kinase (cf. fig. 3a and c). In several experiments, enhanced incorporation of radioactivity into a M_r 145000 band was also observed. It is not clear at present if this band represents cardiac 'C-protein' [22] or some unrelated protein.

The results obtained so far suggested that cGMP-kinase used at a low concentration phosphorylated TN-I and shifted thereby the pCa/force-relationship of skinned fibers to the right. They did not completely rule out the possibility that cGMP-kinase might be able to cause an opposite shift in the force/pCa-relationship by increasing the activity of a protein phosphatase followed by dephosphorylation of TN-I, since the phosphorylation status of TN-I was unknown. For example cGMP-kinase could induce only a leftward shift in the force/pCa-relationship if TN-I was initially phosphorylated by cAMP-kinase. Therefore, skin-

ned fibers were incubated first with the catalytic subunit of cAMP-kinase and then with cGMP-kinase and vice versa. At all submaximal [Ca^{2+}] tested, both enzymes inhibited tension development. Typically, the rightward shift of calcium sensitivity induced by one enzyme could not be reversed by addition of the second enzyme regardless of the order of addition. The relaxing effects of both enzymes were additive if submaximal effective concentrations of enzymes were used. Together this indicates that in detergent skinned cardiac fibers cGMP-kinase phosphorylates directly TN-I and is not involved in a sequence leading to dephosphorylation of myofibrillar proteins.

cGMP-kinase affected contractility of the skinned fibers in a manner which is indistinguishable from that of cAMP or cAMP-kinase. This similarity is not a non-specific effect of cGMP-kinase, since the enzyme was added at a low concentration to the bathing medium and probably only part of the enzyme equilibrated with the interior of the fibers. However, the physiological significance of this similarity is not yet obvious. It is possible that due to the preparation procedure of the muscle fibers cGMP-kinase had access to proteins like TN-I which are not accessible for it in the intact cell.

To obtain some evidence whether or not cGMP-kinase may be involved also in the relaxation process of other muscle types, the effect of the enzyme on tension development of skinned fibers from coronary arteries and tracheal smooth muscle was tested. These two different smooth muscles were used since cAMP relaxes both, whereas solid evidence for cGMP-mediated relaxation has been obtained only for arterial but not for tracheal smooth muscle [23–25]. Under the conditions used which resulted in submaximal contraction tension development of coronary arteries was not affected by addition of cGMP-kinase, whereas that of tracheal smooth muscle was reversibly inhibited (fig. 4).

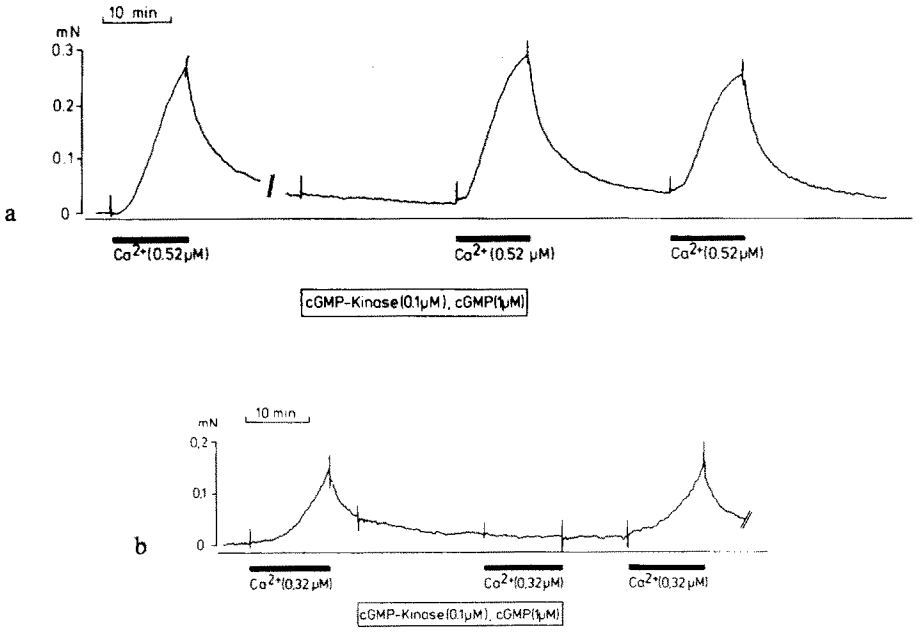

Fig. 4. Effect of cGMP-kinase of tension development in skinned smooth muscle fibers from coronary artery (a) or trachea (b). Skinned fibers were incubated in the same buffer system as used for cardiac muscle fibers containing 7.5 mM Mg·ATP, 1 μM (a) and 0.2 μM (b) calmodulin. Contraction was elicited by raising the [Ca^{2+}] from 0.01 μM to (a) 0.52 or (b) 0.32 μM for the time indicated. The second contraction was carried out in the presence of cGMP-kinase (0.1 μM) and cGMP (1 μM). Note, that cGMP-kinase did not alter tension development in (a) when the calmodulin concentration was lowered to 0.1 μM, and that the effect of cGMP-kinase in (b) is reversed after changing the bath medium.

This indicates that cGMP-kinase does not induce in all muscles relaxation by directly modifying contractile proteins.

This study shows that the use of purified enzymes in combination with skinned fibers might help to unravel the mechanisms by which hormones regulate muscle tone.

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