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Phosphorylation of magainin-2 by protein kinase C and inhibition of protein kinase C isozymes by a synthetic analogue of magainin-2-amide

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Magainins are a family of antimicrobial peptides present in the skin extracts of *Xenopus laevis* Both magainin-1 and -2 do not have any significant effect on the activity of protein kinase C (PKC) Magainin-2 was found to be readily phosphorylated by PKC to 0.5 mol ^{32}P/mol of peptide. Neither magainin-1, which has a sequence of S^{8}AGK and not S^{8}AKK as in the case of magainin-2, nor the magainin-2 analogue with substitution of Ala for Ser^8 was phosphorylated by the kinase, suggesting that Ser^8 is the phosphorylation site of magainin-2 One synthetic analogue of magainin, designated magainin B, which has a greater tendency for α-helix formation in non-aqueous environment than the parent peptide resulting from substitution of Ser^8, Gly^{13}, and Gly^{18} with Ala in magainin-2-amide, is a potent inhibitor of PKC. This peptide inhibits all three PKC isozymes with IC_{50} less than 20 μM. Magainin B also inhibits the binding of [^{3}H]phorbol 12,13-dibutyrate to the kinase. These results suggest that magainin-2 may be modified by PKC through phosphorylation and that certain synthetic analogues of magainins may be used as inhibitors of PKC.

Protein kinase C; Inhibitor; Magainin, Phosphorylation

1. INTRODUCTION

Two closely related magainin peptides with 23 amino acid residues, designated magainin-1 and -2, have been shown to display antimicrobial activity toward Gram-positive and Gram-negative bacteria, fungi, and protozoa [1,2]. Recently, a series of magainin analogues having a greater growth inhibitory activity toward a variety of microbes by one to two orders of magnitude than the parent peptides have been synthesized [3]. Despite considerable effort focused on the therapeutic application of magainins in the treatment of infectious diseases, little is known about their mechanism of action. Because magainins have a sequence typical of an amphiphilic α-helical structure, it has been speculated that these peptides affect membrane function [1,3].

Protein kinase C (PKC) has been implicated in transmembrane signaling to regulate the functions of cell surface receptors, transporters, ion pumps, and ion channels [4]. This enzyme preferentially phosphorylates serine and threonine residues located at the amino- and/or carboxyl-terminus of a cluster of basic amino acids [5]. Several cationic antimicrobial amphiphilic peptides having similar structures as magainins are known inhibitors of PKC [6–8]. To investigate the biological action of magainins, we examined the effect of two magainins and a synthetic analogue on PKC activity under in vitro assay conditions. We found that both magainin-1 and -2 had no significant effect on the PKC activity at concentrations up to 100 μM, whereas a synthetic analogue, named magainin B, was a potent inhibitor of all three PKC isozymes. Magainin-2, which has a substrate-like amino acid sequence motif, S^{8}GKK, was readily phosphorylated by PKC. Although magainin B has the pseudosubstrate-like sequence [9], its inhibitory effect was not relieved by increasing the concentration of a protein substrate, but rather by the addition of phosphatidylserine (PS) and diacylglycerol. Magainin B also inhibited the binding of phorbol ester to the kinase. These results indicate the potential application of magainin B as a potent inhibitor of PKC.

2. MATERIALS AND METHODS

The following materials were obtained from the indicated sources: histone IIIS, protamine sulfate, and EGTA from Sigma; [γ-^{32}P]ATP and [^{3}H]PDBu from DuPont New England Nuclear; PS and diolcoylglycerol (DOG) from Avanti Polar Lipids, Birmingham, AL; PDBu from LC Services Corp., Woburn, MA; magainin-1 (GIGKFLHSAGKFGKAFVGEIMKS), and magainin-2 GIGKFLHSAKKFGKAFVGEIMNS), from Peninsula Laboratories, Belmont, CA; and Nonidet P-40 (NP-40) from Pharmacia LKB Biotechnology; GF/C glass fiber filters and phosphocellulose P-81 paper from Whatman.

Magainin B (GIGKFLHAAKKFAKAFVAEIMNS-NH_2) was synthesized and purified as previously described [3]. Homogeneous rat

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Abbreviations: PKC, protein kinase C; PKM, the catalytic fragment of PKC; PS, phosphatidylserine; DOG, dioleoylglycerol; PDBu, phorbol 12,13-dibutyrate; NP-40, Nonidet P-40

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brain PKC isozymes [10] and the catalytic fragment of PKC (PKM) [11] were prepared as previously described. Phosphorylation of the substrates and measurement of [^{3}H]PDBu binding were done with the mixed micelles assay [12]. The concentrations of the synthetic peptides were calculated from amino acid analysis. Calculations of kinetic constants and ligand binding parameters were carried out by a computer program designed for making statistical analysis of a group of dose-response curves [13].

3. RESULTS

Initial experiments were carried out to determine if magainins and the synthetic analogues of magainins affect PKC activity. Neither magainin-1 nor magainin-2, at their effective antimicrobial concentrations (< 100 μM), have any inhibitory effect on the kinase activity, whereas magainin B is a potent inhibitor of the kinase. We noticed that magainin-2, but not magainin-1, was readily phosphorylated by PKC to approximately 0.5 mol ^{32}P/mol peptide (Fig. 1). The same extent of phosphorylation of the peptide was achieved by PKM. Phosphorylation of magainin-2 by PKC was stimulated by Ca^{2+}, PS, and DOG, whereas the reaction catalyzed by PKM was not affected by these activators. Phosphorylation of magainin-2 by PKC displayed cooperativity with respect to the concentration of the peptide (Fig. 2). At saturating concentrations of PS (100 μg/ml) and DOG (20 μg/ml), the kinetic parameters were: Hill's coefficient, 2.28 ± 0.15; $S_{1/2}$, 20.1 ± 1.39 μM; and V_{max}, 1325 ± 27.7 nmol/min/mg. At a higher concentration of the peptide (greater than 50 μM), the rate of phosphorylation was reduced slightly. Phosphorylation of magainin-2 by PKM in the absence of the activator appears to follow a hyperbolic saturation kinetics. The kinetic parameters were: Hill's coefficient, 1.23 ± 0.05; $S_{1/2}$, 23.4 ± 1.15 μM; and V_{max}, 2133 ± 53.1 nmol/min/mg.

PKC and PKM could not phosphorylate magainin-1 to any significant extent (data not shown). Magainin-1 and -2 differ in their amino acid sequences at residues 10 and 22. Lys^{10} and Asn^{22} in magainin-2 are replaced by Gly^{10} and Lys^{22} in magainin-1. Replacement of Lys^{10} in magainin-2 by Gly abolishes the Lys^{10}-Lys^{11} sequence, a motif recognized by PKC as a substrate. These results suggest that Ser^8 is the phosphorylation site of magainin-2.

Magainin B is an analogue of magainin-2-amide obtained by substitutions of Ser^8, Gly^{13}, and Gly^{18} with Ala, which results in increased propensity to α-helix formation and antimicrobial activity [3]. This modification also abolishes the potential phosphorylation site of PKC. Neither PKC nor PKM phosphorylates magainin B to any significant extent. Although magainin B is not a substrate of PKC, this peptide inhibited PKC isozymes with slightly different potency: the IC_{50} values were 15.3 ± 1.71, 9.9 ± 0.95 and 14.6 ± 1.56 μM for the type I, II, and III isozymes,

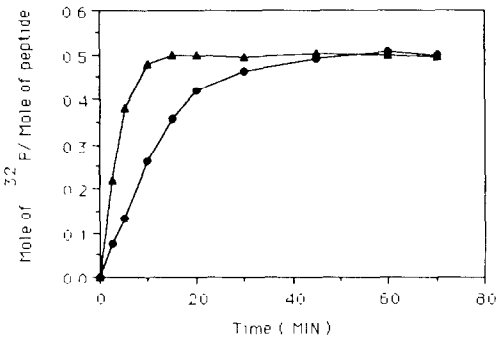

Fig. 1 Stoichiometry of phosphate incorporation into magainin-2 by PKC and PKM. Magainin-2 (25 μM) was incubated with 1.45 μg/ml of type II PKC isozyme (●) or PKM (▲) under the standard assay condition containing 100 μg/ml of PS, 20 μg/ml of DOG (for PKC) or 1 mM EGTA (for PKM). Aliquots were withdrawn at indicated intervals, and phosphate incorporation into the peptide was measured as described in section 2

respectively (Fig. 3A). Despite the fact that magainin B has a similar structural motif as a PKC pseudosubstrate, in which the phosphorylatable Ser^8 is replaced with Ala, inhibition of PKC was not alleviated by increasing concentration of histone IIIS (data not shown). Moreover, PKM is less sensitive to inhibition by magainin B than PKC. At the highest concentration of magainin B (150 μM) tested, greater than 75% of the PKM activity remained. Phosphorylation of protamine sulfate, known as a Ca^{2+}/phospholipid-independent substrate of PKC, was not significantly affected by magainin B at a concentration of 100 μM. Magainin B also inhibited the binding of [^{3}H]PDBu to PKC II with an IC_{50} value of 25.5 ± 4.73 μM (Fig. 3B). These data indicate that the inhibitory action of magainin B is mediated primarily through interaction with the regulatory rather than the catalytic domain of PKC.

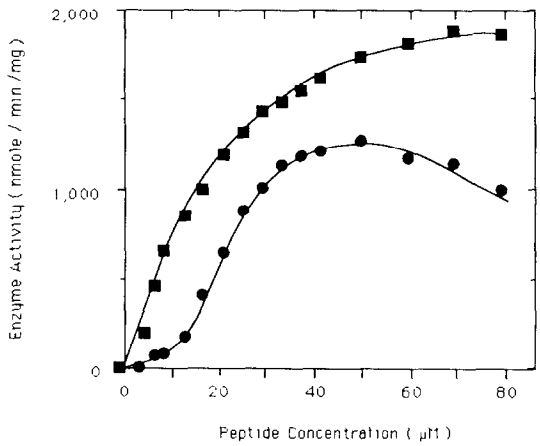

Fig. 2. Comparison of the rate of phosphorylation of magainin-2 by PKC and PKM. Incorporation of ^{32}P into the peptide was measured under standard assay conditions containing 100 μg/ml of PS, 20 μg/ml of DOG (for PKC, ●) or 1.0 mM of EGTA (for PKM, ■). The kinetic parameters were obtained by computer analysis.

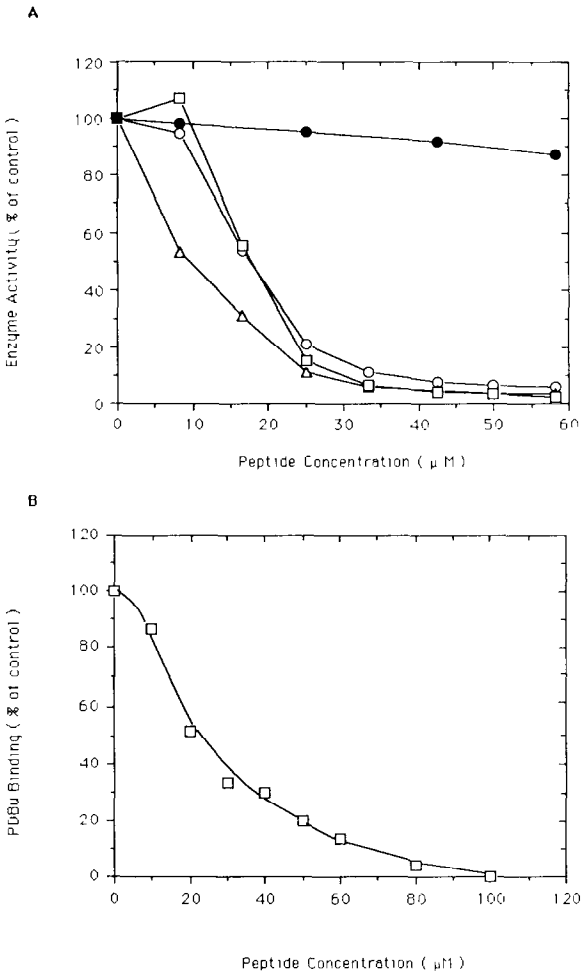

Fig. 3. (A) Inhibition of the three PKC isozymes and PKM by magainin B. The kinase activities of type I (○), II (△) and III (□) PKC and PKM (●) were measured with histone IIIS as substrate in the presence of 1.5 μg/ml of each kinase under the standard assay conditions containing 60 μg/ml of PS and 20 μg/ml of DOG (for PKC) or 1 mM EGTA (for PKM). The kinase activity without magainin B was taken as 100%. (B) Inhibition of [^{3}H]PDBu binding by magainin B. The [^{3}H]PDBu binding was measured with 1.7 μg/ml of type II PKC and the binding without magainin B was taken to be 100%.

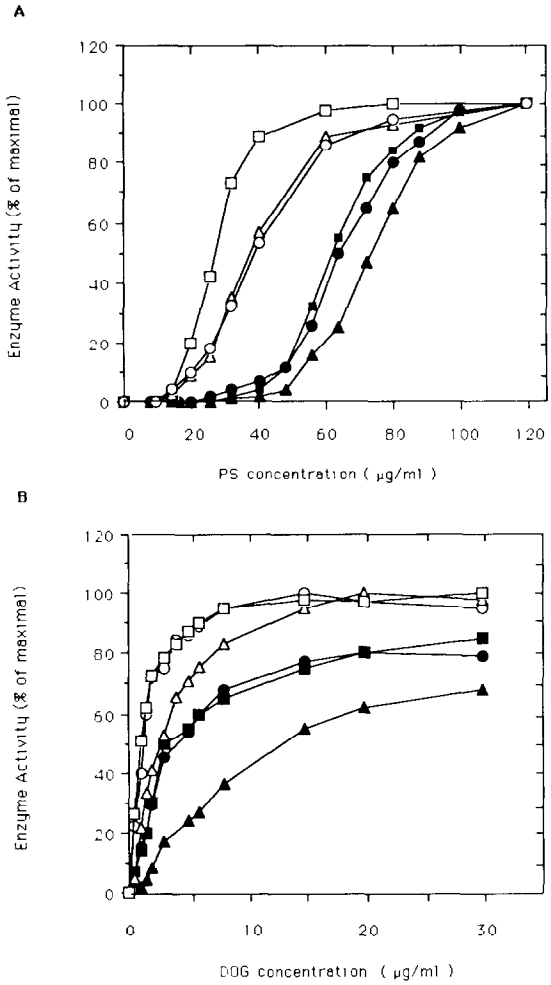

Fig. 4. Effect of magainin B on type I, II, and III PKC activities in the presence of varied concentrations of PS and DOG. PKC I (●,○), II (▲, △), and III (■, □) were assayed in the presence (●, ▲, ■) or absence (○, △, □) of magainin B ((A), 25 μM; and (B) 40 μM) as described in section 2 except for varying concentrations of PS and 20 μg/ml of DOG (A) or for varying concentrations of DOG and 100 μg/ml of PS (B). The highest activity of each isozymes measured in the absence of the magainin B was taken to be 100%.

The effects of PS and DOG on the inhibition of PKC by magainin B were investigated. In the presence of 20 μM of magainin B, the concentrations of PS required for half-maximal kinase activity, $A_{1/2}$, were 67.0 ± 4.74, 77.4 ± 4.67, and 64.4 ± 3.80 μg/ml for PKC I, II, and III, respectively, and those values for PKC I, II, and III in the absence of the peptide were 36.1 ± 1.52, 38.9 ± 0.76, and 24.3 ± 1.87 μg/ml, respectively (Fig. 4A). At PS concentrations less than 40 μg/ml (6.5 mol%), 20 μM of magainin B was sufficient to inhibit all three PKCs for more than 90%. Inhibition of all three PKCs by magainin B was completely reversed by increasing PS concentration. In the presence of 40 μM of magainin B, the $A_{1/2}$ of DOG for all three PKCs was increased by at least 3-fold (Fig. 4B). The $A_{1/2}$ of DOG in the presence of 40 μM of magainin B was 3.35 ± 0.31, 7.63 ± 0.83, and 3.19 ± 0.42 μg/ml for PKC I, II, and III, respectively. In comparison, the $A_{1/2}$ values for PKC I, II, and III in the absence of the peptide were 1.22 ± 0.09, 2.14 ± 0.23, and 1.0 ± 0.04 μg/ml, respectively. Thus, magainin B inhibits PKC by interfering with the interaction of the lipid cofactors with the regulatory domain.

4. DISCUSSION

The antimicrobial activities of magainins and their synthetic analogues appear to be related to their α-helical contents in helicogenic environments, such as cell membranes [3]. Phosphorylation of these com-

pounds by protein kinase may reduce the hydrophobicity and renders them less effective. This presumptive detoxification mechanism apparently is not functioning in the microbes, in which the PKC-like activity has not been identified. Since the naturally occurring magainin-1 and -2 have no inhibitory effect on PKC at concentrations up to 100 μM, we suspect that the effect of magainins on the mammalian cells is not mediated by PKC. However, the PKC-mediated phosphorylation of magainin-2, but not magainin-1, could reduce its hydrophobicity and membrane-disrupting effect on the mammalian cells. Thus, magainin-2 may be a more desirable therapeutic agent than magainin-1.

Magainin-2 is phosphorylated by PKC up to 0.5 mol/mol of the peptide. Since there is only one potential phosphorylation site of PKC, these results indicate that only 50% of the molecule is phosphorylated. This may be due to the existence of multiple conformers of this short chain peptide in the aqueous environment and only some of them are PKC substrates. Another explanation could be that the peptide is present as polymer, similar to melittin [14], resulting in steric hindrance at some of the phosphorylatable serine residue. In vivo, certain actions of PKC seem to result from the phosphorylation of ion pumps and ion channels [15]. It is of interest to determine the effect of phosphorylation of magainin-2 on the conductivity of lipid membrane. Introduction of a negatively charged phosphate group could electrostatically interfere with anion transport.

Magainin B, which has a greater propensity to α-helix formation than the parent peptide and cannot be phosphorylated by PKC, exhibits both antimicrobial and hemolytic activities. Several biologically active peptides which share similar amphiphilic features as magainin B, such as melittin [6], polymyxin B [7], defencins [8], and cytotoxins [16], have been shown to inhibit PKC. Some of these peptides also show antimicrobial activity [7,8]. Magainin B, at concentrations less than 10 μM, inhibits mainly PKC II without significant effct on PKC I and III. At this low concentration, magainin B, unlike melittin and mastoparans, does not seem to inhibit myosin light chain kinase. At higher concentrations, magainin B can also inhibit PKC I and III with IC_{50} near 20 μM as well as myosin light chain kinase with IC_{50} of greater than 60 μM. Inhibition of PKC by magainin B apparently results from interference with the binding of phospholipid and diacylglycerol (or phorbol ester) to the regulatory domains of the kinase. This interference could result from the interaction of magainin B with the lipid vesicles or alternatively a direct interaction of the peptide with the kinase. We noticed that incubation of magainin B with PKC in the absence of lipid caused an inactivation of the kinase as was observed for the acidic phospholipid-induced inactivation [17]. This finding supports the contention that magainin B also interacts directly with PKC and, thus, conferring the unique specificity of inhibition.

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