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Structure and function of a calmodulin-dependent smooth muscle myosin light chain kinase

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Summary. In smooth muscle the M_r 20,000 light chain of myosin is phosphorylated by a calmodulin-dependent protein kinase. It consists of 2 subunits: calmodulin, an acidic protein of M_r 17,000 that binds 4 moles of Ca^{2+}; and a larger protein of M_r circa 130,000. Activation of the kinase is dependent upon their association in the presence of Ca^{2+}. Cyclic AMP-dependent protein kinase phosphorylation of the myosin light chain kinase occurs at 2 sites. It decreases the affinity of the kinase for calmodulin and a reduction in the rate of light chain phosphorylation occurs. The kinase has an overall asymmetric shape composed of a globular head and tail region for the skeletal muscle enzyme. Trypsin digestion of this kinase releases a fragment of M_r 36,000 from the globular region that contains the catalytic and calmodulin binding sites. Chymotrypsin digestion of the kinase from smooth muscle generates a fragment of M_r 80,000 that does not contain the calmodulin binding or cyclic AMP-dependent protein kinase phosphorylation sites. It is a Ca^{2+}-independent form of the kinase that phosphorylates the light chain of myosin. These structural features indicate a regulatory role for the kinase in smooth muscle phosphorylation and contraction.

Key words. Calmodulin; cyclic AMP; myosin; protein kinase; phosphorylation; smooth muscle (gizzards).

The contraction and relaxation cycle of smooth muscle of the cardiovascular system is needed to provide, in part, a supporting wall against the blood pressure and ensure blood flow. In smooth muscle, the contractile protein myosin is subject to a sequence of chemical reactions involving phosphorylation and dephosphorylation. The phosphorylation of myosin is catalyzed by a calmodulin-dependent myosin light chain kinase and there is evidence accumulating that it plays a key role in the regulation of contraction.

Structure of calmodulin-dependent myosin light chain kinase

Calmodulin-dependent myosin light chain kinase has been isolated from several tissue sources including rabbit skeletal[23], chicken[22] and turkey gizzard[3] muscle. The content of the enzyme is 150 mg/kg of gizzard muscle. It catalyzes the phosphorylation of the light chain of M_r 18,500 in rabbit skeletal myosin[31] and the light chain of M_r 20,000 of chicken gizzard myosin[2,29].

Myosin light chain kinase, MLCK, consists of 2 different subunits: a calcium dependent subunit, an acidic protein of M_r 17,000, termed calmodulin, that binds up to 4 moles of calcium per mole of protein[7]; the other subunit is a larger molecule of M_r 90,000 and 130,000 for rabbit skeletal and turkey gizzard muscle, respectively[3]. The differences in size could be due to limited proteolysis; the skeletal protein being more susceptible to proteolytic digestion than its smooth muscle counterpart[2]. However, recent antibody studies on skeletal MLCK suggest that this may not be the case[24]. Activation of kinase activity occurs when calcium, at a concentration no less than 0.1 μM, binds to calmodulin and subsequently forms a ternary complex with the larger subunit[11,38]. The half maximal values for the calcium-dependent activation of the kinase calmodulin complex vary from 0.7 μM to 10 μM for rabbit skeletal muscle[31,38]. These values are, however, consistent with the physiological requirements for contraction, i.e. calcium concentrations for half maximal activation of the ATPase activity of a reconstituted or native rabbit skeletal actomyosin is circa 1 μM[27]. The stoichiometry of the binding of calmodulin to the large kinase subunit appears to be 1 mole of calmodulin per mole of kinase[2,23,32]. The apparent K_m value for the calmodulin-myosin light chain kinase interaction also varies depending upon the experimental conditions employed[10]. For example, there is a dependence on ionic strength among other factors[10]. Values ranging between 1 to 70 nM have been reported but a value close to 1 nM has been recently obtained in the presence of 0.5 mM $CaCl_2$[10]. This is consistent with the calmodulin concentration value of 1 nM that was obtained previously for 50% activation of rabbit skeletal[22] and turkey gizzard myosin kinase[3]. In the presence of calcium the complex formed is in a highly associated state.

Previous reports have revealed some of the properties of the myosin light chain kinase subunit. The kinase from turkey smooth muscle has an M_r of 130,000[3]. Recent studies with the turkey gizzard[37] and bovine stomach[36] MLCK, however, have shown that higher M_r forms of the kinase, e.g. M_r 136,000[1], 141,000[37] and 155,000[36] are present. It was suggested that the M_r 130,000 enzyme may be a proteolytic fragment of a higher M_r species[1] or they may represent different isozymes[37]. The kinase of M_r 130,000 has an amino acid composition that is highlighted by large amounts of glutamate, a larger than expected amount of proline and there are circa 28 cysteine residues per mole of protein[3]. No free amino terminal residue was found. From a calculation of the Stokes radius it appears that MLCK is asymmetric in shape[37]. This could provide for a facilitated orientation of the kinase in relation to its interaction with the substrate myosin molecule which is also quite asymmetric[3]. The chicken gizzard MLCK has a similar amino acid composition and it has a pH optimum of 7.75 to 8.0[22]. The temperature dependence of the kinase was also studied and a Q_{10} value of 2.0 was found; it is much lower than that observed for the Mg^{2+}-ATPase activity of gizzard actomyosin. This indicates that lower activation energies are associated with the phosphorylation reaction catalyzed by the calmodulin-dependent MLCK[22]. In the case of the kinase from rabbit skeletal muscle, it has an M_r of circa 91,000 and is also highly asymmetric[10]. The high proline content found in this protein (9%) is consistent with that found in the smooth muscle enzyme suggesting that there are several perturbations of the helical structure in this molecule[10]. Recent unique trypsin digestion studies suggest that the structure of the rabbit skeletal kinase consists of a head and tail region[20]. A proteolytic fragment of M_r 36,000 corresponds to the head region and reportedly, it contains the catalytic and calmodulin binding sites[20].

Myosin light chain kinase can be phosphorylated by another kinase. Phosphorylation of MLCK from smooth muscle by a cyclic AMP-dependent protein kinase in the absence of calmodulin resulted in the incorporation of 1.4–2.0 moles of phosphate per mole of protein[9]. Concurrently, there was a 10–20-fold decrease in the affinity of calmodulin for the modified light chain kinase and there was a reduction in the rate of light chain phosphorylation by MLCK; the V_{max} value, however, was not affected[9,13]. In the presence of calmodulin 0.4–1.0 mole of phosphate was incorporated but there was no loss of phosphorylating activity by MLCK[20]. There are 2 sites for phosphorylation, sites 1 and 2, and removal of one of the phosphate groups by a rather specific phosphatase[25] restores the ability of the kinase to bind calmodulin tightly and phosphorylate the myosin light chain at a normal rate[25]. This second messenger control by cyclic AMP may explain the relaxation effects on vascular smooth muscle when cyclic AMP levels are elevated by epinephrine[9]. However, some problems remain with this hypothesis. It has yet to be determined if the approx. 20-fold decrease in the affinity of calmodulin for phosphorylated MLCK, in vitro, has any physiological significance. Indeed, phosphorylated MLCK can still phosphorylate the light chain of myosin[9,13] and calmodulin concentrations in muscle are not limiting or changing significantly[8,13,21]. The effective control of MLCK probably depends on Ca^{2+} which is limiting, in vivo. The effect of cyclic AMP on Ca^{2+} levels and the interaction of Ca^{2+} with calmodulin may be more important in mediating relaxation than its effect on cyclic AMP-dependent protein kinase phosphoryla-

tion of MLCK[21]. Secondly, the rates of calcium-calmodulin binding and dissociation to the kinase have not been calculated nor have they been correlated to the rate of cyclic AMP-dependent phosphorylation of site 1 on the kinase[13]. It is noteworthy that cyclic AMP-dependent protein kinease phosphorylation of the skeletal muscle myosin kinase did not alter its activity[12]. One mole of phosphate was incorporated into the skeletal kinase and no change in the affinity for calmodulin was found[27]. It was suggested that the phosphorylation of skeletal MLCK might change its susceptibility to proteolysis and thus provide a mechanism for the control of the kinase via its turnover[12]. This remains to be established.

Activity of calmodulin-dependent myosin light chain kinase

The specificity of the phosphorylation reaction catalyzed by the calcium-calmodulin MLCK is limited to the following: myosin and its isolated light chains of M_r 18,500 and 20,000 for skeletal and smooth muscle sources, respectively[2,29,31]. It will not phosphorylate histone, casein or phosphorylase 'b'[2]. The K_m values for the myosin light chains are of the order of 5 µM to 11 µM for turkey smooth[3] and circa 24 µM for the rabbit skeletal[23] kinases, respectively. It is noteworthy that the isolated light chain of gizzard myosin was phosphorylated about 2–10-fold more rapidly than its myosin[3,22]. The V_{max} values, ranging between 15–30 µmoles P/min/mg kinase, were essentially the same for the smooth and skeletal muscle enzyme[13]. Reportedly, the activity of MLCK is enhanced when synthetic substrate peptides containing lysine and arginine are used[16].

Evidence has been accumulating in support of a role for phosphorylation of myosin in the regulation of smooth muscle contraction. The ATPase activity of myosin in the presence of actin was activated when its light chain of M_r 20,000 was phosphorylated[2,4,13,22,29]. Moreover, MLCK catalyzed phosphorylation induces conformational changes in myosin[5,15,17]. In contrast, phosphorylation of rabbit skeletal myosin was not required for actin activation of the ATPase activity. In this case, calmodulin-dependent myosin light chain kinase phosphorylation may serve to increase the affinity of myosin for actin binding without an effect on the V_{max} of the actin-activated ATPase activity[26]. It may also play a role in post-tetanic potentiation[19,31].

There are also reports that several proteins of rabbit skeletal sarcoplasmic reticulum, SR, and those of a microsomal membrane protein of smooth muscle are phosphorylated by a calmodulin-dependent protein kinase that may resemble the myosin light chain kinase[6,8]. In particular, rapid phosphorylation of a protein of M_r 60,000 was observed. In one report phosphorylation was associated with a change in the Ca^+ transport of SR; the removal of calmodulin or addition of EGTA inhibited the phosphorylation[6]. The calmodulin-dependent protein kinase could be involved in governing Ca^{2+} release in muscle[8].

Modification of calmodulin-dependent protein kinase

Myosin light chain kinase activity is inhibited by trifluoperazine[18] and other drugs[14]. The site of this modification was the calmodulin subunit and it occurred mainly when calcium was bound to calmodulin. This was also the case when the kinase was treated with naphthalene sulfonamide derivatives[33]. The calmodulin antagonist fluphenazine inhibited the phosphorylation of myosin and tension development in intact smooth muscle[28]. Proteolytic digestion of smooth muscle myosin light chain kinase by chymotrypsin produced a proteolytic fragment of M_r 80,000 that did not contain the calmodulin binding site(s) or the 2 sites usually phosphorylated by cyclic AMP[35]. It was a Ca^{2+}-independent form of the kinase that phosphorylated the M_r 20,000 light chain of gizzard myosin similar to that obtained with intact MLCK[34,35]. The Ca^{2+}-independent phosphorylation was found to cause a loss of Ca^{2+} sensitivity in a) a crude actomyosin system assayed for ATPase activity; and b) during tension development in skinned gizzard fibers[35]. In a preliminary report, a Ca^{2+} independent fragment was also isolated from a digest of skeletal muscle MLCK but it contained the phosphorylation sites[30]. Clearly, further characterization of these fragment is required to define the location and role of the sites on MLCK for calmodulin binding and cyclic AMP-dep. phosphorylation. Other chemical modifications using –SH reagents showed a difference in the reactivity of the calmodulin-dependent myosin light chain kinase compared to that of an actomyosin system from smooth muscle[13].

In conclusion, elucidation of the structural and functional relationships of myosin light chain kinase and its catalysis of smooth muscle myosin phosphorylation may provide new insights into studies of clinical abnormalities of the cardiovascular system where the contraction and relaxation cycle may be thrown into disarray.

Note added in proof. It has been shown recently that the Mr 141,000 component is Caldesmon and that the native form of the kinase has an Mr of 136,000 (Ngai, P.K., Carruthers, C., and Walsh, M.P., Isolation of the native form of chicken gizzard myosin light chain kinase. Biochem. J. *218* (1984) 863–870).

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