Intracellular Delivery of Bioactive Peptides to RBL-2H3 Cells Induces β-Hexosaminidase Secretion and Phospholipase D Activation

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This investigation compared the secretory efficacies of a series of peptides delivered to the cytoplasm of RBL-2H3 mast cells. Mimetic peptides, designed to target intracellular proteins that regulate cell signalling and membrane fusion, were synthesised as transportan 10 (TP10) chimeras for efficient plasma membrane translocation. Exocytosis of β-hexosaminidase, a secretory lysosomal marker, indicated that peptides presenting sequences derived from protein kinase C (PKC; C1 H-CRRLSVEIWDWDL-NH_2) and the CB_1 cannabinoid receptor (C3 H-RSKDLRHAFRSMFPSCE-NH_2) induced β-hexosaminidase secretion. Other peptide cargoes, including a Rab3A-derived sequence and a homologue of C3, were inactive in similar assays. Translocated C1 also activated phospholipase D (PLD), an enzyme intimately involved in the regulated secretory response of RBL-2H3 cells, but C1-induced secretion was not dependent upon phosphatidate synthesis. Neither down-regulation of Ca^{2+}-sensitive isoforms of PKC nor the application of a selective PKC inhibitor attenuated the secretory efficacy of C1. These observations indicate that the molecular target of C1 is a protein involved in the regulated secretory pathway that is upstream of PLD but is not a PKC isoform. This study also confirmed that TP10 is a relatively inert cell-penetrating vector and is, therefore, widely suitable for studies in cells that are sensitive to peptidyl secretagogues.

Introduction

Cellular secretion is a consequence of discrete interactions between proteins that modulate intracellular signalling and membrane trafficking. The molecular interfaces and regulatory domains of intracellular proteins are, therefore, attractive targets for mimetic peptides that selectively modulate their function(s). A powerful approach to study and specifically modulate discrete protein – protein interactions within intact viable cells is the employment of cell-penetrating vectors to deliver cargoes to the intracellular compartments. For this purpose, a variety of cell-penetrating peptides (CPPs) have been employed as vectors for the delivery of both peptide and peptide nucleic acid probes to eukaryotic cells (reviewed in ref. [1]).

The RBL-2H3 cell line[2] is a widely studied model of secretory mucosal type mast cells. Numerous basic amphiphilic peptides are reported to promote secretion from mast cells (reviewed in ref. [3]). However, our recent comparative studies of a diverse range of putative peptidyl secretagogues indicate that basic residues per se are not sufficient to promote secretion from RBL-2H3 cells.[4] The same studies[4] identified a range of rationally designed peptides, structural analogues of the tetradecapeptide mastoparan (MP), that differentially stimulate the secretion of either 5-HT or β-hexosaminidase, a secretory lysosomal marker, from RBL-2H3 cells. Thus, the RBL-2H3 line was used in these studies to determine the utility of translocated mimetic peptides as molecular probes of secretion.

Transportan (galanin(1 – 12)-Lys-MP[5]), a chimeric amino-terminal extended analogue of MP, is a synthetic CPP with efficient cell-penetrating properties.[5] Moreover, the deletion analogue transportan 10 (TP10; galanin(7 – 12)-Lys-MP[6]) retains efficient translocating properties but lacks the inhibitory action on GTPases that is characteristic of most other transportan (TP) analogues. Thus, in addition to analysing the secretory efficacies of a range of different peptide cargoes, this study was also designed to further address the utility of TP10 as a vector for applications in cells that are usually responsive to peptidyl secretagogues such as MP. Rationally designed cargoes utilised in this study included sequences designed to target and activate protein kinase C and $G_{\alpha i/o}$, proteins widely reported to modulate regulated secretory pathways.[7, 8] This study also compared the secretory efficacy of a Rab3A effector domain peptide previously reported to stimulate insulin secretion from permeabilised HIT-T15 cells.[9a]

Results

Design and synthesis of cell-penetrant peptides

The synthetic strategy illustrated in Scheme 1 proved reliable for the synthesis of TP10 chimeras bearing four different peptide cargoes (C1 – C4) designed to target proteins with the potential to modulate β-hexosaminidase secretion. C1 (H-CRRLSVEIWDWDL-NH_2) is a sequence from the middle of the

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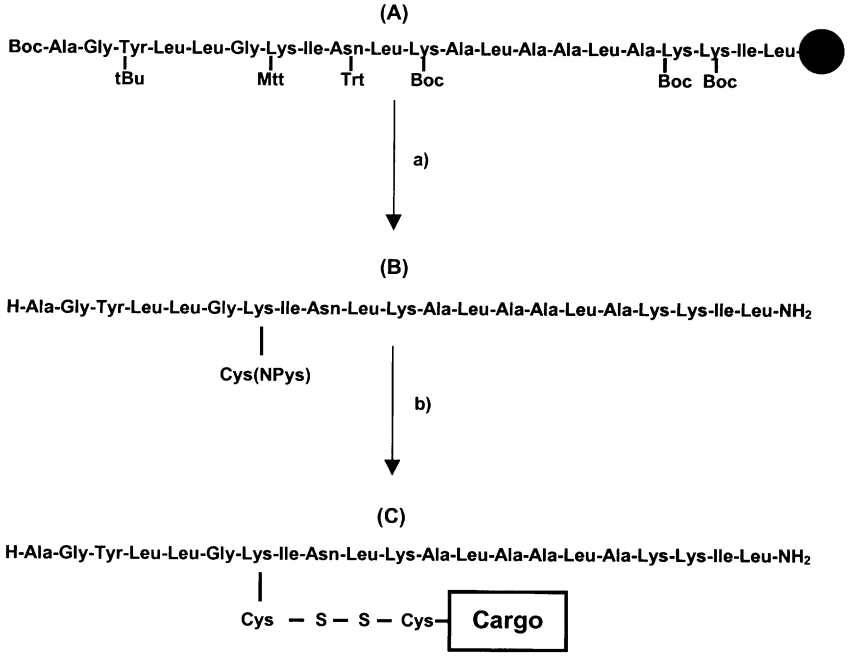

Cargo sequence		Protein source
C1	H-**Cys**-Arg-Arg-Leu-Ser-Val-Glu-Ile-Trp-Asp-Trp-Asp-Leu-NH_2	Cys^0-$PKC^{238-249}$
C2	H-**Cys**-Thr-Pro-Ala-Phe-Val-Ser-Thr-Val-Gly-Ile-Asp-Phe-Lys-Val-Lys-NH_2	Cys^0-$Rab3A^{47-61}$
C3	H-Arg-Ser-Lys-Asp-Leu-Arg-His-Ala-Phe-Arg-Ser-Met-Phe-Pro-Ser-**Cys**-Glu-NH_2	$CB_1^{401-417}$
C4	H-**Cys**-Arg-Ser-Lys-Asp-Leu-Arg-His-Ala-Phe-Arg-Ser-Met-Phe-Pro-Ser-Ser-Glu-NH_2	Cys^0-$[Ser^{416}]CB_1^{401-417}$

Scheme 1. *Synthetic route to chimeric CPPs. TP10 (A) and peptide cargoes (C1 – C4; see below) were manually synthesised (0.1 or 0.2 mmol scale) on Rink amide methylbenzhydrylamine resin by employing an Fmoc protection strategy with HBTU/HOBt activation (Fmoc = 9-fluorenylmethoxycarbonyl, HBTU = 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt = N-hydroxybenzotriazole). Selective removal of the 4-methyltrityl group of Lys7 with trifluoroacetic acid (TFA) (3% (v/v) in dichloromethane (DCM; 2 × 10 min), acylation with Boc-Cys(NPys) (2 equiv) and cleavage with TFA/H_2O/triisopropylsilane (95:2.5:2.5%) yielded the fully deprotected [Lys7$N^{\varepsilon Cys(Npys)}$]TP10 (B). Disulfide-bond formation, to generate TP10 chimeras (C), was achieved by dissolving [Lys7$N^{\varepsilon Cys(Npys)}$]TP10 and individual cargoes (twofold molar ratio) in a minimum volume of DMF/DMSO/$C_2H_3O_2Na$ (0.1 M) pH 5.0 (3:1:1) and mixing overnight.[9b] The sequences of all cargoes and their protein sources are also indicated. Cysteine residues indicated in bold represent the point of attachment of peptide cargoes to [Lys7$N^{\varepsilon Cys(Npys)}$]TP10. a) i) 3% TFA/DCM, ii) 2 equiv Boc-Cys(NPys)/HBTU/HOBt/collidine, iii) TFA/H_2O/TIS, 95:2.5:2.5%; b) 2 equiv Cys-cargo in DMF/DMSO/$C_2H_3O_2Na$ (0.1 M) pH 5.0 (3:1:1).*

C2 region of the regulatory domain of human protein kinase C β1 ($PKC^{238-249}$). For these studies we extended the original sequence of pseudo-RACK1 (SVEIWD; RACK = receptor for activated C kinase) to include an additional β-PKC-derived sequence including one further WD motif and an amino-terminal Cys for attachment to [Lys7$N^{\varepsilon Cys(Npys)}$]TP10.[12]

Cargo C2 mimics residues 47 – 61 of the effector domain of the human Rab3a GTPase. The peptide used in this study included sequences flanking FVSTVGIDF, a Rab3A-derived peptide that can promote exocytosis from a range of permeabilised cells including rat peritoneal mast cells.[9a, 13, 14]

C3 is a predicted juxtamembrane C-terminal sequence, residues 401 – 407, of the rat CB_1 cannabinoid receptor. C4 is a homologue of C3 with a cysteinyl amino-terminal extension and a Ser/Cys substitution at position 416. C4 was included in these studies as this peptide autonomously activates both G_o and G_i.[15a]

We confirmed that TP10 chimeras effectively penetrated RBL-2H3 cells by measuring the intracellular fluorescence of carboxyfluorescein diacetate-conjugated peptide cargoes.[15b] Using this system, we estimated an effective speed of uptake ($t_{0.5}$) of 6 min at 37 °C, a value comparable with that of 8.4 min in the Bowes melanoma cell line.[6] Intracellular fluorescence was essentially maximal by 60 min.

Peptide-induced secretion of β-hexosaminidase

A range of structural analogues of MP induce β-hexosaminidase secretion from RBL-2H3 cells.[4] Thus, one of the aims of this investigation was to determine whether TP10, a chimeric MP-containing construct, was suitably inert for studies with RBL-2H3 cells. As illustrated in Figure 1, [Lys7-$N^{\varepsilon Cys}$]TP10 was a very weak secretagogue active only at a concentration (> 3 μM) above those required for efficient cellular translocation. When tested alone, none of the peptide cargoes (C1 – C4) was a β-hexosaminidase secretagogue (Figure 1). Of the cargoes analysed in this study, translocated C2 and C4 were completely without effect, whilst intracellular delivery of C1 and C3 promoted a concentration-dependent increase in β-hexosaminidase secretion (Figure 1). On the basis of these experiments, cargo C1 was selected for the additional studies detailed below.

Mechanism(s) of C1-induced β-hexosaminidase secretion

The β-hexosaminidase secretory efficacies of peptidyl secretagogues are positively correlated to the activation of phospholipase D in RBL-2H3 cells.[4] Thus, we predicted that the cellular delivery of C1 would also stimulate PLD activity. As indicated in Figure 2, the TP10-C1 chimera activated PLD over the same range of concentrations as those required to promote β-hexosaminidase secretion. Significantly, neither [Lys7$N^{\varepsilon Cys}$]TP10 nor C1 alone had any effect upon PLD activity. Primary alcohols such as butan-1-ol inhibit the synthesis of phosphatidate (PA) by the action of PLD.[4, 11] Thus we used butan-1-ol (1%, *v/v*) and the noninhibitory control butan-2-ol, to determine the contribution of PA synthesis to C1-induced secretion. As reported in Table 1,

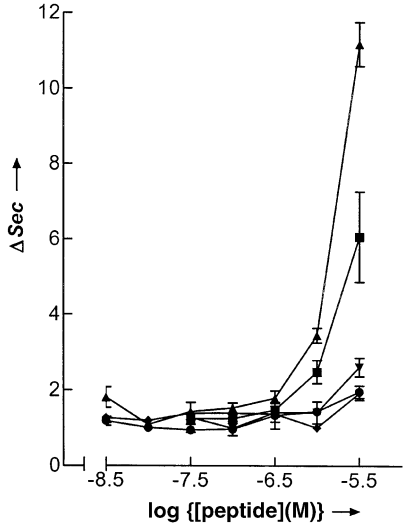

Figure 1. *Comparative secretory efficacies of cell-penetrant peptides. This figure compares the concentration-dependent induction of β-hexosaminidase secretion by [Lys7N$^{\varepsilon Cys(Npys)}$]TP10 and TP10-peptide chimeras. All data are means ± S.E.M. from at least three independent experiments, each performed in triplicate. In these experiments Abs_{405} values ranged from 0.011 (minimum basal) to 0.221 (maximum stimulation). ΔSec indicates changes in β-hexosaminidase secretion and data points are calculated as fold/basal levels. Legend: ▲ TP10-C1, ◆ TP10-C2, ■ TP10-C3, ▼ TP10-C4, ● [Lys7N$^{\varepsilon Cys(Npys)}$]TP10.*

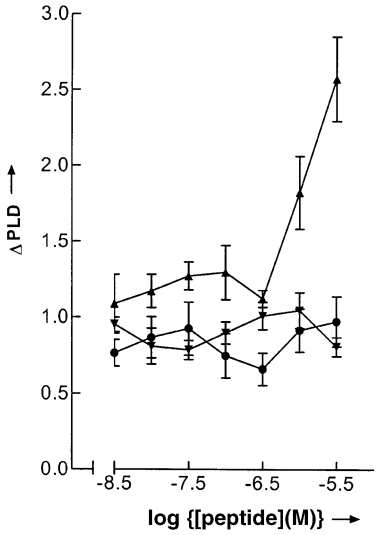

Figure 2. *TP10-C1-induced accumulation of Pbut. RBL-2H3 cells were incubated with peptides for 60 min at 37 °C and Pbut accumulation determined as an index of PLD activity. ΔPLD indicates changes in phosphatidylbutanol accumulation, and data points are calculated as fold/basal levels. Data are means ± S.E.M. of three independent experiments, each performed in triplicate. Legend: ▲ TP10-C1, ● [Lys7N$^{\varepsilon Cys}$]TP10; ▲ C1.*

neither alcohol attenuated TP10-C1-induced β-hexosaminidase secretion; this indicates that this exocytotic response was not dependent upon the de novo synthesis of PA.

Additional studies were designed to determine whether the secretory action of C1 was dependent on PKC activation. As indicated in Table 1, neither down-regulation of Ca^{2+}-sensitive isoforms of PKC with phorbol 12-myristate 13-acetate (100 nM) nor selective inhibition of the catalytic activity of PKC with (2-(8-(dimethylaminomethyl)-6,7,8,9-tetrahydropyridol[1,2-a]indol-3-yl)-3-(1-methylindol-3-yl)maleimide) (Ro-32-0432; 1 μM) produced any significant attenuation of the secretory efficacy of C1.

Table 1. *Normalised indices of TP10-C1-induced β-hexosaminidase secretion.*

Treatment	% β-Hexosaminidase secretion
TP10-C1	100
TP10-C1 + PMA (100 nM, 24 h)	101 ± 15
TP10-C1 + Ro-32-0432 (1 μM)	99 ± 8
TP10-C1 + butan-1-ol (1%, *v/v*)	109 ± 13
TP10-C1 + butan-2-ol (1%, *v/v*)	105 ± 11

Data are means ± S.E.M. from at least three experiments, each performed in triplicate. Ro-32-0432 and alcohol were added to cells 15 min before the addition of TP10-C1 (3 μM) for a further period of 60 min.

Discussion

The selection of TP10 as a cell-penetrating vector for these studies was based on several criteria. Extensive characterisation of transportans[5, 6, 16] has clearly indicated that free cargoes are liberated from the vector by reduction of the disulfide bond following cellular penetration. Moreover, TP10 does not influence the basal GTPase activity of cell membranes; this indicates that it is relatively inert compared with CPPs of the same generic class.[6] We considered this latter feature particularly important as other chimeric MP analogues are potent β-hexosaminidase secretagogues[4] and we were keen to eliminate any effects directly attributable to the CPPs in these studies. Our data confirm that TP10 can effectively penetrate a variety of cell types by a mechanism that is independent of receptor binding and internalisation.[6] Furthermore, [Lys7N$^{\varepsilon Cys}$]TP10 was a very weak secretagogue active at concentrations (3 μM) above those required for the effective delivery of peptide cargoes. Thus, we conclude that TP10 can be utilised for the intracellular delivery of bioactive molecules to cells, such as RBL-2H3, that are particularly responsive to MP and its structural analogues.[4]

The sequence RSKDLRHAFRSMFPSSE, a peptide mimic of a juxtamembrane segment of the carboxyl terminus of the rat CB_1 cannabinoid receptor, autonomously activates $G_{i/o}$ proteins and competitively disrupts interaction of the CB_1 receptor with $G_{\alpha o}$ and $G_{\alpha i3}$.[15, 17, 18] This peptide contains a Ser substitution for the natural Cys416, a strategy originally intended to reduce spontaneous disulfide-bridge formation.[18] The studies reported herein compared a Cys0-extended analogue of this peptide (C4, Cys0-[Ser416]$CB_1^{401-417}$) and the natural sequence of the same segment (C3, $CB_1^{401-417}$ H-RSKDLRHAFRSMFPSCE-NH_2). We chose to study the effects of C3 and C4 since MP, a potent β-hexosaminidase secretagogue,[4] is also a known activator of G proteins, particularly G_i and G_o. Moreover, $G_{\alpha i3}$ has been identified as a specific target for the secretagogue MP in rat peritoneal mast cells[7] and selectively regulates a slow component of MP-induced secretion from melanotrophs.[19] This latter observation is significant as the relatively slow kinetics of β-hexosaminidase secretion induced by TP10 chimeras and other peptidyl secre-

tagogues[4] supports the contention that there are multiple pathways of regulated secretion with distinct kinetics.[19] Intriguingly, we consistently observed that translocated C3 promoted the exocytosis of β-hexosaminidase, whilst C4 was inactive. Thus, Cys416 may be a significant pharmacophore for the interaction of C3 with its intracellular molecular target(s) in RBL-2H3 cells. C4 differs from the biologically active sequence previously reported[7, 15, 18] due to the Cys0-extension and an amidated carboxyl terminus. It is possible that either or both of these modifications could reduce the predicted interaction of C4 with G proteins that modulate secretion. Significantly, more recent studies have indicated that $G_{\alpha i3}$ is unlikely to be a component of the elusive, distally acting exocytotic G protein G_E in mast cells.[20] Thus, we conclude that the molecular target(s) of action underlying the secretory activity of translocated C3 is not $G_{\alpha i3}$ but must be another protein involved in the regulated secretory mechanism leading to β-hexosaminidase exocytosis.

The PKC-derived peptide SVEIWD, conventionally named pseudo-RACK1,[12] is located in the middle of the C2 regulatory domain of β-PKC. Homologous sequences are found in other PKC isoforms and also in four WD40 repeat domains of RACK1, a scaffolding protein that binds a variety of signalling proteins including PKC. The pseudo-RACK1 peptide directly binds PKC and acts as an agonist of PKC in vivo.[12] For the purpose of this study we synthesised an extended homologue of pseudo-RACK1 (C1, H-CRRLSVEIWDWDL-NH_2), a sequence that is conserved in rodent, human and bovine β-PKC, with an amino-terminal Cys for attachment to [Lys7N$^{\varepsilon Cys}$]TP10. Of all the mimetic peptides compared in this study, C1 was the most efficient inducer of β-hexosaminidase secretion. However, our data indicate that the mechanism of action of C1 is not a consequence of the activation of PKC. Though long-term treatment of RBL-2H3 cells with PMA has a profound influence on cell morphology, [21a] the magnitude of the C1-induced secretory response in cells treated with PMA for 24 h was identical to that of normal cells. Thus, C1 still induced secretion even under conditions expected to significantly down regulate Ca^{2+}-sensitive isoforms of PKC in RBL-2H3 cells.[8, 21b, 22] Similarly, treatment of cells with the PKC inhibitor Ro-32-0432 did not effect the magnitude of C1-induced secretion; this indicates that Ca^{2+}-insensitive isoforms of PKC are not molecular targets for this peptide either. Thus, we conclude that the primary intracellular target for C1, leading to β-hexosaminidase secretion from RBL-2H3 cells, is not a PKC isoform. Intriguingly, a database search (SIB BLAST network service) identified a sequence (SPLRVELWDWDM), homologous to C1, that is located within a C2 domain of a rasGAP-related protein.[23] Thus, we speculate that C1 might interfere with the normal function of C2 domains, which is to promote calcium-dependent phospholipid binding.

Further evidence that the locus of action of C1 is a protein component of the regulated secretory machinery was provided by the observation that translocated C1 also activated PLD, an enzyme intimately involved in the antigen-stimulated secretory response of RBL-2H3 cells.[24] We have previously demonstrated a clear correlation between the activity of PLD and the β-hexosaminidase secretory efficacies of MP analogues,[4] and MP directly activates PLD2 in RBL-2H3 membranes independently of PKC.[25] However, β-hexosaminidase secretion is not dependent upon the de novo synthesis of the putative second messenger PA, since butan-1-ol did not attenuate the C1-induced exocytotic response. Thus, the role of PLD in the secretion of β-hexosaminidase may be directly related to the enzymatic modification of lipid membranes rather than a consequence of PA synthesis. It is possible that C1 directly stimulates PLD or interacts with a known modulator of PLD activity that could include small GTPases and ADP-ribosylation factor 6 (ARF6).[26, 27] Our finding that [Lys7N$^{\varepsilon Cys}$]TP10 was without effect on PLD activity further endorses the utility of this construct as an inert cell penetrant vector.

Of all the cargoes designed for this study, we predicted that C2, a sequence corresponding to the effector domain of Rab3A, a small molecular weight GTPase, would be the most likely to promote β-hexosaminidase secretion. The Rab3A-derived peptide FVSTVGIDF is able to promote the complete exocytotic degranulation of rat peritoneal mast cells.[13] Competition with endogenous Rab3A for binding sites on target effector proteins is the most likely explanation for the above phenomenon.[13, 28] Both a cytoplasmic protein doublet[9a] and Rabphilin3A[29] are possible selective targets for Rab3A peptides that are part of the regulated exocytotic machinery. Thus, Rab3A effector domain peptides also promote amylase secretion and activate phospholipase C in permeabilised pancreatic acini[14, 28] and stimulate insulin secretion from HIT-T15 cells.[9a] However, our studies clearly indicated that translocated C2, an extended homologue of FVSTVGIDF, had no secretory activity in RBL-2H3 cells. A possible molecular explanation for the lack of effect of C2 is provided by the observation that a different Rab3 isoform, Rab3D, is specifically localised on the secretory granules of RBL-2H3 cells. Moreover, Rab3D is proposed to control antigen-stimulated exocytosis following translocation to the plasma membrane.[30, 31] Thus, it is possible that Rab3A, and presumably its molecular targets likely to bind C2, do not play a major role in the regulatory secretory mechanism of RBL-2H3 cells.

The activity of any translocated peptide cargo depends upon both its free intracellular concentration and its binding affinity for an accessible protein target. The bioactivities of C1 and C3 studied herein clearly indicate that the TP10 system delivered an intracellular concentration of peptide that was sufficient to promote secretion. We therefore conclude that TP10-mediated delivery of bioactive peptides is a powerful approach to study and modulate the activities of intracellular proteins that regulate cell signalling and exocytosis.

Experimental Section

Materials: *N*-α-*tert*-Butyloxycarbonyl-Cys(3-nitro-2-pyridinesulfanyl)-OH (Boc-Cys(NPys)-OH) (Boc = *tert*-butoxycarbonyl) was from Bachem (UK). All other materials for solid-phase peptide synthesis were from Novabiochem (UK). Cell culture medium was obtained from PAA Laboratories (UK). [9,10(n)-^{3}H]Palmitic acid (51.0 Ci mmol^{-1}) was from Amersham Pharmacia Biotech (UK). PMA was from Sigma (UK) and Ro-32-0432 (2-(8-(dimethylaminomethyl)-6,7,8,9-tetrahydropyridol[1,2-a]indol-3-yl)-3-(1-methylindol-3-yl)maleimide) was from Calbiochem (UK).

Peptide synthesis, purification and analysis: The synthetic route to TP10-conjugated peptides is illustrated in Scheme 1. Peptide conjugates (C) and individual cargoes (C1 – C4) were purified to apparent homogeneity by semipreparative-scale HPLC.[10] The predicted masses of all peptides used in this study (average $[M+H]^+$) were confirmed to an accuracy of ± 1 by matrix-assisted laser desorption ionisation time of flight MS (Kratos *Kompact Probe* operated in positive ion mode).

Analysis of peptide-induced β-hexosaminidase secretion: The RBL-2H3 cell line was maintained in Dulbecco's modified Eagle medium (DMEM) containing L-glutamine (0.1 mg ml^{-1}) and supplemented with foetal bovine serum (10%, *w/v*), penicillin (100 U ml^{-1}) and streptomycin (100 μg mL^{-1}) in a humidified atmosphere of 5% CO_2 at 37 °C. Secreted β-hexosaminidase was assayed in samples of cell medium.[4] To enable maximal delivery of peptide cargoes, confluent cells in 24-well plates were treated with TP10 chimeras for a period of 60 min at a maximum concentration of 3 μM. We confirmed, by using a variety of MP analogues,[32] that this experimental protocol had no adverse effect on cell viability. Samples of medium (5 μL) were transferred into 96-well plates and incubated with ρ-nitrophenyl *N*-acetyl-*β*-D-glucosamide (20 μL, 1 mM in 0.1 M sodium citrate buffer, pH 4.5) for 1 h at 37 °C. Na_2CO_3/$NaHCO_3$ buffer (200 μL, 0.1 M, pH 10.5) was then added, and β-hexosaminidase activity was determined by colorimetric analysis at 405 nm.

Phospholipase D assays: The activities of PLD in RBL-2H3 cells, labelled with [^{3}H]palmitic acid (1 μCi) for 24 h, were determined as previously reported[4] by using a transphosphatidylation assay in the presence of butan-1-ol (0.3%, *v/v*[11]). These assays measured the accumulation of metabolically stable phosphatidylbutanol (PBut) as an index of PLD activity. Radiolabelled cells, in 24-well plates, were treated with TP10-C1 (Scheme 1) for 60 min, and PBut was separated from other the lipids by TLC.[11] The radioactivity in PBut fractions was determined by liquid-scintillation spectroscopy.

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Keywords: enzymes · mast cells · peptidomimetics · secretion · transportan

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