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INHIBITION OF PROTEIN KINASE C BY DEFENSINS, ANTIBIOTIC PEPTIDES FROM HUMAN NEUTROPHILS*

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Abstract—Defensins, human neutrophil peptide (HNP) antibiotics, potently inhibited phospholipid/Ca^{2+} protein kinase (protein kinase C, PKC) and phosphorylation of endogenous proteins from rat brains catalyzed by the enzyme. Of the three defensin peptides, HNP-2 appeared to be more potent than HNP-1 and HNP-3. Kinetic studies indicated that defensins inhibited PKC noncompetitively with respect to phosphatidylserine (a phospholipid cofactor), Ca^{2+} (an activator), ATP (a phosphoryl donor) and histone H1 (a substrate protein) with K_i values ranging from 1.2 to 1.7 μM. Defensins, unlike polymyxin B (another peptide inhibitor of PKC), did not inhibit the binding of [^{3}H]phorbol 12,13-dibutyrate to PKC; however, defensins, like polymyxin B, inhibited the PKC activity stimulated by 12-*O*-tetradecanoylphorbol-13-acetate. Defensins had little or no effect on myosin light chain kinase (a calmodulin/Ca^{2+}-dependent protein kinase) and the holoenzyme or catalytic subunit of cyclic AMP-dependent protein kinase, indicating a specificity of action of defensins. It is suggested that defensins, among the most potent peptide inhibitors of PKC so far identified, may have profound effects on functions of neutrophils and other mammalian cells, in addition to their well-recognized antimicrobial activities.

By participating in membrane-based signal transduction pathways, protein kinase C (PKC) is believed to play an important role in many neutrophil activities [1, 2]. PKC is known to be inhibited by several small cationic peptides, including the antibiotic polymyxin B [3], melittin [4] and cobra venom cytotoxin I [5]. Defensins, abundant in human neutrophils, are small (molecular weight < 3500) cationic peptides, that manifest prominent antimicrobial activity *in vitro* against many bacteria, fungi and viruses [6, 7]. They have been shown recently to have appreciable cytotoxic potential as well [8]. We now report that the human neutrophil peptide (HNP) antibiotics are potent inhibitors of PKC activity *in vitro*, and that the kinetic characteristics of this inhibitory effect differ from those of the previously reported inhibitors.

MATERIALS AND METHODS

Materials. Histone H1, 1,4-piperazinediethanesulfonic acid (Pipes), calmodulin and phosphatidylserine (PS, brain) were purchased from the Sigma Chemical Co. (St. Louis, MO); [^{3}H]phorbol-12,13-dibutyrate (PDBU) (15.8 Ci/mmol) was from New England Nuclear (Albany, MA); 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from the LC Service Corp. (Woburn, MA); and polymyxin B sulfate was from the Upjohn Co. (Kalamazoo, MI).

Methods. Human defensins (HNP-1, HNP-2 and HNP-3) were purified from human blood neutrophils as recently described by Ganz *et al.* [6]. The carboxyamidomethyl derivative of HNP-2 was prepared as previously described [7]. Low and high density granules of human blood neutrophils were prepared as reported recently by Rice *et al.* [9]. PKC was partially purified through the phenyl sepharose step [10]. The enzyme activity was assayed under standard conditions as described elsewhere [10, 11]. Briefly, the reaction mixture (0.2 ml) contained 5 μmol Pipes (pH 6.5), 2 μmol $MgCl_2$, 5 μg PS, 40 μg histone H1, 0.06 μmol ethylene glycol bis(β-aminoethyl-ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.6 nmol [γ-^{32}P]ATP (containing about 1.5×10^6 cpm), with or without 0.1 μmol $CaCl_2$, and various kinds and concentrations of inhibitors as indicated. When present, the final concentration of $CaCl_2$ was 200 μM (in excess of 300 μM EGTA). The reaction was carried out at 30° for 5 min. In experiments (Fig. 2B) that examined the specific stimulation of PKC activity by TPA, the standard assay conditions were modified to contain a low concentration of PS (2 μg/0.2 ml assay volume) and both EGTA and $CaCl_2$ were omitted. Myosin light chain kinase (MLCK) and myosin light chains were purified to homogeneity from rabbit skeletal muscle, and the enzyme activity was assayed using 1 mM ATP as described by Blumenthal and Stull [12]. Cyclic AMP-dependent protein kinase (APK) was partially purified from pig hearts [13], and the cata-

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lytic subunit of APK was purified to homogeneity from bovine hearts [14]; the enzyme activity was assayed using 5 μM ATP as reported previously [13]. Phosphorylation of endogenous proteins from rat brain extracts and subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiograph of the phosphoproteins were carried out as described elsewhere [15]. The binding of [^{3}H]PDBU to PKC was carried out by the method of Sharkey and Blumberg [16]. All experiments reported herein were incubated in triplicate and repeated two to five times to ascertain the reproducibility of the findings. [γ-^{32}P]ATP was prepared as described by Post and Sen [17], and protein was determined by the method of Bradford [18].

RESULTS

Protein kinase activity of neutrophil granules. Although the high density granule fractions (H1–5) from human neutrophils contained a low level of protein kinase activity, it was not stimulated by Ca^{2+} under the standard assay conditions for PKC (Table 1). The low density granule fractions (L1–2 and L3–8), in comparison, displayed greater levels of protein kinase activity but, again, they were not stimulated by Ca^{2+} and were thus distinguishable from PKC.

PKC inhibition by granules and defensins. Table 1 also shows that fractions H1–5 inhibited exogenous PKC more potently than did fractions L1–2 and L3–8. Because fractions H1–5 are especially rich in defensins [19], we examined the effects of purified human defensins on PKC. It was observed that each defensin peptide was indeed an effective inhibitor of the enzyme stimulated by PS and Ca^{2+} (Fig. 1), with a decreasing order of potency and estimated IC_{50} values (concentration causing 50% inhibition) of HNP-2 (1.3 μM) > unfractionated defensins (a 1:1:1 mixture of HNP-1, HNP-2 and HNP-3; 1.8 μM) > HNP-1 (2.7 μM) ≥ HNP-3 (2.9 μM). Each of the defensin peptides contains three intramolecular disulfides [7]. It is of interest to know the importance of these —S—S— bonds in their biological activities. We found that carboxyamidomethyl HNP-2, the alkylated product of the reduced peptide, was totally inactive (data not shown), indicating that intact disulfide linkages in HNP-2 were essential for its anti-PKC activity.

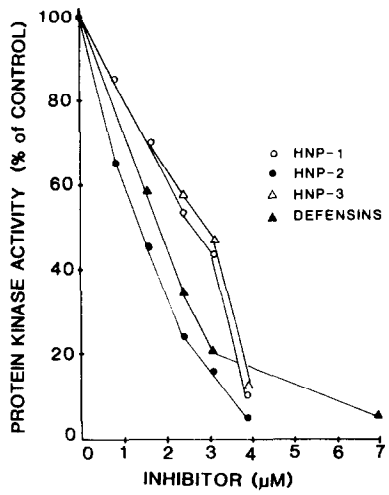

Fig. 1. Inhibition of PKC by HNP-1, HNP-2 or HNP-3, or by defensins (a 1:1:1 mixture of HNP-1, HNP-2 and HNP-3). The enzyme (0.5 μg) was assayed under the standard conditions containing a high concentration of PS (25 μg/ml) and $CaCl_2$ (200 μM in excess of 300 μM EGTA). The Ca^{2+}-stimulated enzyme activity in the absence of defensins (15 pmol P transferred/min) was taken as 100%. Defensins had no effect on the basal enzyme activity seen in the absence of added $CaCl_2$, which amounted to 5–7% of the total activity. For the reason of simplicity, the basal enzyme activity and lack of effect of defensins on the basal activity were not shown here and in subsequent figures where applicable.

We reported previously that polymyxin B, a polypeptide antibiotic of bacterial origin, is a potent and

Table 1. Protein kinase activity and PKC inhibitory activity in low and high density granule fractions isolated from human neutrophils

Addition	Protein kinase activity (pmol P transferred/min) Control	+$CaCl_2$ (200 μM)
PKC (0.5 μg)	0.6	23.9
H1–5 (4 μg)	1.4	1.1
L1–2 (4 μg)	7.2	7.2
L3–8 (4 μg)	6.6	6.3
PKC (0.5 μg) + H1–5 (8 μg)	3.7	4.9
PKC (0.5 μg) + H1–5 (16 μg)	2.2	2.3
PKC (0.5 μg) + L1–2 (8 μg)	8.2	18.6
PKC (0.5 μg) + L3–8 (8 μg)	12.1	21.3

The low density granules (L1–2 and L3–8) and high density granules (H1–5), fractionated from human neutrophils, were sonicated in 50 mM Tris–HCl, pH 7.5. Aliquots of the sonicated granules (0.02 to 0.04 ml, containing 4 or 8 μg protein) were assayed (in 0.2 ml) separately for PKC activity and the activity to inhibit exogenous PKC under the standard incubation conditions. Other experimental procedures were as described under Materials and Methods.

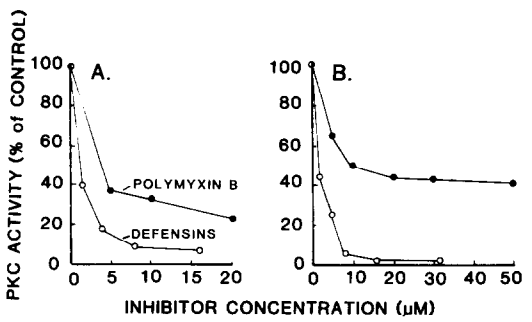

Fig. 2. Comparative inhibition of PKC by defensins and polymyxin B. (A) PKC (0.5 μg) was assayed under the standard conditions as given in Fig. 1. (B) PKC (0.5 μg) was assayed under modified conditions (without added $CaCl_2$ but with a low concentration of PS, 10 μg/ml) in the presence of 50 nM TPA. These modified conditions allowed a detection of maximal stimulation of the enzyme by TPA over and above that stimulated by PS. In (A) and (B), the enzyme activities, respectively, stimulated by Ca^{2+} in the presence of PS (12.7 pmol/min) and by TPA in the presence of PS (12.1 pmol/min), in the absence of the inhibitors, were taken as 100%.

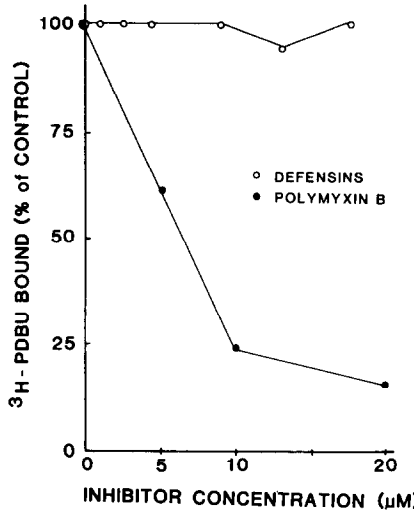

Fig. 3. Comparative effects of defensins and polymyxin B on [^{3}H]PDBU binding to PKC. The enzyme (5 μg) was incubated for 30 min at 37° in the presence of 8 μg/ml of PS, 1 μM $CaCl_2$ and 16 nM [^{3}H]PDBU (17, 323 cpm), with or without 200 μM non-radioactive PDBU, as described [20]. The non-specific binding was less than 10% of the total binding activity. The specific binding (74.8 pmol/mg enzyme preparation) seen in the absence of defensins or polymyxin B was taken as 100%.

specific inhibitor of PKC [3]. Under standard assay conditions (i.e. high concentrations of PS and $CaCl_2$), defensins were even more potent than polymyxin B in inhibiting PKC stimulated by PS/Ca^{2+}, with estimated IC_{50} values of 1.5 and 4.0 μM for defensins and polymyxin B respectively (Fig. 2A). Under modified assay conditions (i.e. with a low PS concentration and without added $CaCl_2$), defensins inhibited the TPA-stimulated enzyme activity with a similar potency (IC_{50} = 1.5 μM), whereas polymyxin B was less effective (IC_{50} = 10 μM) (Fig. 2B).

Phorbol ester binding studies. Whereas polymyxin B effectively inhibited binding of the radiolabeled phorbol ester, [^{3}H]PDBU, to PKC with an IC_{50} of 6.4 μM, defensins were without appreciable effect at a concentration as high as 18 μM (Fig. 3). These data strongly suggested that the inhibition mechanism of defensins was unique in that they did not appear to interact with the site(s) on PKC with which PS and/or phorbol ester directly or indirectly bind.

Inhibition kinetics. Inhibition of PKC by defensins was noncompetitive with respect to PS (Fig. 4A), $CaCl_2$ (Fig. 4B) or ATP (Fig. 5A). The K_i values (mean ± range, if applicable), calculated from the data given in Figs. 4 and 5, were 1.7 ± 0.2, 1.2 and 1.4 ± 0.3 μM respectively. Defensins also appeared to inhibit PKC noncompetitively with respect to histone H1 because the inhibition was not overcome by increasing concentrations of the substrate protein (Fig. 5B). In the presence of defensins, PKC activity was progressively inhibited at higher concentrations of the histone. In contrast, in the absence of defensins, the enzyme activity increased as a function of histone H1 concentration and reached a plateau around 80 μg/ml (Fig. 5B), indicating that defensins may cause an increased affinity of the enzyme for the substrate protein. Because of the unusual and complex kinetics, we were unable to determine the K_i values for defensins by double-reciprocal analysis based upon the data shown in Fig. 5B.

Phosphorylation of rat brain proteins. Histone H1 was used as an artificial substrate protein for assaying PKC in all experiments mentioned above. Therefore, the effects of defensins on phosphorylation of endogenous proteins in extracts of rat brains were examined for comparison (Fig. 6). It was found that defensins inhibited, in a dose-dependent manner, the PS/Ca^{2+}-dependent phosphorylation of several endogenous substrate proteins, notably the species having moelcular weights of 70,000, 46,000, 37,000, 20,000 and 16,000. These findings indicated that inhibition of PKC by defensins was characteristic of direct interactions between defensins with the enzyme and was not affected by the nature of the substrate proteins involved.

Other protein kinases. The specificity of the inhibitory effect of defensins was examined (Fig. 7). Defensins inhibited PKC with an IC_{50} of about 2 μM, a value similar to that seen above in Fig. 1. In comparison, defensins had little or no effect on skeletal muscle MLCK or cardiac APK and its catalytic subunit (Fig. 7), clearly indicating a specificity of action of defensins. It should be noted here that PS inhibited Ca^{2+}-dependent phosphorylation of doublet proteins having a molecular weight of about 16,000 in brain extracts, and that defensins further inhibited their phosphorylation (Fig. 6). The data suggested the presence of a protein kinase that is inhibited by PS or a phosphoprotein phosphatase that is activated by PS, or both. Because of the possibility that defensins could inhibit these enzymes leading to a decreased protein phosphorylation, the absolute specificity of the action of defensins remains to be investigated further.

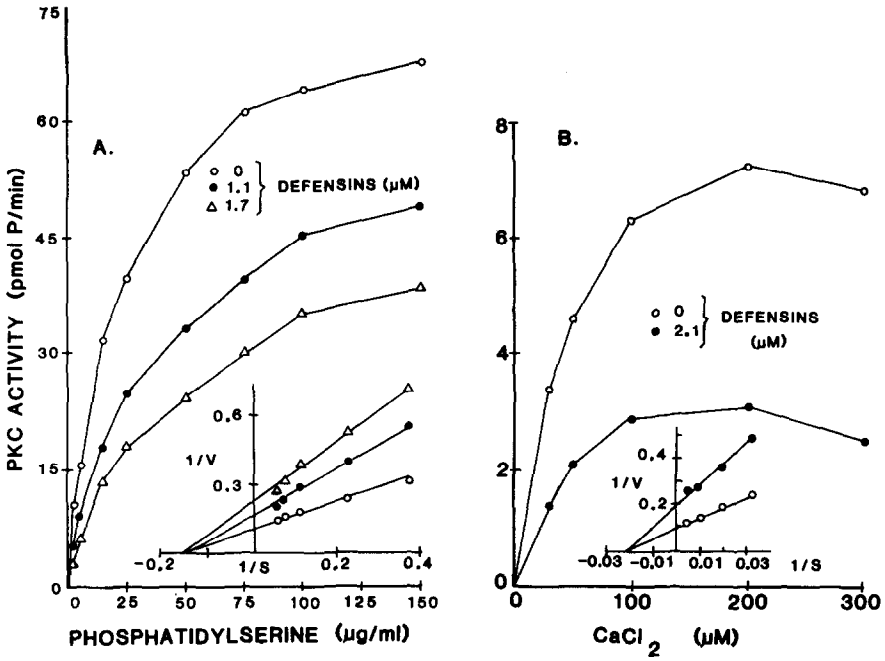

Fig. 4. Inhibition of PKC by defensins as a function of PS and $CaCl_2$ concentrations. (A) The enzyme (1.5 μg) was incubated under the standard conditions except for the various concentrations of PS, with or without defensins, as indicated. (B) The enzyme (0.3 μg) was incubated under the standard conditions except for the various concentrations of $CaCl_2$ (in excess of 200 μM EGTA), with or without defensins, as indicated. The double-reciprocal plots of the data are also shown (insets).

DISCUSSION

We found in the present studies that defensins were effective inhibitors of PKC. The inhibition potency (e.g. K_i of 1.7 μM with respect to PS) was similar to polymyxin B [3]; it was lower than cobra venom cytotoxin I [5], the most potent PKC inhibitor studied to date. Defensins were more potent than other peptides such as melittin [5] and synthetic myelin basic protein analogs [20], hydrophobic organic compounds such as the antipsychotic drug trifluoperazine [21], the antineoplastic agents alkyllysophospholipid [22] and tamoxifen [23], the isoquinoline sulfonamides [24, 25] or the simple inor-

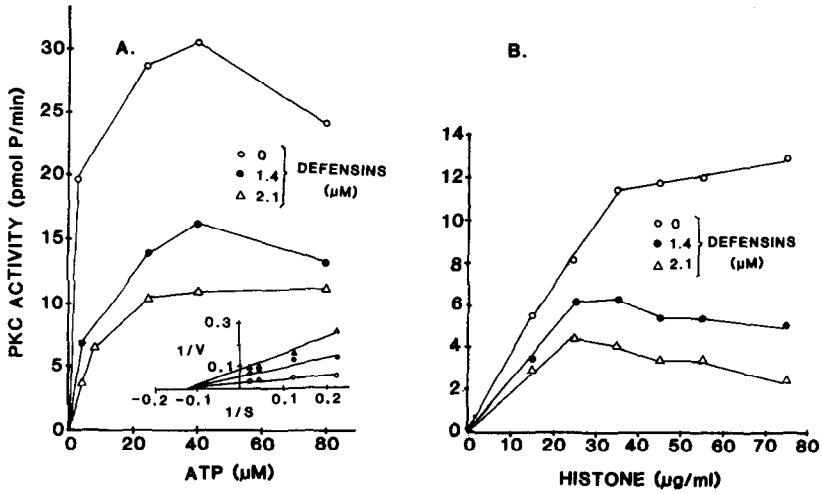

Fig. 5. Inhibition of PKC by defensins as a function of ATP and histone concentrations. (A) The enzyme (1.1 μg) was incubated under the standard conditions except for the various concentrations of ATP, with or without defensins, as indicated. The double-reciprocal plots of the data are also shown (inset). (B) The enzyme (0.5 μg) was assayed under the standard conditions except for the various concentrations of histone, with or without defensins, as indicated.

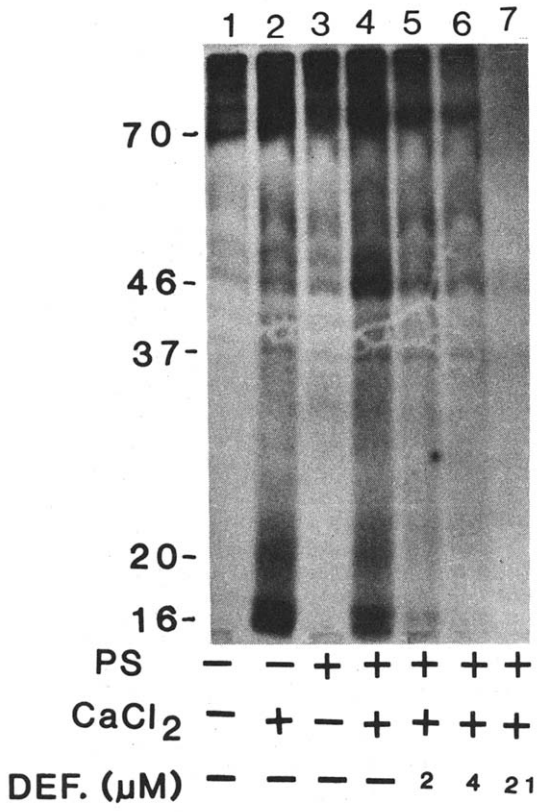

Fig. 6. Autoradiograph showing inhibition by defensins of phosphorylation of endogenous proteins in extracts of rat brains. The extracts (40 μg protein) were incubated in the presence or absence of PS (10 μg/ml), $CaCl_2$ (100 μM) or defensins (2, 4 and 21 μM), as indicated. The numbers indicate the molecular weights ($\times 10^{-3}$) of the phosphoproteins.

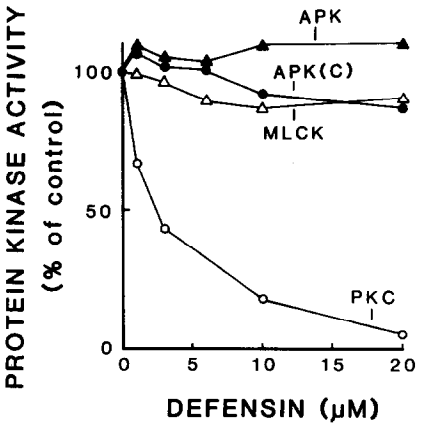

Fig. 7. Comparative effects of defensins on various protein kinases. The control activities (in the absence of added defensins), which were taken as 100%, for PKC, MLCK, APK holoenzyme, and APK catalytic subunit [APK(C)] were 8.5, 43.6, 14.1, and 3.1 pmol P/min respectively.

ganic compound selenium dioxide SeO_2 [26], all of which are inhibitors of PKC. The K_i values and types of inhibition for these agents are summarized and compared with defensins (Table 2). The K_i values for defensins were determined using a mixture (1:1:1) of HNP-1, HNP-2 and HNP-3. Because HNP-2 was at least twice as active as other components (Fig. 1), it, if determined individually, would have a K_i value similar to that for cobra cytotoxin I, and hence would be among the most potent inhibitors so far identified.

One interesting feature of defensin action concerns the type of inhibition. Defensins, along with SeO_2, were the only agents that inhibited PKC noncompetitively with respect to PS [for a review, see Ref. 27]. Polymyxin B, cytotoxin I and melittin all have hydrophilic and hydrophobic domains in their peptide molecules. That these peptides are surface-active, as well as amphipathic, presumably enables them to interact with the phospholipid-binding hydrophobic domain on PKC, inhibiting the enzyme competitively with respect to PS. The ability of some organic agents to inhibit PKC in a similar manner may be also, in part, due to their lipophilicity. Possibly, the noncompetitive nature of inhibition by defensins results from their lesser degree of functional hydrophobicity. The relative lack of hydrophobic interactions between defensins and PKC

Table 2. Summary of inhibition kinetics of PKC for defensins and some other inhibitors

Agent	K_i or IC_{50} values (μM) and types of inhibition with respect to: PS	Ca^{2+}	ATP	Reference
Defensins	1.7 (NC)*	1.2 (NC)	1.4 (NC)	This paper
Polymyxin B	1.8 (C)			3
Cobra cytotoxin I	0.8 (C)			5
Melittin	4.5 (C)	4.5 (NC)	1.4 (NC)	4
Synthetic myelin basic protein peptide analog	<50 (ND)			20
Trifluoperazine	14.0 (C)	9.0 (NC)		21
Alkyllysophospholipid	6.3 (C)			22
Tamoxifen	16.0 (C)	14.0 (NC)		23
Selenium dioxide (SeO_2)	60.0 (NC)	68.0 (NC)		26

* NC, noncompetitive; C, competitive; ND, not determined.

could also explain the observation that they failed to inhibit binding of radiolabeled phorbol ester to the enzyme (Fig. 3). Defensins also inhibited PKC non-competitively with respect to Ca^{2+}, ATP and possibly substrate protein (histone), suggesting that HNP antibiotics may not interact with the binding or active sites for these reactants on the enzyme. It seems that defensins may inhibit PKC by interacting with some undefined sites on the enzyme. The mechanism of action of defensins is intriguing and warrants further investigation.

Another interesting feature of the action of defensins is their ability to specifically inhibit PKC (Fig. 7). Polymyxin B [3] and cobra cytotoxin I [5] are two other PKC-specific inhibitors; all other inhibitors studied to date, including those mentioned above, are non-specific in that they inhibit both PKC (phospholipid/Ca^{2+}-dependent enzyme) and MLCK (calmodulin/Ca^{2+}-dependent enzyme) with similar potencies [27].

It remains to be determined whether the ability of defensins to inhibit PKC or other phospholipid-dependent systems is related in part to their roles as broad-spectrum antimicrobial or cytotoxic agents. Finally, because of a central role played by PKC in leukocyte function, defensins, by potently inhibiting the enzyme, could potentially modulate not only the functions of neutrophils but also a host of activities fundamental to other cell types including transduction of membrane signals, metabolism, growth and differentiation.

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