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DIRECT ACTIVATION OF GTP-BINDING PROTEINS BY VENOM PEPTIDES THAT CONTAIN CATIONIC CLUSTERS WITHIN THEIR ALPHA-HELICAL STRUCTURES

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SUMMARY: Direct interactions of venom peptides that contained a cysteine-stabilized α-helical motif within their internal molecules with αβγ-trimeric GTP-binding proteins (G proteins) were studied in reconstituted phospholipid vesicles. Mast cell-degranulating (MCD) peptide stimulated the steady-state rate of GTP hydrolysis catalyzed by the reconstituted G proteins. Synthetic *D*-MCD peptide, the optical isomer of MCD peptide, was also effective in the activation of G proteins as *L*-MCD peptide. The stimulations by *L*- and *D*-peptides were both abolished in G proteins that had been ADP-ribosylated by pertussis toxin. Charybdotoxin also stimulated, though slightly, the GTPase activity of G proteins. Such a stimulation was, however, not observed upon the incubation of G proteins with other venom peptides such as apamin, sarafotoxin and endothelin. Thus, in comparison of the amino acid sequences of their venom peptides, the extent of the activation of G proteins appeared to be correlated with the number of basic amino acid residues around the α-helix. These results suggest that cationic clusters at one side of the α-helical surface are more important in the direct activation of G proteins than a specific, α-helical structure.

GTP-binding proteins (G proteins) are a family of signal-coupling proteins that play key roles in many hormonal and sensory transduction processes in eukaryotes (1, 2). These proteins composed of α-, β- and γ-subunits carry signals from membrane receptors to effectors such as enzymes or ion channels. Recent reports have indicated that several peptides directly interact with G proteins resulting in the activation of the signal-coupling proteins in manners similar to activated receptors by agonists. Such a first example was mastoparan, a peptide toxin isolated from wasp venoms (3). Other peptide hormones such

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Abbreviations used: G protein, GTP-binding protein; G_i, G protein that mediates inhibition of adenylate cyclase; G_o, a G protein of unknown function purified from brain tissues; MCD peptide, mast cell-degranulating peptide.

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as substance P (4) and bradykinin (5) or synthetic compound 48/80 (4, 6) were later reported to have similar effects on G proteins.

Mast cell-degranulating (MCD) peptide is one of honeybee (*Apis Mellifera*) venoms (7, 8). The toxin contains two disulfide bonds inside the molecules, and its structure is characterized by an α-helix stabilized by the two disulfide bonds and a β-turn in the amino termini (9). MCD peptide, that was first noted for its mast cell-degranulating activity (10), induced long-term potentiation of synaptic transmission in the CA-1 region of hippocampus (11, 12). Fujimoto *et al.* have recently reported that there is a rapid increase in intracellular Ca^{2+} level upon incubation of rat mast cells with the peptide (13). Such an elevation of intracellular Ca^{2+} was also observed with the complete optical isomer of MCD, which was synthesized by replacing all amino acid residues of MCD peptide with *D*-amino acids. Interestingly, the actions of the two isomers (*L*- and *D*-peptides) were both abolished in the cells that had been preincubated with pertussis toxin. These findings suggest that a pertussis toxin-sensitive G protein(s) is involved in the signaling pathway induced by MCD peptides. In this paper, we study the effects of the two *L*- and *D*-MCD peptides together with structurally related some peptides on the GTPase activity of G proteins. Our results suggest that cationic clusters at one side of the α-helical surface are more important than a specific, α-helical structure in terms of the direct activation of G proteins.

MATERIALS AND METHODS

Purification of G proteins from bovine brain membranes and their reconstitutions into phospholipid vesicles

G proteins (G_{i-1} and G_o) serving as the specific substrate of pertussis toxin-catalyzed ADP-ribosylation were purified from bovine brain membranes as described in (14, 15). The proteins that had been incubated with preactivated pertussis toxin (10 μg/ml) and purified azolectin (1 mg/ml in 0.3 % sodium cholate) in the presence (ADP-ribosylated) or absence (the control) of 50 μM NAD at 30°C for 20 min were filtered through a Sephacryl S-300HR (Pharmacia-LKB), and the voided fraction was used as phospholipid vesicles (6) in the following experiments.

Assay of GTPase activity

The vesicle preparations (30-50 nM G proteins) were incubated at 30°C for 30 min with 1 μM [γ-^{32}P]GTP (2-3 x 10^3 cpm/pmol) and the indicated concentration of various compounds in 20 μl of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid/NaOH buffer (pH 7.4) containing 1.1 mM $MgSO_4$ and 1 mM EDTA. The amounts of $^{32}P_i$ released during the incubation were analyzed as described previously (6). The rate of steady-state GTP hydrolysis is expressed as turnover number, which normalized the activity to the maximum amount of [^{35}S]GTPγS binding to G proteins in the phospholipid vesicles.

Miscellaneous

L- and *D*-MCD peptide were synthesized as described in (12, 13). Apamin (purchased from Sigma) was purified as described previously (16). Charybdotoxin, Endothelin 1 and sarafotoxin were purchased from Peptide Institute, Inc. (Osaka, Japan). Other materials and chemicals were obtained from the same sources as described previously (6). All experiments have been achieved at least twice with different batches of reconstituted vesicles, and the results were fully reproducible. Hence, most of the data shown are

averages of duplicate or triplicate determinations with a single batch of reconstituted vesicles that varied within less than 5 %.

RESULTS

Fig. 1 shows the effect of MCD peptide on the steady-state rate of GTP hydrolysis catalyzed by G_o or G_{i-1}. The peptide stimulated the GTPase activity of G_o in a concentration-dependent manner; the maximum stimulation was observed at approximately 1 μM MCD peptide. The peptide-induced stimulation of GTPase activity was also observed with G_{i-1}. However, the concentration of MCD peptide required for the maximum stimulation of the G_{i-1} activity was significantly higher than that of the G_o activity (compare Fig. 1B with Fig. 1A). The effect of pertussis toxin-catalyzed ADP-ribosylation of G proteins on the action of MCD peptide was next investigated, and the results are also illustrated in Fig. 1. The basal GTPase activities observed in ADP-ribosylated and un-modified (control) G proteins, which were measured in the absence of the peptide, were not significantly different from each other. However, MCD peptide-induced stimulation of GTPase activity of G_o or G_{i-1} was completely abolished in G proteins that had been modified by pertussis toxin-catalyzed ADP-ribosylation.

We next investigated the effect of the optical isomer (*D*-form) of MCD peptide, that was synthesized with *D*-amino acids, on the GTPase activity of G proteins. As shown in Table I, *D*-MCD peptide also acted as the stimulator of the GTPase activity of G_o or G_{i-1} as had been observed with *L*-MCD peptide. The concentration-dependent stimulations by *D*-MCD peptide of the GTPase activities of the two G proteins were essentially the same as those observed with *L*-MCD peptide (data not shown). The stimulation by the isomer was, again, not observed in G_o or G_{i-1} modified by pertussis toxin-catalyzed ADP-ribosylation (see Table I).

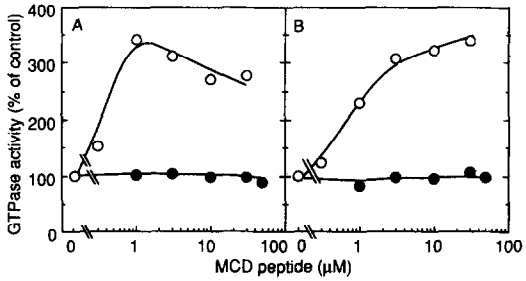

Fig. 1. Effect of *L*-MCD peptide on the steady-state rate of GTP hydrolysis catalyzed by G proteins. Vesicle preparations of G_o (A) or G_{i-1} (B) that had been ADP-ribosylated by pertussis toxin (●) or not (○) were incubated in a reaction mixture containing the indicated concentrations of *L*-MCD peptide as described in MATERIALS AND METHODS. The activities of GTPase are expressed as percentages of values obtained without peptides, which were 0.105 ± 0.02, 0.118 ± 0.04, 0.043 ± 0.007, and 0.035 ± 0.005 mol of $^{32}P_i$ released per min/mol of G protein for control G_o, ADP-ribosylated G_o, control G_{i-1}, and ADP-ribosylated G_{i-1}, respectively.

Table I
Effect of *L*- and *D*-MCD peptides on the GTPase activity of G proteins

G proteins	GTPase activity	
	L-form	*D*-form
	(% of control)	
control G_o	312	297
ADP-ribosylated G_o	103	112
control G_{i-1}	307	309
ADP-ribosylated G_{i-1}	96	120

Vesicle preparations of G proteins (50 nM) were incubated in a reaction mixture containing the indicated isomers of 3 μM MCD peptide as described in MATERIALS AND METHODS. The GTPase activities are expressed as percentages of values obtained without peptides, which were the same activities as shown in Fig. 1.

MCD peptide has been characterized by a cysteine-stabilized α-helical motif formed by two disulfide bonds (9). Such a structure was also found in other peptides (17). They included apamin, another peptide isolated from honeybee venoms, endothelin which was a recently discovered 21 amino acid peptide with a potent vasoconstrictor property (18), sarafotoxin and charybdotoxin found in snake and scorpion venoms, respectively (19, 20). Thus, effects of these peptides on the activity of G proteins were further investigated, together with those of mastoparan and compound 48/80 that had been previously reported as activators of the signal-coupling proteins (3, 4, 6). As shown in Table II, charybdotoxin acted as a stimulator of the GTPase activity of G_o, though the degree of its stimulation was less than those of MCD peptide-induced ones. However, apamin, endothelin 1 or sarafotoxin had no such an effect on the GTPase activity of G_o under the conditions that the other peptides acted as the activator of the G protein. Essentially the same results were obtained with G_{i-1} (data not shown).

Table II
Effects of various compounds on the GTPase activity of G_o

Additions	GTPase activity
	(% of control)
L-MCD peptide (10 μM)	332
charybdotoxin (10 μM)	187
apamin (10 μM)	127
endothelin 1 (10 μM)	109
sarafotoxin (10 μM)	99
mastoparan (10 μM)	885
compound 48/80 (10 μg/ml)	441

Vesicle preparations of G_o (50 nM) were incubated in a reaction mixture containing the indicated compounds as described in MATERIALS AND METHODS. The GTPase activities are expressed as percentages of value obtained without compounds, which were 0.105±0.02 mol of $^{32}P_i$ released per min/mol of G protein.

DISCUSSION

MCD peptide, which is composed of 22 amino acid residues (7), was first noted for its mast cell degranulating activity (10). The peptide also inhibited the activity of a voltage-dependent K^+-channel (21) and induced long term potentiation in hippocampus (11, 12). A high-affinity binding site for the peptide has been found in brain membranes (22) and was supposed to be the K^+-channel (21). However, it is not yet demonstrated that the MCD peptide-induced histamine release or long term potentiation results from this inhibition of the K^+ channel current.

Fujimoto *et al.* recently reported that there was a rapid increase in intracellular Ca^{2+} upon incubation of rat mast cells with synthetic *L*- or *D*-MCD peptide (13). The actions of the two isomers were both abolished by prior treatment of the cells with pertussis toxin, suggesting that the toxin-sensitive G protein(s) was involved in the signaling pathway. The data presented in this paper clearly indicate that both the *L*- and *D*-MCD peptides can directly activate $\alpha\beta\gamma$-trimeric G proteins in terms of their GTPase activities. The activations induced by the two isomers were never observed in G proteins that had been ADP-ribosylated by pertussis toxin. Thus, the previous findings observed in the intact cell-system were confirmed by this report in a well-defined system.

Mastoparan, a peptide from wasp venom, is also known to induce histamine release from mast cells (23) and directly activates G proteins (3, 6). When mastoparan is bound to a phospholipid bilayer, it forms an α-helix with its three positive charges facing outward as have been discussed in the previous paper (24, 25). This conformation was thought to mimic a structure of the interaction site of agonist-liganded receptors with G proteins (3, 26). However, *D*-MCD peptide appeared to activate G proteins in the same manners as the *L*-form. In the present study, we also investigated the effects of other peptides containing the cysteine-stabilized α-helical motif on the GTPase activities of G proteins. Besides the two *L*- and *D*-MCD peptides, charybdotoxin was able to activate G proteins in a lesser extent. As shown in Fig. 2, a structural difference between the active and inactive peptides (such as apamin, endothelin and sarafotoxin) may be characterized by the number

mastoparan	(4-12)	**K**ALAALA**KK**
MCD peptide	(13-20)	**H**I**C**R**K**I**C**G
apamin	(9-15)	AL**C**A**RRC**
charybdotoxin	(11-19)	**KEC**WSV**C**Q**R**
endothelin 1	(9-15)	**KEC**VYF**C**
sarafotoxin	(9-15)	**KEC**LYF**C**

cysteine-stabilized α-helical motif

Fig. 2. Amino acid sequences of the α-helical structure in various peptides. The amino acid sequences of the putative α-helical structure in various peptides are shown by the standard one-letter abbreviation code (data are from refs. 3, 7, 18, 19). Numbers in parentheses correspond to the positions of amino acid residues from amino termini. One-letter codes with bold face represent amino acids positively charged.

of cationic amino acid residues within the α-helix. Although apamin contained two arginine residues within the helix, their basic side chains did not face the same direction (9, 17). These results suggested that cationic cluster at one side of the α-helical surface was more important in the activation of G proteins than a specific, α-helical structure.

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