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Effects of mastoparan B and its analogs on the phospholipase D activity in L1210 cells

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Abstract Mastoparan B (MP-B), an amphiphilic α-helical peptide isolated from hornet venom, and its Ala-substituted analogs were examined for their effectiveness on phospholipase D (PLD) activity in L1210 cells. PLD activity was determined by measuring phosphatidylethanol produced from [^{3}H]myristate-labelled cells in the presence of ethanol. PLD activity was stimulated by MP-B, 4MP-B (Lys^4 → Ala), and 12MP-B (Lys^{12} → Ala), but not by 3MP-B (Leu^3 → Ala) and 9MP-B (Trp^9 → Ala). Other MPs including mastoparan 7 also stimulated the PLD activity, but inactive mastoparan 17 did not. The stimulatory effect of various MP analogs could be correlated with their α-helical contents. The PLD activity stimulated by MP-B was not affected by G-protein blocking chemicals. The extent of PLD stimulation by various MP-Bs, as well as by digitonin and β-escin, correlated with the permeability of the membrane to ethidium bromide. These results suggest that the stimulation of PLD activity by MP-B in L1210 cells is probably coupled with membrane perturbation brought about by the peptide.

Key words: Mastoparan B; Phospholipase D; L1210 cell

1. Introduction

Mastoparan B (MP-B), a cationic tetradecapeptide isolated from the venom of hornet *Vespa basalis* [1], is a homolog of mastoparans (MPs) found in various wasps. MPs exhibit a wide variety of biological effects such as stimulation of histamine release from mast cells, erythrocyte lysis, and antimicrobial activity against bacteria [1–6]. They also affect the activity of phospholipase A_2 [7], C [8], and D [9] and induce the mitochondria permeability transition [10] and cytosolic Ca^{2+} elevation [11]. All these effects have been related to the activation of G-proteins [12–14] and/or induction of membrane pore formation [11,15]. However, the mechanism of MP action remains elusive as more diverse cellular functions are observed.

Various natural and synthetic MP analogs have been developed to study the structure-activity relationship. For example, mastoparan 7 (Mas-7) and mastoparan 17 (Mas-17) are used as an active MP and an inactive MP, respectively [10–14]. Mas-7 has been obtained by substituting Ala for the positive Lys in position 12 and Mas-17 by substituting Lys for the neutral Leu in position 6 [12]. Similarly, Park et al. synthesized a series of Ala-substituted MP-B analogs and studied their interactions with phospholipid bilayers [16,17]. MP-B adopts an amphiphilic α-helical structure, hydrophobic residues providing a nonpolar environment on one side and hydrophilic residues a polar environment on the other side [18]. When residues with hydrophobic side groups (Leu-1, Leu-3, Ile-6, Val-7, Trp-9, Val-13, Leu-14) in the MP-B analogs were substituted with Ala, the hemolytic and antimicrobial activities decreased significantly along with the decreased α-helical contents [16]. On the other hand, when Ala was substituted for hydrophilic residues (Lys-2, Lys-4, Ser-5, Ser-8, Lys-11, Lys-12), the α-helical contents and the biological effects increased substantially [17]. The opposite properties of these series of MP-B analogs suggest that these series could be used to investigate the structure-activity relationship of MP toxins on a broad spectrum of biological processes.

Recently there were several reports on the effects of MP on phospholipase D (PLD) activities in some cell lines. One claimed inhibitory effect in HL60 cells [19], while others reported stimulatory effects in human astrocytoma cells [9] and in green alga *Chlamydomonas eugametos* [20]. PLD catalyzes hydrolysis of phosphatidylcholine to produce phosphatidic acid, a lipid mediator, and the action of PLD is regulated by a variety of hormones, growth factors, and other extracellular signals [21]. Thus the PLD associated with membrane has emerged as one of the major signal-activated phospholipases [22]. In an effort to understand the action of MP, we investigated the effects of MP-B analogs on PLD activity in lymphocytic mouse leukemia L1210 cells. The PLD in L1210 cells has shown to be unique in that it is activated by oleate [23]. In this article, we present results which show that the extent of stimulation of the PLD in L1210 cells by MP-B analogs can be correlated with their capability of membrane perturbation.

2. Materials and methods

2.1. Materials

[9,10-^{3}H(*N*)]myristic acid and [5,6,8,9,11,12,14,15-^{3}H(*N*)]arachidonic acid were purchased from Dupont NEN. Synthesized Mas-7 and Mas-17 were obtained from Peninsula Laboratories (Belmont, CA). Mastoparans (*Vepula lewisii*, M-5280; *Polistes jadwagae*, M-3545), mast cell degranulating peptide HR1, amyloid β-protein (25–35), pertussis toxin, benzalkonium chloride, and β-escin were from Sigma. Daunomycin was obtained from Calbiochem and digitonin was from Merck. RPMI 1640 medium and fetal bovine serum were from GibcoBRL. MP-B and its analogs were synthesized and purified as the method described by Park et al. [16,17]. Purities of synthesized MP-Bs were further characterized by a JASCO HPLC amino acid analysis system and molecular weights were determined by fast atom bombardment mass spectra using a JEOL SX-102A. Indolicidin

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Abbreviations: EtBr, ethidium bromide; G-proteins, guanine nucleotide binding proteins; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; MP-B, mastoparan B; PEt, phosphatidylethanol; PLA_2, phospholipase A_2; PLD, phospholipase D; TES, *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid

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was supplied by Dr. J.S. Park (Seoul National University). PEt was prepared from phosphatidylcholine using cabbage PLD according to the published procedure [24].

2.2. Cell culture and prelabelling

L1210 cells were grown in HEPES-buffered RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and subcultured every 2–3 days so that the cells density did not exceed 1–2×10^6/ml. Cells were labelled with [^{3}H]myristic acid (1.5 μCi/ml, for the measurement of PLD activity) or [^{3}H]arachidonic acid (0.8 μCi/ml, for the measurement of PLA_2 activity) for 3 h according to the method described by Lee et al. [23].

2.3. Assay of PLD and PLA_2 activities

PLD activity in intact L1210 cells was assayed as described previously [23]. Harvested ^{3}H-labelled cells were washed twice with phosphate-buffered saline and were resuspended in an assay medium containing 20 mM HEPES (pH 7.2), 137 mM NaCl, 2.7 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM EGTA, and 1 mg/ml BSA. The final assay mixtures (200 μl) containing 1.5% ethanol with various agents as indicated were incubated for 20 min at 37°C. The reaction was terminated by addition of 2 ml of chloroform/methanol/1 N HCl (100:50:3) and [^{3}H]PEt was separated on a TLC plate using ethylacetate/iso-octane/acetic acid/water (13:2:3:10) solvent system. PLA_2 activity in intact L1210 cells was assayed as above except that, for separation of [^{3}H]arachidonic acid, authentic arachidonic acid was added and heptane/diethylether/acetic acid (60:40:2) solvent system was used. The presented data were normalized to a total radioactivity of 2×10^5 cpm. The counting efficiency of measured radioactivity was 52%. Assays were carried out in duplicate and each set of experiments was repeated 3 to 4 times.

2.4. Measurement of membrane permeability

Membrane permeability was measured with fluorescent dye ethidium bromide (EtBr) as described previously [11]. An aliquot of cells (5×10^5/ml) was resuspended in 1.2 ml of the assay medium containing 25 μM EtBr. After addition of indicated chemicals, the cell suspensions were incubated for 10 min at 37°C and the EtBr influx was determined by measuring changes in fluorescence intensity. The excitation wavelength was 360 nm and the emission wavelength was 580 nm.

3. Results

3.1. Differential stimulation of PLD by MP-B analogs

Fig. 1A shows that different MP-B analogs (Table 1) exhibit different stimulatory effects on the PLD activity in intact L1210 cells. MP-B, 4MP-B, and 12MP-B stimulated the PLD activity, whereas 3MP-B and 9MP-B showed no stimulation. 12MP-B was most effective and enhanced the PLD activity 6-fold at 25 μM concentration. MP-B and 4MP-B increased the PLD activity approximately 4-fold at the 25 to 50 μM concentration range. This all or none stimulatory behavior of MP-B analogs was further observed with other MP homologs (Fig. 1B). Mas-7, MP (*V. lewisii*), and MP (*P. jadwagae*) increased the PLD activity, but Mas-17 did not show any stimulatory effect. Mas-7 seemed to be more potent than 12MP-B, and a maximum enhancement of 15-fold was observed. The natural MPs, MP (*V. lewisii*) and MP (*P. jadwagae*), obtained from corresponding wasp venoms, exerted similar effects at 25 μM concentration, but at 50 μM the effect of MP (*V. lewisii*) surpassed that of MP (*P. jadwagae*).

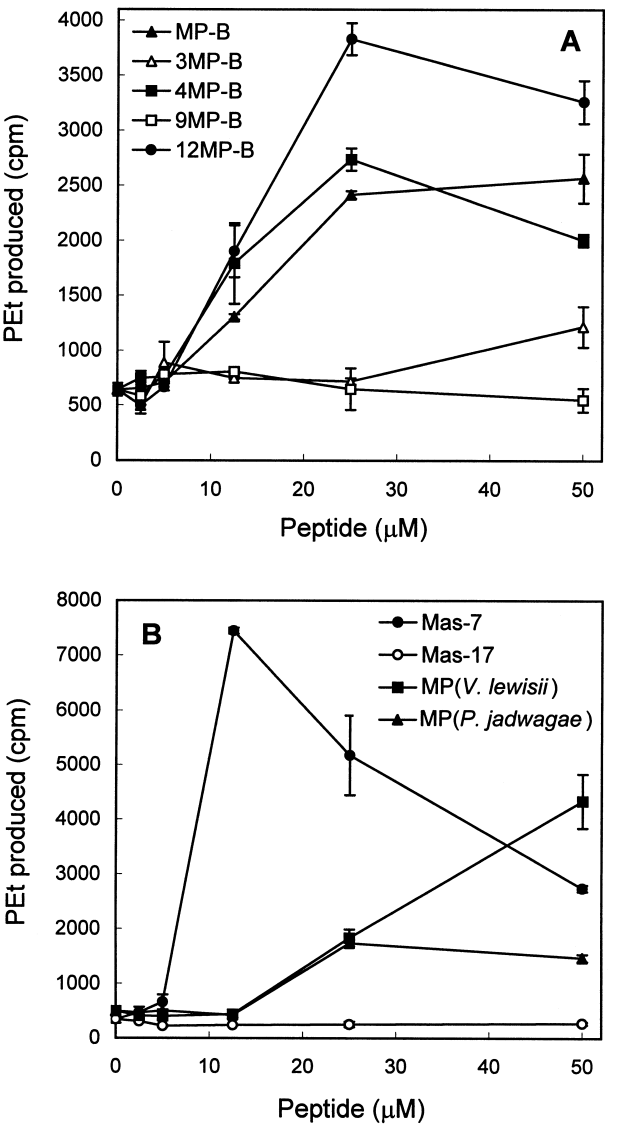

Fig. 1. Effects of various MPs on the PLD activity in L1210 cells. [^{3}H]myristate-labelled L1210 cells were incubated for 20 min with (A) MP-B and its analogs; (B) MPs and its analogs, Mas-7 and Mas-17 as indicated. The radioactivity of [^{3}H]PEt produced was normalized to a total labelled radioactivity of 2×10^5 cpm. Mean of 3–4 determinations and bars indicate the standard deviation.

Other related amphiphilic peptides such as mast cell degranulating peptide HR1 [25], amyloid β-protein (25–35) [26], indolicidin [27], and apidaecin Ib [28] were also examined for

Table 1
Amino acid sequence of MP-B and its analogs

Peptide	Position													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MP-B (*V. basalis*)	Leu	Lys	Leu	Lys	Ser	Ile	Val	Ser	Trp	Ala	Lys	Lys	Val	Leu NH_2
3MP-B	Leu	Lys	*Ala*	Lys	Ser	Ile	Val	Ser	Trp	Ala	Lys	Lys	Val	Leu NH_2
4MP-B	Leu	Lys	Leu	*Ala*	Ser	Ile	Val	Ser	Trp	Ala	Lys	Lys	Val	Leu NH_2
9MP-B	Leu	Lys	Leu	Lys	Ser	Ile	Val	Ser	*Ala*	Ala	Lys	Lys	Val	Leu NH_2
12MP-B	Leu	Lys	Leu	Lys	Ser	Ile	Val	Ser	Trp	Ala	Lys	*Ala*	Val	Leu NH_2

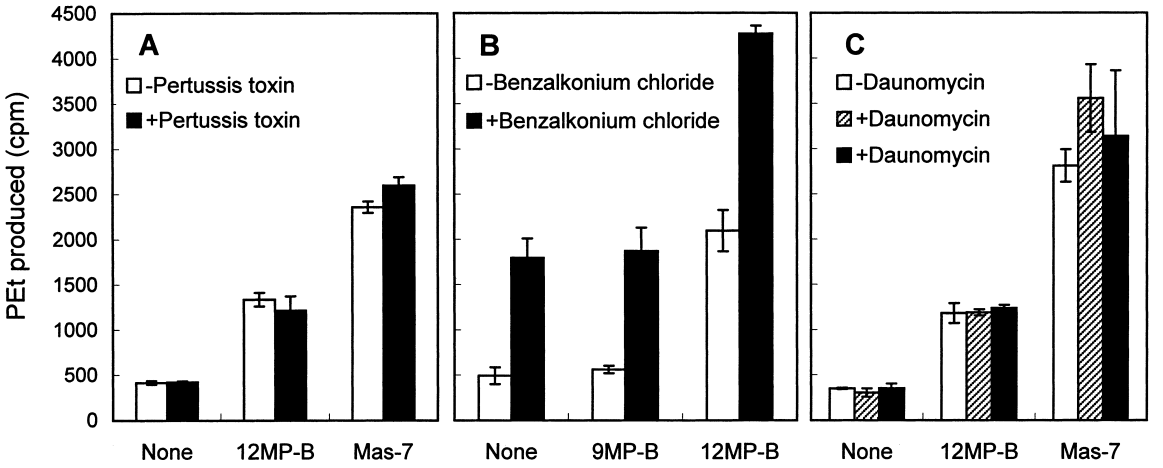

Fig. 2. Effects of chemicals related to G-protein linked pathways on the MP stimulated PLD activity in L1210 cells. [^{3}H]myristate-labelled L1210 cells were incubated for 20 min with MP-Bs (25 μM) or Mas-7 (12.5 μM), in the absence and presence of 2.5 μg/ml pertussis toxin pre-incubated for 10 min (A); in the absence and presence of 10 μg/ml benzalkonium chloride (B); in the absence and presence of 50 μM (hatched) and 200 μM (closed) of daunomycin (C). Mean of 3–4 determinations.

their effectiveness on the PLD in L1210 cells. A significant activation similar to that of MP (*P. jadwagae*) was observed with the mast cell degranulating peptide, but with amyloid β-protein (25–35) and indolicidin only moderate stimulatory effects were detected (data not shown). The apidaecin Ib had no effect on the PLD activity (data not shown).

3.2. Insensitivity of the MP-B stimulated PLD to G-protein related chemicals

To investigate whether the observed stimulation of PLD by MP-B is related with the G-protein, the effect of some agents, such as pertussis toxin known to be a G-protein inhibitor [13,22], on the MP-B stimulated PLD activity was studied. As shown in Fig. 2A, the stimulated PLD activities were not inhibited by the presence of pertussis toxin at 2.5 μg/ml. When benzalkonium chloride, another competitive antagonist for the Mas-7 stimulated GTPase activity of Gi [12], was added to the assay medium of PLD, the PLD activity was enhanced, instead of being inhibited (Fig. 2B). The benzalkonium ion, a hydrophobic quaternary amine, is also known to be an antibacterial chemical [29], and its stimulatory effect seems consistent with that of the hydrophobic MP-Bs. Daunomycin, with a known lipid bilayer stabilizing property, has been suggested as a disruptor of cellular signaling pathways by impairing proper G-protein couplings [30]. Like other G-protein related chemicals tested, daunomycin exerted no effect on the MP-B stimulated PLD up to 200 μM concentration (Fig. 2C). These observations indicate that the stimulatory effect of MP-B has no connection with the G-protein linked pathways.

Table 2
α-Helical content of MP-B and its Ala-substituted analogs

Peptide	% α Helix	
	Neutral liposomes[a]	Acidic liposomes[b]
MP-B	43	57
3MP-B	0	34
4MP-B	48	61
9MP-B	0	27
12MP-B	50	78

The α-helical contents were calculated from CD spectra obtained previously in TES buffer (pH 7.4) [16,17].
[a]Egg-yolk phosphatidylcholine liposomes.
[b]Egg-yolk phosphatidylcholine:phosphatidylglycerol (3:1) liposomes. The ratio of peptide to lipid concentration was 0.1 mM to 1 mM.

3.3. Induction of membrane permeability by MP-B analogs

Numerous actions of MP, including pore formation, have been related to the membrane perturbation induced by interaction of MP with lipid bilayer [10,11,15]. Hence, we compared the PLD stimulating effect of MP-Bs with that of other membrane-perturbing chemicals, digitonin and β-escin (Fig. 3A). The enhancement of PLD activity, nearly 7-fold by digitonin and 4-fold by β-escin, was similar to that by MP-B and 12MP-B. Intrigued by the fact that PLD activity is enhanced by membrane-perturbing agents, we investigated the quantitative relationship between the PLD activity and the extent of pore formation measured by the influx of EtBr into L1210 cells (Fig. 3B). As expected, the active MP-B and 12MP-B allowed the EtBr influx, but inactive 3MP-B did not. The increase in permeability by active forms of MP-B is in good agreement with the reported increase by Mas-7 [11]. The observed correlation between the fluorescence changes, due to pore formation and the EtBr influx, and the enhancement of the PLD activity seems quite remarkable. This correlation suggests that the membrane-perturbing action of the MP-Bs might lead to the stimulation of the PLD activity.

3.4. Effect of MP-Bs on arachidonate release

Generally MP has been reported as stimulators for some phospholipases in various cell types [7–9]. We extended investigation of the MP-B effect to the PLA_2 activity in L1210 cells (Fig. 4). The release of arachidonic acid was stimulated by MP-B, 4MP-B and 12MP-B, all of which stimulate the PLD activity, but not by the inactive analog 3MP-B.

4. Discussion

Earlier results showed that the α-helical content of MP-B and its Ala-substituted analogs is a key structural parameter

in the structure-activity relationship in antimicrobial and hemolytic activities [16,17]. Similarly the α-helical content of the MP-B analogs, summarized in Table 2, appears to correlate with the increased PLD activity in L1210 cells (Fig. 1A). The MP-B analogs with high α-helical content, such as MP-B, 4MP-B, and 12MP-B exerted stimulatory effects on the PLD in L1210 cells. On the other hand, the analogs with low α-helical structure, 3MP-B and 9MP-B, were totally ineffective. This relationship was further reinforced by other natural MPs and MP analogs, Mas-7 and Mas-17 (Fig. 1B). As expected, the natural MPs and analog Mas-7 showed stimulatory effects, whereas Mas-17, known to have a low α-helical content [12], had no effect at all. These results suggest that the high α-helical content of MPs and their analogs might be a critical determinant for the stimulatory effect of the MP on the PLD activity in L1210 cells.

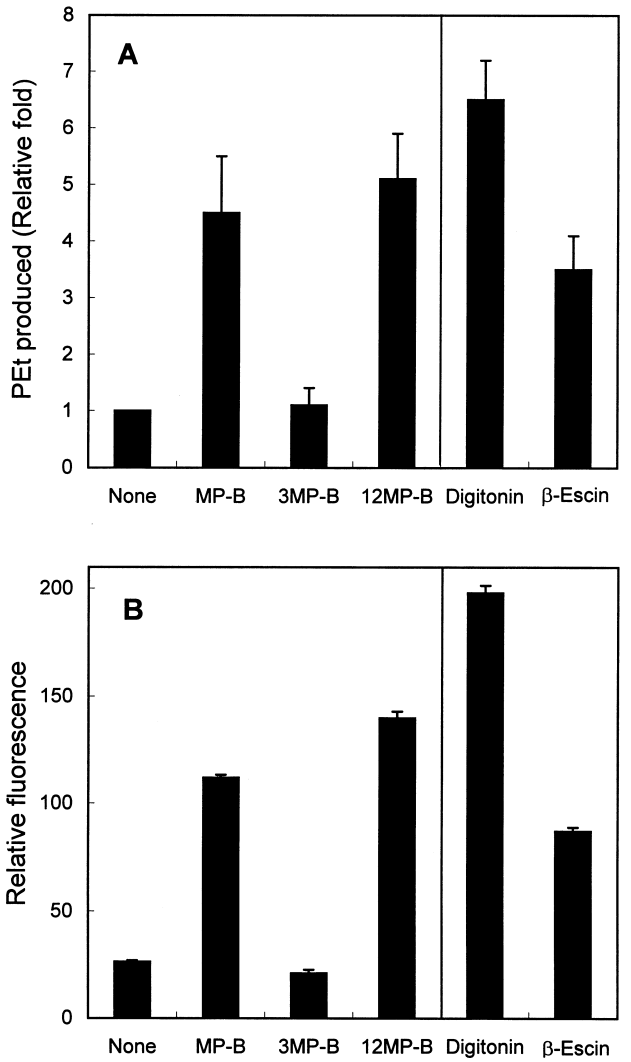

Fig. 3. Effects of membrane-perturbing chemicals on PLD activity in L1210 cells. A: Comparison of effects of MP-Bs and membrane-permeabilizing chemicals on the PLD activity. [^{3}H]myristate-labelled L1210 cells were incubated for 20 min in the presence of MP-Bs (25 μM each), digitonin (30 μg/ml), or β-escin (30 μg/ml). B: Effects of MP-B and its analogs on EtBr permeability in L1210 cells. The EtBr (25 μM) influx was carried out in the presence of 25 μM of MP-Bs. After 10 min incubation the relative fluorescence was determined at wavelengths of 360 nm excitation, 580 nm emission. As a permeability control the permeabilizing chemicals, digitonin and β-escin (30 μg/ml each), were included. Mean of 3–4 determinations.

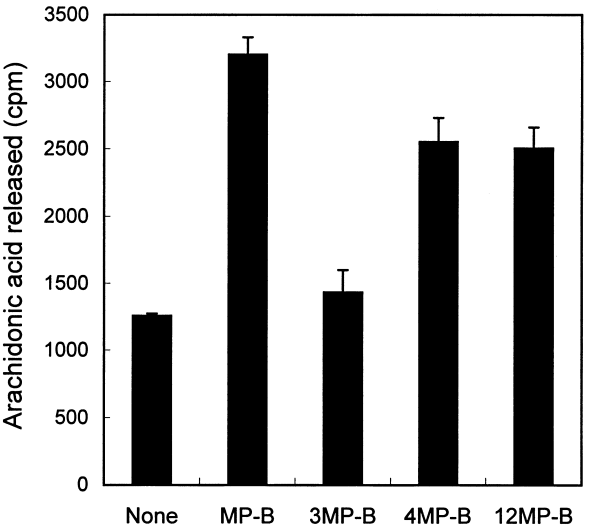

Fig. 4. Effects of MP-B and its analogs on PLA_2 activity in L1210 cells. [^{3}H]arachidonic acid-labelled L1210 cells were incubated for 20 min with MP-B and its analogs at the concentration of 25 μM each. The radioactivity of [^{3}H]arachidonic acid released was normalized to a total labelled radioactivity of 2×10^5 cpm. Mean of 3–4 determinations.

Generally the action of MP has been interpreted in two aspects: one as a modulator of the G-protein function and the other as a membrane-perturbing amphiphilic peptide. Since none of the G-protein blocking chemicals, including pertussis toxin, influenced the effect of MP-B on the PLD activity under the experimental conditions (Fig. 2), the involvement of the G-protein pathway in the MP-B modulated PLD activity in L1210 cells could be ruled out. An earlier finding that the PLD activity in this cell is not dependent on guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) [23] is consistent with the insensitivity of the MP-B action to the G-protein blockers. A similar insensitivity of the PLD stimulation by MP to pertussis toxin was reported in human astrocytoma cells, in which the PLD was not mediated by G-proteins [9]. In contrast with these cell lines, the GTPγS stimulated PLD activity in HL60 cells was inhibited by MP [19]. The PLD in HL60 cells has been shown to be dependent on ADP-ribosylation factor (ARF), a small G-protein [19,23].

The induction of permeability in L1210 cells by MP-B analogs (Fig. 3B) indicates that the active MP-Bs may act as a membrane-perturbing amphiphilic peptide. With respect to the membrane perturbation by MP, much information have been accumulated such as transition in mitochondrial permeability [10], elevation of cytosolic Ca^{2+} through membrane pores [11], and permeability enhancement in various biomembranes [15–17]. It has also been pointed out that the membrane-perturbing action of MPs including dye leakage from lipid vesicles can correlate with their α-helical contents [15–18]. Recently a series of synthetic amphiphilic peptides, which forms α-helical structure in the presence of vesicles, was shown to exhibit a strong membrane-perturbing activity [31]. The permeabilizing action of MP-B analogs with high α-helical contents is most likely through altering lipid bilayer structure of the L1210 cell membrane. This permeabilizing action

of MP-Bs might in turn stimulate the PLD presented in the L1210 cell membranes. The PLD stimulatory effects observed with digitonin and β-escin, permeabilizing agents used frequently, appear to coincide with those of active MP-Bs (Fig. 3A). The similarity in the stimulation behavior between MP-Bs and permeabilizing agents implies that the membrane perturbation is an important factor for stimulating the PLD in L1210 cells. Since the PLD in L1210 is activated by oleate, we speculate that the oleate might act as a detergent to activate the PLD through membrane perturbation and/or permeabilization [23,32].

The membrane-perturbing action of MP-Bs in L1210 cells was related not only with stimulating the PLD activity but also with inducing arachidonic acid release (Fig. 4). The release of arachidonic acid upon exposure to MP-B followed a similar structure-activity relationship except that the extent of stimulation was somewhat less than that of PLD. This result suggests a possibility of multiple effects of MP-B on various phospholipases present in the membrane of L1210 cells. In fact, broader multiple effects of MP beside on phospholipase were observed in human neutrophils [33]. In summary, the stimulation of PLD activity in L1210 cells by MP-B and MP-B analogs with high α-helical contents may be coupled with membrane perturbation. Additionally this study demonstrates that the series of Ala-substituted MP-B analogs provides a valuable tool for investigating the structure-activity relationship of MP homologs.

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