The Model Calmodulin-Binding Peptide Melittin Inhibits Phosphorylase Kinase by Interacting with Its Catalytic Center†

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ABSTRACT: The inhibition by melittin, a model calmodulin-binding peptide, of phosphorylase kinase, which contains an intrinsic calmodulin subunit, has been characterized in detail. The inhibition was competitive with respect to phosphorylase *b* for both the phosphorylase kinase holoenzyme and its isolated catalytic γ-subunit (minus calmodulin), and the ratios of the K_m for phosphorylase to the K_i for melittin were similar for both forms of the kinase. These findings indicate that inhibition of the phosphorylase kinase holoenzyme by melittin is caused predominantly by its interaction with the catalytic subunit of the enzyme, and not with the endogenous calmodulin subunit. Further proof that melittin interacts directly with the catalytic site was obtained when it was observed that melittin was also a substrate for phosphorylase kinase, with a K_m that was less than that for phosphorylase *b*, although the k_{cat}/K_m specificity constant was only $^1/_{200}$th of that for phosphorylase. The apparent tight binding of melittin to the kinase active site could not be readily rationalized by conventional comparison of sequence similarity between melittin and phosphorylase; however, considerable sequence similarity, centered around the convertible seryl residue of phosphorylase, was observed when the sequences were aligned in reversed polarity. The possible regulatory significance of the direct interaction of the catalytic site of this Ca^{2+}-dependent kinase with a calmodulin-binding peptide is discussed.

Phosphorylase kinase is a structurally complex enzyme whose Ca^{2+}-dependent phosphorylation of glycogen phosphorylase *b* couples glycogenolysis with contraction in fast-twitch glycolytic skeletal muscle (Brostrom et al., 1971). The enzyme's Ca^{2+} dependence is conferred by an endogenous calmodulin subunit termed δ (Cohen et al., 1978). Unlike most other calmodulin-dependent enzymes, the δ-subunit does not associate–dissociate, but remains a firmly bound integral subunit of the holoenzyme, even in the presence of high concentrations of Ca^{2+} chelator (Cohen et al., 1978). In addition to the stimulatory δ-subunit, the enzyme also has two other regulatory subunits, α and β, which are phosphorylated by cAMP-dependent protein kinase and during autophosphorylation (Pickett-Gies & Walsh, 1986). The catalytic subunit is termed γ, and although it clearly associates with the δ-subunit, calmodulin (Picton et al., 1980; Chan & Graves, 1982a), and contains two calmodulin-binding domains (Dasgupta et al., 1989), additional calmodulin-binding regions have also been identified on the regulatory α- and β-subunits (James et al., 1991). Within the holoenzyme, it is not known whether calmodulin associates with only the γ-subunit or with γ plus a calmodulin-binding domain on the α- or β-subunits, as has recently been hypothesized (Newsholme et al., 1992). It is also not known if the δ-subunit interacts with different calmodulin-binding domains on the α-, β-, and γ-subunits at different times depending on the activation state of the kinase or the concentration of Ca^{2+} ions. Even the extent of exposure of the δ-subunit on the surface of the hexadecameric kinase holoenzyme, $(\alpha\beta\gamma\delta)_4$, is not known, although it may have a predominantly surface location because it is the only subunit that is specifically dissociated from the hexadecamer under precise, mildly denaturing conditions (Paudel & Carlson, 1990).

Calmodulin-binding peptides have been previously utilized in attempts to elucidate the mechanism for activation of the phosphorylase kinase holoenzyme by Ca^{2+} ions and the underlying interactions between the intrinsic calmodulin subunit, δ, and the catalytic subunit, γ. In an early report, a peptide corresponding to the calmodulin-binding domain of myosin light chain kinase was found not to inhibit phosphorylase kinase activity, although that peptide was evaluated at only one concentration (Blumenthal et al., 1988). In a later study by Newsholme et al. (1992), a variety of calmodulin-binding peptides, including the two calmodulin-binding domains from the carboxyl-terminal region of the catalytic γ-subunit, were found to inhibit activated phosphorylase kinase, and it was concluded that the peptides inhibited by functionally reversing the activation process, as opposed to inhibiting the intrinsic catalytic ability of the γ-subunit. The relative inhibitory effectiveness of the peptides correlated with their calmodulin-binding abilities, although inhibition required from 2 to 5 orders of magnitude greater concentrations of the peptides than the concentrations required to effectively complex free calmodulin. The authors hypothesized that the peptides inhibited phosphorylase kinase activity by binding to its δ-subunit, calmodulin, which in some manner then caused perturbations in the interactions between the catalytic γ-subunit and the regulatory α- and/or β-subunits. In contrast, activity measurements with the isolated γ-subunit in the absence of calmodulin have indicated that removal of the catalytic subunit's carboxyl-terminal calmodulin-binding domains gives rise to its activation, suggesting an autoinhibitory interaction between the active site and the calmodulin-binding regions of the γ-subunit (Harris et al., 1990; Cox & Johnson, 1992). Because of the noncorrespondence of the results from

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the two studies discussed above that evaluated the effects of calmodulin-binding peptides on the activity of the phosphorylase kinase holoenzyme, and because of the studies with the free γ-subunit suggesting that calmodulin-binding peptides may inhibit by interacting with the active site to cause autoinhibition, we decided to evaluate in greater detail the interactions between a calmodulin-binding peptide and phosphorylase kinase. The calmodulin-binding peptide we chose to concentrate on in this study is the model calmodulin target melittin.

Melittin is a 26 amino acid, basic, cytotoxic polypeptide from bee venom that binds calmodulin in a Ca^{2+}-dependent manner to form a 1:1 complex with a nanomolar dissociation constant (Comte et al., 1983; Maulet & Cox, 1983). The interaction between calmodulin and melittin is thought to be similar to the interaction between calmodulin and its binding domains on target proteins (Comte et al., 1983; Erickson-Viitanen & DeGrado, 1987), and an antibody raised aainst melittin binds to calmodulin-binding proteins (Kaetzel & Dedman, 1987). Because of its high specificity and high affinity for binding to calmodulin, melittin has been used in competition experiments to study the interaction between calmodulin and its binding domains on target proteins. To gain further insight regarding the potential targets of calmodulin-binding peptides among the subunits of phosphorylase kinase, we have examined in detail the interaction of melittin with the homoenzyme and with the isolated catalytic subunit. Like Newsholme et al. (1992), we have found that the calmodulin-binding peptide melittin is an inhibitor of phosphorylase kinase; however, unlike their findings with calmodulin-binding peptides, we conclude that the inhibition by melittin is caused almost completely by interactions with the enzyme's catalytic center, and not with its intrinsic calmodulin subunit, δ. This finding may have regulatory significance in identifying the catalytic center as a potential target for one or more of the enzyme's calmodulin-binding domains. This work has been previously presented in abstract form (Paudel et al., 1991).

EXPERIMENTAL PROCEDURES

Proteins and Peptides. Nonactivated phosphorylase kinase was purified from the back skeletal muscles of New Zealand White rabbits through the DEAE-cellulose chromatography step (Cohen, 1973), and its catalytic γ-subunit was isolated by gel filtration chromatography of denatured enzyme as described (Paudel & Carlson, 1987). When it was advantageous or necessary to use relatively high concentrations of phosphorylase kinase holoenzyme in phosphorylation assays, namely, those of Figures 3–5 and Table II, then autophosphorylated enzyme was used instead of nonactivated kinase to avoid high background counts of ^{32}P incorporation into protein that could otherwise be caused by autophosphorylation during the assay. The nonradioactive, autophosphorylated phosphorylase kinase was prepared by allowing autophosphorylation at pH 8.2 to proceed for 15 min at 30 °C in a reaction mixture containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes),[1] 5 mM $Mg(CH_3CO_2)_2$, 0.4 mM ATP, 0.2 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 2 mg/mL phosphorylase kinase. The reaction was stopped by addition of an equal volume of a saturated solution of $(NH_4)_2SO_4$. Following centrifugation, the pellet was dissolved in 50 mM Hepes (pH 6.8)/0.1 mM EDTA/10% sucrose and dialyzed extensively at 4 °C against the same buffer. Following dialysis, the kinase was stored frozen at −80 °C in small aliquots until used.

Phosphorylase *b* was purified as described (Fischer & Krebs, 1958), and residual AMP was removed by treatment with coconut charcoal. Bee venom melittin was from Serva Biochemicals. Bovine brain calmodulin and bovine serum albumin were from Sigma. Calmodulin–agarose (Affi-Gel calmodulin) and agarose (Bio-Gel A-1.5m) were from Bio-Rad Laboratories. The synthetic tetradecapeptide alternative substrate (SDQEKRKQISVRGL) corresponding to the convertible region of phosphorylase *b* was prepared by the Molecular Resource Center of St. Jude Children's Research Hospital of Memphis, TN, and was further purified by Cm-cellulose ion-exchange chromatography (Farrar & Carlson, 1991).

The concentrations of phosphorylase kinase, phosphorylase *b*, and calmodulin were determined spectrophotometrically using their respective absorbance indexes (Cohen, 1973; Kastenschmidt et al., 1968; Maulet & Cox, 1983). Melittin's concentration was also determined spectrophotometrically using the extinction coefficient of its single tryptophan residue as described (Maulet & Cox, 1983). The concentration of the γ-subunit was determined as before using the Bio-Rad protein assay with bovine serum albumin as the standard (Paudel & Carlson, 1987). The concentrations of tetradecapeptide substrate solutions were based on amino acid analysis performed by the Harvard Microchemistry Facility.

Phosphorylation Assays. Unless otherwise stated, the phosphorylation activity of phosphorylase kinase was measured at pH 8.2 and 30 °C with product formation detected using a phosphocellulose paper assay (Roskoski, 1983). For the phosphorylation of phosphorylase *b* or tetradecapeptide by the kinase holoenzyme, the final concentrations in the assays were 50 mM Hepes buffer, 10 mM $Mg(CH_3CO_2)_2$, 1.5 mM [γ-^{32}P]ATP (ICN), 0.3 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 μg/mL kinase, and either 3 mg/mL phosphorylase or 100 μM tetradecapeptide. At appropriate times, aliquots were removed for determination of ^{32}P incorporation into substrate.

The activity of the free γ-subunit was assayed as previously described (Paudel & Carlson, 1987), but with some modifications. The γ-subunit in 8 M urea, 0.1 M H_3PO_4 (pH 3.3), 1 mM EDTA, and 1 mM dithiothreitol was diluted 7-fold into dilution buffer (50 mM Hepes, 0.1 mM EDTA, and 0.1 mM dithiothreitol, pH 8.2) and then diluted an additional 5-fold into a mixture containing all components of the phosphorylase conversion assay except MgATP. After 15 min, the reactions were initiated by the additon of MgATP. The final concentrations of the assay components were 50 mM Hepes buffer (pH 8.2), 10 mM $Mg(CH_3CO_2)_2$, 1.5 mM [γ-^{32}P]ATP, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5 μg/mL γ-subunit, and 3 mg/mL phosphorylase. Aliquots were removed after 3 h for determination of ^{32}P incorporation into phosphorylase.

For the phosphorylation of melittin by the phosphorylase kinase holoenzyme, the final concentrations in the assays were 50 mM Hepes buffer (pH 8.2), 10 mM $Mg(CH_3CO_2)_2$, 1.5 mM [γ-^{32}P]ATP, 0.3 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25 μg/mL autophosphorylated phosphorylase kinase, and 0.2 mM melittin. At appropriate times, aliquots were removed and analyzed for ^{32}P incorporation into melittin by phosphocellulose paper assay or by SDS–PAGE/autoradiography. Background controls were generated from phosphorylation mixtures containing all components of the assay

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

except the kinase, and except melittin. Phosphoamino acid analyses were performed on phosphomelittin bands excised from SDS–PAGE gels and relied on two-dimensional electrophoresis to resolve individual amino acids (Hildebrandt & Fried, 1989).

SDS–PAGE of Melittin and Phosphomelittin. Melittin and phosphomelittin were electrophoresed in a mini-slab gel apparatus using, with minor modification, the method of Swank and Munkres (1971) for low molecular weight polypeptides. Samples were mixed (2:1, v/v) with denaturing buffer (0.1 M Tris-HCl, pH 8.0, 2% SDS, 8 M urea, 4% mercaptoethanol, and 0.1% bromophenol blue) and loaded onto a discontinuous upper stacking and lower separating gel. The separating gel contained 0.1 M H_3PO_4, pH 6.8 (adjusted with solid Tris), 0.1% SDS, 8 M urea, 10% acrylamide, 1% bis(acrylamide), 0.075% Temed, and 32.5 μg/mL ammonium persulfate. The stacking gel contained all of the above components, but the concentration of the acrylamide was 4%. The electrophoresis was carried out for 3 h at 100 V in 0.1 M H_3PO_4, pH 6.8 (adjusted with solid Tris), and 0.1% SDS. After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue R-250/0.5% $CuSO_4$/40% methanol/10% acetic acid and destained with 5% methanol/7% acetic acid. Autoradiography was performed with Kodak XAR-5 film.

Calmodulin–Agarose Treatment of Melittin and Phosphomelittin. The calmodulin–agarose and agarose beads were washed once with water and twice with 50 mM Hepes (pH 8.2) containing 0.5 mM EGTA and then equilibrated with 50 mM Hepes (pH 8.2) containing 0.5 mM $CaCl_2$. An aliquot of 0.2 mM melittin or phosphomelittin in 50 mM Hepes (pH 8.2), 10 mM $Mg(CH_3CO_2)_2$, 1.5 mM [γ-^{32}P]ATP, 0.3 mM $CaCl_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol was added to an equal volume of calmodulin–agarose or agarose and incubated with gentle mixing for 5 min at room temperature. Following centrifugation, an aliquot of the supernatant was mixed with 0.5 volume of SDS–PAGE denaturation buffer and subjected to electrophoresis as described above.

Mobility Shift Assay for Calmodulin. The binding of melittin and phosphomelittin to calmodulin was assayed by the shift in calmodulin's mobility on 12.5% polyacrylamide gels containing 4 M urea and 0.1 mM $CaCl_2$ (Erickson-Viitanen & DeGrado, 1987) in the discontinuous system of Laemmli (1970). The binding mixture contained 50 mM Hepes (pH 8.2), 9.5 mM $Mg(CH_3CO_2)_2$, 1.4 mM [γ-^{32}P]-ATP, and 0.34 mM melittin or phosphomelittin and was incubated for 1 h at room temperature. Two volumes of this mixture were then mixed with 1 volume of dilution buffer (50 mM Hepes, pH 8.2, 0.1 mM $CaCl_2$, 0.1 mM EDTA, 25% glycerol, and 0.1% bromophenol blue), loaded onto the polyacrylamide gel, and electrophoresed for 4 h at 50 V. The gel was then stained with 0.25% Coomassie blue R-250, destained, and subjected to autoradiography.

Kinetic Data. Using the Fortran program of Cleland (1979), the kinetic data were analyzed to determine the pattern of inhibition (Figure 2) and all of the kinetic parameters except the K_i.

RESULTS

Inhibition of Phosphorylase Kinase Activity by Melittin. When the hexadecameric phosphorylase kinase holoenzyme was assayed in the presence of increasing concentrations of melittin, its catalytic activity was progressively inhibited (Figure 1). Because the δ-subunit of the kinase is an intrinsic molecule of calmodulin, one might conclude that the inhibition could be due to an interaction between melittin and the

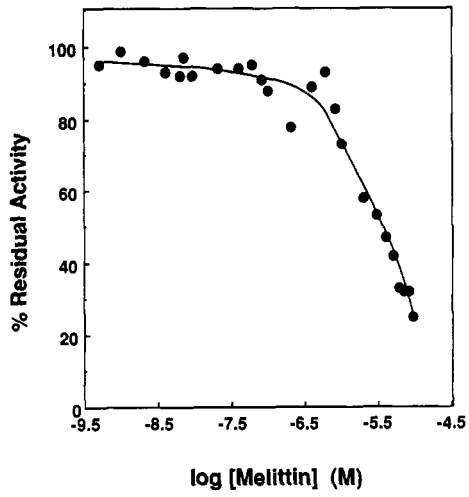

FIGURE 1: Inhibition of phosphorylase kinase activity by melittin. The phosphorylase *b* conversion activity of nonactivated phosphorylase kinase (0.1 μg/mL) was measured at pH 8.2 in the presence of the indicated concentrations of melittin. Aliquots were removed after 6 min to determine ^{32}P incorporation into phosphorylase.

δ-subunit; however, the nature of the dose response curve caused us to question whether this straightforward interpretation could be the sole mechanism for inhibition. Melittin inhibited kinase activity in a biphasic manner with a slight inhibition (≃10%) occurring at nanomolar concentrations of melittin, which is equivalent to the dissociation constant for a calmodulin–melittin complex (Comte et al., 1983; Maulet & Cox, 1983); however, the predominant inhibition occurred at micromolar concentrations of the inhibitor (Figure 1). Thus, although the slight inhibition by nanomolar melittin was consistent with the expected affinity for an interaction between melittin and the δ-subunit of phosphorylase kinase, it was unclear whether the predominant inhibition by micromolar melittin was also caused by such an interaction. We therefore decided to further characterize the strong inhibition caused by micromolar concentrations of melittin. It might be noted that the corresponding K_i values for the calmodulin-binding peptides, including melittin, characterized by Newsholme et al. (1992) ranged upward from 16 μM.

When the kinase activity of the holoenzyme was measured at different concentrations of melittin with phosphorylase *b* as the variable substrate, melittin acted as a competitive inhibitor against phosphorylase (Figure 2A). Because melittin and the large phosphorylase *b* dimer could in theory compete for binding to the kinase at a substrate docking site distinct from the active site, phosphorylase *b* was replaced in the assays by an alternative tetradecapeptide substrate that corresponds to the region surrounding the convertible serine of phosphorylase and that has been shown to mimic phosphorylase in its phosphorylation by phosphorylase kinase (Carlson et al., 1976; Farrar & Carlson, 1991). Melittin was also found to be a competitive inhibitor against this alternative peptide substrate (Figure 2B). When similar experiments were performed using ATP as the variable substrate, double-reciprocal plots of the data showed melittin to be a noncompetitive inhibitor against ATP (data not shown).

The results shown in Figure 2A,B suggested that melittin might be an active-site-directed inhibitor of phosphorylase kinase that competes with the protein substrate. If so, then the predominant inhibition of the holoenzyme by micromolar melittin in Figure 1 could be caused by its interaction with the catalytic γ-subunit, as opposed to an interaction with the integral calmodulin subunit (δ). To test this possibility, we

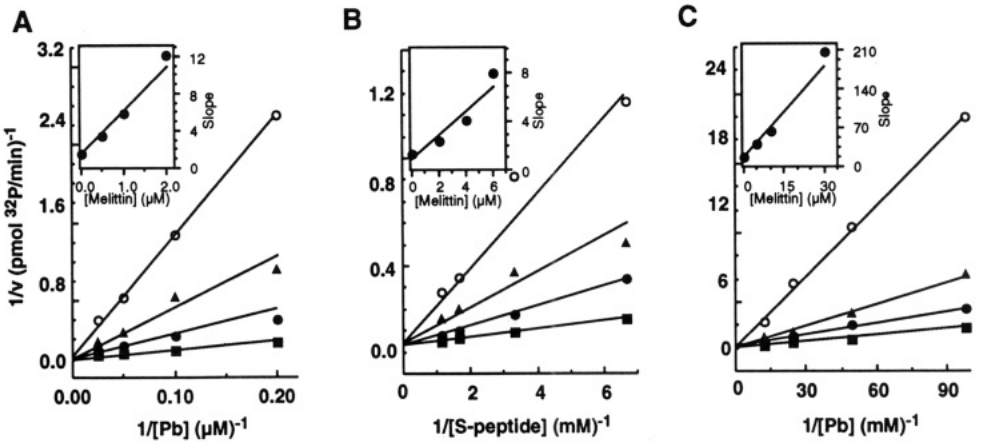

FIGURE 2: Patterns of inhibition by melittin of the phosphorylase kinase holoenzyme and its isolated catalytic γ-subunit. The activities of nonactivated phosphorylase kinase (A and B) and its isolated γ-subunit (C) were assayed at pH 8.2 in the presence of a fixed concentration of ATP (1.5 mM), variable concentrations of phosphorylase b (A and C) or tetradecapeptide alternative substrate (B), and fixed variable concentrations of melittin. The melittin concentrations were 0 (■), 0.5 (●), 1.0 (▲), and 2.0 (○) μM in (A); 0 (■), 2 (●), 4 (▲), and 6 (○) μM in (B); and 0 (■), 5 (●), 10 (▲), and 30 (○) μM in (C). The insets of each panel show a replot of the slopes against the concentration of melittin.

Table I: Comparison of the K_m's for Phosphorylase *b* and the K_i's for Melittin of Phosphorylase Kinase and Its Catalytic γ-Subunit[a]

enzyme form	K_m (μM)	K_i (μM)	K_m/K_i
phosphorylase kinase	27.6 (25.4, 29.8)	0.95 (0.79, 1.1)	29.0
γ-subunit	82.4 (79.5, 85.4)	3.6 (4.2, 3.0)	19.2

[a] The K_m values of phosphorylase kinase and the γ-subunit are from Figure 2A,C at zero melittin concentration using the Fortran program of Cleland (1979). To determine the K_i values, the slopes of the lines in Figure 2A,C were plotted on the ordinate against the corresponding concentrations of melittin on the abscissa, and the K_i was obtained from the abscissa intercept of the resulting line. The values shown for K_m and K_i are the average of two independent determinations, with the values from the separate determinations given in parentheses.

examined the effect of melittin on the activity of the free catalytic γ-subunit, which has a low level of phosphotransferase activity that is independent of calmodulin (Chan & Graves, 1982b). When the activity of the free γ-subunit was measured in the presence of various concentrations of melittin with phosphorylase *b* as the variable substrate, melittin was once again found to be a competitive inhibitor with respect to phosphorylase (Figure 2C). If the predominant inhibition of the holoenzyme by melittin is caused by its interaction with the catalytic γ-subunit within the $\alpha\beta\gamma\delta$ protomers, then one would expect that the ratios of the K_m for phosphorylase to the K_i for melittin would be similar for the holoenzyme and for the isolated γ-subunit. This was in fact the case, as the ratio was 29.0 for the holoenzyme and 19.2 for the catalytic γ-subunit (Table I).

Melittin Is a Substrate for Phosphorylase Kinase. Because the above data suggested that melittin may bind to the active site of phosphorylase kinase and because melittin is a basic peptide that contains seryl and threonyl residues, we evaluated whether it could serve as a substrate for phosphorylase kinase, which has basic residues as part of its substrate recognition determinants [reviewed in Pickett-Gies and Walsh (1986)]. When melittin was incubated with the kinase holoenzyme, Mg/[γ-^{32}P]ATP, and the other components of the phosphorylation assay described under Experimental Procedures, there was a linear increase of phosphorylated product with time as detected by the phosphocellulose paper assay (Figure 3A). To obtain sufficient amounts of phosphorylated product for accurate analyses, greater concentrations of kinase were required than for phosphorylase conversion. Because all of the components necessary for phosphorylase kinase auto-

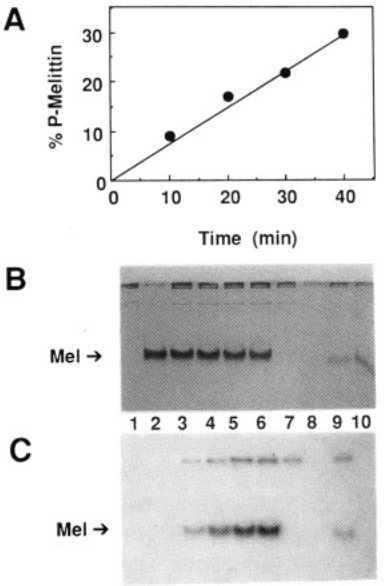

FIGURE 3: Phosphorylation of melittin by phosphorylase kinase. The reaction mixture contained all components of the melittin phosphorylation assay described under Experimental Procedures, except that the concentration of autophosphorylated phosphorylase kinase was 0.1 mg/mL. Aliquots were removed at various times and spotted onto phosphocellulose paper for determination of ^{32}P incorporation into product (A) or mixed with SDS–PAGE denaturing buffer in preparation for electrophoresis. The final aliquot was removed from the phosphorylation reaction mixture after 60 min and treated with calmodulin–agarose or control agarose beads as described under Experimental Procedures. (B) Coomassie blue-stained SDS–PAGE gel of the phosphorylation reaction mixture and effect of its treatment with calmodulin–agarose. Lane 1, phosphorylase kinase control (2 μg); lane 2, melittin control (11 μg) incubated for 45 min with all assay components but phosphorylase kinase; lanes 3–6, phosphorylation assay aliquots removed after 10, 20, 30, and 40 min, respectively; lanes 7 and 9, phosphorylation assay aliquots removed after 60 min and treated with calmodulin–agarose and agarose, respectively; lanes 8 and 10, control samples of a phosphorylation mixture containing all components except phosphorylase kinase treated with calmodulin–agarose and agarose, respectively. (C) Autoradiograph of gel in (B). The bands in (C) that barely migrate into the gel correspond to the α/β-subunits of phosphorylase kinase and indicate that only a trace of autophosphorylation capacity remains in the previously autophosphorylated phosphorylase kinase used for melittin phosphorylation.

phosphorylation were present in the melittin phosphorylation assay, phosphorylase kinase that had been previously autophosphorylated with nonradioactive ATP was used as the catalyst for melittin phosphorylation in order to avoid high control backgrounds of ^{32}P incorporation into protein. The phosphorylated products formed during the phosphorylation assay in Figure 3A were analyzed by SDS–PAGE and autoradiography. Although a trace amount of kinase autophosphorylation still occurred (top band in lanes 3–7 and 9 in Figure 3C), most of the radioactive product was in a band that comigrated with melittin, and the amount of radioactivity incorporated increased with time (Figure 3C, lanes 3–6). The extent of melittin phosphorylation achieved with autophosphorylated kinase normally reached a plateau at slightly above the level shown in Figure 3A. A melittin control containing all components of the phosphorylation assay except phosphorylase kinase showed no incorporation of radioactivity, even after 45 min of incubation (Figure 3C, lane 2).

To ensure that the phosphorylated product that comigrated with melittin was actually phosphomelittin, as opposed to a phosphorylated contaminant, we evaluated, using two independent approaches, whether the phosphorylated product could bind to calmodulin. In the first approach, an aliquot from the melittin phosphorylation mixture was withdrawn after 60 min and then incubated for 5 min with either calmodulin–agarose or agarose beads. Following centrifugation to pellet the beads, the supernatant was analyzed by SDS–PAGE and autoradiography. Melittin, either from the phosphorylation mixture or as a nonphosphorylated control, was removed from solution by treatment with the calmodulin–agarose beads (Figure 3B, lanes 7 and 8), but not by control agarose beads (Figure 3B, lanes 9 and 10). The diminution of the bands in lanes 9 and 10 compared to the other lanes in Figure 3 is simply caused by the dilution that occurs in the expanded protocol using the agarose beads. The radioactivity that comigrates with melittin was also removed by calmodulin–agarose (Figure 3C, lane 7), but not by control agarose (Figure 3C, lane 9).

The second approach to show that the phosphorylated peptide was phosphomelittin relied on calmodulin mobility shift assays, because the binding of peptides to calmodulin can alter its mobility during PAGE in 4 M urea (Erickson-Viitanen & DeGrado, 1987). Melittin was phosphorylated by the standard procedure for 60 min, and an aliquot was examined by SDS–PAGE and autoradiography to ensure that phosphorylation had occurred (Figure 4A,B, lane 2). Another aliquot was incubated with calmodulin and subjected to the mobility shift assay described under Experimental Procedures. As expected, nonphosphorylated control melittin slowed the migration of calmodulin (Figure 4C, lane 4) compared to control calmodulin without melittin (Figure 4C, lane 3). When calmodulin was incubated with the phosphorylated melittin, three bands with different mobilities were observed (Figure 4C, lane 5). The fastest migrated with free calmodulin and the slowest with the complex of calmodulin and nonphosphorylated control melittin; the remaining band had a migration between the other two. Because this middle band was also radioactive (Figure 4D, lane 5), it must represent a complex between calmodulin and phosphomelittin. In control experiments, both phosphorylated and nonphosphorylated phosphorylase kinases were found not to affect the mobility of calmodulin in these assays (data not shown). From these results, we conclude that the phosphorylated species that comigrates with melittin in Figure 3C is indeed phosphomelittin and that it binds to calmodulin.

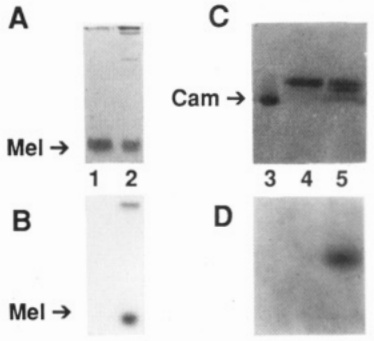

FIGURE 4: Mobility shift of calmodulin upon binding to melittin and phosphomelittin. Melittin was incubated for 60 min plus or minus phosphorylase kinase in phosphorylation reaction mixtures as described in the legend to Figure 3. Aliquots were removed and subjected to SDS–PAGE and to the mobility shift assay for calmodulin binding as described under Experimental Procedures. (A) Coomassie blue-stained SDS–PAGE gel of phosphorylation reaction mixtures. Lane 1, melittin (15 μg) incubated without phosphorylase kinase; lane 2, complete phosphorylation reaction mixture containing 10 μg of melittin. (B) Autoradiograph of gel in (A). (C) Coomassie blue-stained calmodulin mobility shift gel with 5 μg of calmodulin loaded in each lane. Lane 3, calmodulin control containing all phosphorylation mixture components except melittin; lane 4, calmodulin incubated with an aliquot of the phosphorylation reaction mixture containing all components except phosphorylase kinase; lane 5, calmodulin incubated with an aliquot of the complete phosphorylation reaction mixture. (D) Autoradiograph of the gel in (C).

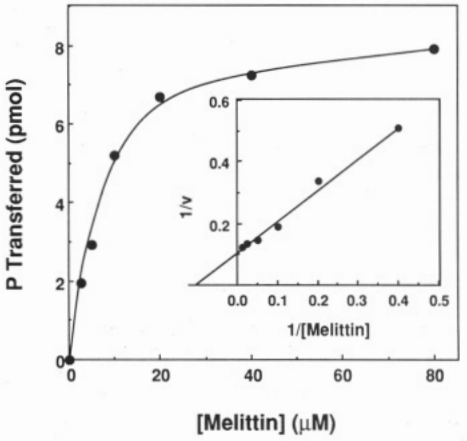

FIGURE 5: Concentration dependence of melittin's phosphorylation. Melittin was phosphorylated at pH 8.2 by autophosphorylated phosphorylase kinase (25 μg/mL) as described under Experimental Procedures except that progressively increasing concentrations of melittin were used. Aliquots were removed after 15 min to determine ^{32}P incorporation into product. Inset: Double-reciprocal replot of the data showing a K_m in this experiment of 7.6 μM.

Phosphoamino acid analysis of phosphomelittin excised from SDS–PAGE gels showed the major phosphorylated amino acid to be phosphoserine, although large amounts of phosphothreonine were also present.

Kinetic Parameters for Melittin as a Substrate. Because the above results indicated that melittin was a substrate for phosphorylase kinase, it was of interest to compare the K_m for melittin's phosphorylation to its previously determined K_i as an inhibitor of phosphorylase conversion. As before, autophosphorylated phosphorylase kinase was used for melittin phosphorylation. The rate of phosphorylation at increasing concentrations of melittin showed saturation kinetics (Figure 5); double-reciprocal plots of this and replicate data indicated a substrate K_m for melittin of 9.8 ± 3.1 μM (Table II), which is 10 times greater than its K_i determined with nonactivated kinase (Table I).

Table II: Kinetic Parameters of Phosphorylase Kinase for Various Substrates[a]

substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\times 10^6$ M^{-1} min^{-1})
phosphorylase *b*	29.0 (24.7, 33.3)	3423 (3397, 3449)	118.0
tetradecapeptide	308.5 (284, 333)	1415 (1493, 1337)	4.6
melittin	9.8 (7.6, 12.0)	5.9 (6.4, 5.5)	0.6

[a] The activity of autophosphorylated phosphorylase kinase toward various concentrations of the indicated substrates was measured as described under Experimental Procedures. The assay conditions for all substrates were identical, except that with melittin the kinase concentration was higher, as described. The K_m and k_{cat} values were derived using the Fortran program of Cleland (1979). The values given are the average of two independent determinations, with the values from the separate determinations given in parentheses.

To further evaluate melittin as a substrate for phosphorylase kinase, we compared the kinetic parameters for this substrate to those for two well-characterized substrates, phosphorylase *b* and the tetradecapeptide. The phosphorylation of progressively increasing concentrations of all three substrates by autophosphorylated kinase was carried out under identical conditions, except for kinase concentration, as described under Experimental Procedures and in the legend to Table II. Melittin had the lowest K_m value (Table II), suggesting that it binds with the highest affinity to phosphorylase kinase. The turnover number (k_{cat}) for melittin, however, was by far the lowest of the three substrates at only $^1/_{580}$th that for phosphorylase *b*. Comparison of the k_{cat}/K_m ratios for the three substrates (Table II) indicates that overall melittin is the worst substrate for phosphorylase kinase, with its tight binding being offset by its poor k_{cat}.

The K_m for ATP with melittin as substrate was also determined and compared to the value obtained at the same time with tetradecapeptide as substrate. At pH 8.2 with autophosphorylated phosphorylase kinase as the enzyme, the respective K_m values for ATP were very similar at 70 and 88 μM.

DISCUSSION

Despite melittin's high affinity for calmodulin (Comte et al., 1983; Maulet & Cox, 1983) and despite the fact that all four subunits of phosphorylase kinase are capable of binding melittin (Jarrett & Madhavan, 1991), the sum of our data indicates that the predominant inhibitory effect of melittin on the phosphorylase kinase holoenzyme is caused by its interaction with the enzyme's catalytic γ-subunit and not with its intrinsic calmodulin subunit, δ. This conclusion is in contrast to that of Newsholme et al. (1992), who hypothesized that the inhibitory calmodulin-binding peptides they characterized inhibited phosphorylase kinase by binding to its δ-subunit. Although melittin was screened in the study by Newsholme et al. (1992) and found to be inhibitory with an IC_{50} of 18 μM (compared to the K_i of 0.95 μM in this report), the mechanism of its inhibition was not evaluated. As discussed below, whether the target of calmodulin-binding peptides is the γ-subunit or the δ-subunit of the phosphorylase kinase hexadecamer has different implications regarding the possible autoregulation of this complex enzyme. We do think it is possible that a small amount of the inhibition caused by melittin may be due to its interaction with the δ-subunit, because the slight inhibition ($\simeq$10%) occurring at nanomolar concentrations of melittin (Figure 1) corresponds to the dissociation constant for melittin–calmodulin.

FIGURE 6: Comparison of the amino acid sequences of melittin and the phosphorylation site of phosphorylase. The N→C sequence of phosphorylase (Nolan et al., 1964) around its phosphorylation site (residues 7–19 with the convertible Ser-14 underlined) is aligned with residues 25–15 (C→N) of the C-terminal half of melittin. Maximum alignment of the 26-residue polypeptide melittin (Habermann & Jentsch, 1967; Jentsch 1969) was achieved by aligning it in the C→N direction and inserting a gap of two residues.

On the basis of its K_i and K_m values (Tables I and II), melittin binds to phosphorylase kinase with relatively high affinity. In fact, their respective K_m values suggest that melittin may bind slightly better to activated kinase than does phosphorylase *b* and that it binds significantly better than the alternative tetradecapeptide substrate (Table II). It is possible that some of melittin's high-affinity-binding ability may be attributable to its amphipathic α-helical conformation (Maulet & Cox, 1983) inasmuch as the majority of the high-affinity-binding region of a peptide derived from the heat-stable protein kinase inhibitor has this secondary structure when bound to the catalytic subunit of cAMP-dependent protein kinase (Knighton et al., 1991), to which phosphorylase kinase is homologous (Reimann et al., 1984).

In hopes of understanding the underlying basis for melittin being a substrate for phosphorylase kinase, we compared the amino acid sequence of melittin with the region surrounding the convertible Ser-14 of glycogen phosphorylase, which includes substrate recognition determinants for the very specific phosphorylase kinase (Tessmer et al., 1977; Tabatabai & Graves, 1978; Viriya & Graves, 1979). No similarity was apparent when the two sequences were compared conventionally beginning with the N-terminus of each; however, when the sequences were aligned antiparallel, that is N→C of one versus C→N of the other, sequence similarity was observed that was centered around the convertible Ser-14 of phosphorylase (Figure 6). Although the notion that melittin and phosphorylase might bind to the catalytic site of phosphorylase kinase in opposite polarity is experimentally unproved, the question of whether a peptide might bind to phosphorylase kinase in either polarity has been raised previously as a possible explanation for the second-site phosphorylation of a symmetrical peptide with seryl residues flanking a single basic cluster (Chan et al., 1982). Residues 11–16 of phosphorylase (Figure 6), which sandwich the convertible serine on either side with hydrophobic followed by basic residues, are considered to be particularly important specificity determinants [reviewed in Carlson et al. (1979), Pickett-Giess and Walsh, (1986), Kemp (1988), and Pearson and Kemp (1991)]. The reversed-polarity alignment for melittin in Figure 6 provides the same structural features, except that it is missing a basic residue corresponding to position 16 of phosphorylase. It should be noted, however, that glycogen synthase, which is an excellent substrate for phosphorylase kinase, also lacks a basic residue at this position (Chan et al., 1982; Kemp, 1988). The Ile at position 17 immediately adjacent to the convertible serine of melittin may also contribute to melittin's tight binding, because a peptide analogue of phosphorylase in which Ile replaced Val-15 showed a 63% decrease in K_m (Viriya & Graves, 1979). It is also possible, however, that melittin binds with the same polarity as phosphorylase, with one of the basic residues in the cluster 21–24 of melittin fulfilling the role of Arg-16 of phosphorylase. Evaluation of these two alternative possibilities will require collection of more data concerning

the relative importance of the polypeptide backbone polarity in the context of side-chain specificity determinants.

In addition to phosphoserine, phosphothreonine was also observed in the melittin phosphorylated by phosphorylase kinase, which is not without precedent inasmuch as troponin I has also been reported to be phosphorylated on a threonyl residue by this kinase (Huang et al., 1974; Moir et al. 1974). There are only two threonyl residues in melittin, and they occur in the sequence K_7VLTTGLPAL$_{16}$ (Habermann & Jentsch, 1967; Jentsch, 1967), which is similar to the sequence R_4TLSVSSLPGL$_{14}$ surrounding the underlined convertible Ser-7 that is phosphorylated in glycogen synthase by phosphorylase kinase (Embi et al., 1979). On this basis of sequence similarity, the first of the two threonines, Thr-10, is the more likely threonyl phosphorylation site.

The major question raised by these current observations with melittin is whether they are germane to the mechanism for the Ca^{2+}-dependent activation of the phosphorylase kinase holoenzyme, i.e., in the absence of Ca^{2+} ions, does a calmodulin-binding region of the holoenzyme directly interact with the active site? In this simple model, the active site and calmodulin (the δ-subunit) would compete for a particular region of primary structure, and, thus, activation by Ca^{2+} ions would result from deinhibition caused by the binding of this domain to calmodulin instead of to the active site. This mechanism of activation would be comparable to the regulatory models proposed for the calmodulin-dependent protein kinases myosin light chain kinase and calmodulin-dependent protein kinase II (Lukas et al., 1986; Kemp et al., 1987; Kennelly et al., 1987; Payne et al., 1988; Colbran et al., 1989), and the autoinhibitory and calmodulin-binding domains could be either overlapping (as in the case of melittin) or adjacent. Certainly there is no shortage of such potential regulatory domains in the phosphorylase kinase holoenzyme, in that calmodulin-binding regions have been identified on the α-, β-, and γ-subunits (Dasgupta et al., 1989; James et al., 1991; Newsholme et al., 1992). Evidence consistent with the catalytic γ-subunit itself supplying a calmodulin-binding, autoinhibitory domain is the previous report that a proteolytic fragment of the γ-subunit missing both calmodulin-binding domains had a greater specific activity than the γ-calmodulin complex (Harris et al., 1990). However, when Newsholme et al. (1992) characterized the inhibition patterns of peptides corresponding to the two calmodulin-binding regions of the γ-subunit, they classified both as noncompetitive inhibitors with respect to phosphorylase, although this classification was not clear-cut for one of the peptides. The simple model under discussion here would predict competitive, rather than noncompetitive, patterns of inhibition; however, it might be noted that sometimes a seemingly minor alteration of a peptide, such as C-terminal amide capping, can change its pattern of inhibition (Salerno et al., 1990). Another candidate subunit for supplying a calmodulin-binding, autoinhibitory domain is the β-subunit, because its conformation is closely associated with the activity level of the kinase (Fitzgerald & Carlson, 1984; Trempe & Carlson, 1987; Cheng et al., 1988) and because an autoinhibitory region from residues 420 to 436 of this subunit has been identified (Sanchez & Carlson, 1993), although it is not yet known if this domain binds calmodulin. Because of the complexity of phosphorylase kinase and its multiplicity of calmodulin-binding sites, if its catalytic site is directly regulated by an autoinhibitory, calmodulin-binding domain, conclusive proof of that will presumably require evidence that is direct, such as chemical cross-linking of appropriate regions of the holoenzyme to the active site. For this and related enzymes, activity-based studies utilizing peptides to evaluate potential models of enzyme regulation provide indirect evidence that is limited to being only consistent with, or inconsistent with, a particular model. With these caveats in mind, the current results with melittin are consistent with the direct regulatory model described above and are inconsistent with the indirect regulatory model proposed by Newsholme et al. (1992) that was based on studies with other calmodulin-binding peptides.

A further aspect of the simple, direct regulatory model is the question of whether the autoinhibitory region surrounding the calmodulin-binding domain interacts with the active site because it is an alternative substrate or a pseudosubstrate [reviewed by Soderling (1990)]. Melittin, being a tight-binding but poor substrate, could be argued to act in this manner, although despite its being a substrate the strongest argument for its homology with previously characterized substrates requires its binding to the active site in reverse-polarity to the natural substrate. One of the inhibitory, calmodulin-binding peptides evaluated by Newsholme et al. (1992) corresponds to a site of autophosphorylation on the enzyme's β-subunit, and thus by definition is a substrate, and it is possible, on the basis of their sequences, that some of the other calmodulin-binding peptides screened in that study may also be substrates. Even glycogen phosphorylase, the natural substrate of phosphorylase kinase, is known to bind calmodulin (Villar-Palasi et al., 1983); in fact, we have found that a peptide corresponding to residues 9–30 of glycogen phosphorylase, which contains the convertible Ser-14, not only is a good substrate for phosphorylase kinase but also binds tightly to calmodulin.[2] Moreover, it has recently been found that phosphorylase kinase phosphorylates the calmodulin-binding regions of the neuronal proteins neuromodulin and neurogranin (Paudel et al., 1993). Thus, there are a minimum of five distinct protein/peptide substrates for phosphorylase kinase that also bind calmodulin, which suggests that the active site of phosphorylase kinase may share similarities with calmodulin in the manner that it binds target peptides. This would be analogous to the postulated flip-flop model for the regulation of calmodulin-dependent enzymes (Jarrett & Madhavan, 1991), but with the calmodulin-like-binding site being the active site. In this regard, it might be noted that other basophilic protein kinases, such as cAMP-dependent protein kinase, protein kinase C, and calmodulin-dependent protein kinase II, often phosphorylate target proteins within or adjacent to regions that are also binding sites for calmodulin. Among these target proteins are caldesmon (Bartegi et al., 1990; Ikebe & Reardon, 1990), calcineurin (Kincaid et al., 1988; Martensen et al., 1989), MARCKS protein (Graff et al., 1989; Stumpo et al., 1989), and calmodulin-dependent protein kinase II (Payne et al., 1988; Schworer et al., 1988). In the case of cAMP-dependent protein kinase, it was suggested early on that this kinase may recognize calmodulin-binding sequences (Malencik et al., 1982, 1986; Malencik & Anderson, 1982).

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