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Structural Determinants Required for the Bioactivities of Prokineticins and Identification of Prokineticin Receptor Antagonists

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ABSTRACT

Prokineticins are cysteine-rich secreted proteins that regulate diverse biological processes, including gastrointestinal motility, angiogenesis, and circadian rhythms. Two closely related G protein-coupled receptors that mediate signal transduction of prokineticins have recently been cloned. The structural elements required for prokineticins' bioactivities are still unknown. We show here that both the N-terminal hexapeptide (AVITGA) and C-terminal cysteine-rich domains are critical for the bioactivities of prokineticins. Substitutions, deletions, and insertions to the conserved N-terminal hexapeptides result in the loss of agonist activity. Mutant prokineticins with the substitution of the first N-terminal alanine with methionine or the addition of a methionine to the N terminus inhibit the activation of prokineticin receptors and thus are considered as antagonists of prokineticin receptors. We have further shown that mutations in selected cysteine residues in the C-terminal domain result in prokineticins without biological activity. The essential role of C-terminal domain is reinforced by two observations: that peptides without the carboxyl domain and proteins with the N-terminal hexapeptide fused to the carboxyl domains of colipase or dickkopf are devoid of biological activity. We demonstrate that limited structural changes of C-terminal cysteine-rich regions of prokineticins are tolerable because chimeric prokineticins with swapped cysteine-rich domains between prokineticin 1 and prokineticin 2, as well as a splice variant of prokineticin 2 that contains extra 21 residue insertion in its C-terminal domain, are biologically active.

Prokineticins (PKs) are newly identified secreted cysteine-rich proteins that possess diverse biological functions. Originally identified as potent agents that contract smooth muscle of gastrointestinal tract (Li et al., 2001), PKs have also been shown to promote angiogenesis, particularly in a number of endocrine organs (LeCouter et al., 2001, 2003). The frog homolog of PKs, Bv8, increases pain sensitization by reducing nociceptive threshold to thermal and mechanical stimuli (Mollay et al., 1999; Negri et al., 2002). Recently, we have identified prokineticin 2 as an output signal from the circadian clock of the suprachiasmatic nucleus (Cheng et al., 2002). Thus, prokineticins are involved in regulating diverse biological processes that include gastrointestinal motility, angiogenesis, and circadian rhythms. Based on pharmacological evidence, we have previously concluded that the receptors for prokineticins belong to the family of G protein-coupled receptors (GPCR) (Li et al., 2001). Recent molecular cloning studies have confirmed that PK receptors (PKRs) are composed of two very closely related GPCRs, PKR1 and PKR2 (Lin et al., 2002a; Masuda et al., 2002; Soga et al., 2002). PK1 and PKR1 are expressed in various peripheral organs, whereas PK2 and PKR2 expression is mostly confined to the central nervous system (Cheng et al., 2002; Lin et al., 2002a).

Sequence alignment suggests that PKs possess distinct N- and C-terminal domains. The N-terminal domain contains six amino acids (AVITGA) that are absolutely conserved among PKs from mammalian and nonmammalian species (Mollay et al., 1999; Schweitz et al., 1999; Li et al., 2001). The C-terminal region contains 10 cysteine residues that are predicted to form five pairs of disulfide bonds (Boisbouvier et al., 1998; Li et al., 2001). The same cysteine motif has been found in several other secreted proteins. Of these, the dickkopfs, a family of proteins that play an important role in early embryonic development (Glinka et al., 1998; Aravind and Koonin, 1998; Krupnik et al., 1999; Monaghan et al., 1999; Mao et al., 2001), and colipase, a cofactor for intestinal lipid digestive enzyme lipase, have the same cysteine configuration, although conservation among noncysteine residues

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C.M.B. and J.-D.L. contributed equally to this work.

ABBREVIATIONS: PK, prokineticin; GPCR, G-protein-coupled receptor; PKR, prokineticin receptor; CHO, Chinese hamster ovary; AEQ, aequorin; GI, gastrointestinal.

is limited (Lowe, 1997; Boisbouvier et al., 1998). Our study delineates the structural elements that are essential for bioactivities of PKs.

Materials and Methods

Production of Mutant and Chimeric Prokineticins. Mutations were made with QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The chimeric prokineticin constructs were made by a combination of site-directed mutagenesis to create restriction endonuclease sites and subcloning of appropriate components of cDNAs in frame. As to chimera 21, the N-terminal 47 amino acids of prokineticin 2 (GenBank accession number AF333025) were fused to residues 48 to 86 of prokineticin 1 (GenBank accession number AF333024), and chimera 12 was made by fusing the N-terminal 47 residues of prokineticin 1 to residues 48 to 81 of prokineticin 2. AVITGA-colipase was generated by fusing AVITGA to residues 34 to 112 of human colipase (GenBank accession number BT006812), and AVITGA-DKK4 was made by fusing AVITGA to residues 145 to 224 of human dickkopf-4 (GenBank accession number NM_014420). All cDNA constructs were confirmed by sequencing.

The cDNAs for mutant and chimeric prokineticins were cloned into the prokaryotic expression vector pGEX-3X (Pharmacia, Piscataway, NJ). All cDNAs included sequences for a glutathione *S*-transferase tag for translation initiation, a factor Xa protease digestion site upstream of the mature N terminus, as well as a hexahistidine tag at the C terminus, allowing the fusion proteins to be purified via nickel-nitriloacetic acid chromatography (QIAGEN, Valencia, CA). The purification and refolding of recombinant prokineticins were carried out as described previously (Li et al., 2001). Briefly, BL21 *Escherichia coli* cells were transformed with the appropriate plasmid, grown to an optical density at 600 nm of 0.8 and induced with 600 μM isopropyl β-D-thiogalactoside for 3 to 5 h at 37°C with shaking. Cells were pelleted and lysed overnight in lysis buffer (6 M guanidine HCl, 100 mM NaH_2PO_4, and 10 mM Tris, pH 8, and 10 nM β-mercaptoethanol). The lysate was centrifuged and the supernatant was incubated for several hours in a nickel-nitriloacetic acid slurry, and then washed extensively with wash buffers at pH 6.3 followed by pH 5.9 (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris). Protein-bound beads were then treated overnight with Factor Xa in digestion buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM $CaCl_2$). Factor Xa was washed with wash buffer at pH 5.3 and the peptide was eluted with wash buffer at pH 4.5. Eluted protein was refolded by dilution to 100 μg/ml in ice-cold refolding buffer (2 M urea, 100 mM oxidized glutathione, 10 mM reduced glutathione, 0.02% Tween 20, 10% glycerol, 10 mM Tris, pH 8, 150 mM NaCl, and 100 mM NaH_2PO_4, pH 8.3). The refolded proteins were then desalted and concentrated on a spin column (QIAGEN).

Establishment of Stable CHO Cell Clones Coexpressing PKR1 or PKR2 and Aequorin. Chinese hamster ovary (CHO) cells stably expressing photoprotein aequorin (CHO/AEQ) were grown in α-modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were maintained in a humidified 5% CO_2 incubator at 37°C. Human PKR1 or PKR2 cDNA in pcDNA3.1-Zeo (Invitrogen, Carlsbad, CA) were transfected into CHO/AEQ using LipofectAMINE (Invitrogen). Clones of cells stably expressing PKR1 or PKR2 were selected in 100 μg/ml Zeocin (Invitrogen) for about 2 weeks. Colonies were picked by cloning cylinders, expanded, and tested in a Ca^{2+} mobilization assay in response to 10 nM PK1.

Ca^{2+} Mobilization Assay. An aequorin-based luminescent assay for calcium mobilization was used to measure mobilization of intracellular Ca^{2+} (Lin et al., 2002a). CHO cells stably expressing the photoprotein aequorin and PKR1 or PKR2 were tested by this method. Briefly, the cells were charged in Opti-MEM (Invitrogen) containing 8 μM coelenterazine *cp* at 37°C for 2 h. Cells were detached by brief trypsinization and maintained in Hank's balanced salt solution plus 10 mM HEPES, pH 7.5, and 0.1% bovine serum albumin at about 5×10^5 cells/ml. Luminescence measurements were made using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

All proteins were diluted in Hank's balanced salt solution plus 10 mM HEPES, pH 7.5, and 0.1% bovine serum albumin. To test the agonist activity, 100 μl of cells were injected into the tubes with 20 μl of wild-type or mutant prokineticins. For antagonist assays with coinjection method, 100 μl of cells were injected into the tubes containing 100 μl of 10 nM PK1 or PK2 with or without various concentrations of mutant proteins. In the preincubation assays, 80 μl of cells were incubated in the tubes with 20 μl of different concentrations of antagonists at room temperature for 20 min, and then 100 μl of 10 nM PK1 or PK2 was injected into these tubes. For Schild analysis, the antagonistic activities of A1MPK1 and MetPK1 were measured with the coinjection method.

Bioassay for Contraction of Gastrointestinal Smooth Muscle. Bioassays of smooth muscle contraction were performed as described previously (Li et al., 2001). Briefly, guinea pigs were euthanized with CO_2, and 2- to 3-cm sections of ileum, 10 cm rostral to the cecum, were removed, washed in Krebs-Ringer-bicarbonate buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$ and 10 mM glucose), and mounted longitudinally in an organ bath containing Krebs-Ringer-bicarbonate buffer. The ileum was allowed to rest for 30 min and then contracted three times with 40 nM oxotremorine-M, with washing between each contraction to ensure adequate and reproducible contractions. The tissue was then allowed to rest for an hour before testing recombinant PKs. Isometric contractions were measured with a force-displacement transducer and polygraph.

[^{3}H]Thymidine Incorporation Assays. CHO cells stably expressing human PKR1 were seeded at 5×10^5 cells per well in 24-well plates. After 36 h, the cells were placed in serum-free medium for 16 h. Recombinant proteins were then added at various concentrations for 8 h, followed by the addition of 5 μCi/ml of [^{3}H]thymidine (76 Ci/mmol) for another 16 h. Cells were then washed carefully with 1 ml of ice-cold phosphate-buffered saline, followed by the addition of 1 ml of ice-cold 5% tricholoroacetic acid. After leaving at 4°C for 30 min, the cells were washed once with phosphate-buffered saline, lysed with 0.5 ml of 0.5 M NaOH/0.5% SDS, and counted on a scintillation counter.

Results

Generation of Mutant and Chimeric Prokineticins. Sequence analysis revealed that both the human and mouse PK1 and PK2 genes contain three exons (data not shown). The first exon encodes a signal peptide and the first five amino acids of the mature protein, whereas exons 2 and 3 encode the cysteine-rich domain (Li et al., 2001). The complete conservation of N-terminal hexapeptide (AVITGA) among mammalian and nonmammalian species suggests their potential functional significance. To address the functional importance of these N-terminal residues as well as that of the C-terminal domain, we generated several mutant PKs (Table 1), including chimeras that have the cysteine-rich C-terminal domains of PK1 and PK2 swapped. Except for two peptides that were synthesized chemically, all mutant structures were cloned into expression vector pGEX-3X and recombinant proteins were purified and refolded.

Essential Role of N-Terminal Hexapeptide (AVITGA) for Bioactivities of Prokineticins. We first examined the N-terminal mutants of human PK1 for their abilities to mobilize Ca^{2+} in an aequorin assay with CHO cells that stably express PKR1 or PKR2. As shown in Table 2 and Fig. 1, most

N-terminal mutants, including A1MPK1, MetPK1, and Ala6PK1, were devoid of any biological activities at concentrations up to 1 μM when tested on both PKR1 and PKR2. Consistent with these observations, no activities were detected in the GI smooth muscle contraction assay with concentrations up to 300 nM of N-terminal mutant PKs (Table 2). Because activation of PKRs promotes cell proliferation (Lin et al., 2002b), we examined the activities of mutant PKs in a thymidine incorporation assay with CHO cells that stably express PKR1. Figure 2A shows that the two mutants, A1MPK1 and MetPK1, had no activity, whereas PK1 promoted proliferation. These results revealed the crucial role of the N-terminal hexapeptide of PKs in PKR activation.

Whereas most mutants resulted in a similar effect on both PKR1 and PKR2, one mutant protein, delAPK1, displayed a differential effect on PKR1 and PKR2. As shown in Fig. 3, delAPK1 maintained residual activity for PKR1, albeit with a potency 150-fold lower than that of PK1, whereas it exhibited negligible activity for PKR2. This result suggests that the binding pockets of highly conserved PKR1 and PKR2 might be slightly different.

C-Terminal Cysteine-Rich Domains Are Also Essential for Bioactivities of PKs. A striking feature of the carboxyl domain of PKs is the existence of 10 conserved cysteines that form five pairs of disulfide bridges (Li et al., 2001). We sought to investigate whether this cysteine-rich domain is crucial for PK bioactivities. We first generated mutant PKs in which two cysteines (cysteines 18 and 60) were substituted with a serine and arginine, respectively (Table 1). Aequorin assays revealed that these mutants failed to activate either PKR1 or PKR2 (Table 2). Thus, the C-terminal cysteine-rich domain seems to be important for the bioactivities of PKs. To test whether similar cysteine-rich domain from colipase or dickkopf can substitute for the ones from PKs, we generated chimeric proteins in which the conserved N-terminal hexapeptide (AVITGA) was fused to the cysteine-rich domains from colipase or dickkopf 4. Functional assays revealed that these two chimeras displayed no activity on PKRs (Table 2). These results indicate that C-terminal cysteine-rich domains from other proteins cannot substitute for the ones from PKs. Similarly, two synthetic peptides that contain the N-terminal hexapeptide were inactive (Table 2). Together with results from N-terminal mutants, our observations indicate that the N-terminal hexapeptide of PKs, although essential, are not sufficient for activating PKRs.

PK1 and PK2 are about 50% identical to each other (Li et al., 2001). To address the degree of tolerance of structural changes for a functional PK, we generated two chimeric PKs by swapping cysteine-rich domains between PKs (chimeras 12 and 21). As shown in Fig. 1 and Table 2, these two chimeras displayed activities comparable with wild-type PKs, although with some reduction (5- to 10-fold) in potency as measured by aequorin assays. Similar observations were also made in the assay of GI smooth muscle contraction (Fig. 4 and Table 2). These results indicate that the C-terminal cysteine-rich domains from PK1 and PK2 are exchangeable. Taken together, our results imply that conserved residues in the C-terminal cysteine-rich domains of PK1 and PK2 may also contribute to binding to PKRs.

Pharmacological Activity of a PK2 Splice Variant. A testis-specific splice variant of PK2, containing 21 extra highly charged amino acids inserted between exon 2 and 3, has recently been detected (Jilek et al., 2000; our unpublished observations). To examine whether such a splice vari-

TABLE 1
Structures of mutant prokineticins

Name	Structure
A1MPK1	Substitute alanine 1 of PK1 with methionine
MetPK1	Add a methionine to the N terminus of PK1
Ala6PK1	Mutate the N-terminal AVITGA of PK1 to AAAAAA
delAPK1	Delete the alanine 1 of PK1
C18SPK1	Substitute cysteine 18 of PK1 with serine
C60RPK1	Substitute cysteine 60 of PK1 with arginine
AVITGA-colipase	Fuse AVITGA to the residue 34–112 of human colipase
AVITGA-DKK4	Fuse AVITGA to the residue 145–224 of human dickkopf-4
Peptide 1	AVITGA
Peptide 2	AVITGACERDVQ
Chimera 12	Chimera of PK1 (1–47) and PK2 (48–81).
Chimera 21	Chimera of PK2 (1–47) and PK1 (48–86).
PK2 insert	PK2 containing 21 amino acids being inserted between the residue 47 and residue 48

TABLE 2
Agonist activity of prokineticins and related mutants tested with Ca^{2+} mobilization and ileum contraction assays
Data of Ca^{2+} mobilization assay are shown as average ± S.E. of at least three independent experiments done in duplicate. The EC_{50} values for ileum contraction assays are averages from two independent experiments.

Proteins	EC_{50} of Ca^{2+} Mobilization		EC_{50} of Ileum Contraction
	PKR1	PKR2	
	nM		*nM*
PK1	2.64 ± 0.294	2.66 ± 0.76	0.60
PK2	3.23 ± 1.21	3.00 ± 0.76	0.56
A1MPK1	>1000	>1000	>300
MetPK1	>1000	>1000	>300
Ala6PK1	>1000	>1000	>300
delAPK1	444.7 ± 2.1	>1000	>300
C18SPK1	>1000	>1000	>300
C60RPK1	>1000	>1000	>300
AVITGA-colipase	>1000	>1000	>300
AVITGA-DKK4	>1000	>1000	>300
Peptide 1	>1000	>1000	>300
Peptide 2	>1000	>1000	>300
Chimera 12	9.7 ± 3.56	6.95 ± 3.30	0.75
Chimera 21	17.0 ± 10.6	15.0 ± 1.01	2.02
PK2 insert	498.6	>1000	>300

ant can produce biologically active protein, we produced a recombinant protein of this splice variant (PK2 insert). Figure 1 and Table 2 show that PK2 insert could activate both PKR1 and PKR2, but with potency about 150-fold lower than that of PK2. This result, with exchangeability of cysteine-rich domains between PK1 and PK2, suggests that tolerable changes in the C-terminal domain for functional PKs are likely.

Two N-Terminal Mutants Are PKR Antagonists. Antagonists for PKRs have obvious values for understanding the physiological functions of PK system. Consequently, we examined the antagonist activities of PK mutants that we have generated. The antagonist activities of mutant PKs were examined against 10 nM PKs (~EC_{70}) with CHO/AEQ cells that stably express PKR1 or PKR2. Although most of the mutant PKs displayed little or no antagonist activity at concentration up to 1 μM (data not shown), the two N-terminal mutants (A1MPK1 and MetPK1) displayed potent antagonistic activities (Fig. 5). A1MPK1 and MetPK1 antagonized the signaling of both PKR1 and PKR2 when activated by either PK1 or PK2. In the coinjection regimen, the IC_{50} values for A1MPK1 ranged from 27.6 to 208 nM. The potencies of MetPK1 were similar, ranging from 27 to 98 nM (Table 3). We also examined the potency of A1MPK1 and MetPK1 when they were added 20 min before the delivery of agonists. These preincubation experiments showed that the potency of these antagonists were increased by 1.7- to 14.7-fold, dependent upon ligand and receptor combination (Table 3).

We also examined the inhibitory activity of A1MPK1 in the thymidine incorporation assay. As shown in Fig. 2B, A1MPK1 (200 nM) abolished PK1 (30 nM)-stimulated proliferation activity. To further determine whether these antagonists are competitive antagonists, we performed Schild analysis. Figure 6 shows that in the presence of increasing concentrations of A1MPK1 or MetPK1, the dose-response curves of PK1 were shifted to the right but without changes to the maximum responses. Hence, A1MPK1 and MetPK1 are both competitive antagonists for PKR1 or PKR2. The

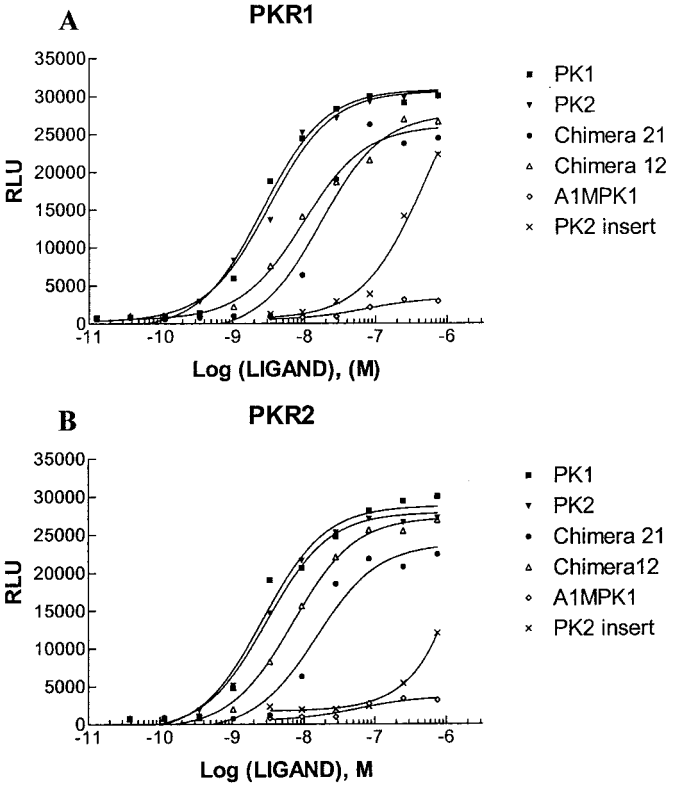

Fig. 1. Calcium mobility activity of PKR1 (A) and PKR2 (B) stimulated by wild-type and mutant prokineticins as measured by an aequorin-based assay. Data represents the average of at least three independent experiments done in duplicate. RLU, relative luminescence unit.

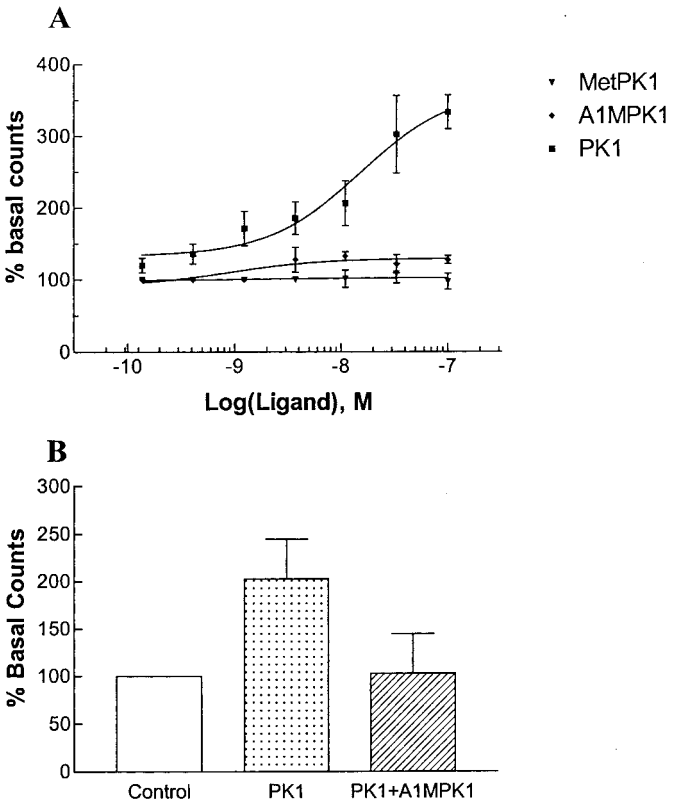

Fig. 2. [^{3}H]Thymidine incorporation assays in CHO cells stably expressing PKR1. A, stimulatory effect of PK1, A1MPK1, and MetPK1 on thymidine incorporation. B, antagonistic effect of A1MPK1 (200 nM) on PK1 (30 nM)-stimulated thymidine incorporation. Results are shown as a percentage of basal counts and represent the average ± S.E. of three independent experiments done in duplicate.

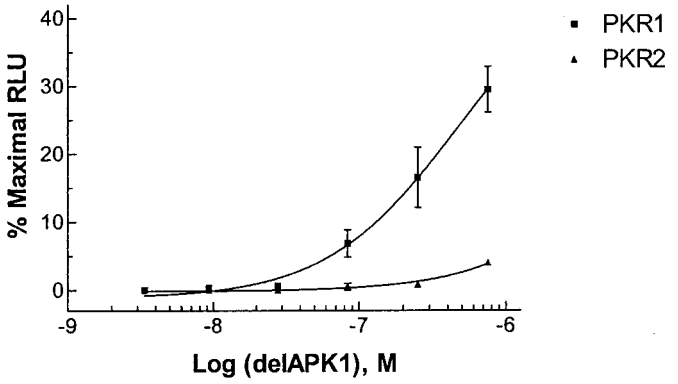

Fig. 3. Differential effects of N-terminal alanine deletion of PK1 on the activation of PKR1 and PKR2. The Ca^{2+} responses were measured with an aequorin assay. Percentage of maximal relative luminescence unit (RLU) is shown as the percentage of luminescence units from cells treated with delAPK1 at various concentrations to that of cells activated with 2 μM PK1. Results represent the average ± S.E. of at least three independent experiments done in duplicate.

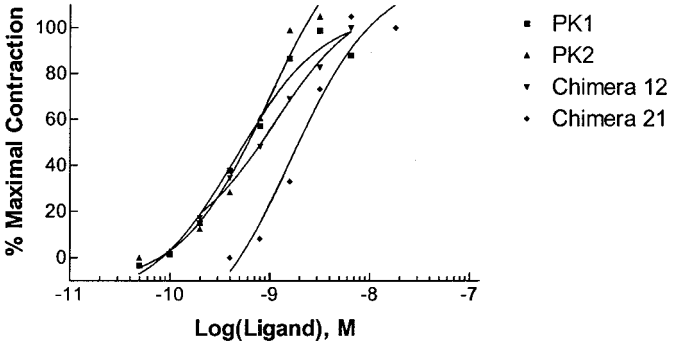

Fig. 4. Dose-response curves of wild-type and chimeric prokineticins assayed with guinea pig ileum contraction. Results are given as a percentage of maximum contractility and represent the average of two independent experiments.

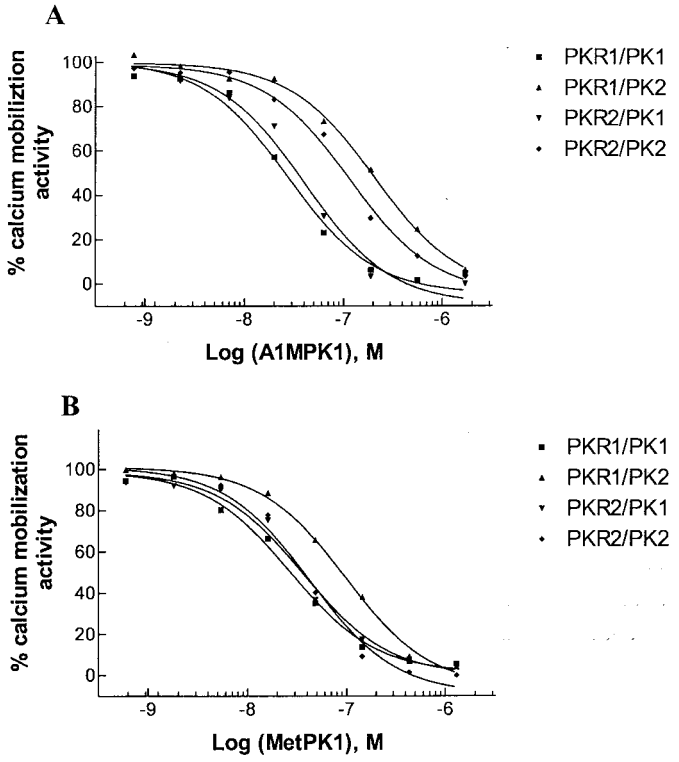

Fig. 5. Antagonist activities of A1MPK1 and MetPK1. The antagonist activities of MetPK1 and A1MPK1 were measured in CHO/AEQ cells that stably express human PKR1 or PKR2. A, cells are treated with 10 nM PK1 or PK2 together with various concentrations of A1MPK1. B, cells are treated with 10 nM PK1 or PK2 together with various concentrations of MetPK1. Results are shown as a percentage of relative luminescence units in response to 10 nM PK1 or PK2 and represent the average of at least three independent experiments done in duplicate.

dissociation constants (K_b) of A1MPK1 were 116.1 ± 27.2 nM (n = 3) and 37.8 ± 10.5 nM (n = 3) for PKR1 and PKR2, respectively. The dissociation constants (K_b) of MetPK1 for PKR1 and PKR2 were similar at 260.7 ± 135 nM (n = 3) and 48.9 ± 32.1 nM (n = 3), respectively.

Discussion

Our studies unequivocally demonstrate that the N-terminal residues (AVITGA) of PKs are critical for their bioactivities. The addition or substitution of one amino acid at the N terminus resulted in mutated PKs that were completely devoid of bioactivities. Yet the N-terminal functional domain is not sufficient for the bioactivities of PKs. Synthetic peptides, corresponding to the N-terminal 6 or 12 amino acids of human PK1, were almost unable to contract GI smooth muscle preparation or activate prokineticin receptors (Table 1 and Table 2), suggesting that an intact C-terminal domain is indispensable for receptor activation. Further underscoring the structural importance of the C-terminal domain, the fusion proteins AVITGA-colipase and AVITGA-DKK4 failed to activate prokineticin receptors. Parallel observations were made when cysteines were mutated in the native PK1. Although the complete conservation of the N-terminal domain (AVITGA) is essential, some changes in the C-terminal domain are tolerable, as demonstrated by the exchangeability of the C-terminal domain between PK1 and PK2. Thus, the bioactivities of PKs require a completely conserved N-terminal domain and moderate conservation of the C-terminal domain.

The binding profiles of smaller molecules, such as the monoamines, to their cognate GPCRs have been more extensively investigated than their counterpart peptide ligands to their receptors (Berthold and Bartfai, 1997). Despite their large size and less-defined secondary and tertiary structures, excellent structure activity relationship studies for several peptide ligands have been successfully carried out. Alanine scanning analysis of the vasoactive intestinal peptide, for example, shows that the residues most critical for binding to the VPAC1 receptor are also critical for receptor activation; i.e., there is a linear relationship between log K_i and the log EC_{50} of the vasoactive intestinal peptide analogs (Nicole et al., 2000). These residues are scattered throughout the 28-amino acid primary sequence. Many of these residues, although certainly not all, are also critical for maintaining the predicted secondary structure of the peptide. Other protein ligands, such as corticotropin and the opioid peptides, show a more modular organization; different regions of the molecule have different functions in receptor binding and activation

TABLE 3

Antagonistic potency of MetPK1 and A1MPK1

Data are shown as average ± S.E. of at least three independent experiments done in duplicate. The procedure is described in the legend to Fig. 5. The potency increase (-fold) after preincubation is shown in parentheses below the IC_{50} values.

Antagonists	IC_{50} without Preincubation				IC_{50} with Preincubation			
	PKR1		PKR2		PKR1		PKR2	
	PK1	PK2	PK1	PK2	PK1	PK2	PK1	PK2
	nM				*nM*			
MetPK1	27 ± 4.54	98 ± 4.4	36.4 ± 4.56	42.7 ± 3.52	10.2 ± 4.63 (2.6)	29.98 ± 6.66 (3.3)	3.6 ± 1.29 (10.1)	4.49 ± 0.54 (9.5)
A1MPK1	27.6 ± 5.03	208 ± 83.75	40.7 ± 6.5	116 ± 91.53	16.1 ± 2.89 (1.7)	32.8 ± 2.6 (6.3)	8.2 ± 2.61 (5.0)	7.9 ± 5.59 (14.7)

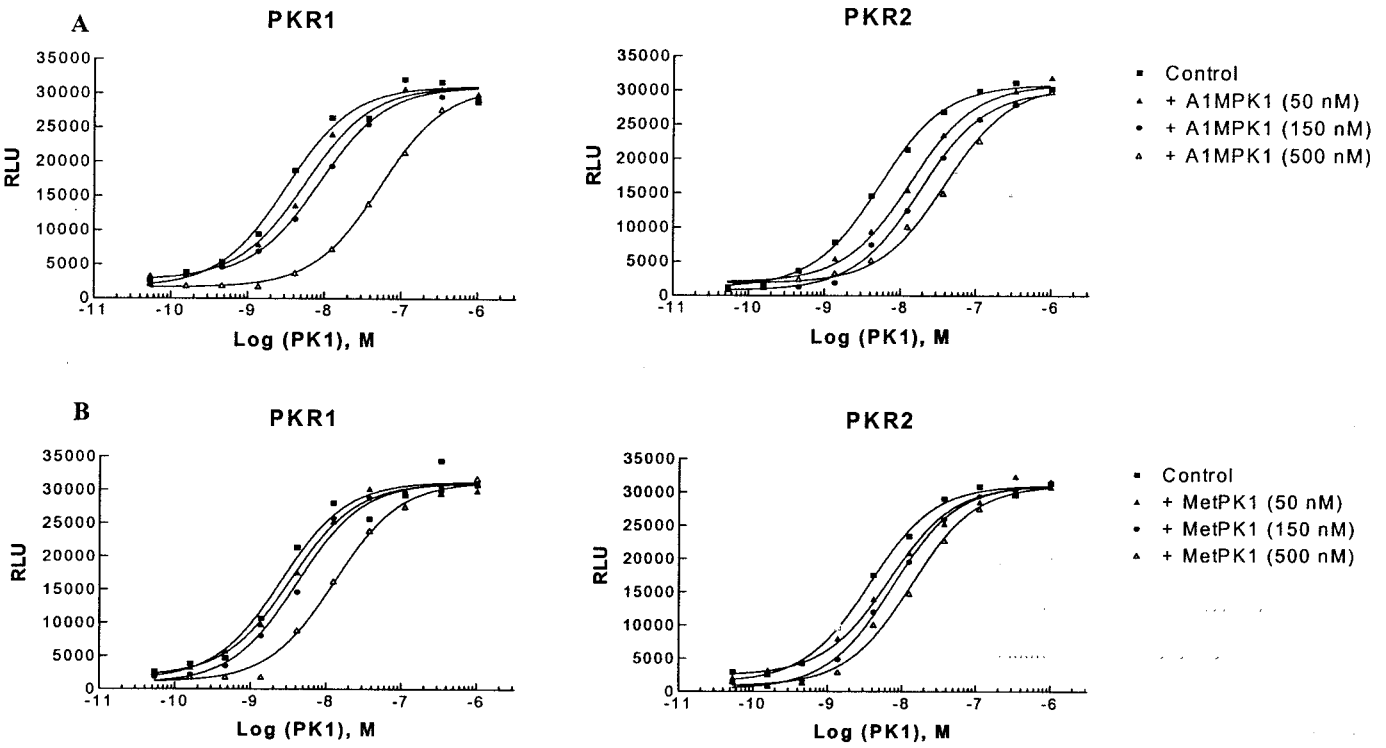

Fig. 6. Schild analyses of the antagonistic effects of A1MPK1 (A) and MetPK1 (B) on PKR1 and PKR2. The antagonist activities of A1MPK1 and MetPK1 were measured in CHO/AEQ cells that stably express human PKR1 or PKR2. Representative dose-response curves of PK1 in the presence of increasing concentration of A1MPK1 or MetPK1 (50, 150, and 500 nM) are shown.

(Schwyzer, 1977; Chavkin and Goldstein, 1981; Reinscheid et al., 1998). Such organization has fostered the "message-address" concept, in which one region of the protein (the "message") is by itself sufficient for full receptor activation, whereas another region of the protein (the "address") enhances the potency of the protein (by enhancing binding) and/or confers selectivity for a receptor or receptor subtype. For example, the N-terminal 10 amino acids of corticotropin (Schwyzer, 1977) and the N-terminal 4 amino acids of the Dynorphin A are sufficient for full activation of their respective receptors. Residues in the carboxyl region of both proteins, however, enhance their potency, and in the case of dynorphin A, enhance selectivity for the κ opioid receptor over the μ and δ receptors (Chavkin and Goldstein, 1981). Our studies here show that both N- and C-terminal domains of PKs contribute to their bioactivies. However, it is difficult to assign them to the "message-address" concept because neither domain is sufficient for receptor activation.

The importance of N-terminal residues of PKs in PKR activation is further strongly supported by observations that adding or substituting only a single amino acid resulted in mutant PKs (A1MPK1 and MetPK1) that possess potent antagonist activity. The apparent sensitivity of PKs to N-terminal residue deletion may also suggest possible molecular mechanisms of in vivo ligand inactivation. We and others (Boisbouvier et al., 1998; Li et al., 2001) have shown that human PKs or their snake homologs are resistant to degradation by proteases such as trypsin; therefore, the likely PK inactivation may thus involve the removal of one or more N-terminal residues by N-terminal peptidase. The different sensitivity of PKR1 and PKR2 to the deletion of the first alanine, on the other hand, suggests the binding pocket for PKs might be slightly different between these highly conserved receptors.

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