Release of Ca^{2+} from intracellular organelles by peptide analogues: evidence against involvement of metalloendoproteases in Ca^{2+} sequestration by the endoplasmic reticulum

Margaret A. BROSTROM,* Wai Lam WONG LING,† Debra GMITTER and Charles O. BROSTROM

Department of Pharmacology, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, U.S.A.

N-Benzyloxycarbonyl-Gly-Phe-amide (Z-Gly-Phe-NH₂), a competitive substrate for metalloendoproteases, mobilizes intracellular Ca^{2+} and suppresses protein synthesis and processing in a Ca^{2+} -dependent, reversible manner. To ascertain whether Z-Gly-Phe-NH₂ acts at Ca^{2+} -storing organelles, effects of the dipeptide on Ca^{2+} sequestration by saponin-porated GH₃ pituitary cells were examined. Porated preparations sequestered Ca^{2+} into two compartments with different Ca^{2+} affinities. Ca^{2+} accumulation at nM concentrations of free Ca^{2+} was inhibited by thapsigargin and inositol 1,4,5-triphosphate [Ins(1,4,5)P₃], enhanced by oxalate and unaffected by oligomycin. Cation accumulation at μM concentrations of free Ca^{2+} was sensitive to oligomycin but not to thapsigargin. Z-Gly-Phe-NH₂ reduced Ca^{2+} sequestration by both compartments. The dipeptide mobilized Ca^{2+} from the high-affinity compartment within 1–2 min without affecting Ca^{2+} uptake. Ca^{2+} was mobilized more rapidly by Z-Gly-Phe-NH₂

and thapsigargin together than by either agent alone. The presence of a thiol-reducing agent was required for Ca^{2+} mobilization by Z-Gly-Phe-NH₂ but not by thapsigargin or Ins(1,4,5)P₃. Ca^{2+} mobilization by Z-Gly-Phe-NH₂ could not be attributed to effects on anion-permeability or to actions at Ins(1,4,5)P₃ or ryanodine receptors. Results with assorted peptide analogues did not favour suppression of metalloendoprotease activity in the Ca^{2+} -mobilizing action of Z-Gly-Phe-NH₂. The more hydrophobic analogue Z-L-Tyr-*p*-nitrophenyl ester was 60–80-fold more potent in mobilizing Ca^{2+} from intact and porated cells and perturbed the high-affinity Ca^{2+} -sequestering compartment selectively. Z-Gly-Phe-NH₂ and Z-L-Tyr-*p*-nitrophenyl ester are proposed to release Ca^{2+} from the endoplasmic reticulum through an ion pore with affinity for hydrophobic molecules containing internal peptide bonds.

INTRODUCTION

A variety of evidence supports the involvement of metalloendoproteases in structuring of the extracellular matrix, cell-to-cell fusion and exocytosis [1–4]. Metalloendoproteases of the endoplasmic reticulum (ER), plasmalemma and mitochondria have also been variously suggested to function in the processing of newly synthesized polypeptides, inactivation of peptide hormones and hydrolysis of membrane-associated proteins [4]. Much of the current understanding of metalloendoprotease function in cellular processes has been generated from studies utilizing synthetic peptides that, upon cellular penetration, compete with endogenous substrates for enzymic action. For example, the observation that certain benzyloxycarbonyl-dipeptide-amides (Z-dipeptide-amides) such as Z-Gly-Phe-NH₂ block all steps in intracellular protein transport in human hepatoma cells [5] is thought to favour the involvement of these enzymes in post-translational processing of secretory proteins. The findings [6–8] that these peptides perturb intracellular Ca^{2+} homeostasis are also viewed as being compatible with a role for metalloendoprotease activity in promotion of organellar Ca^{2+} sequestration.

Ca^{2+} sequestered by the ER functions in the maintenance of optimal rates of protein synthesis and early protein processing (reviewed in [9]). Mobilization of sequestered cation with Ca^{2+} ionophores or chelators or with the ER-specific Ca^{2+} -ATPase inhibitor thapsigargin produces a rapid blockade of mRNA

translation at the initiation step [10–12]. The inhibition of initiation is associated with phosphorylation of the α -subunit of initiation factor eIF-2 and with the loss of ability to resynthesize eIF-2·GTP complexes [13]. The same Ca^{2+} -mobilizing drugs cause retention of high mannose forms of secretory glycoproteins within the ER [14–17] and promote the induction of GRP78, a chaperone of the ER lumen thought to function in protein translocation, folding and assembly [18–20] and in translational accommodation to chronic depletion of ER Ca^{2+} stores [21,22].

At concentrations that block ER to Golgi transport of secretory proteins [5,15], Z-Gly-Phe-NH₂ caused eIF-2 α to be phosphorylated and suppressed translational initiation concurrently with mobilization of cell-associated Ca^{2+} [8,13]. Induction of GRP78 and its mRNA occurred during extended (3 h) incubations with the dipeptide [8]. Inhibition of translation, interruption of glycoprotein processing and induction of GRP78 mRNA by Z-Gly-Phe-NH₂ were each reversed as extracellular Ca^{2+} was increased [8,15]. However, in contrast to Ca^{2+} ionophores which mobilize Ca^{2+} from sequestered sites to the cytosol and increase the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [23,24], Z-Gly-Phe-NH₂ was found over several min to lower $[\text{Ca}^{2+}]_i$ in either previously unchallenged or ionomycin-treated cells. The peptide also overturned the increase in $[\text{Ca}^{2+}]_i$ produced by high extracellular K⁺ and other secretagogues [6–8]. It was therefore unclear whether promotion of plasmalemmal Ca^{2+} transport to the extracellular fluid by the peptide was the cause or the

Abbreviations used: ER, endoplasmic reticulum; Z-, benzyloxycarbonyl-; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration.

† Present address: Department of Anatomy and Structural Biology/Developmental Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461, U.S.A.

* To whom correspondence should be addressed.

consequence of mobilization of Ca^{2+} stores. The involvement of metalloendoprotease(s) in the maintenance of intracellular Ca^{2+} sequestration also remained to be demonstrated. The present investigation examines the effects of peptides on organellar Ca^{2+} storage in GH_3 cells, porated with saponin to dismiss plasmalemmal Ca^{2+} transport systems from consideration as variables and to allow the introduction of peptide analogues that cannot cross the intact plasmalemma.

EXPERIMENTAL

General methodology

Procedures for culture, harvest and Ca^{2+} depletion of GH_3 pituitary tumour cells [10] and for measurements of ^{45}Ca associated with intact GH_3 cells [21] have been described previously. Leucine incorporation into protein was measured as described [25]. ATP and GTP contents were determined on 10% trichloroacetic acid-extracts of variously treated intact cell preparations by h.p.l.c. as described [15].

Permeabilization of cells

A modification of the procedure of Bian et al. [26] was used to prepare porated GH_3 cells. Ca^{2+} -depleted cells were suspended to 5.5×10^5 cells/ml of ice-cold 'uptake medium' comprised of 140 mM KCl, 10 mM NaCl, 2.5 mM MgCl_2 , 0.3 mM EGTA, 10 mM Hepes, pH 7.4, and, unless indicated otherwise, 10 mM dithiothreitol. Freshly prepared saponin (1% in uptake medium) was added to a concentration of 0.002%. After 2 min incubation on ice, >99% of cells were porated by the criterion of permeability to Trypan Blue. Longer incubations on ice or incubations at 30 °C or 37 °C were avoided because resulting preparations exhibited decreased ability to accumulate ^{45}Ca into both the oligomycin-insensitive and the thapsigargin-insensitive Ca^{2+} compartments. Pored cells were washed three times by centrifugation at 10000 *g* (5 min) and resuspension in uptake medium lacking saponin at 4 °C. The saponin-free porated cells were held on ice for no longer than 30 min before measurements of Ca^{2+} accumulation.

Measurement of Ca^{2+} accumulation

Pored cells (1×10^6 per ml) were suspended in uptake medium containing 1 mM ATP, 5 mM phosphocreatine, 20 units/ml creatine phosphokinase and, unless indicated otherwise, 20 μM potassium phosphate. Agents of interest or solvent (<0.5% Me_2SO) were added 1 min before or 25 min after addition of $^{45}\text{CaCl}_2$ (4×10^6 d.p.m./ml). Reactions were initiated by placing suspensions at 30 °C. At appropriate times, aliquots (100 μl of suspension) were withdrawn and the porated cells collected by filtration with the Millipore 96-well MultiScreen Assay System. Filters were washed with ice-cold uptake buffer containing 1 mM LaCl_3 , air dried, inserted into scintillation vials with a Millipore MultiScreen Punch, and analysed for radioactivity. Results are presented as the mean of values obtained for quadruplicate incubation samples within a single experiment. Standard error was usually less than 10%, and typical variation within a single experiment is presented in the Tables. Ca^{2+} accumulation observed in the absence of ATP, phosphocreatine and creatine phosphokinase was considered to constitute the blank. Such blank values were routinely 3%–5% of that observed in the presence of the complete regenerating system and 106 nM free Ca^{2+} and less than 0.5% of that observed in the presence of the complete regenerating system and 29 μM free Ca^{2+} . Significant degrees of Ca^{2+} sequestration were not achieved with ATP alone.

Measurement of free Ca^{2+} concentrations

Quin2 free acid (5 μM) was added to aliquots of the reconstituted Ca^{2+} accumulation system containing various concentrations of added Ca^{2+} . Fluorescence was recorded at 30 °C using a Perkin-Elmer MPF-66 spectrophotometer at $\lambda_{\text{ex.}} = 339$ nm, $\lambda_{\text{em.}} = 492$ nm, slit-widths each 5 mm. Calculation of free Ca^{2+} concentrations assumed a K_D of quin2 for Ca^{2+} of 115 nM, a value determined previously for buffers of comparable ionic composition and comparable incubation conditions [27]. Minimum fluorescence values were obtained upon addition of 1 mM EGTA.

MATERIALS

Saponin, inositol 1,4,5-triphosphate [$\text{Ins}(1,4,5)\text{P}_3$], heparin (molecular mass 5000), oligomycin, glutathione, dithiothreitol, ryanodine, caffeine, procaine and Ruthenium Red were purchased from Sigma. Thapsigargin was from LC Services Corp., Woburn, MA, U.S.A. $^{45}\text{CaCl}_2$ (11.92 mCi/mg) was obtained from Dupont NEN. Z-Ser-Leu- NH_2 was from Novabiochem and Z-L-Tyr-*p*-nitrophenyl ester was from ICN. Z-Gly-Phe- NH_2 and other modified peptides and amino acids were purchased from Sigma. Stock solutions (0.6 M) of modified peptides or amino acids were prepared in Me_2SO . Stock solutions of peptides or amino acids containing a free carboxylic acid group were diluted with an equal volume of uptake medium and buffered to pH 7.4 with KOH. Quin2 free acid was obtained from Molecular Probes, Inc., Eugene, OR, U.S.A.

RESULTS

Effects of Z-Gly-Phe- NH_2 on Ca^{2+} sequestration by high- and low-affinity Ca^{2+} compartments

In initial experiments, Ca^{2+} accumulation by saponin-pored GH_3 cells was examined at varying free Ca^{2+} concentrations with or without exposure to agents known to inhibit Ca^{2+} sequestration by the ER or mitochondrial compartments (Table 1). Accumulation of the cation increased as a function of increasing free Ca^{2+} concentrations with greater degrees of accumulation observed at μM concentrations. Thapsigargin, which selectively inhibits the Ca^{2+} -ATPase of the ER, was highly effective in suppressing Ca^{2+} accumulation at low free Ca^{2+} concentrations, with 90% inhibition observed at 0.1 μM free cation. Thapsigargin

Table 1 Ca^{2+} accumulation by porated GH_3 cells in the absence or presence of oligomycin, thapsigargin, or Z-Gly-Phe- NH_2

Pored cells were suspended in EGTA-containing medium (see Experimental section) adjusted to 4×10^6 d.p.m./ml $^{45}\text{CaCl}_2$ and with CaCl_2 to provide the indicated free Ca^{2+} concentrations. Preparations were incubated at 30 °C for 25 min with no further additions or with thapsigargin (30 nM), Z-Gly-Phe- NH_2 (2.5 mM) or oligomycin (3 μM). Ca^{2+} accumulation was determined as described in the Experimental section. Results are expressed as the average $\pm$ range of values obtained for quadruplicate incubation samples from a single experiment.

Addition	Ca^{2+} accumulation (nmol/ 10^6 cells)			
	106 nM Ca^{2+}	224 nM Ca^{2+}	4 μM Ca^{2+}	25 μM Ca^{2+}
None	0.92 $\pm$ 0.04	1.21 $\pm$ 0.07	12.3 $\pm$ 0.3	32.1 $\pm$ 0.1
Thapsigargin	0.09	0.26 $\pm$ 0.02	9.95 $\pm$ 0.2	34.9 $\pm$ 1.7
Z-Gly-Phe- NH_2	0.39 $\pm$ 0.02	0.67 $\pm$ 0.06	5.20 $\pm$ 0.27	17.1 $\pm$ 0.7
Oligomycin	0.92 $\pm$ 0.04	1.07 $\pm$ 0.13	0.91 $\pm$ 0.06	0.92 $\pm$ 0.07
Oligomycin + thapsigargin	0.09	0.16 $\pm$ 0.02	0.21 $\pm$ 0.02	0.53 $\pm$ 0.03
Oligomycin + Z-Gly-Phe- NH_2	0.32 $\pm$ 0.04	0.40 $\pm$ 0.04	0.40 $\pm$ 0.03	0.49 $\pm$ 0.03

Figure 1 Time dependence of Ca^{2+} accumulation by porated GH_3 cells in the absence or presence of thapsigargin or Z-Gly-Phe- NH_2

Porated cells were suspended in medium adjusted to 106 nM free Ca^{2+} , 3 μM oligomycin and 4×10^6 d.p.m./ml $^{45}\text{CaCl}_2$ and with no further addition (●) or with 30 nM thapsigargin (○) or 2.5 mM Z-Gly-Phe- NH_2 (▲). Suspensions were incubated at 30 °C, and at the indicated times samples were withdrawn for measurements of Ca^{2+} accumulation as described in the Experimental procedures section. Findings have been reproduced on two separate occasions.

Figure 2 Thapsigargin and Z-Gly-Phe- NH_2 concentration dependences of inhibition of Ca^{2+} accumulation in the absence and presence of dithiothreitol

Porated GH_3 cell suspensions were prepared in the absence (○) or presence (●) of 10 mM dithiothreitol. Preparations were adjusted to 106 nM free Ca^{2+} and 3 μM oligomycin and with the indicated concentrations of thapsigargin or Z-Gly-Phe- NH_2 . ^{45}Ca accumulation was determined after 25 min of incubation. Findings have been reproduced on three separate occasions.

was not inhibitory at 30 μM free Ca^{2+} . By contrast oligomycin, an inhibitor of mitochondrial Ca^{2+} uptake only when it is driven by ATP, reduced accumulation minimally at 0.2 μM free cation while providing prominent inhibitions at 4 μM and 30 μM free Ca^{2+} concentrations. In the presence of oligomycin, thapsigargin was found to reduce Ca^{2+} accumulation at all free Ca^{2+} concentrations examined although the inhibitor was most effective at 0.1 μM free cation.

Z-Gly-Phe- NH_2 (2.5 mM) reduced accumulation of Ca^{2+} by porated GH_3 cells at all concentrations of free Ca^{2+} tested regardless of the presence of oligomycin (Table 1). Somewhat larger (65 % versus 46 %) inhibitions by dipeptide were routinely observed at low (0.1–0.2 μM) free Ca^{2+} concentrations in the presence of oligomycin than were observed at high (30 μM) free Ca^{2+} concentrations in the absence of oligomycin.

Table 2 Ca^{2+} accumulation by porated GH_3 cells exposed to Ca^{2+} -mobilizing agents in the presence of glutathione or dithiothreitol

Porated GH_3 cell suspensions were prepared without reducing agent or in the presence of 1 mM glutathione or 1 mM dithiothreitol. Preparations were adjusted to 106 nM free Ca^{2+} and 3 μM oligomycin with no further additions or with thapsigargin (30 nM), Z-Gly-Phe- NH_2 (2.5 mM), Ins(1,4,5) P_3 (10 μM) and GTP (10 μM), or A23187 (1 μM). ^{45}Ca accumulation was determined after 25 min of incubation. Results are expressed as the average $\pm$ range of values obtained for quadruplicate incubation samples from a single experiment. ND = not detectable

Additions	Ca^{2+} accumulation (nmol/ 10^6 cells)		
	– Reductant	+ Glutathione	+ Dithiothreitol
None	0.88 ± 0.04	0.89 ± 0.03	0.93 ± 0.03
Thapsigargin	0.14 ± 0.01	0.11 ± 0.01	0.14 ± 0.02
Z-Gly-Phe- NH_2	0.72 ± 0.03	0.48 ± 0.05	0.39 ± 0.04
Ins(1,4,5) P_3 + GTP	0.36 ± 0.03	0.35 ± 0.11	0.33 ± 0.02
A23187	ND	ND	0.03

Inhibition by Z-Gly-Phe- NH_2 of Ca^{2+} accumulation by the high-affinity Ca^{2+} compartment

Ca^{2+} accumulation at 0.1 μM free cation was taken to represent that by the ER since such accumulation was exceptionally sensitive to thapsigargin and insensitive to oligomycin. The time dependence of cation accumulation at the low free Ca^{2+} concentration was examined in the absence or presence of thapsigargin or Z-Gly-Phe- NH_2 (Figure 1). Optimal Ca^{2+} accumulation in the absence of drug was routinely obtained at 20–25 min of incubation and was sustained for at least 12 additional min of incubation (Figure 4). Thapsigargin profoundly inhibited accumulation throughout the incubation period (Figure 1). Z-Gly-Phe- NH_2 , in contrast, did not slow Ca^{2+} accumulation during the first 5 min of incubation, consistent with the inability to block Ca^{2+} uptake. With longer exposures to the dipeptide however, Ca^{2+} accumulation was observed to decline such that after 20 min of incubation cation retention was reduced to 38 % of the untreated control.

The importance of a reducing environment for Ca^{2+} accumulation by the high-affinity Ca^{2+} compartment and for the inhibitory effects of varying concentrations of thapsigargin and Z-Gly-Phe- NH_2 was determined (Figure 2). Omission of dithiothreitol during preparation of the porated cells and from the uptake medium did not affect Ca^{2+} accumulation in the absence of Ca^{2+} -mobilizing drugs. As observed with intact GH_3 preparations [12], sub-nanomolar to nanomolar concentrations of thapsigargin were effective in inhibiting Ca^{2+} sequestration. Half-maximal inhibition of accumulation was observed at 0.7 nM drug concentration regardless of the absence or presence of dithiothreitol. The Z-Gly-Phe- NH_2 concentration dependence for inhibition of Ca^{2+} accumulation was similar to that for Ca^{2+} mobilization from intact GH_3 cells [8] provided that dithiothreitol (1–10 mM) was included in the uptake medium. In the absence of reducing agent, Z-Gly-Phe- NH_2 concentrations in excess of 2 mM were required to inhibit accumulation. The weaker reductant, glutathione, supported the inhibition by Z-Gly-Phe- NH_2 almost as well as dithiothreitol without influencing Ca^{2+} accumulation in the absence or presence of other Ca^{2+} -mobilizing agents (Table 2).

The non-mitochondrial Ca^{2+} -sequestering compartment of porated smooth muscle cells is characterized by permeability to oxalate anion and by increased degrees of Ca^{2+} -accumulation in the presence of the anion [26,28]. Ins(1,4,5) P_3 and thapsigargin

Table 3 Ca^{2+} accumulation by porated GH_3 cells exposed to Ca^{2+} -mobilizing agents in the absence or presence of oxalate

Porated cells were suspended in medium with or without 10 mM potassium oxalate and with no further additions or with thapsigargin (30 nM), Z-Gly-Phe-NH₂ (2.5 mM), Ins(1,4,5) P_3 (10 μM), GTP (10 μM), or A23187 (1 μM). Free Ca^{2+} was adjusted to 10 nM and oligomycin to 3 μM , and ^{45}Ca accumulation was measured after 25 min of incubation. Results are expressed as the average $\pm$ range of values obtained for quadruplicate incubation samples from a single experiment. Findings were reproduced on two separate occasions.

Additions	Ca^{2+} accumulation (nmol/10 ⁶ cells)	
	— Oxalate	+ Oxalate
None	0.99 $\pm$ 0.09	5.13 $\pm$ 0.11
Thapsigargin	0.11	0.11
Z-Gly-Phe-NH ₂	0.35 $\pm$ 0.03	0.32 $\pm$ 0.02
Ins(1,4,5) P_3	0.62 $\pm$ 0.05	1.08 $\pm$ 0.07
GTP	0.87 $\pm$ 0.07	7.11 $\pm$ 0.11
Ins(1,4,5) P_3 + GTP	0.46 $\pm$ 0.02	1.38 $\pm$ 0.11
Z-Gly-Phe-NH ₂ + thapsigargin	0.11	0.11
Z-Gly-Phe-NH ₂ + Ins(1,4,5) P_3 + GTP	0.25	0.32 $\pm$ 0.02
A23187	0.07	0.07

Table 4 Ca^{2+} accumulation by porated GH_3 cells exposed to Ca^{2+} -mobilizing agents in the presence of various phosphate concentrations

Porated preparations were adjusted to 106 nM free Ca^{2+} , 3 μM oligomycin and either 20 μM , 1 mM or 10 mM potassium phosphate, pH 7.4, and with no further additions or with thapsigargin (30 nM), Z-Gly-Phe-NH₂ (2.5 mM), Ins(1,4,5) P_3 (10 μM) and GTP (10 μM), or A23187 (1 μM). ^{45}Ca accumulation was determined after 25 min of incubation. Results are expressed as the average $\pm$ range of values obtained for quadruplicate incubation samples from a single experiment.

Additions	Ca^{2+} accumulation (nmol/10 ⁶ cells)		
	20 μM Phosphate	1 mM Phosphate	10 mM Phosphate
None	0.83 $\pm$ 0.04	1.03 $\pm$ 0.08	0.39 $\pm$ 0.03
Thapsigargin	0.09 $\pm$ 0.03	0.12 $\pm$ 0.02	0.04 $\pm$ 0.01
Ins(1,4,5) P_3 + GTP	0.23 $\pm$ 0.02	0.34 $\pm$ 0.03	0.12 $\pm$ 0.01
Z-Gly-Phe-NH ₂	0.30 $\pm$ 0.02	0.33 $\pm$ 0.03	0.13 $\pm$ 0.03
A23187	0.01	0.06	0.02 $\pm$ 0.02

each suppress Ca^{2+} sequestration by this compartment, suggesting that the oxalate-permeable pool is the ER. Ca^{2+} accumulation by the high-affinity compartment of porated GH_3 cells was similarly increased in the presence of oxalate (Table 3). The effects of various Ca^{2+} -mobilizing drugs, including Z-Gly-Phe-NH₂, on Ca^{2+} accumulation by preparations incubated in the absence or presence of oxalate were also examined. As expected thapsigargin, Ins(1,4,5) P_3 and ionophore A23187 reduced accumulation in the presence or absence of oxalate. In the absence of oxalate, GTP alone did not affect sequestration but was required for maximal inhibition of accumulation by Ins(1,4,5) P_3 . Also, in accord with previous observations, GTP increased Ca^{2+} accumulation by the oxalate-sensitive pool, an action that has been attributed to Ca^{2+} translocation across thapsigargin-sensitive compartments or sub-compartments of the ER [29]. Like thapsigargin and A23187, Z-Gly-Phe-NH₂ reduced Ca^{2+} accumulation to the same value in incubations with or without oxalate. Inhibitions by Z-Gly-Phe-NH₂ in combination with thapsigargin or in combination with Ins(1,4,5) P_3 and GTP were not additive regardless of the presence of oxalate.

The possibility that Z-Gly-Phe-NH₂ reduced sequestration of

Table 5 Ca^{2+} accumulation by porated GH_3 cells exposed to Ca^{2+} -mobilizing agents in the absence or presence of heparin

Porated cells were suspended in medium with or without 20 $\mu\text{g}/\text{ml}$ heparin and with no further additions or with thapsigargin (30 nM), Z-Gly-Phe-NH₂ (2.5 mM), or Ins(1,4,5) P_3 (10 μM) and GTP (10 μM). Free Ca^{2+} was adjusted to 106 nM and oligomycin to 3 μM and ^{45}Ca accumulation was measured after 25 min of incubation. Results are expressed as the average $\pm$ range of values obtained for quadruplicate incubation samples from a single experiment. Findings were reproduced on two separate occasions.

Additions	Ca^{2+} accumulation (nmol/10 ⁶ cells)	
	— Heparin	+ Heparin
None	0.97 $\pm$ 0.09	1.01 $\pm$ 0.04
Thapsigargin	0.14	0.14
Z-Gly-Phe-NH ₂	0.39	0.37
Ins(1,4,5) P_3 + GTP	0.30 $\pm$ 0.02	0.92 $\pm$ 0.02
Z-Gly-Phe-NH ₂ + Ins(1,4,5) P_3 + GTP	0.30 $\pm$ 0.02	0.41 $\pm$ 0.02

Ca^{2+} within the high-affinity compartment by decreasing the availability of a precipitating counter-ion was considered. Increasing the concentration of phosphate anion from 20 μM to 1 mM, however, neither increased Ca^{2+} accumulation notably nor relieved the inhibition of Ca^{2+} accumulation observed with Z-Gly-Phe-NH₂ or other Ca^{2+} -mobilizing drugs (Table 4). Addition of 10 mM phosphate suppressed Ca^{2+} accumulation in the absence of drugs, with further suppression provided by Z-Gly-Phe-NH₂ or other Ca^{2+} -mobilizing agents. Heparin, a potent competitive antagonist of Ins(1,4,5) P_3 at its receptor [30] blocks Ca^{2+} release through the Ins(1,4,5) P_3 -sensitive channel of permeabilized cells [31] but does not affect the Ca^{2+} -mobilizing actions of thapsigargin in such systems [26]. In porated GH_3 cells, heparin overturned the inhibition of Ca^{2+} sequestration by Ins(1,4,5) P_3 but did not relieve the inhibitions provided by thapsigargin or Z-Gly-Phe-NH₂ (Table 5). The inhibition observed with Z-Gly-Phe-NH₂ in combination with Ins(1,4,5) P_3 was reduced by heparin to that observed with Z-Gly-Phe-NH₂ alone.

The possibility that Z-Gly-Phe-NH₂ acted as an agonist at ryanodine receptors was examined (Table 6). Caffeine, which opens ryanodine-sensitive Ca^{2+} channels of the sarcoplasmic reticulum, and in some cases the ER, to promote Ca^{2+} release [32–34], did not suppress Ca^{2+} accumulation by porated GH_3 preparations. Although ryanodine modestly (39%) reduced cation accumulation, inhibitions were neither potentiated by caffeine nor overturned by agents, e.g. procaine or Ruthenium Red, which inhibit Ca^{2+} fluxes through ryanodine/caffeine-sensitive Ca^{2+} channels. Inhibitions by Z-Gly-Phe-NH₂ and the more potent analogue Z-L-Tyr-*p*-nitrophenyl ester (see below) were not overturned by procaine or Ruthenium Red. Ruthenium Red alone caused significant suppression of Ca^{2+} sequestration which was attributable to inhibition of Ca^{2+} uptake (Table 6).

Ca^{2+} release from the high-affinity Ca^{2+} compartment by Z-Gly-Phe-NH₂

The inability of Z-Gly-Phe-NH₂ to block Ca^{2+} uptake (Figure 1) prompted an investigation of the Ca^{2+} -releasing properties of the dipeptide. The time courses of Ca^{2+} mobilization from the high-affinity compartment by Ins(1,4,5) P_3 or Z-Gly-Phe-NH₂ were compared for porated cells that were preloaded with ^{45}Ca for 25 min prior to drug addition (Figure 3). Z-Gly-Phe-NH₂ provoked Ca^{2+} release but in a manner distinct from that of Ins(1,4,5) P_3 . Approximately 67% of sequestered Ca^{2+} was

Table 6 Ca^{2+} accumulation by porated GH₃ cells exposed to peptide analogues and agents that modulate Ca^{2+} release through caffeine/ryanodine-sensitive channels

Porated cells were suspended in medium with no further additions or with procaine (1 mM), Ruthenium Red (10 μM), caffeine (5 mM), ryanodine (10 μM), Z-Gly-Phe-NH₂ (2.5 mM), or Z-L-Tyr-*p*-nitrophenyl ester (50 μM). Free Ca^{2+} was adjusted to 106 nM and oligomycin to 3 μM , and ^{45}Ca accumulation was measured either after 25 min (Experiment I) or at the indicated times (Experiment II) of incubation. Results are expressed as the average $\pm$ range of values obtained for quadruplicate incubation samples from a single experiment.

Experiment I Additions	Ca^{2+} accumulation (nmol/ 10^6 cells)	Additions	Ca^{2+} accumulation (nmol/ 10^6 cells)
None	0.82 ± 0.08	Ryanodine + caffeine	0.48 ± 0.03
Procaine	0.75 ± 0.04	Ryanodine + caffeine + procaine	0.50 ± 0.03
Ruthenium Red	0.24 ± 0.03	Ryanodine + caffeine + Ruthenium Red	0.19 ± 0.02
Caffeine	0.95 ± 0.04	Z-Gly-Phe-NH ₂	0.33 ± 0.05
Caffeine + procaine	0.72 ± 0.04	Z-Gly-Phe-NH ₂ + procaine	0.31 ± 0.02
Caffeine + Ruthenium Red	0.21 ± 0.02	Z-Gly-Phe-NH ₂ + Ruthenium Red	0.13 ± 0.01
Ryanodine	0.51 ± 0.03	Z-L-Tyr- <i>p</i> -nitrophenyl ester	0.32 ± 0.03
Ryanodine + procaine	0.48 ± 0.04	Z-L-Tyr- <i>p</i> -nitrophenyl ester + procaine	0.32 ± 0.03
Ryanodine + Ruthenium Red	0.18 ± 0.01	Z-L-Tyr- <i>p</i> -nitrophenyl ester + Ruthenium Red	0.14 ± 0.02
Experiment II			
	Ca^{2+} accumulation (nmol/ 10^6 cells)		
Time (min)	— Ruthenium Red	+ Ruthenium Red	
1	0.16 ± 0.01	0.06	
3	0.35 ± 0.05	0.14 ± 0.02	
5	0.40	0.21 ± 0.02	
7	0.65 ± 0.06	0.21 ± 0.01	
29	0.92 ± 0.05	0.32 ± 0.04

Figure 3 Mobilization of Ca^{2+} from porated GH₃ cells by inositol 1,4,5-trisphosphate or Z-Gly-Phe-NH₂: effects of dithiothreitol

Porated cells were loaded with ^{45}Ca at 106 nM free Ca^{2+} for 25 min in the presence (a) or absence (b) of 10 mM dithiothreitol. Preparations were then challenged for the indicated times with solvent (Δ), with 10 μM inositol 1,4,5-trisphosphate and 10 μM GTP ($\circ$), or with 2.5 mM Z-Gly-Phe-NH₂ ($\bullet$). ^{45}Ca retained by the porated cells was determined as described in the Experimental procedures section. Findings have been reproduced on three separate occasions.

mobilized within 0.5 min by Ins(1,4,5) P_3 whereas the dipeptide mobilized approximately 50% of the sequestered cation within 2 min (Figure 3a). Greater degrees of mobilization were observed upon continued incubation with the dipeptide but not with Ins(1,4,5) P_3 (Figures 3 and 4). Additionally, Ca^{2+} mobilization by Z-Gly-Phe-NH₂ depended on the presence of a reducing agent whereas release of Ca^{2+} by Ins(1,4,5) P_3 did not (Figure 3b). The time dependence of mobilization of ^{45}Ca from preloaded preparations by Z-Gly-Phe-NH₂ was also compared with that by thapsigargin. As observed in other porated cell systems [26],

addition of the Ca^{2+} uptake inhibitor resulted in relatively slow Ca^{2+} mobilization (Figure 4a and b). Approximately 50% of the cation was mobilized after 6–7 min of incubation with thapsigargin as compared with 2 min with the dipeptide (Figure 4a and c). When thapsigargin and Z-Gly-Phe-NH₂ were added in combination (Figure 4a), extensive (80%) mobilization of sequestered Ca^{2+} occurred within 2 min and did not diminish upon continued incubation. Similar degrees of mobilization (75%) were seen during short treatments with Ins(1,4,5) P_3 ; some attenuation of the Ca^{2+} -releasing effect of Ins(1,4,5) P_3 occurred at later times (Figure 4b and c). Mobilization of Ca^{2+} following exposure to Ins(1,4,5) P_3 with thapsigargin (Figure 4b) or Ins(1,4,5) P_3 with dipeptide (Figure 4c) was equivalent to that observed after 2 min with Ins(1,4,5) P_3 alone. However, no attenuation of Ca^{2+} mobilization was observed when Ins(1,4,5) P_3 was added in combination with the other Ca^{2+} -mobilizing drugs.

Effects of modified peptides and amino acids

Synthetic peptides and peptide analogues recognized to serve as competitive substrates for, or as inhibitors of, metalloendoproteases were examined for effects on Ca^{2+} accumulation by the high-affinity Ca^{2+} compartment (Table 7). Analogues that do not interact with metalloendoproteases served as controls. Results obtained with certain derivatives were in accord with the hypothesis that interaction with the endopeptidase mediates Ca^{2+} mobilization by Z-Gly-Phe-NH₂. For example neither Z-Gly-Phe nor Gly-Phe-NH₂, which are not recognized by proteinases that cleave internal peptide bonds [2,4], mobilized Ca^{2+} . Additionally Z-Gly-Gly-NH₂, which does not interact with metalloendoproteases and is often employed to identify non-specific or toxic effects of Z-modified dipeptide amides on intact cells [2,4], was not effective. On the other hand only modest effects were observed with the competitive substrates Z-Ser-Leu-NH₂ and Z-Gly-Leu-NH₂ as well as with the more hydrophobic non-

Figure 4 Mobilization of Ca^{2+} from porated GH_3 cells by Z-Gly-Phe-NH_2 alone or in combination with thapsigargin or inositol 1,4,5-trisphosphate

Porated cells were loaded with ^{45}Ca at 106 nM free Ca^{2+} for 25 min and then challenged for the indicated times with solvent ($\circ$), with 30 nM thapsigargin ($\blacktriangle$), with 2.5 mM Z-Gly-Phe-NH_2 ($\bullet$) or with 10 μM inositol 1,4,5-trisphosphate and 10 μM GTP ($\triangle$). Additional preparations were challenged with Z-Gly-Phe-NH_2 and thapsigargin (**a**, $\blacktriangledown$), with inositol 1,4,5-trisphosphate, GTP, and thapsigargin (**b**, $\blacktriangledown$), or with Z-Gly-Phe-NH_2 , inositol 1,4,5-trisphosphate, and GTP (**c**, $\blacktriangledown$). ^{45}Ca remaining associated with the porated cells was determined as described in the Experimental Procedures section. (**a**), (**b**) and (**c**) represent three separate experiments; findings from each have been reproduced at least once.

substrate peptides $\text{Z-Leu-Leu-methyl ester}$ and $\text{Z-Val-Leu-methyl ester}$. Lack of an amide or ester function rendered all of these peptides inactive. Z-L-Phe , a general metalloendoprotease inhibitor [4] and phosphoramidon, which potently inhibits selec-

Figure 5 Ca^{2+} accumulation by oligomycin-insensitive and thapsigargin-insensitive Ca^{2+} compartments of porated GH_3 cells in the presence of various concentrations of Z-Gly-Phe-NH_2 or $\text{Z-L-Tyr-p-nitrophenyl ester}$

Porated cell suspensions were adjusted to 106 nM free Ca^{2+} and 3 μM oligomycin ($\circ$) or to 29 μM free Ca^{2+} and 30 nM thapsigargin ($\bullet$) and with the indicated concentrations of Z-Gly-Phe-NH_2 or $\text{Z-L-Tyr-p-nitrophenyl ester}$. ^{45}Ca accumulation was determined after 25 min of incubation. Findings have been reproduced on two separate occasions.

ted forms of metalloendoprotease [1–4], were also inactive. Although Z-L-Met and several Z -modified dipeptides with free carboxylic acid groups provoked no inhibitions, $\text{Z-L-S-benzylcysteine}$ inhibited Ca^{2+} accumulation to the same degree as Z-Gly-Phe-NH_2 . To our knowledge the cysteine analogue has not been previously observed to suppress endoprotease activities.

The possibility that positioning of aromatic rings in Z -modified peptides or amino acids correlated with the capacity to mobilize sequestered Ca^{2+} was considered. $\text{Z-L-Tyr-p-nitrophenyl ester}$, a tyrosine analogue possessing three aromatic rings, proved 80-fold more potent than Z-Gly-Phe-NH_2 in inhibiting Ca^{2+} accumulation by the high-affinity Ca^{2+} compartment of porated GH_3 cells (Figure 5), with half-maximal inhibition observed at 20 μM . This analogue was also compared with Z-Gly-Phe-NH_2 for ability to block Ca^{2+} sequestration by the low-affinity, thapsigargin-insensitive Ca^{2+} compartment. Z-Gly-Phe-NH_2 inhibited Ca^{2+} accumulation by both low- and high-affinity compartments at comparable (mM) concentrations (Figure 5a). The tyrosine analogue, by contrast, did not inhibit accumulation by the low-affinity compartment at concentrations significantly

Table 7 Ca^{2+} accumulation by porated GH_3 cells exposed to various modified peptides and amino acids

Porated cells were suspended in medium without or with the indicated peptides or amino acids (2.5 mM). Free Ca^{2+} was adjusted to 106 nM and oligomycin to 3 μM , and ^{45}Ca accumulation was measured after 25 min of incubation.

Experiment I		Experiment II	
Addition	Ca^{2+} accumulation (% of control)	Addition	Ca^{2+} accumulation (% of control)
None	100	None	100
Z-Gly-Phe-NH_2	36	Z-Gly-Phe-NH_2	28
Z-Gly-Phe	90	Z-Ser-Leu-NH_2	44
Z-Phe-Gly	91	Z-Gly-Leu-NH_2	57
Gly-Phe-NH_2	98	$\text{Z-Leu-Leu-methyl ester}$	65
Z-L-Phe	94	$\text{Z-Val-Leu-methyl ester}$	62
Z-Gly-Gly-NH_2	100	Z-Gly-Leu	99
Z-L-Met	99	Z-Val-Leu	99
$\text{Z-L-S-benzylcysteine}$	35	Phosphoramidon	99

Figure 6 Z-L-Tyr-*p*-nitrophenyl ester concentration dependencies of ^{45}Ca mobilization and inhibition of leucine incorporation in intact GH_3 cells

GH_3 cells in Hams F-10 medium containing 100 μM leucine and 0.2 mM CaCl_2 were pretreated for 90 min without (○) or with (●) 4 $\mu\text{Ci/ml}$ ^{45}Ca . Prelabelled preparations were challenged for 30 min with the indicated concentrations of Z-L-Tyr-*p*-nitrophenyl ester, and cell-associated ^{45}Ca was determined. Unlabelled preparations were challenged similarly, [^3H]leucine (4 $\mu\text{Ci/ml}$) was added and incorporation of labelled amino acid into protein was measured after an additional 15 min of incubation. Findings were reproduced on one separate occasion.

suppressive to sequestration by the high-affinity compartment (Figure 5b).

Z-L-Tyr-*p*-nitrophenyl ester was also found to mobilize ^{45}Ca from intact GH_3 cells that had been preloaded with labelled cation and to inhibit leucine incorporation by these preparations (Figure 6). Ca^{2+} mobilization and inhibition of protein synthesis were observed at comparable (μM) concentrations of the tyrosine analogue. Higher (60-fold) concentrations of Z-Gly-Phe- NH_2 produced these effects in intact GH_3 cells [8]. Z-L-Tyr-*p*-nitrophenyl ester was also compared with the dipeptide for effects on ATP and GTP contents of GH_3 cells (Table 8). In accord with previous findings [12] treatment with Z-Gly-Phe- NH_2 resulted in significant lowering of the nucleotides at concentrations that suppressed protein synthesis more than 70%. The tyrosine analogue, however did not lower ATP or GTP contents at concentrations that fully suppressed amino acid incorporation. Z-L-Tyr-*p*-nitrophenyl ester was insoluble at concentrations exceeding 100 μM .

DISCUSSION

Results of the present study with porated GH_3 cells confirm that Z-Gly-Phe- NH_2 and certain related analogues depress organellar Ca^{2+} sequestration. Free Ca^{2+} concentrations were controlled within narrow ranges utilizing EGTA buffer systems such that two distinct compartments sequestering Ca^{2+} in an ATP-dependent manner and possessing the predicted properties of the ER and mitochondria were preserved. Accumulation of ^{45}Ca at low (100–200 nM) free Ca^{2+} concentrations was sensitive to thapsigargin and $\text{Ins}(1,4,5)\text{P}_3$, was facilitated by oxalate and was insensitive to oligomycin in accord with Ca^{2+} sequestration by the ER [27,35–37]. In contrast, the accumulation of cation occurring at μM free Ca^{2+} concentrations was sensitive to oligomycin but was not affected by thapsigargin, as would be characteristic of Ca^{2+} sequestration by mitochondria [28,37,38]. Z-Gly-Phe- NH_2 inhibited Ca^{2+} accumulation by both compartments whereas Z-L-Tyr-*p*-nitrophenyl ester affected the high-affinity compartment selectively.

Ca^{2+} sequestration by porated GH_3 cells at physiological resting $[\text{Ca}^{2+}]_i$ resembled that of porated lacrimal cells [39] more than that of smooth muscle cells [26,29] in that a single Ca^{2+} -sequestering compartment appeared to be functional. Accumulation by this compartment was almost completely (90%) blocked by thapsigargin. At saturating concentrations of thapsigargin, the further addition of $\text{Ins}(1,4,5)\text{P}_3$ and/or Z-Gly-Phe- NH_2 did not produce additional effects on Ca^{2+} accumulation either with or without oxalate in the incubation. No evidence indicating the existence of a distinct oxalate-impermeable pool was obtained. Rather, the increment in Ca^{2+} accumulation in response to the addition of oxalate appeared to reflect only the formation within the ER of an insoluble Ca^{2+} -oxalate complex as the two ions exceed their solubility product. In incubations conducted without oxalate, free Ca^{2+} clearly accumulates within the ER to a steady-state value whereat the rate of Ca^{2+} influx is balanced by the rate of efflux. Similar influx rates would be anticipated in oxalate-containing incubations. In the event that steady-state rates of Ca^{2+} influx and efflux exceed rates of deposition of the oxalate salt, steady-state concentrations of free cation within the lumen would be comparable for both oxalate-treated and untreated porated cells. Assuming that the Ca^{2+} -oxalate complex dissociates slowly [35], Ca^{2+} releasable by a combination of Ca^{2+} ionophore and Ca^{2+} -uptake blocker would represent the mobilizable cation pool(s). As predicted from this reasoning, total

Table 8 ATP and GTP contents and of intact GH_3 cells treated with Z-Gly-Phe- NH_2 or Z-L-Tyr-*p*-nitrophenyl ester

Cells were treated for 15 min with solvent or with Z-Gly-Phe- NH_2 or Z-L-Tyr-*p*-nitrophenyl ester at the indicated concentrations. Aliquots were taken for measurements of leucine incorporation as described in the legend to Figure 6 or for preparation of extracts for analysis of ATP and GTP contents by high-pressure liquid chromatography. 10^6 GH_3 cells contain 215 μg of protein. Findings were reproduced on two occasions.

Treatment	Leucine incorporation (nmol/ 10^6 cells)	ATP (nmol/ 10^6 cells)	GTP (nmol/ 10^6 cells)
Control	0.69	5.1	1.1
Z-Gly-Phe- NH_2 , 1 mM	0.40	5.1	1.1
Z-Gly-Phe- NH_2 , 1.5 mM	0.20	5.3	1.1
Z-Gly-Phe- NH_2 , 2 mM	0.06	4.1	0.8
Z-Gly-Phe- NH_2 , 2.5 mM	0.03	2.9	0.7
Z-L-Tyr- <i>p</i> -nitrophenyl ester, 20 μM	0.37	5.2	1.1
Z-L-Tyr- <i>p</i> -nitrophenyl ester, 30 μM	0.16	5.3	1.1
Z-L-Tyr- <i>p</i> -nitrophenyl ester, 40 μM	0.05	5.2	1.1
Z-L-Tyr- <i>p</i> -nitrophenyl ester, 60 μM	0.04	4.9	1.0

Ca^{2+} released from preloaded GH_3 preparations in response to addition of A23187 in combination with thapsigargin was identical for preparations loaded in the absence and presence of oxalate (M. A. Brostrom, unpublished observation).

The results of this study support the concept that Z-Gly-Phe-NH₂ acts to release Ca^{2+} from, rather than block its uptake into, the ER. Accumulation of Ca^{2+} during the first several minutes of incubation, which should primarily reflect uptake mediated by Ca^{2+} -ATPase activity, was blocked by thapsigargin but was unaffected by the dipeptide. The more rapid mobilization of Ca^{2+} from preloaded preparations by Z-Gly-Phe-NH₂ in combination with thapsigargin as compared with the dipeptide alone was consistent with different mechanisms of action of the two substances. Mobilization of Ca^{2+} by Z-Gly-Phe-NH₂ however, was not attributable to cation release through the $\text{Ins}(1,4,5)\text{P}_3$ -regulated channel. Unlike release by dipeptide, Ca^{2+} release by $\text{Ins}(1,4,5)\text{P}_3$ was maximal within seconds and could be antagonized by heparin. The observation that a thiol-reducing agent was required for Ca^{2+} mobilization by Z-Gly-Phe-NH₂ but not by thapsigargin or $\text{Ins}(1,4,5)\text{P}_3$ is also consistent with the action of the dipeptide being mediated by protein(s) or receptor(s) other than the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive channel or the Ca^{2+} -ATPase. Neither the ER Ca^{2+} pump nor the $\text{Ins}(1,4,5)\text{P}_3$ receptor is known to require free sulphhydryl groups for function. Sulphydryl inhibitors have been reported to increase the affinity of the $\text{Ins}(1,4,5)\text{P}_3$ receptor for ligand [40]. Functional caffeine/ryanodine-sensitive Ca^{2+} release channels were not detectable in the porated preparations.

The Ca^{2+} -mobilizing action of Z-Gly-Phe-NH₂ in porated, as in intact [8], GH_3 cells was inherently slower than that of either Ca^{2+} ionophore or $\text{Ins}(1,4,5)\text{P}_3$ and exhibited a latent period of approximately 1 min. The delayed response could be generated by drug actions at either enzymic or non-enzymic sites. The requirement for both amino- and carboxylic acid-blocking groups for Ca^{2+} mobilization was consistent with endoprotease inhibition by Z-Gly-Phe-NH₂ whereas findings with various other Z-modified analogues was not. Although other dipeptide substrates for metalloendoproteases mobilized Ca^{2+} from the high-affinity compartment, the observed order of potency of such agents for mobilization was inappropriate for involvement of these enzymes. For example, Z-Ser-Leu-NH₂, which has significantly higher affinity for metalloendoproteases than does Z-Gly-Phe-NH₂ [2,4], mobilized Ca^{2+} less effectively than Z-Gly-Phe-NH₂. Furthermore Z-L-Phe, a substrate analogue thought to inhibit all forms of metalloendoprotease [4], did not mobilize Ca^{2+} whereas Z-L-S-benzylcysteine and Z-L-Tyr-*p*-nitrophenyl ester, which are not recognized as metalloendoprotease inhibitors, were as effective as Z-Gly-Phe-NH₂ in suppressing Ca^{2+} accumulation.

The characteristics of Ca^{2+} release by Z-modified peptides were compatible with the opening of ER pores or channels exhibiting considerable specificity for molecular structures mimicking certain types of peptides. Z-Gly-Gly-NH₂ and various Z-blocked peptides/amino acids with free carboxylic acid groups were completely ineffective. However the finding that Z-Gly-Phe-NH₂, Z-L-S-benzylcysteine and Z-L-Tyr-*p*-nitrophenyl ester were equally efficacious in releasing Ca^{2+} was consistent with selective recognition and opening of a channel by compounds resembling internal regions of peptides and possessing substantial degrees of hydrophobicity at critical positions. Such a manipulatable pore would necessarily possess affinity for structures with internal peptide bond(s) or Z-modified amino group(s), additional aromatic residue(s) and blocked or unrecognizable N-terminal ends. The most reasonable candidate for such a pore is the translocon, the channel through which nascent proteins destined for modification by ER enzymes are inserted co-

translationally [41–43]. Cleavable signal peptides characterized by central hydrophobic regions are required for recognition and translocation by this channel. Recent electrophysiological studies confirm that ion conductance is associated with translocon function [44]. Both translocon function [45] and Ca^{2+} release by Z-modified peptides appear to involve sulphhydryl requirements. The hydrophobicity of Ca^{2+} -mobilizing Z-modified analogues should favour permeation to perturbant binding sites within the translocon such that the channel would open to allow Ca^{2+} conductance from the ER to the cytoplasm. The observation that Z-Gly-Phe-NH₂ also disturbs mitochondrial Ca^{2+} accumulation is in accord with this hypothesis. Translocation of proteins into mitochondria also occurs through proteinaceous channels [46].

That Z-Gly-Phe-NH₂ lowers $[\text{Ca}^{2+}]_i$ in intact GH_3 cells while affecting $[\text{Ca}^{2+}]_i$ minimally in other cells [6,7] is not entirely surprising. Unlike most cultured cells, GH_3 cells do not respond to thapsigargin or arachidonic acid with an elevation in $[\text{Ca}^{2+}]_i$ [12,47]. Thapsigargin mobilizes Ca^{2+} stores of GH_3 cells with unusual potency (1–3 nM), attenuates the rise in $[\text{Ca}^{2+}]_i$ due to ionomycin and, at 90 nM, decreases $[\text{Ca}^{2+}]_i$. The increase in $[\text{Ca}^{2+}]_i$ following treatment with Ca^{2+} ionophores attenuates uncommonly rapidly in GH_3 cells [8,23,24]. Nevertheless ionomycin, arachidonate, thapsigargin and Z-Gly-Phe-NH₂ effectively mobilize Ca^{2+} associated with the GH_3 cell to the extracellular fluid. It was therefore proposed [12] that the plasmalemmal Ca^{2+} -ATPase, which transports cytosolic free Ca^{2+} to the extracellular fluid, is present in atypically high concentrations or is unusually efficient in GH_3 cells. EGTA depletes Ca^{2+} stores of GH_3 cells rapidly and extensively [9,25]. Z-Gly-Phe-NH₂ mobilizes sequestered Ca^{2+} of GH_3 cells more rapidly but lowers $[\text{Ca}^{2+}]_i$ more slowly than does EGTA [8,23,24], consistent with depression of organellar Ca^{2+} sequestration by the dipeptide. Further studies will be needed to ascertain whether Z-Gly-Phe-NH₂ also acts at the plasmalemma to enhance Ca^{2+} transport from the cytosol to the extracellular fluid.

This work was supported by National Institutes of Health Grant DK 35393 and National Science Foundation Grant BNS 90-21616.

REFERENCES

- Bond, J. S. and Beynon, R. J. (1985) *Int. J. Biochem.* **17**, 565–574
- Strittmatter, W. J., Couch, C. B. and Mundy, D. I. (1987) in *Cell Fusion* (Sowers, A. E., ed.), pp. 99–121. Plenum Press, NY
- Bond, J. S. and Butler, P. E. (1987) *Annu. Rev. Biochem.* **56**, 333–364
- Lennarz, W. J. and Strittmatter, W. J. (1991) *Biochim. Biophys. Acta* **1071**, 149–158
- Strous, G. J. A. M., van Kerkhof, P. and Schwartz, A. (1988) *J. Biol. Chem.* **263**, 18197–18204
- Harris, B., Cheek, T. R. and Burgoyne, R. D. (1986) *Biochim. Biophys. Acta* **889**, 1–5
- Leikes, P. I. and Pollard, H. B. (1987) *J. Biol. Chem.* **262**, 15496–15505
- Brostrom, M. A., Prostko, C. R., Gmitter-Yellen, D., Grandison, L. J., Kuznetsov, G., Wong, W. L. and Brostrom, C. O. (1991) *J. Biol. Chem.* **266**, 7037–7043
- Brostrom, M. A. and Brostrom, C. O. (1993) in *Nutrition and Gene Expression* (Berdanier, C. and Hargrove, J., eds.), pp. 117–141. CRC Press, Inc., Boca Raton, FL
- Chin, K. V., Cade, C., Brostrom, C. O., Galuska, E. M. and Brostrom, M. A. (1987) *J. Biol. Chem.* **262**, 16509–16514
- Brostrom, C. O., Chin, K. V., Wong, W. L., Cade, C. and Brostrom, M. A. (1989) *J. Biol. Chem.* **264**, 1644–1649
- Wong, W. L., Brostrom, M. A., Kuznetsov, G., Gmitter-Yellen, D. and Brostrom, C. O. (1993) *Biochem. J.* **289**, 71–79
- Prostko, C. R., Brostrom, M. A., Malara, E. M. and Brostrom, C. O. (1992) *J. Biol. Chem.* **267**, 16751–16754
- Lodish, H. F. and Kong, N. (1990) *J. Biol. Chem.* **265**, 10893–10899
- Kuznetsov, G., Brostrom, M. A. and Brostrom, C. O. (1992) *J. Biol. Chem.* **267**, 3932–3939
- Lodish, H. F., Kong, N. and Wikstrom, L. (1992) *J. Biol. Chem.* **267**, 12753–12760
- Kuznetsov, G., Brostrom, M. A. and Brostrom, C. O. (1993) *J. Biol. Chem.* **268**, 2001–2008
- Haas, I. G. (1991) *Curr. Topics Microbiol. Immunol.* **167**, 71–82

- 19 Lee, A. S. (1992) *Curr. Opin. Cell Biol.* **4**, 267–273
- 20 Gething, M.-J. and Sambrook, J. (1992) *Nature (London)* **355**, 33–45
- 21 Brostrom, M. A., Cade, C., Prostko, C. R., Gmitter-Yellen, D. and Brostrom, C. O. (1990) *J. Biol. Chem.* **265**, 20539–20546
- 22 Prostko, C. R., Brostrom, M. A. and Brostrom, C. O. (1991) *J. Biol. Chem.* **266**, 19790–19795
- 23 Albert, P. R. and Tashjian, A. H., Jr. (1984) *J. Biol. Chem.* **258**, 15350–15363
- 24 Albert, P. R. and Tashjian, A. H., Jr. (1986) *Am. J. Physiol.* **251**, C887–C891
- 25 Brostrom, C. O., Bocckino, S. B. and Brostrom, M. A. (1983) *J. Biol. Chem.* **258**, 14390–14399
- 26 Bian, J., Ghosh, T. K., Wang, J.-C. and Gill, D. L. (1991) *J. Biol. Chem.* **266**, 8801–8806
- 27 Tsien, R. and Pozzan, T. (1989) *Methods Enzymol.* **172**, 230–262
- 28 Gill, D. L. and Chueh, S.-H. (1985) *J. Biol. Chem.* **260**, 9289–9297
- 29 Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. and Gill, D. L. (1989) *Nature (London)* **340**, 236–239
- 30 Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S. and Snyder, S. H. (1987) *J. Biol. Chem.* **262**, 12132–12136
- 31 Ghosh, T. K., Eis, P. S., Mullaney, J. M., Ebert, C. L. and Gill, D. L. (1988) *J. Biol. Chem.* **263**, 11075–11079
- 32 Galione, A. (1992) *Trends Pharmacol. Sci.* **13**, 304–306
- 33 Sorrentino, V. and Volpe, P. (1993) *Trends Pharmacol. Sci.* **14**, 98–103
- 34 McPherson, P. S. and Campbell, K. P. (1993) *J. Biol. Chem.* **268**, 13765–13768
- 35 Walz, B. and Baumann, B. (1989) *Prog. Histochem. Cytochem.* **20**, 1–45
- 36 Tsien, R. W. (1990) *Annu. Rev. Cell Biol.* **6**, 715–760
- 37 Meldolesi, J., Madeddu, L. and Pozzan, T. (1990) *Biochim. Biophys. Acta* **1055**, 130–140
- 38 Rutter, G. A., Theler, J.-M., Murgia, M., Wollheim, C. B., Pozzan, T. and Rizzuto, R. (1993) *J. Biol. Chem.* **268**, 22385–22390
- 39 Bird, G. S. J., Obie, J. F. and Putney, J. W., Jr. (1992) *J. Biol. Chem.* **267**, 18382–18386
- 40 Hilly, M., Pietri-Rouxel, F., Coquil, J.-F., Guy, M. and Mauger, J.-P. (1993) *J. Biol. Chem.* **268**, 16488–16494
- 41 Rapoport, T. A. (1991) *FASEB J.* **5**, 2792–2798
- 42 Sanders, S. L. and Schekman, R. (1992) *J. Biol. Chem.* **267**, 13791–13794
- 43 Rapoport, T. A. (1992) *Science* **258**, 931–936
- 44 Simon, S. M. and Blobel, G. (1991) *Cell* **65**, 371–380
- 45 Nicchita, C. V. and Blobel, G. (1990) *Cell* **60**, 259–269
- 46 Pfanner, N. and Neupert, W. (1990) *Annu. Rev. Biochem.* **59**, 331–353
- 47 Rotman, E. I., Brostrom, M. A. and Brostrom, C. O. (1992) *Biochem. J.* **282**, 487–494