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Membrane interactions of mastoparan analogues related to their differential effects on protein kinase C, Na,K-ATPase and HL60 cells

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Membrane interactions of tetradecapeptide toxin mastoparan (MP) and analogues (MP-3, MP-X and polistes MP), as indicated by inhibition of various enzymatic and cellular activities, were investigated. MP-3 was found to be the least active in inhibiting protein kinase C (PKC; activated by phosphatidylserine vesicles, synaptosomal membranes or phorbol ester), synaptosomal membrane Na,K-ATPase and proliferation and viability of leukemia HL60 cells. MP-3, however, was as active as others in inhibiting PKC activated by arachidonate monomers and phorbol ester binding. The unique properties of MP-3, the [des-Ile^1-Asn^2]- analogue of MP, might be related to its low functional amphiphilicity compared to others and useful in further delineating biological activities associated with or regulated by membranes.

Mastoparan and analogues; Protein kinase C; Na,K-ATPase; HL60 cell

1. INTRODUCTION

MP, an amphiphilic tetradecapeptide, is a wasp venom toxin shown to possess a variety of biological activities, such as activation of phospholipase A_2 [1,2] and phospholipase C [3,4], degranulation of mast cells and release of histamine [1], binding to calmodulin [5] and troponin C [6], and inhibition of calmodulin-dependent enzymes [7,8]. More recently, MP has also been reported to catalyze nucleotide exchange on G proteins in a manner similar to that of receptors [9] and, like other membrane-active polypeptides, inhibit PKC, Na,K-ATPase (Na pump) and TPA-induced HL60 cell differentiation [8]. Structure–activity relationship studies [10], with the use of various synthetic MP analogues, indicate that hydrophobicity or hydrophobic moment of the peptides is related to their potency or ability to stimulate GTPase of G proteins, although relative contribution of the two parameters is unclear. In the present studies, we examined effects of MP and some analogues on PKC (activated by various lipid cofactors), synaptosomal membrane Na,K-ATPase and HL60 cells in order to gain some insights into functional interactions of these amphiphilic polypeptides with membranes. We observed that the toxin analogues differentially modified several biochemical and cellular parameters.

Abbreviations: MP, mastoparan; P-MP, polistes mastoparan; TPA, 12-*O*-tetradecanoylphorbol-13-*O*-acetate; PDBu, phorbol 12,13-dibutyrate; PS, phosphatidylserine; PKC, protein kinase C; PKM, protein kinase M; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine; IC_{50}, concentration causing 50% inhibition.

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2. MATERIALS AND METHODS

2.1. *Materials*

MP and its analogues were purchased from Peninsular Laboratories (Belmont, CA); TPA was from LC Services (Wolburn, MA); [^{3}H]thymidine and [γ-^{32}P]ATP were from ICN Radiochemicals (Irvine, CA); human leukemia cell line HL60 was from American Type Culture Collection (Rockville, MD); media and supplies for cell culture were from Gibco (Grand Island, NY); PS, arachidonate, histone HI (Type III-S) and ouabain were from Sigma Chemical Co. (St. Louis, MO).

2.2. *Enzyme preparations and assays*

PKC was purified from pig brain extracts as described [11]; the enzyme preparation was devoid of other contaminating protein kinase activities. Type I (γ), II (β) and III (α) isoforms of PKC were prepared by hydroxyapatite chromatography as described by Huang et al. [12]. PKC was assayed under the standard conditions [8,11]. Briefly, the reaction mixtures (0.2 ml) contained 5 μmol of Tris-HCl (pH 7.5), 2 μmol of $MgCl_2$, 2 μg of sonicated PS, 40 μg of histone HI, either 0.8 μmol of EGTA or 0.06 μmol of $CaCl_2$ (300 μM final concentration), 1 nmol of [γ-^{32}P]ATP (containing about 1×10^6 cpm), and appropriate amount of the enzyme. The reaction, started with [γ-^{32}P]ATP, was carried out for 5 min at 30°C. PKC was also assayed under modified conditions in which arachidonate (125 μM), or rat brain synaptosomal membrane (2 μg protein/0.2 ml), instead of PS, were used to activate the enzyme in the presence of 300 μM $CaCl_2$. In some experiments, PKC was assayed under a suboptimal condition in the presence of PS (2 μg/0.2 ml) and $CaCl_2$ (20 μM) in order to maximize the net enzyme activity that was specifically stimulated by 32 nM TPA. PKM was prepared by tryptic digestion of PKC and its PS/Ca^{2+}-independent activity was assayed as described [13]. Na,K-ATPase was purified from rat cerebral cortex synaptosomes by extraction with sodium dodecyl sulfate and discontinuous sucrose density centrifugation, as described [14–16]. Mg-ATPase activity in this membrane preparation

(which was also used in PKC activation assay as an alternative phospholipid cofactor as mentioned above) was less than 4% of the total ATPase activity. Na,K-ATPase activity was assayed as described [16], using 3 mM [γ-^{32}P]ATP (containing 2–8×10^5 cpm) to start the reaction. The activity of all enzymes studied were linear as a function of incubation time and enzyme amount under the experimental conditions. All experiments concerning effects of the agents on the enzymes and HL60 cells (see below) were repeated two to four times to ascertain reproducibility of the findings reported herein.

2.3. *Other methods*

Bovine cardiac TnI and TnT, purified as reported recently [17], were kindly provided by Dr. Thomas A. Noland of this laboratory. [^{3}H]PDBu binding to PKC was carried out as reported [18]. HL60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum. Cells at the mid-log phase were used in all experiments. [^{3}H]Thymidine uptake by the cells was determined as reported [18].

3. RESULTS

The structures of tetradecapeptide toxin MP and its analogues are shown (Table I). MP-3 is [des-Ile^1-Asn^2]MP whereas MP-X and P-MP, compared to MP, have different sequences in 5 and 12 amino acid residues, respectively. The hydrophobicities (Hϕ) of the polypeptides shown in Table I were calculated using values of Kytes and Doolittle [19] without correction of free N-terminal groups.

Because PKC isozymes α, β and γ were inhibited, with similar potencies, by MP (data not shown) and by other PKC inhibitors [8], the present PKC preparation, which had not been resolved into the individual isozymes and presumably a mixture of all isoforms originally present in the pig brain extract, was used in all studies reported herein. The concentration-dependent inhibition by MP and its analogues of PKC activated by PS vesicles (Fig. 1A) or by rat brain synaptosomal membranes (Fig. 1B) showed that MP-3 was appreciably less potent than others (MP, MP-X and P-MP). Such a difference in the inhibitory potency, however, was not observed when the enzyme was activated by arachidonate monomers (Fig. 1C). The dose-dependent inhibition of PS/Ca^{2+}-dependent PKC activity by ET-18-OCH_3, an anticancer agent [8,20] was also included for comparison (Fig. 1A). The IC_{50} values estimated from these experiments were summarized (Table II). Histone H1, a highly basic and cationic protein, was

Table I

Structures and average hydropathy (Hø) of mastoparan and analogues

Peptide toxin	Amino acid sequence	Hø
Mastoparan (MP)	INLKALAALAKKIL	1.16
Mastoparan 3 (MP-3)	------------	1.27
Mastoparan X (MP-X)	--W-GI--M---L-	0.53
Polistes mastoparan (P-MP)	VDW-KIGQHILSV-	0.35

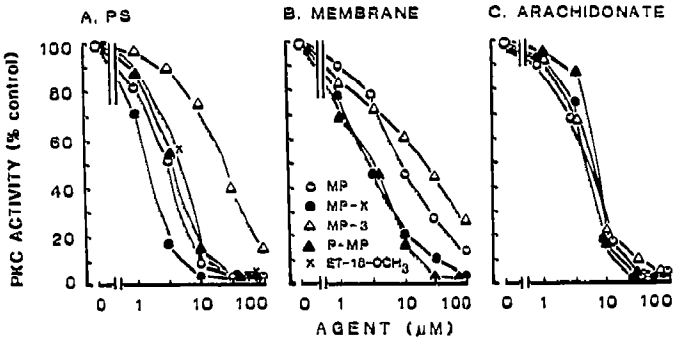

Fig. 1. Inhibition of PKC by MP analogues and ET-18-OCH_3. The enzyme was activated, in the presence of 200 μM $CaCl_2$, by (A) PS vesicles (10 μg/ml), (B) rat brain synaptosomal membrane (10 μg protein /ml) and (C) arachidonate monomers (125 μM). The control activity values obtained in the absence of the inhibitors for (A), (B) and (C) were 13.8, 11.9 and 14.4 pmol/min, respectively, which were taken as 100%. In this and subsequent figures, all data points presented were averages of duplicate incubations, with assay errors being less than 5%; the results were confirmed in 2–4 other separate experiments.

used as a standard substrate protein for PKC throughout the present studies. In order to ascertain that the findings reflected the true mode of inhibition of the toxins, bovine cardiac TnI (pI = 9.5) and TnT (pI = 7.3) were used as substrates instead of histone H1 in parallel experiments in which PKC was activated under standard assay condition in the presence of PS and Ca^{2+} (figures not shown). The IC_{50} values for MP and its analogues, also compared in Table II, clearly indicated that the peptide toxins had similar order of inhibitory potency regardless of the kinds of substrate proteins used. TnI and TnT have been shown previously to be excellent PKC substrates in various contractile protein preparations [17,21]. It was also noted that MP-3 was less potent than MP in inhibiting TPA-stimulated PKC activity (Table II).

Effects of MP and analogues on [^{3}H]PDBu binding to PKC (in the presence of 2 μg/0.25 ml of PS and 20 μM $CaCl_2$) were examined. It was observed, unexpectedly, that all agents inhibited the binding with similar IC_{50} values of 2–3 μM (Fig. 2; Table II), despite the different potency of MP (IC_{50} = 5 μM) and MP-3 (IC_{50} = 40 μM) in inhibiting TPA-stimulated PKC activity shown above in Table II. ET-18-OCH_3 was much less potent inhibitor for [^{3}H]PDBu binding than MP and analogues (Fig. 2), consistent with its low potency in inhibiting TPA-stimulated PKC activity (Table II).

We reported previously that MP and other amphiphilic protein toxins inhibited PKC competitively with respect to PS [8], indicating that they interacted with sites on the hydrophobic domain of the enzyme which were also shared by PS. Consistent with the above notion, we observed that MP and MP-3 were practically without effect on the activity of PKM, the PS/Ca^{2+}-independent catalytic fragment of PKC, with an estimated IC_{50} value of >200 μM for both (Table II).

Next, we examined effects of MP and analogues on Na,K-ATPase activity in the rat brain synaptosomal membrane in order to further illustrate a functional consequence of their interactions with a biomembrane. The same membrane preparation was used in the present (Fig. 1B) and previous studies [8] in PKC activation. Ouabain potently inhibited the activity, with an IC_{50} of about 1 μM; it inhibited about 80% of the activity at 100 μM, indicating that about 20% of the ATPase activity was ouabain-resistant (Fig. 3). Although less potent than ouabain and ET-18-OCH_3, MP and its analogues inhibited Na,K-ATPase activity in the biomembrane, with MP-3, again, being the least effective (Fig. 3; Table II).

Finally, membrane effects of MP and its analogues were examined using intact HL60 cells. MP-3 was practically inactive at the highest concentration (50 μM) tested, P-MP was moderately active, and MP and MP-X were most active in inhibiting proliferation (thymidine uptake) and in causing loss of viability of the leukemia cells (Table II). MP and its analogues consistently had higher IC_{50} values for causing viability loss than for inhibition of thymidine incorporation, suggesting that cell death due to cytotoxicity of the peptides might be secondary to impaired proliferation.

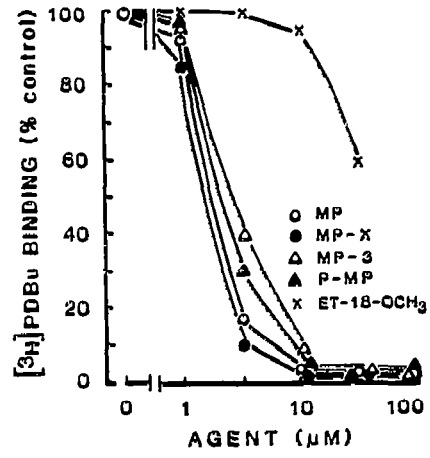

Fig. 2. Inhibition of [^{3}H]PDBu binding to PKC by MP analogues and ET-18-OCH_3. The enzyme was incubated for 30 min at 37°C in the presence of PS (2 μg/0.25 ml), $CaCl_2$ (20 μM), [^{3}H]PDBu (32 nM, 17,000 cpm), with or without non-radioactive PDBu (200 μM), and varying concentrations of the inhibitors, as indicated. The non-specific binding was less than 10% of the total binding. The specific binding (70 pmol/mg protein) obtained in the absence of the inhibitors was taken as 100%.

4. DISCUSSION

The present studies showed that MP and analogues regulated PKC and other biological activities via complex and yet discrete interactions with membranes. Two points seem worth noting in this respect. First, MP-3, among the MP analogues, was the weakest inhibitor of PKC when it was activated by membranes (PS vesicles or synaptosomal membranes), but it was as effective as other inhibitors when the enzyme was activated by arachidonate monomers (see Table II for summary). Although each of these three lipid preparations activated PKC to a similar extent in the presence of Ca^{2+} (Fig. 1), the physical interactions between the hydrophobic, regulatory domain of PKC and the phospholipid membranes (PS and bio-membrane) would be distinct from those with the fatty acid (arachidonate) monomers. Accordingly, interactions of the fatty acid with PKC could be relatively simplistic and could not afford complex, spatial arrangements that would serve as determinants for specificity of inhibition. Strynadka and James [22], using MP and melittin as models, suggested that binding of the amphiphilic helical peptides to hydrophobic

Table II

Summary of IC_{50} values for MP and analogues of various parameters (taken from Figs. 1-3 and other experiments)

	IC_{50} (μM)				
Parameter	MP	MP-3	MP-X	P-MP	ET-18-OCH_3
PKC					
Activated by PS	3 (8)[a](2)[b]	25 (30)[a](25)[b]	2 (3)[a](2)[b]	3 (9)[a](4)[b]	6
Activated by synaptosomal membrane	9	20	3	3	12[c]
Activated by arachidonate	5	6	6	8	16[c]
Activated by TPA	5	40			100
[^{3}H]PDBu binding	2	3	2	2	50
PKM	>200[d]	>200[d]			
Synaptosomal Na,K-ATPase	60	~200	40	80	16
HL60 cells					
[^{3}H]Thymidine uptake	8	~50	7	16	10
Viability	10	~200	10	25	12

[a] PKC was assayed using bovine casdiac TnI (instead of histone H1) as substrate.
[b] PKC was assayed using bovine casdiac TnT (instead of histone H1) as substrate.
[c] Taken from [8].
[d] PKM was inhibited only about 10% at the highest concentration (100 μM) tested.

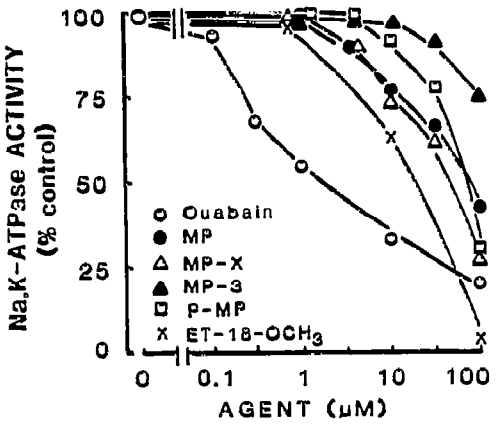

Fig. 3. Inhibition of rat brain synaptosomal membrane Na,K-ATPase by ouabain, ET-18-OCH_3 and MP analogues. The membrane preparation (10 μg protein/ml) was incubated for 30 min at 37°C with various concentrations of the inhibitors, as indicated. The standard reaction mixture contained 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA and 3 mM [γ-^{32}P]ATP (2×10^5 cpm). The control activity obtained in the absence of the inhibitors (9 μmol P released/min/mg protein) was taken as 100%.

cleft of troponin C or calmodulin requires not only hydrophobic but also electrostatic interactions. MP-3 is [des-Ile^1-Asn^2]MP, which lacks isoleucin-1 and asparagine-2 residues at the N-terminal of MP. Based upon helical wheel analysis [23], MP, MP-X and P-MP all have greater amphiphilicity than MP-3. It is likely, therefore, that MP-3 could not interact as effectively as other MP analogues with hydrophobic cleft or domain on PKC unmasked by phospholipid membranes and Ca^{2+}, hence MP-3 was the weakest PKC inhibitor among MP analogues. The differential activity potency of MP analogues, MP-3 being the least active, was also evident in the inhibition of synaptosomal Na,K-ATPase and HL60 cell proliferation and viability (Table II), where biomembranes are involved. It was noted that alkllysophospholipid ET-18-OCH_3 inhibited PKC with a similar potency regardless of the kind of lipid cofactors being used to activate the enzyme (Table II), suggesting lack of rigid structural requirement, relative to peptide inhibitors, for its interaction with hydrophobic cleft (domain) of PKC.

The second point to be discussed concerns the observation that, while being as active as MP and other analogues in inhibiting [^{3}H]PDBu binding to PKC/PS/Ca^{2+} complex, MP-3 was much less active than MP in inhibiting TPA-stimulated PKC (Table II). One possible explanation for this discrepancy is that MP-3, the [des Ile^1-Asn^2] analogue, was able to selectively inhibit the binding more potently than the activation process subsequent to binding, consistent with the observation that MP-3 was also much less potent than MP in inhibiting PKC activity stimulated by PS/Ca^{2+} (Table II). Clearly, hydrophobic interactions of PKC with PS/Ca^{2+} was required for action of MP-3 as in the case of MP, because both peptides were practically inactive toward PKM, the catalytic domain of PKC devoid of the regulatory, hydrophobic domain (Table II). Huang et al. [13] reported that phorbol ester binding and activation of PKC correlated well for the isozyme type I (γ), but for isozymes type II (β) and III (α) its maximal binding failed to maximally activate the enzyme. The findings suggested that phorbol ester binding-activation is not necessary tightly coupled, lending support to the notion that the two events, although interrelated, could be differentiated by certain agents such as MP-3. In this respect, it is of interest that defensins, the cationic peptide antibiotics produced by human neutrophils, potently inhibited PKC activated by PS/Ca^{2+} or by TPA (IC_{50}, 1–2 μM), but was inactive in inhibiting [^{3}H]PDBu binding to PKC (IC_{50}, >50 μM) [24]. In most cases, however, the peptide PKC inhibitors, as ET-18-OCH_3, inhibited equipotently both phorbol ester binding and activation of PKC; [8].

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