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Inhibition of protein kinase C by melittin: antagonism of binding interactions between melittin and the catalytic domain by active-site binding of MgATP

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Abstract—Melittin inhibits the lipid cofactor-independent activity of protein kinase C (PKC) by directly binding to the catalytic domain in a MgATP-sensitive manner. The catalytic domains of certain PKC isozymes have a consensus sequence for a second nucleotide binding site outside their active site regions. In this report, we show that PKC isozymes containing the second nucleotide binding site motif (α, β) and an isozyme lacking the motif (ε) all have MgATP-sensitive binding interactions with melittin. Our results support a mechanism of PKC inhibition by melittin in which active-site binding of MgATP antagonizes binding interactions between PKC and melittin.

Key words: protein kinase C, melittin, protein kinase C isozymes, protein kinase C inhibitors

The toxic peptide melittin is a cationic-amphiphilic molecule that consists of an amphiphilic α-helical domain attached to a cationic tail [1, 2]. Because the physicochemical properties of melittin are well-defined and have been exploited in designing analogs of the hexacosapeptide, melittin is a valuable probe of membranes and isolated proteins that interact with it [2–5]. Protein kinase C (PKC)* is a family of at least ten closely related phospholipid-dependent isozymes [6]. Limited proteolysis of PKC produces a fully active, phospholipid-independent catalytic fragment that represents the catalytic domain [7]. Melittin inhibits PKC by two mechanisms. Inhibition of Ca^{2+}- and phosphatidylserine (PS)-dependent PKC activity is achieved by interference with PS in the activation of the enzyme ($IC_{50} = 4\ \mu M$) [3, 8]. Melittin inhibits the lipid cofactor-independent activity of PKC by directly binding to the catalytic domain in a MgATP-sensitive manner ($IC_{50} = 25\ \mu M$) [9]. The MgATP-sensitive binding interactions between melittin and the catalytic domain of PKC were demonstrated by the selective elution of PKC and its catalytic fragment from melittin-agarose with MgATP [9]. Elution of the catalytic fragment indicated a lack of involvement of the PS binding sites of the regulatory domain in the MgATP-sensitive binding interactions between PKC and melittin [9]. Because some PKC isozymes have in common a consensus sequence for a second nucleotide binding site outside of the active site region [7, 10], it remained unclear whether occupation of the active site or the putative second nucleotide binding site by MgATP antagonized the binding of melittin to the catalytic domain [9]. Furthermore, the kinetics of inhibition of the catalytic fragment by melittin were mixed competitive/noncompetitive with respect to ATP [9], precluding a straightforward kinetic interpretation of the inhibitory mechanism. In this report, we demonstrate that both PKC isozymes that contain the consensus sequence for the putative second nucleotide binding site and PKC isozymes that lack critical features of the consensus sequence bind to immobilized melittin in a MgATP-sensitive manner. Our results rule out an essential role for the putative second nucleotide binding site in the MgATP-sensitive binding interactions between PKC and melittin and support a mechanism of PKC inhibition by melittin in which active-site binding of MgATP antagonizes binding of the inhibitory peptide melittin to PKC.

To ascertain the relative importance of the nucleotide substrate binding site and the second putative nucleotide binding site in the catalytic domain of PKC [7, 10] to the MgATP-sensitive nature of inhibitory binding interactions between melittin and the catalytic domain [9], we compared the abilities of rat brain PKC isozymes that contain the consensus sequence for a second nucleotide binding site (cPKC-α and cPKC-β) and rat brain PKC isozymes in which critical residues of the consensus sequence are not conserved (cPKC-γ and nPKC-ε) [7, 10] to bind immobilized melittin in a MgATP-sensitive manner. We previously reported that rat brain PKC quantitatively binds melittin-agarose and can be recovered by elution with MgATP [9]. By this chromatographic procedure, we purified a semi-crude rat brain PKC preparation to near-homogeneity [9]. In this report, we subjected the melittin-agarose-purified rat brain PKC preparation to immunoblot analysis with isozyme-specific rabbit polyclonal antibodies for cPKC-α, cPKC-β, cPKC-γ, and nPKC-ε (GIBCO BRL, Gaithersburg, MD), using previously described methods [11, 12]. Immunoreactive bands were detected by enhanced chemiluminescence (ECL) using a kit from the Amersham Corp. (Arlington Heights, IL). In Fig. 1, the immunoblot analysis shows that cPKC-α, cPKC-β, cPKC-γ, and nPKC-ε were recovered from melittin-agarose by elution with MgATP. For each isozyme, an immunospecific band was observed at the molecular weight corresponding to the rat brain isozyme (cPKC-α, 82-kDa; cPKC-β, 82-kDa; cPKC-γ, 81-kDa; nPKC-ε, 91-kDa) [6, 11, 12]. The antigenic peptide completely blocked immune reactions between the isozymes cPKC-α (82-kDa), cPKC-β (82-kDa), cPKC-γ (81-kDa), and nPKC-ε (91-dKa) and the corresponding isozyme-specific antibodies. No other major immunospecific bands were detected.

The Gly triad motif Gly-X-Gly-X-X-Gly (GXGXXG) is a hallmark of nucleotide binding sites [13]. Although different three-dimensional structures form protein kinase nucleotide substrate binding sites and nucleotide binding sites shared by dehydrogenases and GTP-binding proteins, the Gly triad motif is nearly invariant and plays an essential role in the formation of both types of binding sites [13]. In addition, an invariant Lys residue is associated with the Gly triad motif in both types of sites [13]. The sequences corresponding to the active-site regions of all of the PKC isozymes examined (α, β, γ, ε) are very closely related and have in common the nucleotide binding site motif Gly-X-Gly-X-X-Gly...Lys (GXGXXG...K) [7, 10]. This motif also occurs at the putative second nucleotide binding sites of cPKC-α and cPKC-β [7, 10]. In contrast, critical residues of the motif are not conserved in the corresponding

* Abbreviations: ECL, enhanced chemiluminescence; PKC, protein kinase C; and PS, phosphatidylserine.

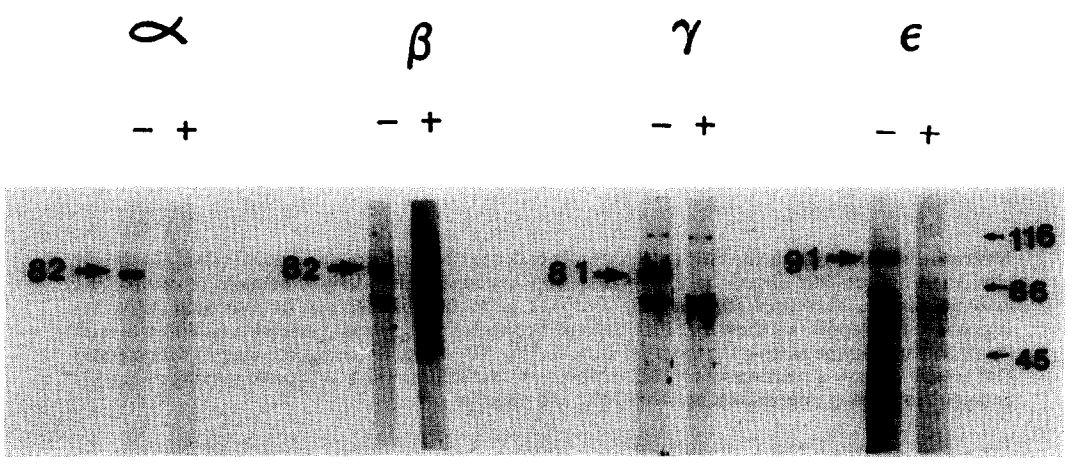

Fig. 1. Identification of PKC isozymes that bind melittin-agarose in a MgATP-sensitive manner. Rat brain PKC bound to melittin-agarose was eluted from the resin with 10 mM $MgCl_2$ plus 1 mM ATP, as previously described [9], and then subjected to immunoblot analysis (40 ng protein/lane), using GIBCO BRL isozyme-specific anti-PKC polyclonal antibodies (0.5 μg/mL) and previously described immunoblotting procedures and conditions [11, 12]. Immunoreactive bands were detected by ECL. For each isozyme examined (PKC-α, β, γ, ε), paired immunoblots done in the absence (−, left lane) and presence (+, right lane) of antigenic peptide (0.25 μg/mL) are shown. The immunospecific bands are indicated by arrows at the left of immunoblot pairs. Molecular weight markers (116-, 66-, and 45-kDa) are indicated at the right-hand side of the figure.

sequences that occur in cPKC-γ (GXGXXG...R) and nPKC-ε (GXVXXXXG...L) [7, 10] (for a comparison of sequences, see Ref. 14). Neither the spacing of the Gly residues of the motif nor the Gly residues themselves are conserved at the putative second nucleotide binding site of nPKC-ε [7, 14]. The associated Lys is replaced by Arg in the corresponding sequence of cPKC-γ [7, 10], suggesting some conservation of function based on reported MgATP binding activities of mutated nucleotide binding sites [15], and it is replaced by Leu in nPKC-ε [14]. In view of the marginal conservation of the Gly triad motif and associated Lys residue in nPKC-ε, it is highly unlikely that its putative second nucleotide binding site is functional. Although the abilities of PKC isozymes to bind nucleotides at their putative second nucleotide binding sites have not been measured [7, 10], the profound structural differences implied by the sequences at the putative second nucleotide binding site in nPKC-ε and the other isozymes examined rule out the possibility that this site produces the nucleotide binding properties shared among the isozymes. We conclude that the MgATP-sensitive nature of melittin binding to PKC [9] is a consequence of the binding of MgATP at the active site of the enzyme. Our results lend support to a mechanism of PKC inhibition by melittin in which binding of melittin to PKC and active-site binding of MgATP are mutually exclusive events. Thus, melittin may be a valuable probe of interactions between nucleotide substrate and the active site of PKC.

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