Calmodulin-Dependent Autophosphorylation of Smooth Muscle Myosin Light Chain Kinase: Intermolecular Reaction Mechanism via Dimerization of the Kinase and Potentiation of the Catalytic Activity Following Activation†

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ABSTRACT: In the presence of Ca^{2+} and calmodulin (CM), purified smooth muscle myosin light chain kinase (MLCKase) was found to undergo autophosphorylation at a rate that was about 200-fold slower than its catalytic activity. Up to 1.7 mol of phosphate were incorporated per mole of kinase. Lower levels of incorporation could be correlated with the presence of an endogenous protein phosphatase which could be inhibited with okadaic acid or Microcystin-LR. The major autophosphorylation site was identified as Thr-863 or Thr-865 and was located on the 24-kDa C-terminal fragment of the kinase. In addition, there was a relatively low and variable contribution of a Ca/CM-independent autophosphorylation at Ser-814 or Ser-815. The initial autophosphorylation rates and maximal incorporation levels were highest at a molar ratio of 2 MLCKase to 1 CM and were inhibited at higher CM levels. This indicated that binding of one molecule of the kinase apoenzyme by a CM−kinase complex was necessary for the reaction to occur. Kinetic analysis of the autophosphorylation reaction was consistent with this interpretation and indicated a second-order intermolecular process that included MLCKase dimerization or oligomerization. In contrast, the low Ca/CM-independent contribution was of intramolecular type since it did not depend on the kinase concentration. The autophosphorylation appeared to be involved in a relatively slow modification of the oligomeric properties of the kinase leading to a 2−4-fold amplification of the kinase catalytic activity which followed its activation by CM. Oligomerization and dimerization of the kinase was independently demonstrated by light scattering measurements.

Myosin light chain kinases (MLCKases)[1] have been found in all muscle and nonmuscle motile systems: of these, only the smooth muscle kinase has been shown to be directly involved in Ca-dependent regulation of the actin−myosin interaction. The key regulatory role of the latter enzyme was established by extensive *in vitro* studies on smooth muscle actomyosin [for reviews, see Marston (1982) and Small and Sobieszek (1980)] as well as *in vivo* studies on whole muscle or muscle strips (De Lanerolle & Paul, 1991; Kamm & Stull, 1985). The former studies demonstrated that in the presence of Ca^{2+} and calmodulin (CM) the kinase phosphorylates the regulatory light chain of myosin (LC_{20}), permitting the cyclic interaction between myosin heads and thin filaments necessary for contraction. The relaxation, that is, dissociation of actomyosin, is attained via dephosphorylation of the myosin heads by a myosin light chain phosphatase (MLCPase). Several phosphatases which dephosphorylate the isolated regulatory light chain of smooth muscle myosin have been identified or purified [for reviews, see Pato and Kerc (1988) and Cohen (1989)]. Some of these appear to be specific for intact smooth muscle myosin [see Tulloch and Pato (1991)] while others are specific for MLCKase (Pato & Adelstein, 1983). It is not clear, however, which phosphatase is directly involved in the relaxation of smooth muscle.

In view of its obvious physiological relevance, the structure and function of MLCKase have been extensively studied. The primary structure and domain identification of the chicken gizzard enzyme has been established [Guerriero et al., 1986; see also Gallagher et al. (1991)]. The catalytic domain of the 107-kDa molecule extends from residue 526 to 762, with the ATP-binding domain occupying the first 22 residues. The CM-binding region has been defined as a 192 amino acid fragment located very close to the COOH-end of the molecule (Olson et al., 1990). A particularly significant finding was that a synthetic peptide representing residues 796−813 bound CM with an affinity similar to the native enzyme (Lukas et al., 1982), while those mimicking residues 787−807 exhibited significant similarity to the myosin light chain substrate (Kemp et al., 1982). Thus, the peptide analog to this pseudosubstrate region serves as an antagonist of light chain phosphorylation.

From the known sequence of the gizzard kinase, the CM-binding region in general, and the overlapping region in particular, there are several serine and threonine residues that could undergo phosphorylation and play a role in the regulation of the kinase activity. Two of these serines were phosphorylated by cAP kinase in the absence of Ca/CM with a concomitant 10-fold reduction of the MLCKase affinity for CM. In the presence of CM, only one site could be phosphorylated while the other one appeared to be blocked

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[1] Abbreviations: MLCKase, myosin light chain kinase; MLCPase, myosin light chain phosphatase; cAP-kinase, cAMP-dependent protein kinase; MLCK-phosphatase, endogenous protein phosphatase contaminating MLCKase preparations; CM, calmodulin; LC_{20}, 20-kDa light chain of smooth muscle myosin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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(Conti & Adelstein, 1981). Similar observations were made for protein kinases from other sources (Miller et al., 1983; Walsh et al., 1982; Higashi et al., 1983; Bhalla et al., 1982) as well as for the phosphorylation by protein kinase C (Ikebe et al., 1985; Nishikawa et al., 1985) and CM-dependent protein kinase II (Hashimoto & Soderling, 1990). In the latter case there were three (and not two) sites involved which were not necessarily identical with those phosphorylated by cAP kinase.

From the data accumulated so far, it was concluded that the regulatory domain of the kinase is phosphorylated at multiple sites [see Stull et al. (1990)]. The autophosphorylation noted earlier (Foyt & Means, 1985) is undoubtedly relevant to the elucidation of the regulatory pathways of the smooth muscle MLCKase. The present study characterizes the role of CM in this autophosphorylation and demonstrates that dimerization of the kinase is involved in the autocatalytic reaction.

MATERIALS AND METHODS

Chemicals. Radioactive [γ-^{32}P]ATP (3000 Ci/mmoe) was purchased from NEM, Boston, MA, in amounts of 1 mCi each time before a series of experiments. Upon arrival, the radioactive ATP was diluted up to 0.5−1.0 mL of the "AA" buffer (Sobieszek, 1991a,b) containing, in addition, 8.75 mM "cold" MgATP and was kept frozen at −30 °C. The composition of AA buffer was as follows (in mM): KCl, 60; $MgCl_2$, 2; dithiothreitol, 0.5; and imidazole, 10, with the pH adjusted to 7.0 at 25 °C. In order to minimalize ATP hydrolysis and its radioactive degradation, this 8−8.5 mM [γ-^{32}P]ATP mix was defrosted only for taking out the amounts needed for a particular experiment. The other chemicals were of analytical grade and unless otherwise stated were purchased from E. Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Protein Preparations. Turkey gizzard or pig stomach MLCKase was used throughout these studies. The kinases were purified as described in detail previously (Sobieszek & Barylko, 1984; Sobieszek, 1990). For some preparations the initial gel filtration step was omitted or applied as the penultimate purification step. For other preparations an additional intermediate passage through a Phenyl-Sepharose column (Pharmacia, Upsalla, Sweden) was included. In all cases the 40−55% (turkey gizzard) or 42.5−52.5% (pig stomach) ammonium sulfate fraction was diluted 3−4-fold with cold water and directly applied to a relatively long (30−40 cm) DEAE-Sepharose 6B-CL ionic exchange column. The column was eluted with a 160−360 mM KCl gradient in AA buffer (see above) containing 0.5 mM EGTA. After identification of the kinase containing tubes by SDS gel electrophoresis, 0.6 mM $CaCl_2$ was added to the kinase pool and applied directly onto a CM-affinity column. After washing of the column at high ionic strength, the kinase was eluted with AA buffer containing 2 mM EGTA. For the autophosphorylation assays it was convenient and necessary to concentrate the kinase preparations further. Accordingly, the kinase containing tubes were collected and precipitated at 60% ammonium sulfate saturation. The dissolved ammonium sulfate pellet was dialyzed over 12−24 h against two or three changes of the AA buffer containing 25% glycerol. This resulted in an additional 2−3-fold concentration of the kinase; the preparations stored at −70 °C were thus at 250−400 μM. The MLCKase concentration was calculated from its absorption at 278 nm using the extinction coefficient of 1.1 (Adelstein & Klee, 1981) and molecular weight of 108.5 kDa (Olson et al., 1990).

CM was purified from the same two smooth muscle sources and used with the kinase of the same origin, although no difference in their activation properties was noted. They were purified from the first muscle wash as described recently (Sobieszek, 1991b). The CM concentration was determined by absorption measurements using $A^{278}_{0.1\%} = 0.18$ (Adelstein & Klee, 1982), and these measurements agreed well with the Biuret method (Gornall et al., 1949).

The regulatory (phosphorylatable) myosin light chain (LC_{20}) was isolated and purified from turkey or pig stomach myosins dissolved initially in 5 M urea and 2 M guanidine hydrochloride as described in detail previously (Sobieszek, 1988). One of the standard substrates for Ca/CM-dependent kinase II, autocamtide-3, was received from Dr. T. Meyer of Duke University (Durham, NC). The other substrate, caldesmon, was purified essentially according to Bretscher (1984). The catalytic subunit of cAP kinase used in these studies was a generous gift of Prof. E. G. Krebs of Howard Hughes Medical Institute, Seattle. Histone IIIS was used as the standard substrate for testing protein kinase C activity and was purchased from Sigma Chemical Co. (St. Louis, MO).

^{32}P-Incorporation Measurements. Phosphorylation assays were carried out under the same conditions (e.g., AA buffer and 25 °C) as described previously [see Sobieszek (1991a,b)]. Intact myosin or its isolated LC_{20} light chain was used as the substrate at concentrations, between 20−40 and 200−300 μM, respectively. The reaction was initiated by addition of the radioactive ATP stock (see above) diluted further 25−5-fold with the 8.75 mM cold ATP mix to obtain the required level of "cpm" and with the AA buffer to obtain 0.3−0.5 mM final ATP concentration.

Autophosphorylation measurements were simpler since they required no substrate and otherwise they were done as the phosphorylation assays. However, they required [γ-^{32}P]-ATP of 10−20-fold higher specific activity. Initially, the position of the radioactively labeled band(s) was checked using SDS−PAGE and autoradiography. For intact kinase preparations 125-kDa band incorporated all the radioactivity. Some incorporation was also found in 115- and 33-kDa C-terminal fragments of the kinase if they were detectable on the gels (e.g., see Figure 5E). Unless otherwise specified, CM concentration was approximately 2-fold lower than that of the kinase.

The computer analysis of the kinetic data was carried out as previously described [see Sobieszek (1991a,b)] using a nonlinear regression software which included computer modeling and plotting capacity.

RESULTS

CM Sensitivity of Autophosphorylation and Its Character. It was previously reported (Foyt & Means, 1985) that smooth muscle MLCKase may undergo apparent autophosphorylation and that this is inhibited by the addition of Ca and CM. In contrast, we formerly observed a Ca/CM-dependent autophosphorylation (Sobieszek, 1991b), and this process is here analyzed in more detail. As shown in Figure 1, the autophosphorylation rates and corresponding maximal incorporation levels were 3−7-fold higher in the presence of Ca/CM compared to the levels observed in the absence of

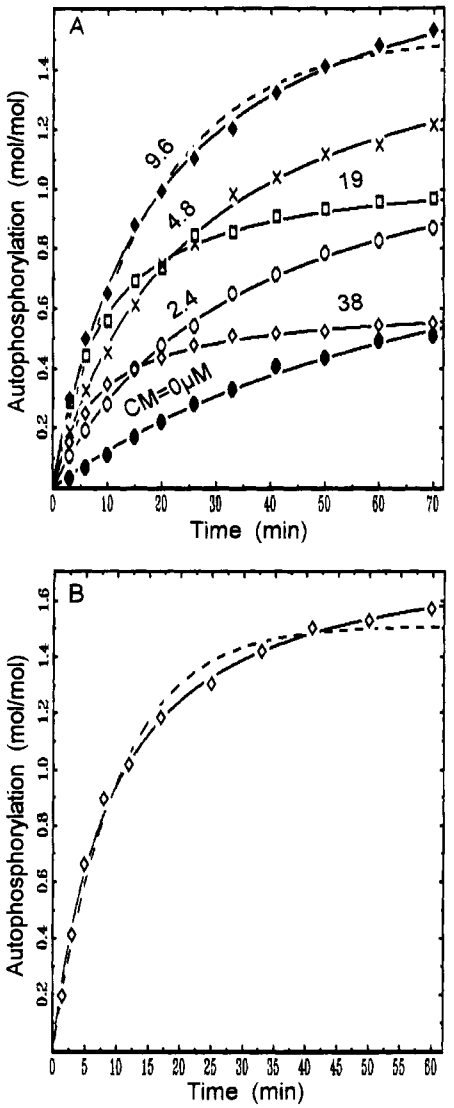

FIGURE 1: (A) Progress of turkey gizzard kinase autophosphorylation at different CM concentrations. The CM concentrations are given on each progress curve, and the kinase concentration was 18 μM. Note that the maximal ^{32}P incorporations and highest initial rates were attained at an about 2-fold lower CM concentration relative to that of the kinase (upper-most curve; CM = 9.6 μM). At higher CM to kinase molar ratios these rates were inhibited and the maximal levels were comparable to those observed with no CM added (filled circles). (B) Hyperbolic character of the autophosphorylation progress curves. In the experiment the kinase (pig stomach) concentration was 32 μM and that of CM was 18 μM. Note that the time points (open diamonds) fitted better to a second-order progress formula (solid curve) than to a first-order progress function (broken curve) in both cases E_o and k being the variable parameters. For more details see text.

CM or after removal of Ca^{2+} by EGTA. The observed autophosphorylation rates did, however, show a strong dependence on MLCKase concentration (see below). These rates were on the order of 200-fold lower than the kinase phosphorylation rates (catalytic activity) observed in the presence of substrates (myosin or its LC_{20} light chain).

Initially, it was demonstrated that no phosphorylation by an endogenous kinase was involved in the observed autophosphorylation. Correspondingly, our MLCKase preparations were free of the endogenous Ca/CM-kinase II or protein kinase C as well as cAP kinase because they could not phosphorylate standard substrates for these three kinases under the assay conditions used. Kemptide and histone IIIS were used respectively for the cAP kinase and for the protein kinase C and autocamtide-3 and caldesmon for Ca/CM-dependent kinase II.

Consistent with an autophosphorylation reaction, the autophosphorylation progress curves exhibited hyperbolic shape; therefore, we concluded that a second order intermolecular reaction was here involved (Figure 1A). This hyperbolic shape was apparent after application of our nonlinear regression software to the time points of the autophosphorylation progresses. The points fitted better to a second-order progress formula (i.e., $P = E_o^2kt/(E_okt + 1)$ than to a first-order progress function (i.e., $P = E_o[1 - \exp(-kt)]$), in both cases E_o and k being the variable parameters. The relevant feature of these curves is the difference in the relative times at which their maximal levels are attained. As demonstrated in Figure 1B, an exponentially rising curve initially increases somewhat slower but attains its maximal level faster. The opposite is true for the second-order progress curve.

CM to Kinase Ratio for Optimal Autophosphorylation and Maximum Stoichiometry. In the presence of saturating Ca^{2+} concentrations (0.1 mM) an optimum CM to kinase molar ratio should exist at which autophosphorylation is maximal. This ratio was established for autophosphorylation progress curves obtained at different CM levels (Figure 1) as well as for simple initial rate measurements (Figure 2). As shown in Figure 1A, the highest initial rates and maximal incorporations were obtained at CM concentrations substantially lower than the concentration of the MLCKase. At much higher CM concentrations the rates and maximal incorporations were low and often comparable to those observed in the absence of CM or with Ca^{2+} removed by EGTA.

The biphasic character of CM action was most clearly seen for the autophosphorylation rate measurements and over a very wide range of CM concentrations (Figure 2A). At the lower range of CM concentrations (Figure 2B) this relationship exhibited a hyperbolic character, with an almost ideal fit to a hyperbola. The observed 1 to 2 optimal saturation molar ratio of CM to MLCKase indicated that dimerization of the kinase was involved in the autophosphorylation reaction.

Parabolic Rate Dependence Confirms Intermolecular Autophosphorylation Mechanism. Measurements of the autophosphorylation rate as a function of MLCKase concentration are normally used to distinguish between intramolecular autophosphorylation, i.e., a kinase molecule phosphorylating itself, and intermolecular phosphorylation, i.e., a kinase molecule phosphorylating other kinase molecules. In the first case the rate is independent of the enzyme concentration, while in the second case the dependence is parabolic.

As shown in Figure 3, the rate concentration dependence was parabolic, consistent with intermolecular autophosphorylation. The parabolic relationship indicates a true second order reaction and allows an evaluation of the apparent K_m value for the autophosphorylation reaction. From kinetic considerations (see Segel, 1975; p.72) the following formula was derived describing the relationship between the rate and enzyme (MLCKase) concentration: $v = k[(2E_t + K_m) - \sqrt{\{K_m(4E_t + K_m)\}}]$. Its application to some curves of the type shown in Figure 3 together with the nonlinear fitting procedure (see Material and Methods) gave a K_m value of 25 ± 5 μM. This indicated that the kinase−kinase interaction involved in the autophosphorylation process had an

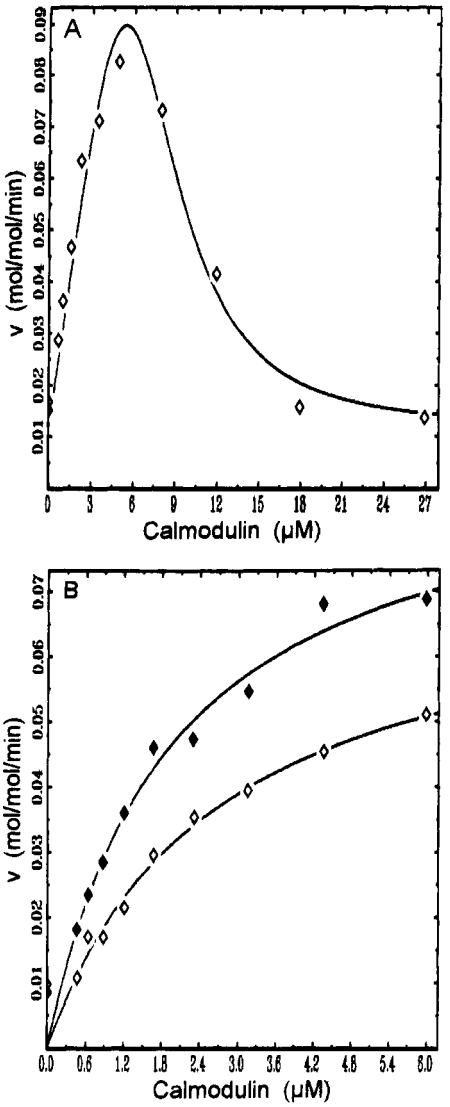

FIGURE 2: (A) Biphasic effect of CM on the initial autophosphorylation rate. Note that the maximal rate was attained at 6 μM CM and this rate was reduced 8-fold at 3−4-fold higher CM concentration. The kinase (turkey gizzard) concentration was 12 μM. (B) A hyperbolic-type relationship between initial autophosphorylation rate and total CM concentration at low CM to kinase ratios. Pig stomach (solid diamonds) and turkey gizzard (open diamonds) MLCKases were used at 16 and 12 μM, respectively.

affinity in the micromolar range, as expected for substrate−enzyme type interactions [see Sobieszek (1991a)].

CM-Independent Autophosphorylation. The kinase preparations exhibited low and rather variable contributions of the CM-independent autophosphorylation rates and the corresponding maximal incorporation levels. As shown by Figure 3A this contribution could be evaluated by computer analysis of the rate versus concentration relationship (broken line) or by direct measurements of the rates in the absence of Ca^{2+} or CM (open diamonds). The linearity of the relationship indicated that an intramolecular reaction was responsible for this contribution and therefore the corresponding specific rate did not depend on the kinase concentration.

As a result of the low rate of the incorporation, the relative contribution of the CM-dependent autophosphorylation was affected by the time period used in the rate measurements. This was apparent from the autophosphorylation progress curves obtained at different molar ratios of CM to kinase (Figure 1). We also noted that this contribution was not

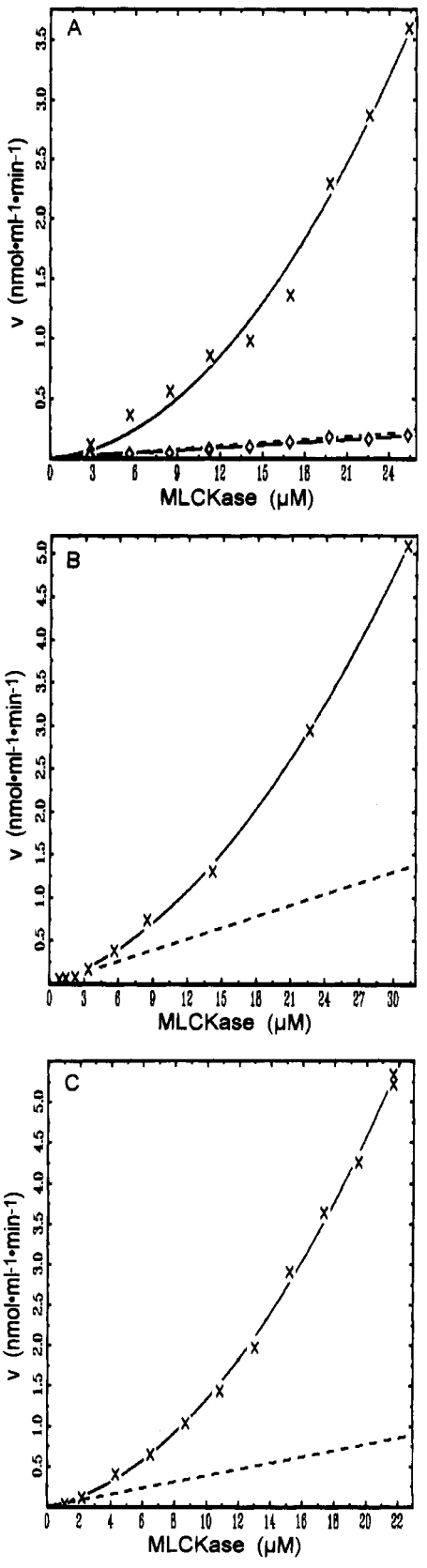

FIGURE 3: Parabolic relationship between the initial autophosphorylation rate and MLCKase concentration. Note that although the relationship was parabolic there was a significant contribution of a linear term (broken line) which was identical with the rate measured in the absence of CM (A; open diamonds). These linear contributions were obtained from the computer analysis which included contribution from all the points. Pig stomach (B) and turkey gizzard (A and C) MLCKase preparations were used and their molar ratios to CM were kept constant at 2 to 1.

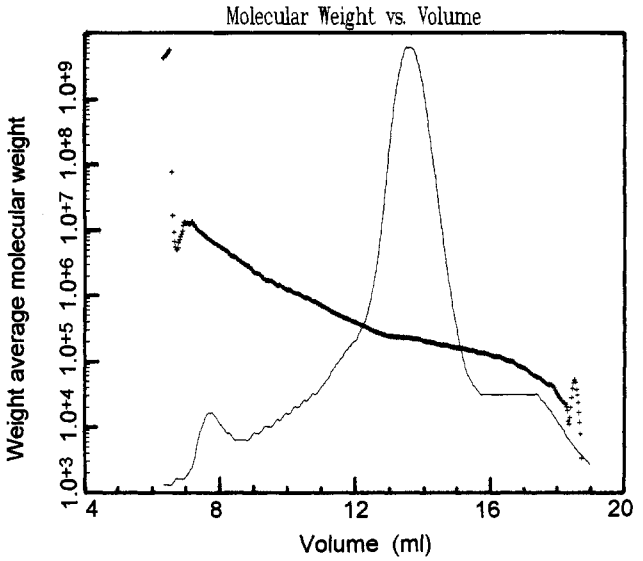

FIGURE 4: Weight average molecular weight distribution of MLCKase during gel filtration chromatography. Note that in the region of the main peak (absorption; thin solid line), the kinase was present as a dimer since the M_w measured by light-scattering (solid line formed from crosses) was approximately 250 kDa. The ascending fractions of the peak contained oligomers of progressively increasing M_w while in the descending fractions only monomers were present ($M_w \approx 105$ kDa). Turkey gizzard kinase (0.5 mL at 25 μM) was applied onto a Superose 6 column (1.0 × 30 cm) using a standard FPLC system connected to a multiangle laser light scattering DAWN instrument (Wyatt Technology, Santa Barbara, CA).

constant for a particular kinase preparation and appeared to depend on the oligomeric state of the kinase. In view of these complications and the relatively low levels observed the CM-independent autophosphorylation was not investigated further.

Dimerization and/or Oligomerization of MLCKase. Aggregation of the kinase was noted in very early stages of this study during simple absorption measurements. Although apparently soluble as a concentrated stock solution (see Materials and Methods), the kinase was slightly insoluble when diluted 10−20-fold just before the assays. This was apparent from the very fine suspension that formed upon dilution. The suspension disappeared, or the kinase solution became clear, after addition of at least 100−150 mM NaCl (or KCl).

Prompted by this simple observation, we took advantage of a new multiangle laser light scattering instrument (Wyatt Technology GmbH, Schlangeband, Germany) provided for demonstration by Dr. C. Johann. This instrument (Dawn Model F) together with its corresponding software allows continuous weight average molecular weight (M_w) determination during the course of column chromatography. Thus, in addition to the protein concentration profile, the instrument provides the transitory M_w distribution of proteins during their elution from a column. Such a distribution was obtained for the kinase applied to Superose 6 linked to a Pharmacia FPLC system (Figure 4). As shown in the figure, at physiological ionic strength (AA buffer plus 120 mM NaCl) and at relatively low kinase concentration, the majority of the molecules were present as dimers since the M_w of the kinase in the peak region was approximately 250 kDa. The M_w distribution obtained also demonstrates that the dimers were in dynamic equilibrium with both oligomeric and monomeric forms of the kinase. The same conclusion was made in an independent study (Babiychuk et al., 1995) in which the kinase oligomeric properties were investigated by a zero-length covalent cross-linker (see Figure 5D).

Endogenous Protein Phosphatase and Autophosphorylation Stoichiometry. Direct measurements of the autophosphorylation stoichiometry indicated that it was variable and depended on the type of MLCKase used. For the preparations not subjected to the CM-affinity chromatography this stoichiometry was very low (not more than 0.2 mol/mol) while for the kinase purified on a Phenyl Sepharose column (followed by the CM-affinity one) up to 1.7 mol of phosphate was incorporated per mole of kinase. This variability arose from different levels of contamination by an endogenous protein phosphatase (MLCK-phosphatase). Even though most of the endogenous protein phosphatase was removed during the standard final purification on a CM-affinity column [see Sobieszek and Barylko (1984)], the remaining MLCK-phosphatase could decrease the autophosphorylation stoichiometry as much as 2−3-fold. (A corresponding increase in the autophosphorylation level was observed after inhibition of the phosphatase by okadaic acid or Microcystin-LR.)

Under the conditions employed, the maximal autophosphorylation levels of pig stomach and gizzard kinase preparations (purified by Phenyl- and CM-affinity chromatography) were as high as 1.7 mol/mol. Since somewhat higher stoichiometry was obtained in the presence of 2.5 μM okadaic acid, it was apparent that there were at least two autophosphorylation sites; one Ca/CM-dependent and the other CM-independent. This was independently demonstrated by a comparison of the autophosphorylation levels to those obtained by phosphorylation of the kinase by cAP protein kinase (Figure 6A).

Identification of the Autophosphorylation Sites. The phosphorylation of MLCKase (containing the endogenous phosphatase) by cAP kinase permitted some conclusions to be drawn concerning the type of site involved in the autophosphorylation. As illustrated in Figure 6B, this type of kinase preparation was phosphorylated by the cAP kinase at at least three sites with two of them being blocked by Ca/CM. The interesting aspect of these experiments was that none of these three sites appeared to be dephosphorylated by the endogenous MLCK-phosphatase. Thus, it may be concluded that at least the major autophosphorylation site was different from the one phosphorylated by the cAP kinase. This was confirmed by phosphoamino acid analysis of ^{32}P-labeled kinase which showed that about 75−85% of the radioactivity migrated in the position of phosphothreonine and the rest was identified as phosphoserine (data not shown). It is well established that the cAP kinase as well as other protein kinases phosphorylate the regulatory domain of the MLCKase exclusively at serine residues (see Discussion). In agreement with this conclusion, the 24-kDa C-terminal trypsin resistant fragment of the kinase was shown to include the major autophosphorylation site(s) (Figure 5E,F), and this fragment includes no residues of the regulatory domain.

The autophosphorylation site was independently identified as the Thr-863 or Thr-865 by amino acid analysis. Turkey gizzard kinase was labeled with ^{32}P and subsequently cleaved with CNBr after alkylation of the SH groups by iodoacetamide. The resulting peptides were fractionated on Sephadex G-50 showing major and minor radioactivity peaks eluting at an M_r in the region of 5 and 2 kDa, respectively. Analysis of phosphoamino acid showed that the 5-kDa peak contained

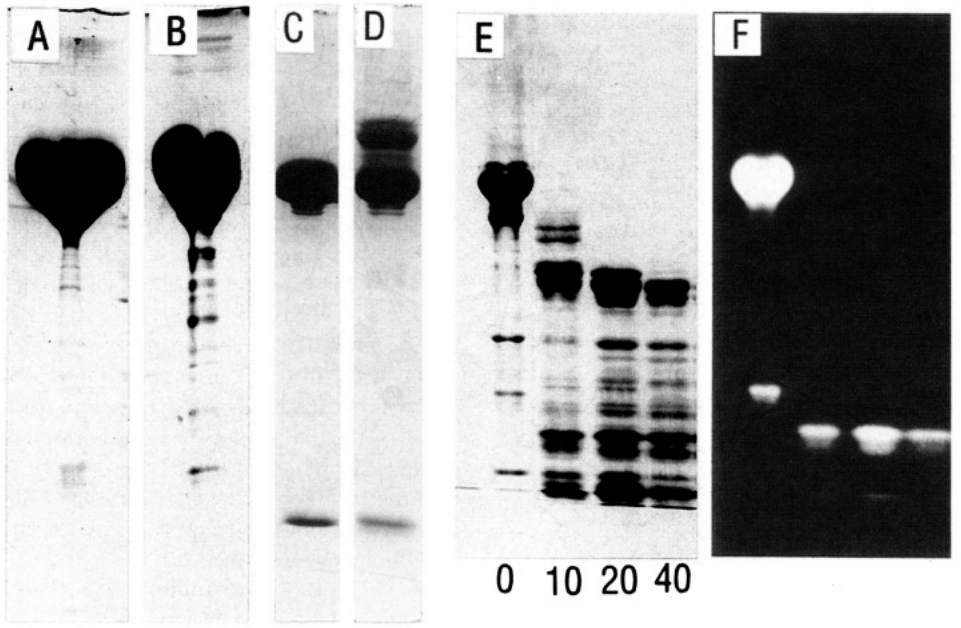

FIGURE 5: SDS−PAGE of the MLCKase preparations used in the present study (A and B), of the kinase cross-linkage (C and D), and identification of its tryptic fragment containing the autophosphorylation site (E and F). (A) Turkey gizzard (TG) kinase purified by ionic exchange and CM-affinity chromatography; B, pig stomach (PS) kinase purified as (A) but with an additional purification step on phenyl affinity column; (D) TG kinase cross-linked with 5 mM EDC [1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide for 20 min and its untreated control (C). The fast migrating band at the gel bottom corresponds to CM added to kinase during cross-linking. Note presence of a strong band corresponding to the kinase dimer and weak bands representing oligomers. (E) Autophosphorylated TG kinase (0) was digested at 10, 20, and 40 μg/mL trypsin for 10 min; (F) corresponding autoradiogram. Note that the ^{32}P label present predominantly in the kinase (125-kDa) band was found only in the trypsin-stable 24-kDa C-terminal fragment of the kinase. In contrast, very little label can be detected in the larger fragments which are known to contain the regulatory and catalytic domains.

peptide(s) phosphorylated exclusively at threonine while in the 2-kDa peak only phosphoserine was present. After further purification by IDA-Fe^{3+} Sepharose affinity chromatography and on C18 reverse-phase HPLC column, only two radioactive phosphopeptides were present. Their initial amino acid sequences were determined as SGRKASGSSPTS-PI with no phosphoamino acids present. This region commenced at Ser-823 of the known sequence of chicken gizzard kinase (Olson et al., 1990). After further digestion of the peptide with V-8 protease and sequence analysis of the resulting peptides, it was concluded that only a Thr-863 or Thr-865 could be phosphorylated, indicating a high degree of similarity between the two putative autophosphorylation sites.

Identification of the sites was also carried out for the minor 2-kDa radioactivity peak resolved on Sephadex G-50 which contained radioactive serine. In this case a similar analysis showed that either Ser-814 or Ser-815 site could be phosphorylated. It should be noted that this minor peak was significant only in the CNBr digests of the kinase preparations which exhibited a low degree of CM sensitivity of autophosphorylation.

Autophosphorylation and MLCKase Activity. Several experiments were designed and implemented to investigate possible effects of the autophosphorylation on MLCKase catalytic activity or on its affinity for CM. In one type of experiment small aliquots of the kinase undergoing autophosphorylation were added at regular time intervals into tubes containing the LC_{20} light chain and radioactive ATP, with or without Ca^{2+} (added 2 mM EGTA). In this way the CM-dependent and CM-independent kinase activities (phosphorylation rates) were measured, at regular time intervals, during the course of autophosphorylation. These experiments showed no immediate modification of either the Ca/CM-dependent or the Ca/CM-independent rates. Similarly, no differences were observed for an autophosphorylated kinase in comparison to the same unphosphorylated kinase after subjection to an identical gel filtration step on a Sephacryl S-200 column. The evaluated kinetic parameters, such as K_{MA}, K_{MBA}, and K_{Ia}, were essentially the same and very close to the values obtained in recent kinetic studies (Sobieszek, 1991a). By measuring the activities of the same two kinase preparations as a function of CM concentrations, no significant effect of the autophosphorylation on affinity of the kinase for CM could be detected.

Autophosphorylation in the Presence of Myosin Filaments. The results described were obtained using the isolated light chains as substrate, but it was considered important to perform similar experiments with intact myosin, which is the native substrate. At kinase concentrations that were optimal for autophosphorylation (>5 μM) the phosphorylation rates were too high for their convenient measurements. They could be measured if the kinase undergoing phosphorylation was diluted several fold upon addition to myosin. Since the initial phosphorylation of the myosin was not zero, the rate measurements have to be called transitory. Figure 7A (filled diamonds) shows the control experiments used in the evaluation of the extent of kinase autophosphorylation under exactly the same conditions as used in the parallel transitory rate measurements (Figure 7B). The figure shows that at about 30% of the initial phosphorylation, the rate increase was 3−4-fold, and there was a hyperbolic relationship between this transitory rate and the extent of the kinase autophosphorylation. In this case nonphosphorylated myosin was employed as substrate which underwent phosphorylation up to the required level during the initial incubation with the kinase and low concentration of unlabeled (cold) ATP. As demonstrated in the figure, similar increases in the intermediate phosphorylation rate were observed not only for the turkey gizzard kinase (with its native myosin) but

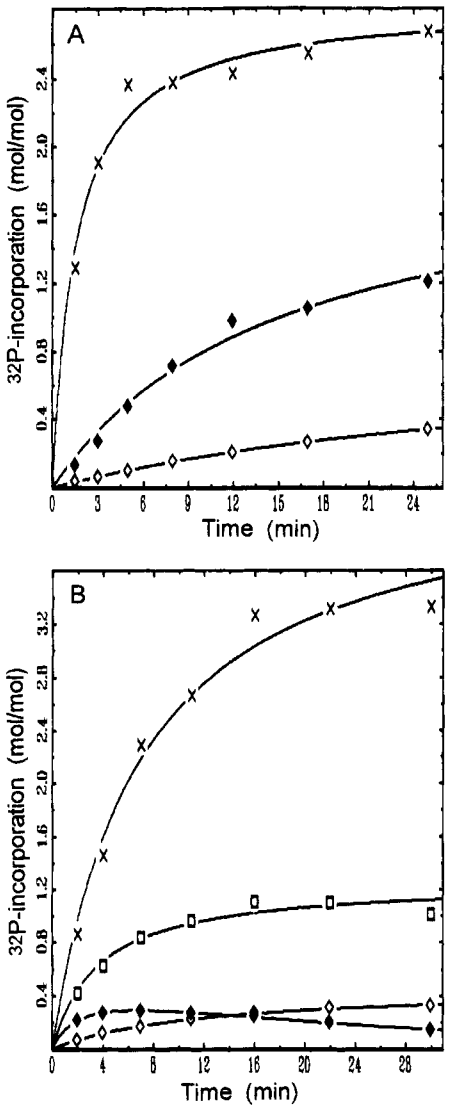

FIGURE 6: Phosphorylation and autophosphorylation of MLCKase. In panel A pig stomach kinase was phosphorylated by cAP protein kinase (50 μg/mL) in the absence of CM and the maximal level of phosphorylation (×−×) compared to that obtained for the autophosphorylation in the presence (filled diamonds) and absence (open diamonds) of CM, respectively. In panel B a similar experiment was done for a turkey gizzard kinase containing relatively high contaminations of an endogenous protein phosphatase. Note that the phosphatase did not dephosphorylate the cAP kinase sites (upper and middle curves) although it was very active toward the autophosphorylation (lower curve). The middle curve (open squares) represents phosphorylation by CAP-kinase in the presence of CM.

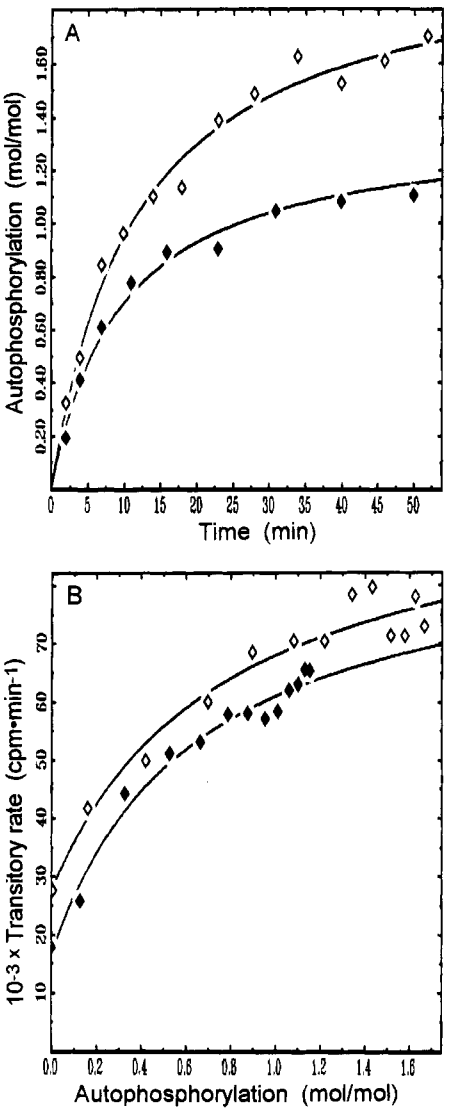

FIGURE 7: Relationship between transitory phosphorylation rate (catalytic activity) and the extent of MLCKase autophosphorylation. In panel A control autophosphorylation progress curves are shown obtained with radioactive ATP under the conditions as used in panel B. They were necessary for determination of the autophosphorylation level of the kinase in a parallel experiment (B) where cold ATP was used for the autophosphorylation. Aliquots of this slowly autophosphorylating kinase were added to tubes containing unphosphorylated myosin as a substrate. Fifteen or thirty seconds later, radioactive ATP was added to the tubes, and ^{32}P incorporations into the myosin during the next 15 s were measured. Note that upon autophosphorylation there was a 3−4-fold increase of the transitory rate. In the experiments marked with open diamonds MLCKase (5 μM) and myosin (40 μM) were from pig stomach muscle while for the other curves (filled diamonds) they were from turkey gizzard.

also for the kinase and myosin obtained from pig stomach muscle. Thus, it is apparent that autophosphorylation of the kinase may enhance the *in vivo* transitory rate of myosin phosphorylation following activation. A simplest kinetic explanation of such an effect would be a reduction of the product (phosphorylated myosin) inhibition.

DISCUSSION

Evidence for an Autocatalytic Process. The present study provides the first direct demonstration of Ca/CM-dependent autophosphorylation of smooth muscle MLCKase. In a previous report (Foyt & Means, 1985) a Ca/CM-independent autophosphorylation was described which was inhibited by high CM concentrations; however, relatively low autophosphorylation rates were observed. In that report, phosphorylation by a contaminating endogenous MLCK-kinase could not be rigorously excluded, and therefore the authors referred to "an apparent autophosphorylation" (Foyt and Means, 1985).

The evidence for autophosphorylation can be listed as follows. Firstly, we observed that the initial autophosphorylation rates were relatively constant for all the MLCKase preparations used provided that the endogenous protein phosphatase had been inhibited or removed by the modifications of the purification procedure. An endogenous protein kinase would, as was seen with the analogous protein phosphatase, contaminate the MLCKase to different extents which would result in markedly different specific autophos-

phorylation rates. Secondly, the autophosphorylation progress curves were of the hyperbolic type, indicating a second-order autocatalytic process. Thirdly, both the initial rates and the maximal incorporation levels were optimal at a constant CM to kinase ratio of approximately 1 to 2. For an endogenous Ca/CM-dependent kinase, this ratio would have to be much lower. Finally, the relationship between the reaction rate and the enzyme concentration was parabolic, which is characteristic of an intermolecular autophosphorylation reaction.

Autophosphorylation Site. From the experiments on the phosphorylation of MLCKase by cAP kinase it was apparent that only one site was involved in CM-dependent autophosphorylation and this was different from those phosphorylated by the cAP protein kinase. This conclusion is based on the fact that the endogenous MLCK-phosphatase did not dephosphorylate the phosphorylated kinase even though its activity toward the autophosphorylated kinase was high. Phosphorylation of the MLCKase by cAP kinase also results in a 10-fold reduction in its affinity for CM (Conti & Adelstein, 1981). Since no detectable effect of the autophosphorylation on CM affinity was observed, we conclude that the sites are not only different but that their phosphorylation must differ during MLCKase regulation.

The different autophosphorylation sites have also been suggested by Foyt and Means (1985), who established that autophosphorylated kinase contains phosphothreonine and phosphoserine residues. From our phosphoamino acid analysis it is clear that the threonine residue was the major site influencing the kinetic analysis. Significantly the sites have been identified as Thr-863 or Thr-865 separated by a lysine residue which agrees well with the highest stoichiometry observed (0.9−1.1 mol/mol).

From the known domain structure of the kinase it is apparent that the autophosphorylation site is localized between the regulatory and the third (carboxyl end) titin-like domains of the kinase [see Olson et al. (1990)]. I therefore suggest that this domain may be responsible for the interaction of the kinase with myosin, a role which may not be detectable with the isolated light chain normally used as the kinase substrate (see below). In accord with this suggestion, significant effects of the autophosphorylation were observed only when intact myosin was used as the substrate and not with the isolated LC_{20} light chain.

Implications for the Regulatory Mechanism. In this first attempt to establish a kinetic analysis of the autophosphorylation reaction the data show that it is second order, as expected for an intermolecular process. Thus intermolecular autophosphorylation appears to represent an alternative Ca/CM-dependent regulatory mechanism of the kinase which exists in addition to the intramolecular autophosphorylation or phosphorylation previously shown for this kinase [see Edelman et al. (1987)]. Intermolecular autophosphorylation has the advantage of providing a more flexible response to CM. Accordingly, the catalytic and autocatalytic activities of an intermolecularly regulated kinase would depend on different CM concentrations, whereas for an intramolecularly regulated kinase these two activities would involve the same CM concentration. The autophosphorylation shown here occurs in the micromolar range of CM concentrations and at subsaturating molar ratios of this activator to the kinase. At the same time it is generally recognized that the catalytic activity of MLCKase requires a nanomolar range of CM concentration and this activation is positively cooperative [see Sobieszek (1991b)] and therefore is most effective after saturation of the kinase by CM.

The established 1 to 2 CM to kinase optimum ratio for the autophosphorylation reaction is particularly suggestive. This stoichiometry indicates that one kinase molecule and a CM/kinase complex associate in order for the autophosphorylation reaction to occur. Formation of such a trimer requires kinase at 4 μM or greater concentrations, which is close to the *in vivo* concentration [see Hartshorne (1987)], and the trimer is a complex of an enzyme-substrate type. Our former results obtained by gel filtration (Sobieszek, 1991b; Sobieszek et al., 1993), light scattering (Figure 4), and cross-linking (Babiychuk et al., 1995) experiments show that dimer formation was facilitated by the presence of CM while oligomerization appeared to be CM-insensitive. This process is therefore better described as a dimerization.

Consistent with the report of Foyt and Means (1985) the autophosphorylation reaction was inhibited by CM, but this inhibition was observed only at CM concentrations in excess of the kinase. The inhibition was interpreted as involving CM-dependent blocking of the autophosphorylation sites and demonstrates again that low affinity CM-MLCKase interactions were taking place. A similar blocking has been observed for the phosphorylation of the serine sites by the three protein kinases known to phosphorylate MLCKase [Hashimoto & Soderling, 1990; Ikebe & Reardon, 1990; see also Edelman et al. (1987)]. Thus, although different from the sites targeted by exogenous kinases, the autophosphorylation site is located within or very closely to the same regulatory domain of the kinase.

Oligomerization of MLCKase. The dimerization or oligomerization of MLCKase represents a novel property of smooth muscle MLCKase not previously considered. Coincidentally, a mechanism for the dimer or oligomer formation has been already proposed. According to Jarrett and Madhavan (1991), CM-dependent enzymes possess a CM-binding and a CM-like binding sites. The binding of the authentic CM-binding domain of one molecule to the CM-like domain of another may result in oligomerization or dimerization. Such domains have in fact been shown to be present on smooth muscle MLCKase (Jarrett & Madhavan, 1991; Sobieszek, unpublished observations), but location of the CM-like domain remains to be established.

Trypsin fragments of MLCKase involved in the dimerization have recently been identified as the 24-kDa C-terminal polypeptide which includes a titin-like (II-3) domain [see Babiychuk et al. (1995)] and a 36-kDa N-terminal polypeptide which is rapidly lost during trypsin digestion. In addition, there is a clear indication that dimerization is also involved in an interaction (or at least close proximity) of the 24-kDa fragment and a 3-kDa amino acid stretch at the C-terminal end of the larger (64-kDa) central fragment of the kinase which includes the catalytic core and CM-binding domain (Babiychuk et al., 1995). From these data and significant protection of the 36-kDa fragment within a cross-linked dimer, it can be concluded that a parallel dimer with overlapping catalytic and the 24-kDa regions represents a kinase form which can elongate to oligomers. Incorporation of the steric blocking hypothesis [see Kemp et al. (1982)] into this simple model makes possible to predict that these dimeric and/or oligomeric forms could readily be blocked at their end(s). From the cross-linking experiments it is apparent that such a blocked dimer is present at relatively high concentration and has reduced affinity for CM. After

binding of CM the dimer would undergo intermolecular autophosphorylation at the observed optimal rate at 1 to 2 CM to kinase molar ratio. In agreement with our previous observations (Babiychuk et al., 1995) the model is also consistent with the effects of CM on the equilibrium between oligomeric and monomeric forms of the kinase.

We have shown recently that dimerization of the kinase may also be responsible for the cooperative activation of the kinase catalytic activity by CM (Sobieszek et al., 1993). This cooperativity has been explained by a reduced apparent affinity of the kinase dimer for CM. Indeed an approximately 6-fold reduction of the CM "off" rate constant was observed at low CM to kinase ratios compared with the rate observed at a 1:1 ratio of CM to kinase (Meyer & Sobieszek, unpublished observations). The rate constants were obtained from fluorescence anisotropy measurements of the type used for demonstration of CM-trapping by Ca/CM-dependent kinase II following its autophosphorylation at Thr-286 (Meyer et al., 1992).

Comparison to the Skeletal Muscle Kinase. Recently, Gao et al. (1992) have described a similar Ca/CM-dependent autophosphorylation of skeletal muscle MLCKase. Thus, this type of covalent modification is evidently not restricted to the smooth muscle enzyme. In contrast to the present study, no clear effects of autophosphorylation on the activity and CM affinity of the skeletal muscle kinase were observed. However, one would not expect a close analogy between these two MLCKases in view of their differences in molecular size and amino acid sequence: the smooth muscle kinase is considerably larger due to the presence of the three titin-like segments each containing about 100 amino acids (Olson et al., 1990). It is apparent from the present study that the domain II-3 of this type may be involved in a more specific interaction of the kinase with intact myosin and may be responsible for the tight association of the kinase with myosin filaments (Sobieszek, 1985, 1990). This latter property, not observed for the skeletal muscle enzyme (Sobieszek, unpublished observation), and a different localization of the autophosphorylation sites (Gao et al., 1993) imply a major functional difference between these two kinases.

The relevance of the observation of the Ca/CM-dependent autophosphorylation lies in its possible involvement in the regulation or modulation of MLCKase activity. In view of the relatively low autophosphorylation rates observed, compared with those observed for myosin or its regulatory LC_{20} light chain, a modulatory rather than regulatory role for the autophosphorylation is suggested. It is unlikely that a rather slow autophosphorylation reaction could regulate an approximately 200-fold faster process, namely, the phosphorylation of myosin. The modulatory effects demonstrated in the present study are consistent with this mechanism, namely, that after activation the existing phosphorylation rate can be further potentiated by autophosphorylation of the kinase.

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