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Novel Mastoparan Analogs Induce Differential Secretion from Mast Cells

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Summary

Cationic amphiphilic peptides stimulate secretion via a receptor-independent action upon G proteins [1]. We have previously utilized chimeric analogs of mastoparan (MP), including galparan (galanin(1–13)-MP [2]), as molecular probes of secretion [3, 4]. Here, we further resolve the structure-activity relationship of peptidyl secretagogs, including rationally designed chimeric MP analogs. The secretory efficacies of 10 MP analogs were significantly higher than 45 unrelated basic peptides. Comparative studies identified MP analogs that are differential secretagogs for 5-hydroxytryptamine (5-HT) and β-hexosaminidase. Peptide-induced activation of phospholipase D (PLD), an enzyme intimately involved in regulated exocytosis [5], correlated with the secretion of β-hexosaminidase but not 5-HT. Thus, these data indicate that different mechanisms are responsible for the exocytosis of 5-HT and β-hexosaminidase, respectively. Moreover, mastoparan analogs are novel tools for probing the molecular details of exocytosis and other biological phenomena.

Introduction

Galparan (galanin(1–13)-MP; GWTLNSAGYLLGPINLKA LAALAKKIL-NH_2 [2]) was synthesized as part of a program aimed at developing selective ligands for galanin receptors. In addition to being a galanin receptor ligand, galparan displays unique biological activities, independent of receptor binding, that include the activation of rat brain Na^+,K^+-ATPase [2] and the inhibition of GTPase activity [6]. Moreover, galparan is a powerful insulin-releasing peptide, acting at a distal site distinct from the locus of action of MP, in the B cell secretory pathway [3]. The chimeric construct M391 ([phenylacetyl-*D*-Tyr(Me)^{2}Arg^{6}Tyr^9]AVP-εAhx-MP), combining a linear vasopressin receptor antagonist [8] and MP separated by a flexible aminohexanoic-acid linker, selectively binds with high affinity to the V_{1a} vasopressin receptor [7]. Like galparan, M391 can also influence biological function by mechanisms that are independent of G protein-coupled receptor interaction and is a potent insulin secretagog [4]. These findings indicate that chimeric analogs of MP can specifically modify the function of both extracellular and intracellular proteins and provide valuable tools to study and modulate biological phenomena.

In order for peptidyl secretagogs to bind G proteins or other intracellular targets, there is clearly a fundamental requirement that the peptide can translocate across the plasma membrane. In the case of MP, interaction with biological membranes and activation of G proteins appear to be largely dependent upon amphiphilicity and the adoption a helical structure in a lipidic environment [9, 10]. Clear evidence that chimeric peptides such as galparan can penetrate eukaryotic cells is provided by studies with the structural homolog transportan (galanin(1-12)-Lys-MP [11]). Biophysical and biochemical studies indicate that transportan, and a variety of deletion analogs, penetrate eukaryotic cells by a nonendocytotic mode of transport [12, 13]. In addition to a complex influence on GTPase activity (see Discussion), other putative intracellular targets for MP analogs include the Ca^{2+}-ATPase and the ryanodine receptor of the endoplasmic reticulum [14]. Intriguingly, MP, galparan, and M391 inhibit Ca^{2+}-ATPases and activate the ryanodine receptor, leading to net Ca^{2+}-release from endoplasmic reticulum in vitro [14].

The above observations, along with many other examples of the ability of MP and galparan to influence cellular activity [15], prompted this investigation, in which we further address the utility of structurally diverse MP analogs as secretagogs. For this purpose, we utilized RBL-2H3, a well-documented model of secretory mucosal mast cells [16]. To facilitate the detailed comparison of MP analogs with other cationic peptides, we synthesized three additional chimeric MP analogs by using sequences reported to modulate secretion as amino-terminal extensions [17, 18, 19]. Our comparative studies, utilizing a total of 56 peptides, revealed a majority of peptides that did not influence the exocytosis of either 5-HT or the secretory lysosomal marker β-hexosaminidase. These studies clearly endorse the unique properties of the MP sequence and its chimeric constructs that interact with multiple intracellular sites to regulate secretory pathways and activate PLD. Moreover, our studies reveal that certain MP analogs are differential secretagogs that could prove to be valuable probes of other cellular systems, such as the pancreatic B cell.

Results

Comparative Secretory Efficacies of Basic Peptides

Earlier reports (reviewed in [1]) have indicated that polybasic hydrophilic peptides can induce secretion in a

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Table 1. Primary Sequences of Peptides Compared in This Study

Sequences Related to Small Peptide Hormones and Neuropeptides	
Bradykinin (BK)	RPPGFSPFR-OH
Des-Arg^{9}BK	RPPGFSPF-OH
[Aib^7]BK	RPPGFS*Aib*FR-OH
[Thi^{5,8}*D*-Phe^7]BK	RPPG*Thi*Sf*Thi*R-OH
Lys^{0}BK	KRPPGFSPFR-OH
H_2N-(*R*)-BK-ac	CH_3CO-rfpsfGppr-NH_2
Arginine vasopressin (AVP)	CYFQNCPRG-NH_2
[Aib^4]AVP	CYF*Aib*NCPRG-NH_2
[Aib^7]AVP	CYFQNC*Aib*RG-NH_2
AVP-BK	CYFQNCPRGRPPGFSPFR-OH
RGDF-ϵAhx-*D*-Arg^0[Hyp^3*D*Phe^{7}Leu^8]BK	RGDFϵ*Ahx*rRPHypGFSfLR-OH
[Pa-*D*-T-yr^{2}Val^{4}Arg^{6}Aib^{7}Arg(NH$_2$)^9]AVP	Pa-yFVNR*Aib*RR-NH_2
[Pa-*D*-Tyr^{2}Aib^{4}Arg^{6}Arg(NH$_2$)^9]AVP	Pa-yF*Aib*NRPRR-NH_2
[Phaa-*D*-Tyr(Et)^{2}Aib^{4}Arg^{6}Tyr(NH$_2$)^9]AVP	Phaa-y(Et)F*Aib*NRPRY-NH_2
[Phaa-*D*-Tyr(Et)^{2}Arg^{6}Aib^{7}Tyr(NH$_2$)^9]AVP	Phaa-y(Et)FQNR*Aib*RY-NH_2
[Phaa-*D*-Tyr(Et)^{2}Arg^6]AVP(1-8)-ϵAhx-RGDF-NH_2	Phaa-y(Et)FQNRPRϵ*Ahx*RGDF-NH_2
[Phaa-*D*-Tyr(Et)^{2}Arg^6]AVP(1-9)-DF-NH_2	Phaa-y(Et)FQNRPRGDF-NH_2
[d(CH$_2$)$_5$-*D*-Tyr(Et)^{2}Lys^{3}Val^{4}Arg(NH$_2$)^9]AVP	d[CH$_2$)$_5$-y(Et)KVNCPRR-NH_2
[d(CH$_2$)$_5$-*D*-Tyr(Et)^{2}Lys^{3}Val^4]AVP	d(CH$_2$]$_5$-y(Et)KVNCPRG-NH_2
BK(1)-AVP(2-5)-BK(6-9)	RYFQNSPFR-OH
H_2N-(*R*)-ATII	fphiyvrd-NH_2
Oxytocin	CYIQNCPLG-NH_2
Neurotensin	*Pyr*LYENKPRRPYIL-OH
Endothelin-1	CSCSSLMDKECVYFCHLDIIW-OH
Bombesin	*Pyr*QRLGNQWAVGHLM-NH_2
Substance K	HKTDSFVGLM-NH_2
Substance P	RPKPQQFFGLM-NH_2
Salmon calcitonin	CSNLSTCVLGKLSQELHKLQTYPRTNTGSGTP-NH_2
M32, Gal(1-13)-NPY(25-36)	GWTLNSAGYLLGPRHYINLITRQRY-NH_2
M88, Gal(1-12)-Ala-NPY(25-36)	GWTLNSAGYLLGARHYINLITRQRY-NH_2
M35, Gal(1-13)-BK(2-9)-NH_2	GWTLNSAGYLLGPPPGFSPFR-NH_2
M58, Gal(1-13)-BK(2-9)	GWTLNSAGYLLGPPPGFSPFR-OH
M59, Gal(1-13)-BK(2-8)-NH_2	GWTLNSAGYLLGPPPGFSPF-NH_2
M69A, Gal(1-13)-Lys-[ϵNH-Gly-NPY(4-1)]-NPY(25-36)	GWTLNSAGYLLGPK(ϵNH-GKSPY)RHYINLITRQRY-NH_2
M120, Gal(1-13)-NPY(14-36)	GWTLNSAGYLLGPAEDLARYYSALRHYINLITRQRY-NH_2
Mastoparan (MP)-Containing Sequences	
MP	INLKALAALAKKIL-NH_2
MP S	INWKGIASM*Aib*RQVL-NH_2
QLKK-MP	QLKKINLKALAALAKKIL-NH_2
SFLLR-MP	SFLLRINLKALAALAKKIL-NH_2
ZGF-MP	ZGFINLKALAALAKKIL-NH_2
M391, [Phaa*D*Tyr(Me)^{2}Arg^{6}Tyr^9]AVP-ϵAhx-MP	Phaa-y(Me)FQNRPRYϵ*Ahx*INLKALAALAKKIL-NH_2
M432, MP-hCGRP(28-37)	INLKALAALAKKILVPTNVGSKAF-NH_2
M435, hCGRP(1-15)-MP	ACDTATCVTHRLAGLINLKALAALAKKIL-NH_2
M436, MP-hCGRP(8-18)(28-37)	INLKALAALAKKILVTHRLAGLLSRVPTNVGSKAF-NH_2
Galparan, galanin(1-13)-MP	GWTLNSAGYLLGPINLKALAALAKKIL-NH_2
Receptor-derived sequences	
Rat V_{1a} receptor$^{9-24}$, ($rV_{1a}R^{9-24}$)	DRSVGNSSPWWPLTTE-NH_2
$rV_{1a}R^{39-52}$	DSPLGDVRNEELAK-NH_2
$rV_{1a}R^{185-194}$	EVNNGTKTQD-NH_2
Rat V_2 receptor$^{100-111}$	DATDRFHGPDAL-NH_2
Human P2Y$_2$ receptor$^{14-27}$ ($hP2Y_2R^{14-27}$)	GTWDGDELGRYCRF-NH_2
$hP2Y_2R^{94-107}$	RGDHWPFSTVLCKL-NH_2
$hP2Y_2R^{174-187}$	SARGGRVTCHDTSA-NH_2
$hP2Y_2R^{180-193}$	VTCHDTSAPELFSR-NH_2
$hP2Y_2R^{271-284}$	RSLDLSCHTLNAIN-NH_2
L312, Cys-VE cadherin$^{625-637}$	CRKQARAHGKSVPE-NH_2
L313, Cys-VE cadherin$^{755-766}$	CVDYDFLNDWGPR-NH_2

(*continued*)

Table 2. Biological Activities of Peptidyl Secretagogs

Peptide	Secretion (Fold/Basal)		Efficacy Index		Secretory Efficacy Ratio	PLD Activity (Fold/Basal)	Efficacy Index PLD
	5-HT	β-Hexosaminidase	5-HT	β-Hexosaminidase			
MP	12.86 ± 0.51	7.64 ± 1.06	1.00	1.00	1.00	2.85 ± 0.60	1.00
M432	3.07 ± 0.36	1.70 ± 0.11	0.16	0.11	1.45	1.36 ± 0.14	0.19
M435	14.26 ± 2.29	7.73 ± 0.32	1.12	1.01	1.11	1.75 ± 0.19	0.41
M436	13.97 ± 1.09	4.31 ± 0.76	1.09	0.50	2.18	1.20 ± 0.09	0.11
Galparan	4.89 ± 0.86	7.97 ± 0.95	0.33	1.05	0.31	5.39 ± 1.43	2.37
M391	5.65 ± 0.68	6.93 ± 0.47	0.39	0.89	0.44	5.07 ± 1.56	2.20
MP S	2.72 ± 0.23	10.68 ± 1.52	0.15	1.46	0.10	2.07 ± 0.39	0.58
QLKK-MP	1.78 ± 0.11	3.17 ± 0.43	0.07	0.33	0.21	1.84 ± 0.23	0.45
SFLLR-MP	2.89 ± 0.34	7.81 ± 0.62	0.16	1.03	0.16	4.21 ± 0.78	1.74
ZGF-MP	3.54 ± 0.45	9.75 ± 1.02	0.21	1.32	0.16	5.08 ± 0.36	2.21
M120	1.87 ± 0.29	0.85 ± 0.11	0.07	n.a.	n.a.	0.81 ± 0.01	n.a.
Substance P	3.28 ± 1.17	1.06 ± 0.13	0.19	0.01	19.00	0.90 ± 0.07	n.a.

Data indicating secretion (fold/basal) and PLD activation (fold/basal) are mean ± SEM of three independent determinations at a final peptide concentration of 3×10^{-5} M. Efficacy indices express the above basal activities of secretagogs as a fraction of the activity of MP, which is ascribed a value of 1.00 in all assays. Secretory efficacy ratios are calculated as the efficacy ratio 5-HT/efficacy ratio β-hexosaminidase. Throughout the text the term "selective" is used to describe a peptidyl secretagog with an efficacy ratio of >2 or <0.5.

range of cell types. Our initial studies were designed to reassess this phenomenon by evaluating the secretory efficacies of 56 peptides that, with the exception of oxytocin, contain one or more basic residues (Lys or Arg; Table 1). As indicated in Table 1, the chosen peptides included 35 monomeric and chimeric sequences related to hormones and neuropeptides, ten MP-related sequences, and a group of 11 peptides representing sequences from extracellular domains of G protein-coupled receptors and the intracellular domains of VE cadherin. The secretory efficacies of all peptides, at concentrations of 1–100 μM, were determined via assays of both 5-HT and β-hexosaminidase exocytosis. These studies clearly endorsed the unique properties of MP and selected analogs as potent peptidyl secretagogs of both 5-HT and β-hexosaminidase (Table 2; Figure 1). Significantly, all other sequences displayed weak (substance P and M120, Table 2) or zero secretory efficacies in either assay system.

Mastoparan Analogs Are Differential Secretagogs

Data presented in Figure 1 and summarized in Table 2 clearly indicate that analogs of MP displayed a range of secretory efficacies that can surpass that of MP in either assay system. The composite effects of both amino- and carboxy-terminal extension of MP in chimeric constructs were dependent on both the secretory mechanism and the sequence. Significantly, M435 and M436, analogs of MP extended by sequences of hCGRP at the amino- and carboxy-temini, respectively, displayed similar potencies to MP in 5-HT secretion assays (Table 2). All other amino-terminally extended chimeric mastoparans, combining MP with sequences known to modify secretion, were relatively weak 5-HT secretagogs (efficacy indices < 0.5, Table 2).

Comparative data measuring β-hexosaminidase secretion revealed a distinctly different pattern of results. Intriguingly, MP S, a reported G_s-selective analog of MP, proved to be the most potent and selective activator of β-hexosaminidase secretion (efficacy ratio 0.10, Table 2). Of the chimeric constructs, both galparan and ZGF-MP were more potent and selective activators of β-hexosaminidase secretion than MP (Table 2).

Figure 2 directly compares the potencies of peptidyl secretagogs for both 5-HT and β-hexosaminidase secretion and further reveals a lack of correlation between the two data sets. This lack of correlation is confirmed by statistical analysis (Spearman $r = 0.210$; $p = 0.514$). These comparative data revealed three broad classes of peptidyl secretagogs. Class 1, including QLKK-MP, M432, and substance P, were relatively weak secretagogs (efficacy indices < 0.5) of both 5-HT and β-hexosaminidase. Despite its relatively low potency, substance P, with an efficacy ratio of 19 (Table 2), was the most selective activator of 5-HT secretion. Class 2, the quantitatively dominant group, contained MP S and four chimeric constructs that were selective activators of β-hexosaminidase secretion (β-hexosaminidase efficacy indices > 0.5, 5-HT efficacy indices < 0.5). Class 3 contained MP, M435, and M436, peptides that were potent activators (efficacy indices ≥ 0.5) of both β-hexosaminidase and 5-HT secretion. With an efficacy ratio of 2.18, M436 represents the only potent peptidyl secretagog that was relatively selective for 5-HT.

Table 1 continued.

Details of the synthesis, purification, and biological properties of many of these peptides are reported in references [2, 4, 7, 20–30]. Basic residues are underlined. Abbreviations are as follows: ϵAhx, 6-aminohexanoic acid; Aib, α-aminoisobutyric acid; AVP, [Arg^8]vasopressin; d$(CH_2)_5$, β-mercapto-β,β-cyclopentamethylene propionic acid; BK, bradykinin; Gal, galanin; hCGRP, human calcitonin gene-related peptide; MP, mastoparan; NPY, neuropeptide Y; Pyr, L-pyroglutamic acid; Thi, β-thienyl-L-alanine; *D*Tyr(Me), *O*-methyl-D-tyrosine; *D*Tyr(Et), *O*-ethyl-D-tyrosine; Pa, propionic acid; Phaa, phenylacetic acid; and Z, benzyloxycarbonyl. D amino acids are prefixed by *D* when triplet nomenclature is used, and they are presented as lowercase symbols when single-letter nomenclature is used. (*R*) indicates a retro-inverso peptide synthesized in reverse sequence from mostly D amino acids.

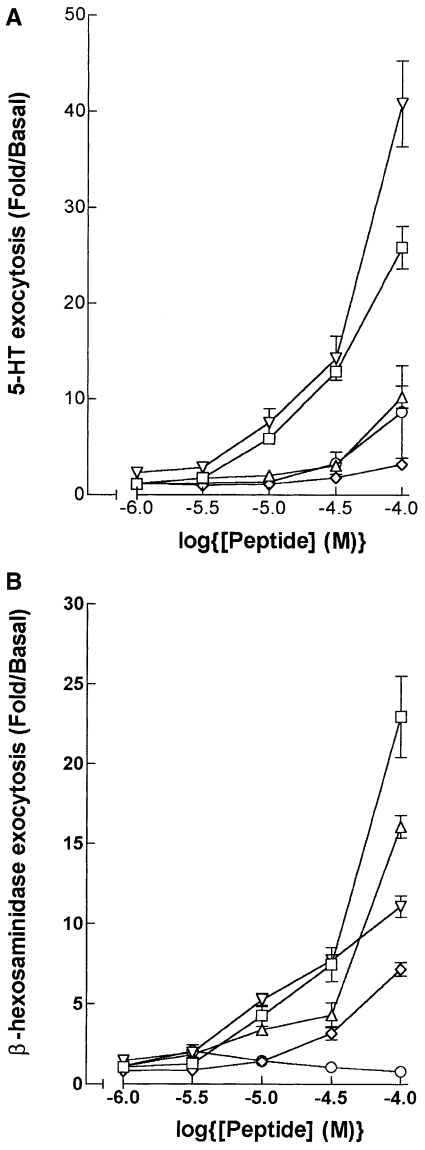

Figure 1. Peptide-Induced Secretion of 5-HT and β-Hexosaminidase

This figure indicates the ranges of peptide-induced exocytotic responses when the secretion of 5-HT (A) and that of β-hexosaminidase (B) are measured. Data are means ± SEM from single experiments performed in triplicate. In these experiments maximal secretory responses released approximately 80%–90% of the total cellular content of either 5-HT or β-hexosaminidase. Symbols are as follows: (A) squares, MP; right-side-up triangles, M432; upside-down triangles, M435; diamonds, QLKK-MP; circles, substance P. (B) squares, MP; triangles, M391; upside-down triangles, M435; diamonds, QLKK-MP; circles, substance P.

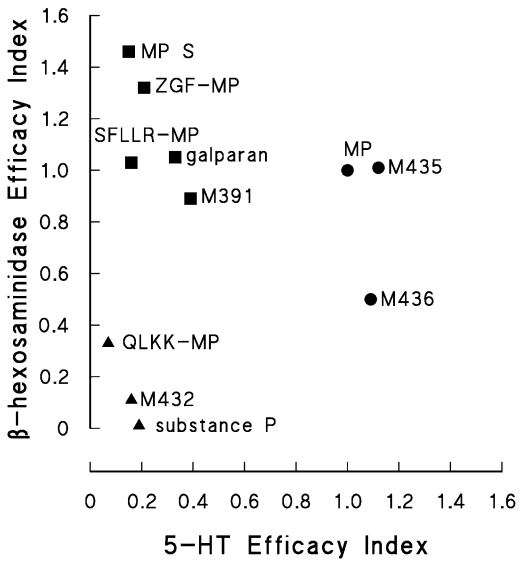

Figure 2. Comparison of the Efficacy Indices of Peptidyl Secretagogs

Symbols used to specify three different classes of peptidyl secretagog (see text) indicate: (squares) selective, high-efficacy β-hexosaminidase secretagogs; (circles) Non-selective, high-efficacy secretagogs; and (triangles) relatively low-efficacy secretagogs. This figure also indicates the lack of correlation between the secretory indices of individual secretagogs.

Is There a Causative Relationship between PLD Activation and Peptide-Induced Secretion?

The activity of PLD has been implicated in the regulated secretory mechanism linking IgE receptor crosslinking to the degranulation of RBL-2H3 [5]. Thus, to provide further insight to the molecular mechanisms responsible for the differential activities of peptidyl secretagogs, we measured phosphatidylbutanol accumulation as an index of PLD activation. Figure 3 indicates that the activation of PLD by peptidyl secretagogs was a complex and concentration-dependent phenomenon. As summarized in Table 2, the rank order of potency for PLD activation was galparan > ZGF-MP = M391 > SFLLR-MP > MP > QLKK-MP = M435 > M432 = M436. Both substance P and M120 were weak antagonists of PLD at 30 μM (Table 2).

Comparative data indicated fundamental differences in the contribution of PLD activity to the secretion of 5-HT or β-hexosaminidase (Figure 4). No causal relationship was observed when PLD activation and 5-HT secretion were compared (Figure 4A), a result confirmed by Spearman correlation analysis ($r = 0.182$; $p = 0.573$). Indeed, the majority of peptides with 5-HT secretory efficacies of less than 0.5 displayed efficacies for PLD activation that broadly ranged from 0.19 (M432) to 2.37 (galparan). Moreover, although M436 and M435 were more potent 5-HT secretagogs than MP, these chimeric constructs were relatively weak activators of PLD.

In marked contrast to the above comparisons, we observed a clear correlation when we compared efficacies for PLD activation and β-hexosaminidase secretion (Figure 4B). This correlation was statistically confirmed (Spearman $r = 0.776$; $p = 0.004$). When we considered β-hexosaminidase, the properties of chimeric constructs were most illuminating. Thus, the relatively potent β-hexosaminidase secretory efficacies of galparan, M391, SFLLR-MP, and ZGF-MP correlated with PLD activation efficacies, which were greater than 1.5. MP S, the most potent activator of β-hexosaminidase secretion, was also a relatively potent activator of PLD (efficacy index, 0.58). Significantly, the least potent β-hexosaminidase

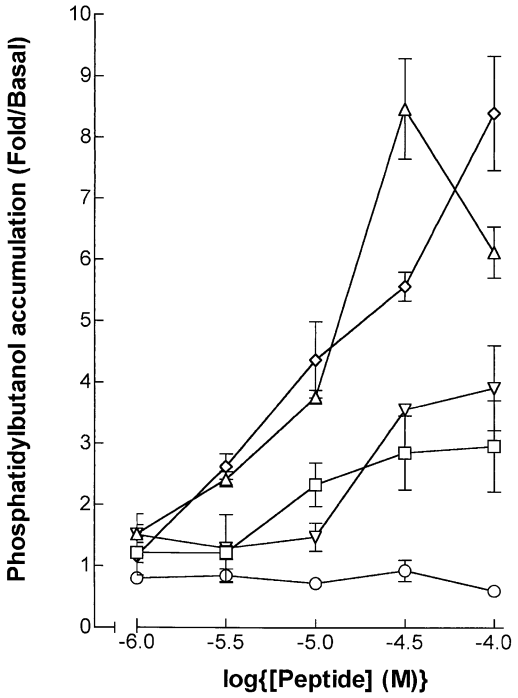

Figure 3. Peptide-Induced Accumulation of Phosphatidylbutanol

This figure indicates the range of efficacies for peptide-induced phosphatidylbutanol accumulation used as an index of PLD activity. Data are means ± SEM from single experiments performed in triplicate. Symbols are as follows: squares, MP; right-side-up triangles, galparan; upside-down triangles, MP S; diamonds, ZGF-MP; circles, substance P.

secretagogs, M436, M432, and QLKK-MP, all displayed PLD activation efficacies of less than 0.5. These data indicate that PLD activity does participate in the exocytotic mechanism leading to peptide-induced β-hexosaminidase release from RBL-2H3.

Discussion

Numerous basic amphiphilic peptides are reported to promote secretion from mast cells [1, 31–34]. A common mechanism that might explain the receptor-independent actions of structurally diverse peptidyl secretagogs is their interaction with intracellular heterotrimeric G proteins. Clearly, such a mechanism would require the efficient membrane translocation of peptides in order to enable access to their intracellular targets. Studies with a fluorescent analog of substance P have endorsed the above conclusion and have revealed a rapid receptor- and energy-independent uptake of the peptide into rat peritoneal mast cells [31]. Subsequent to membrane translocation, a specific interaction of substance P with G protein α subunits is believed to induce degranulation [31]. Significantly, this study identified only two peptide secretagogs, M120 and substance P, which are structurally unrelated to MP and are capable of stimulating secretion from RBL-2H3. Both M120 and substance P displayed relatively low 5-HT secretory efficacies and low or zero efficacy to promote β-hexosaminidase exocytosis. These findings further endorse the unique properties of MP as a peptidyl secretagog and indicate that the presence of one or more basic residues is not a reliable indicator of secretory efficacy in RBL-2H3.

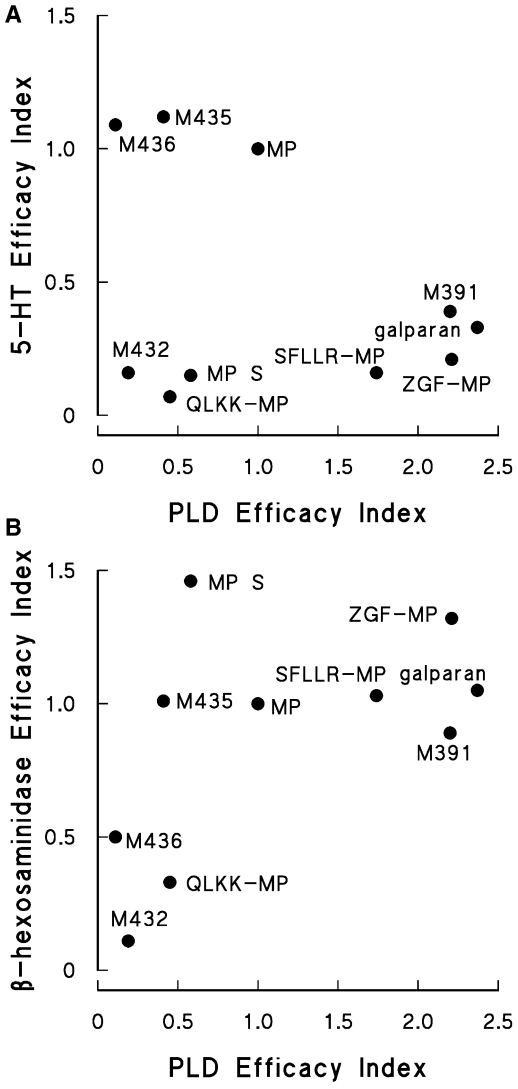

Figure 4. Comparison of Efficacies for Secretion and PLD Activation

This figure reveals the statistically verified correlation between PLD activation and the secretion of β-hexosaminidase but not 5-HT.

The design of chimeric constructs SFLLR-MP, QLKK-MP, and ZGF-MP utilized sequences known to modulate secretion [17–19] as amino-terminal extensions of MP. SFLLR is an agonist motif of the tethered ligand of the PAR-1 thrombin receptor [17]. Moreover, the pentapeptide SFLLR-NH_2 induces histamine release from rat peritoneal mast cells [35]. QLKK is the amino terminal of QLKKLKEICEKEKKELKKKMDKKRQEKITEAK, a highly basic peptide derived from the G protein-interacting region of Gαq. This sequence, spanning Gln^{1053}–Lys^{1084} in the carboxyl terminus of Gαq, blocks GTP-dependent activation of phospholipase C-β1 (PLC-β1) by Gαq in vitro [18]. We utilized the sequence QLKK to introduce an additional pair of basic residues to the MP sequence. ZGF-NH_2 is a dipeptide-competitive metalloendoproteinase inhibitor that is reported to modify Ca^{2+}-depen-

dent membrane fusion, release of Ca^{2+} from intracellular organelles, and stimulus-secretion coupling in a variety of cell types [19, 36]. All of these amino-terminal extensions had a profound influence on the secretory efficacies of chimeric constructs. As indicated by the properties of QLKK-MP, the di-basic sequence QLKK was clearly deleterious to peptide-induced secretion of both 5-HT and β-hexoasaminidase and PLD activation. A possible explanation for the pronounced effect of QLKK is a direct influence upon the membrane-associated secondary structure of the chimera. Indeed, there is ample evidence that MP adopts a highly amphipathic helix with all charged residues in a hydrophilic site when it interacts with biological membranes to activate G proteins [reviewed in 15]. However, in this regard it is noteworthy that the sequence SFLLR also provides a single charged residue adjacent to Ile in SFLLR-MP. Further experiments to determine whether QLKK-MP has any direct action upon the activity of PLC-β1 are obviously justified. As β-hexoasaminidase secretagogs, both SFLLR-MP and ZGF-MP were potent and selective, properties that group these novel constructs together with MP S, galparan, and M391. SFLLR-MP and ZGF-MP were also efficient activators of PLD, a common property of potent β-hexoasaminidase secretagogs. Based on our previous studies with galparan and M391 [3, 4], we predict that SFLLR-MP and ZGF-MP will be potent insulin secretagogs. Moreover, these novel chimeric constructs could prove to be valuable probes for studying the roles of thrombin receptors and metalloproteinases, respectively.

Characterization of the secretory efficacies and PLD-activating potency of MP S, a presumed G_s-selective analog of MP [37], provided intriguing data. MP is a relatively specific activator of G_i/G_o [10] and, since $G_{i\alpha 3}$ has been identified as a specific target for MP in rat peritoneal mast cells [38], we predicted that MP S would possess relatively low secretory efficacy or indeed act as a "secretory antagonist." However, our data clearly indicate that, of all the peptides studied herein, MP S is the most potent and selective β-hexoasaminidase secretagog. These data may indicate that the activation of G proteins per se is not a sole determinant of the secretory efficacies of peptidyl secretagogs. This conclusion is supported by our previous study [6], in which we have shown galparan to be a non-competitive inhibitor of GTPases in rat brain cortical membranes, an effect that is reversed by MP. Moreover, we have also demonstrated [4] that M391 has a complex, concentration-dependent action upon GTPase activity in Rin m5F cell membranes. Individual components of this novel effect of M391 are most likely the selective activation of $G\alpha_i/G\alpha_o$ and suppression of other G proteins, particularly $G\alpha_s$ [4]. Data presented herein provide additional evidence that analogs of MP interact with G proteins at different loci and that these activities are not reliable indicators of secretory efficacy.

Collectively, the chimeric MP analogs used in this study include constructs that bind to receptors for AVP, galanin, and CGRP. However, in vitro all of these constructs are functional antagonists, and peptides such as galparan and M391 are reported to stimulate insulin secretion by a receptor-independent mechanism [3, 4]. Moreover, our extensive studies with RBL-2H3 have failed to detect any endogenously expressed receptors for peptide hormones or neuropeptides. We therefore conclude that the differential activities of structural analogs of MP are a consequence of their selective interactions with multiple intracellular loci of action. In addition to heterotrimeric G protein α subunits, intracellular targets for MP analogs with the potential to modulate both secretion and PLD activity, include the small GTPases Rac, Rho, and members of the Rab family [39–41]. Moreover, MP, galparan, and M391 mobilize Ca^{2+} in vitro by a selective interaction with both Ca^{2+}-ATPases and the ryanodine receptor [14]. Thus, effects upon Ca^{2+} homeostasis will probably also contribute to the diverse biological activities of MP analogs.

Significance

Our findings clearly support the unique properties of MP as a precursor for the synthesis of peptidyl secretagogs that selectively activate individual secretory pathways. We introduce the term *differential secretagog* to describe this unique class of biological agents. Basic residues per se are not minimal secretory pharmacophores in RBL-2H3. Structural modifications of MP, including amino- and carboxy-terminal chimeric extensions, have a profound influence upon secretory efficacies. A quantitatively dominant group of MP analogs, including MP S, ZGF-MP, and galparan, selectively activate the secretion of β-hexosaminidase. Chimeric constructs combining MP with sequences of human CGRP are relatively potent, non-selective 5-HT and β-hexosaminidase secretagogs. Comparison of the secretory activities of MP analogs revealed fundamental differences in the molecular regulation of the exocytosis of 5-HT and β-hexosaminidase. Significantly, statistical analyses of comparative data confirmed that peptide-induced activation of PLD correlates with the exocytosis of β-hexosaminidase but not 5-HT. We conclude that structurally modified MP constructs are unique probes for studying the molecular regulation of biological phenomena.

Experimental Procedures

Materials

BK, Des-Arg^{9}BK, [Thi^{5,8}*D*-Phe^7]BK, Lys^{0}BK, AVP, oxytocin, neurotensin, endothelin-1, bombesin, mastoparan, substance P, and substance K were from Bachem (UK) Salmon calcitonin was purchased from Sigma. Cell culture media were obtained from PAA Laboratories (Kingston upon Thames, UK). 5-hydroxy[G-^{3}H]tryptamine creatinine sulfate ([^{3}H]5-HT, 10.2 Ci/mmol) and [9,10(n)-^{3}H]palmitic acid (51.0 Ci/mmol) were from Amersham Pharmacia Biotech UK (Little Chalfont, UK).

Peptide Synthesis

Details of the syntheses and biological properties of custom-synthesized peptides are contained in references [2, 4, 7, 20–30]. Materials for peptide synthesis were from Novabiochem (UK). MP S [37], QLKK-MP, SFLLR-MP, and ZGF-MP were manually synthesized (50 μM scale) on Rink amide MBHA resin by employing an N-α-Fmoc protection strategy with HBTU/HOBt activation. Crude peptides were purified to apparent homogeneity by semipreperative scale HPLC [42], and their predicted masses were confirmed by MALDI TOF mass spectrometry.

Maintenance and Labeling of RBL-2H3

The RBL-2H3 cell line was obtained from M.J.O. Wakelam, University of Birmingham. Cells were maintained in DMEM containing 0.1 mg/ml L-glutamine and supplemented with 10% v/v fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For the majority of experiments, subconfluent cells in 24-well plates were washed with and transferred into a labeling medium consisting of Hams medium supplemented with 0.1% w/v BSA and 10 mM HEPES. Cells were incubated for 24 hr in 0.5 ml/well labeling medium containing 0.1 μCi/ml [^{3}H]5-HT.

Secretion Assays

[^{3}H]5-HT-labeled cells were washed twice, and peptides were added for a period of 15 min at 37°C in a final volume of 0.25 ml. The medium was collected, and cell debris was removed by centrifugation. Secreted [^{3}H]5-HT was determined in media samples by scintillation spectroscopy.

Secreted β-hexosaminidase was assayed in samples of cell medium. Cellular β-hexosaminidase activity was determined in 0.1% v/v Triton X-100 extracts of cell monolayers. For both assays, 5 μl samples were transferred into 96-well plates and incubated with 20 μl of 1 mM ρ-nitrophenyl N-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer for 1 hr at 37°C. Two hundred microliters of a 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 10.5) was then added, and β-hexosaminidase activity was determined by colorimetric analysis on a microtitre plate reader at 405 nm.

Phospholipase D Assays

The activities of PLD in RBL-2H3 cells labeled with [^{3}H]palmitic acid were determined with our previously reported transphosphatidylation assay in the presence of 0.3% v/v butan-1-ol [43].

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