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Cell calcium signalling induced by endogenous lectin carbohydrate interaction in the Jurkat T cell line

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The effects of the β-galactoside-binding lectin from human placenta (HPL14) on intracellular calcium concentration ($[Ca^{2+}]_i$) were examined in the human Jurkat T cell line. The lectin induces a concentration dependent increase in $[Ca^{2+}]_i$. This calcium signalling effect is clearly mediated through complementary cell surface galactoglycoconjugates because it can be blocked by β-galactosides. The observed Ca^{2+}-response involves both the release of calcium from intracellular stores and a calcium influx from the extracellular space. It is sustained in the presence of 1 mM extracellular calcium whereas it becomes transient when the influx of extracellular calcium was blocked by calcium chelation to EGTA. Voltage-sensitive calcium channel blockers like verapamil and prenylamine were without effect on the action of HPL14. Protection of the sugar binding activity of HPL14 in the absence of a thiol-reducing reagent by carboxamidomethylation (CM-HPL14) or by substitution Cys2 with serine (C2S) results in lectin proteins with considerably decreased calcium signalling efficiency. The recombinant lectin (Rec H) and the mutant protein obtained by substitution of highly conservative Trp68 with tyrosine (W68Y) induce lower levels of $[Ca^{2+}]_i$ compared to wild type lectin.

Keywords: β-galactoside-binding lectin, cell calcium signalling, Jurkat T cells

Abbreviations: $[Ca^{2+}]_i$, concentration of intracytoplasmic free calcium; CM, carboxamidomethylation; CRD, carbohydrate recognition domain; C2S, mutant lectin protein in which Cys2 was replaced by serine; EGTA, ethyleneglycol-bis(2-aminoethylether)-*N,N,N′, N′*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; HPL14, human β-galactoside-binding placental lectin; Rec H, recombinant human 14 kDa lectin; W68Y, mutant lectin protein in which Trp68 was substituted to tyrosine.

Introduction

A large number of animal lectins have been identified that interact with complementary glycoconjugates on cell surfaces, in extracellular matrices or attached to soluble glycoproteins. Because of their ability to discriminate among complex carbohydrate structures it is conceivable that lectins mediate these interactions by decoding the information present in selected saccharides. On the basis of the amino acid sequences of their carbohydrate recognition domains (CRDs) animal lectins recently have been classified into two main types [1–3]. The C-type lectins have primary structures typical of extracellular proteins or membrane-anchored proteins and extracellular located CRDs always bind to carbohydrate containing ligands in a calcium ion dependent manner [4]. The galectins formerly designated S-type or S-lac lectins are a family of related proteins that bind to β-galactoside containing ligands in a Ca^{2+} independent manner and some of them require the presence of a thiol-reducing reagent to retain carbohydrate recognition *in vitro* [5–7]. They have characteristics of cytoplasmic proteins such as the lack of a hydrophobic signal sequence, loss of glycosylation even when they have a potential glycosylation site, together with the absence of a disulfide bond in spite of the presence of several highly conserved cysteine residues, and acetylated N-terminal amino groups [3]. This protein family has been subdivided into three groups

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according to their size and structure. The most common lectins having a single CRD are 14 kDa proteins (Proto type). They usually form noncovalent dimers.

The β-galactoside binding lectins are found inside and outside of cells. Secretion of galectins is obviously necessary for extracellular function. It has been shown that some members are externalized by a non-classical secretory pathway [8, 9]. According to their preferential binding to *N*-acetyllactosamine, it has been suggested that they may be involved in cell-cell and cell-matrix interaction by binding to extracellular matrix proteins such as laminin, fibronectin, and in binding to lysosomal-associated membrane proteins containing glycans with poly-*N*-acetyllactosamine units [10, 11]. They can induce various biological effects such as immunosuppressive effects in experimental autoimmune diseases [12, 13], inhibition of α(1-2)-fucosyltransferase action [14], and may function as an autocrine negative growth factor [15]. A variety of β-galactoside-binding lectins have been detected in human leukocytes [16–18]. Their secretion is of interest because the expression of cell surface lactosaminoglycans characteristic of leukocytes was found to be differentiation dependent [19]. As demonstrated histochemically, the β-galactoside-binding lectin from human placenta (HPL14) is preferentially localized in syncytiotrophoblast and trophoblastic cells forming a continuous barrier at the fetomaternal interface [21]. It is conceivable that HPL14 belongs to a group of regulatory molecules which are able to prevent allosensitization. If that is the case, a signal for the down regulation of the response in T cells should be detectable. In this work the human Jurkat T cell line has been used as a model to look for calcium signalling induced by HPL14. The carboxyamidmethylated derivative (CM-HPL14), a recombinant human lectin (RecH) as well as the mutant lectin proteins C2S and W68Y were included to study the influence of selected amino acids on the biological function of HPL14.

Material and methods

Cells

The human Jurkat T cell line (clone E6-1) used in these experiments was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The cells were cultured in RPMI 1640 (Gibco BRL, Eggenstein, Germany) supplemented with 10% (v/v) fetal-calf serum and 10 $\mu g\,ml^{-1}$ kanamycin.

Preparation of the human placenta lectin (HPL14)

The β-galactoside-binding lectin was extracted from human placenta homogenate with 0.1 M lactose solution under dissociating conditions and purified to homogeneity by sequential affinity chromatography on asialofetuin-Sepharose 4B [20] followed by lactosedivinylsulfone-agarose [21].

Preparation of the carboxyamidmethylated HPL14 (CM-HPL14)

Carboxamidomethylation was performed by elution of the lactosyl-Sepharose 4B bound HPL14 with 0.05 M lactose, 0.1 M iodoacetamide in a 0.5 M potassium phosphate buffer, pH 8.4 [22]. To change the buffer the mixture was passed through a phosphate buffered saline (PBS), pH 7.4, equilibrated Bio-Gel P6 column. CM-HPL14 migrated as a single band as detected by SDS-PAGE.

Production of the recombinant and mutant human 14 kDa β-galactoside-binding lectin proteins

The recombinant lectin (Rec H) was produced by insertion of the cDNA for a 14 kDa β-galactoside-binding lectin into a plasmid carrying a Taq promotor and expressed in *E. coli* as previously described [23]. N-terminal amino acid sequence analysis showed that the recombinant lectin was not blocked at its N-terminus in contrast to the placental lectin. The production of ten mutant lectin proteins by site-directed mutagenesis experiments is described in detail by Hirabayashi and Kasai [24]. One of the mutants in which Cys 2 was substituted with serine (C2S) became more stable asialofetuin binding activity under non-dissociating conditions. Substitution of highly conservative Trp68 with tyrosine (W68Y) resulted in a slightly reduced lactose binding ability by the lectin protein.

Measurement of intracellular calcium

Measurements of intracytoplasmic free Ca^{2+} levels were performed with fura-2 AM. Jurkat T cells (2×10^6) were washed twice and loaded with 5 μM of fura-2 AM in 1 ml RPMI 1640 medium for 30 min at 37 °C. After the addition of 3 ml RPMI 1640 medium, cells were reincubated for 20 min at 37 °C. Then fura-2 loaded cells were washed three times with 10 mM Na-HEPES buffer, pH 7.4, supplemented with 137 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 1 mM Na_2HPO_4 and 1 g $BSAl^{-1}$ and adjusted to 1×10^6 cells ml^{-1}. Viability of cells as detected by Trypan blue exclusion was higher than 95%. The cellular suspension (1.5 ml) was transferred in quartz cuvettes thermostatically controlled at 37 °C and the fluorescence was monitored with a Shimadzu RF-5001 PC spectrofluorimeter. The cell suspension was excited alternatively at 339 and 380 nm and the fluorescence measured at 490 nm. Graphic representations of $[Ca^{2+}]_i$ were calculated by converting the ratio of 339/380 to $[Ca^{2+}]_i$ using a K_d of 224 nM according to the equation

$$[Ca^{2+}]_i = 224\ (R-R_{min})/(R_{max}-R) \times Sf\ 380/Sb\ 380,$$

previously published by Grynkiewicz *et al.* [25]. R_{max} and

R_{min} were evaluated in 1 mM Ca^{2+}-containing medium by lysing the cells with 0.5% Triton X100 for R_{max}, followed by the addition of an excess of EGTA for R_{min}.

Results

The concentration dependent effects of HPL14 on changes in $[Ca^{2+}]_i$ in Jurkat T cells are shown in Fig. 1. HPL14 induced an increase in $[Ca^{2+}]_i$ with an appreciable lag. The onset of the increase and the peak values in $[Ca^{2+}]_i$ are strongly dependent on the lectin concentration used. When 13 $\mu g\,ml^{-1}$ of the lectin were used to elicit a calcium response, concentrations of 596 ± 78 nM were obtained which represent a nine-fold increase over the basal $[Ca^{2+}]_i$ levels of 66 ± 19 nM. As shown in Fig. 1a, preincubation of fura-2 loaded cells in the presence of 8 mM lactose reduced the calcium response induced by 13 $\mu g\,ml^{-1}$ HPL14 to basal levels. This experiment clearly shows that the lectin induced increase in $[Ca^{2+}]_i$ is mediated by interaction with complementary cell surface glycoconjugates. However, in contrast to Jurkat T cells, HPL14 has no effect on $[Ca^{2+}]_i$ in HL-60 human promyeloleukaemic cells and in THP-1 human monocytic leukaemia cells (data not shown). To study the roles of selected amino acid residues of human β-galactoside-binding lectin, the

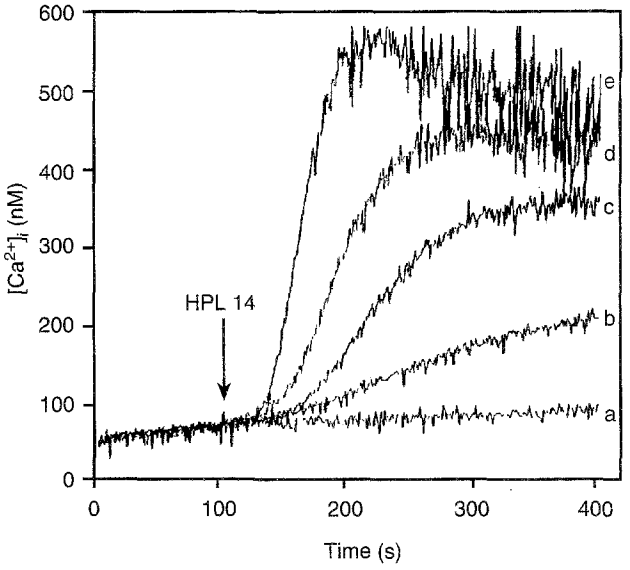

Figure 1. Concentration dependent effects of HPL14 on $[Ca^{2+}]_i$ in Jurkat T cells. $[Ca^{2+}]_i$ levels were measured in a suspension of 1×10^6 cells ml^{-1}, utilizing the fluorescent properties of fura-2 in HEPES-buffer containing 1 mM $CaCl_2$ as described in the Materials and methods section. HPL14 was added as indicated by the arrow at 1.3 $\mu g\,ml^{-1}$ (b), 3 $\mu g\,ml^{-1}$ (c), 6 $\mu g\,ml^{-1}$ (d), 13 $\mu g\,ml^{-1}$ (e), and at 13 $\mu g\,ml^{-1}$ in the presence of 8 mM lactose (a). Lactose was added to the cell suspension at time 0.

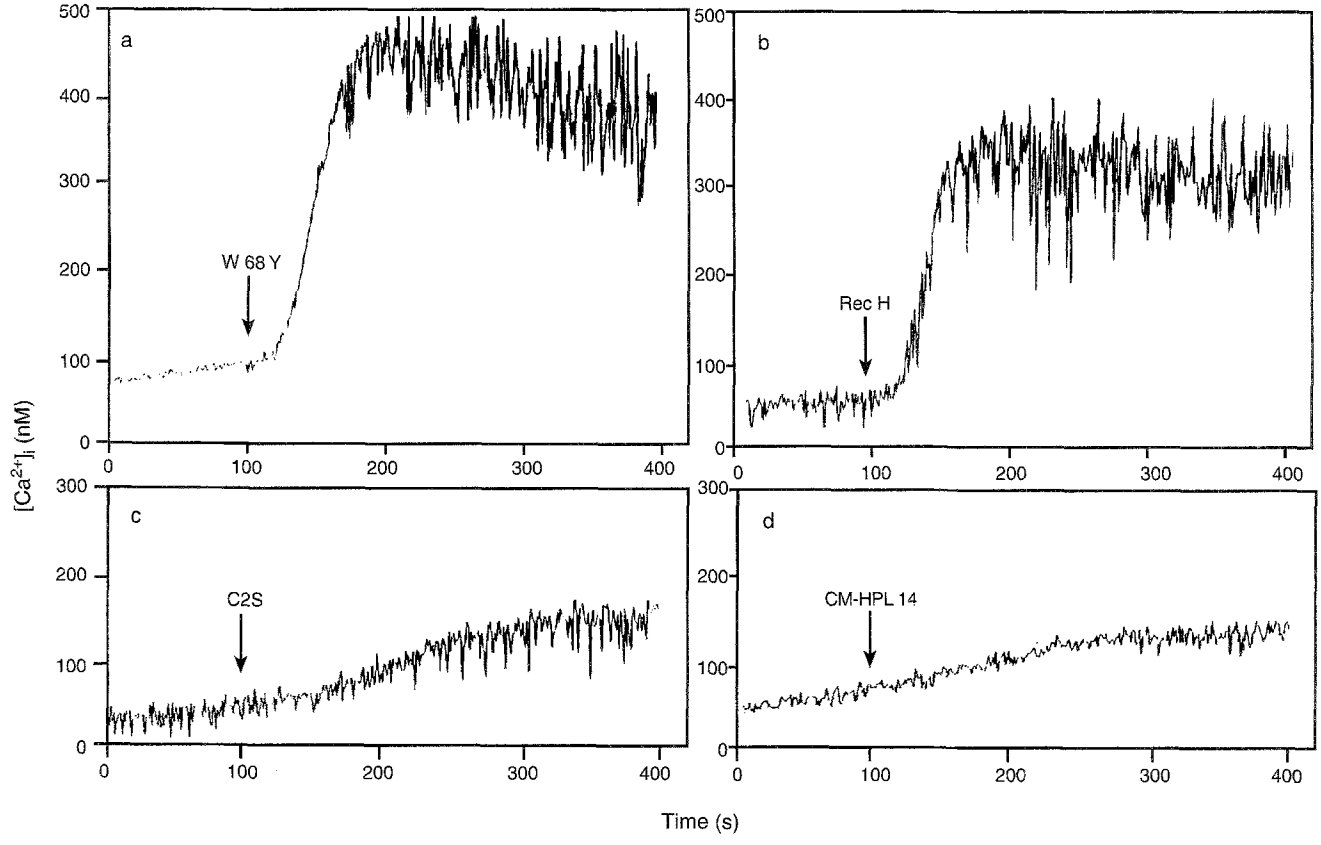

Figure 2. Effects of W68Y (a), RecH (b), C2S (c), and CM-HPL14 (d), each lectin protein at 13 $\mu g\,ml^{-1}$, on $[Ca^{2+}]_i$ in Jurkat T cells in the presence of 1 mM $CaCl_2$.

Table 1. Basal and lectin-induced $[Ca^{2+}]_i$-values in Jurkat T cells. $[Ca^{2+}]_i$-values in nM for HPL14, RecH, W68Y, C2S and CM-HPL14 at the indicated concentrations were calculated as described in the Materials and methods section. Data shown are mean values ±SD from five separate experiments.

Lectin	*HPL 14*					*Rec H*	*W68Y*		*C2S*	*CM-HPL 14*
Concentration ($\mu g\,ml^{-1}$)	0	1.3	3.0	6.0	13.0	13.0	1.3	13.0	13.0	13.0
$[Ca^{2+}]_i$ (nM)	66	199	338	430	596	353	109	451	144	112
± SD	±19	±32	±51	±36	±78	±47	±27	±63	±31	±29

mutant lectin proteins C2S and W68Y as well as the carboxyamidmethylated derivative (CM-HPL14) were included. Because oxidation of Cys 2 is a key process in the inactivation of HPL14 (20), C2S has considerably more stable β-galactoside-binding activity in the absence of a thiol-reducing reagent. We also protected HPL14 by treatment with monoiodoacetamide. As shown in Fig. 2c and 2d, C2S as well as CM-HPL14 elicit considerably decreased $[Ca^{2+}]_i$ values in Jurkat T cells compared to the native lectin (Fig. 1). To exclude a decrease in binding activity, Jurkat T cell lysates were electrophoretically separated and blotted on nitrocellulose membranes. The binding pattern of the biotinylated wild-type lectin and that of biotinylated C2S and CM-HPL14 in the absence of a thiol-reducing reagent were identical (not shown). By applying the mutant lectin protein W68Y which has a significantly weaker lactose-binding affinity [28] and the recombinant human lectin, decreased $[Ca^{2+}]_i$ levels are also obtained in comparison to the wild-type lectin (Fig. 2a, 2b). The basal and lectin-induced $[Ca^{2+}]_i$ values in Jurkat T cells in the presence of 1 mM $CaCl_2$ in the buffer are summarized in Table 1. According to the calculated $[Ca^{2+}]_i$ values for the lectins at 13 $\mu g\,ml^{-1}$ the following sequence of decreasing Ca^{2+}-inducing activity were obtained HPL14 > W68Y > Rec H > C2S > CM-HPL14. To study the carbohydrate specificity of the HPL14 induced rise in $[Ca^{2+}]_i$ in Jurkat T cells, we tested the effects of different saccharidic inhibitors up to 8 mM. Saccharides such as galactose, glucose, mannose, *N*-acetylglucosamine, α and β-methylgalactoside, maltose (Glcα1-4Glc) and gentiobiose (Glcβ1-6Glc) do not show any inhibitory effects (data not shown). As indicated in Table 2, GalNAc itself has only low inhibitory activity. Saccharidic inhibitors containing non-reducing terminal β-galactosyl residues such as lactose (Galβ1-4Glc), *N*-acetyllactosamine (Galβ1-4GlcNAc), lactulose (Galβ1-4Fru), and Galβ1-4Man inhibit the cellular Ca^{2+}-response elicited by the lectin. In contrast, cellobiose (Glcβ1-4Glc) which is identical to lactose except for the equatorial orientation of the 4-hydroxyl group of the non-reducing Glc and melibiose (Galα1-6Glc) are inactive. Asialofetuin containing a triantennary oligosaccharide with three Galβ1-4GlcNAc sequences and terminal non-reducing Gal residues [26] fully inhibits the Ca^{2+}-response in Jurkat T cells at 2 $mg\,ml^{-1}$ induced by 13 $\mu g\,ml^{-1}$ HPL14 (not shown here).

Table 2. Concentration dependent inhibition of HPL14-induced rise of cytosolic $[Ca^{2+}]_i$ in Jurkat T cells by different saccharides. The inhibitory activity of each compound is expressed relative to the $[Ca^{2+}]_i$-values induced by 13 $\mu g\,ml^{-1}$ HPL14 in the absence of a saccharide.

Formula of saccharides	*Relative Ca^{2+} – inducing activity of 13 μg HPL 14 per ml in the presence of the corresponding saccharide at:*					
	0 mM	0.5 mM	1 mM	2 mM	4 mM	8 mM
GalNAc	1.0	1.0	0.96	0.98	0.95	0.90
Galβ1-4Glc	1.0	0.54	0.34	0.29	0.21	0.09
Galβ1-4GlcNAc	1.0	0.38	0.27	0.14	0.07	0.02
Galβ1-4Fru	1.0	0.58	0.48	0.14	0.10	0.08
Galβ1-4Man	1.0	0.48	0.40	0.29	0.11	0.08
Glcβ1-4Glc	1.0	1.0	0.98	0.97	0.97	1.0
Galα1-6Glc	1.0	0.96	0.99	1.0	1.0	1.0

Since the increase in $[Ca^{2+}]_i$ could originate from calcium entry and/or from intracellular stores we investigated the rise in $[Ca^{2+}]_i$ in calcium-free medium. As illustrated in Fig. 3, HPL14 induces a transient calcium signal with peak values of around 200 nM. Reconstitution of the medium with $CaCl_2$ (1 mM) results in a second phase that is sustained by an influx of extracellular calcium. When the calcium influx is blocked by an excess of EGTA (0.5 mM) the second phase is completely absent. Identical patterns of calcium responses as shown for HPL14 in Fig. 3 were also recorded for RecH and W68Y (not shown). To obtain further data on the nature of the influx pathway, we studied the effects of the organic calcium channel blockers verapamil (20 μM) and prenylamine (25 μM). The action of both varapamil (Fig. 4b) and prenylamine (not shown here) give increased levels of $[Ca^{2+}]_i$ of about 110 nM. However, these effects are absent when extracellular calcium is blocked by calcium chelation to EGTA (not shown). Neither of the calcium channel blockers affected $[Ca^{2+}]_i$

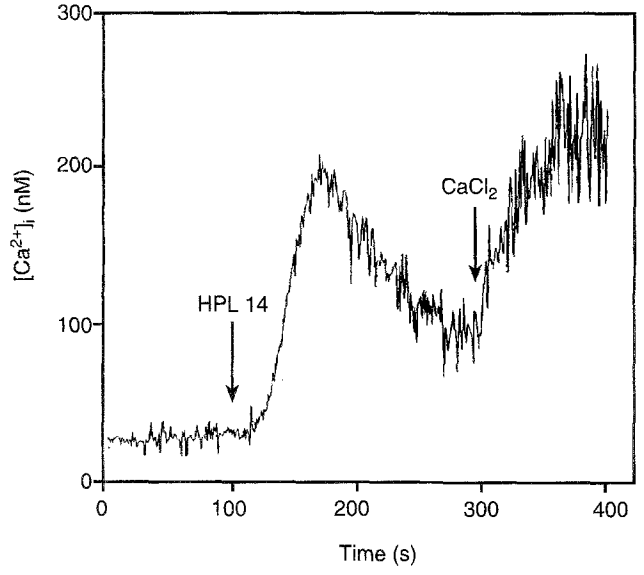

Figure 3. Effects of 13 $\mu g\,ml^{-1}$ HPL14 on calcium transients in Jurkat T cells in a calcium-free medium. EGTA (1.5 mM) was added at time 0. The addition of HPL14 and $CaCl_2$ (1.5 mM) is indicated by arrows.

in Jurkat T cells stimulated by HPL14 as indicated for varapamil (Fig. 4b) and prenylamine.

Discussion

The results of the present study show that HPL14 promotes a concentration dependent rise in $[Ca^{2+}]_i$ in Jurkat T cells. This lectin effect on cellular calcium response was found to be efficiently suppressed by β-galactosidic inhibitors such as Galβ1-4Glc, Galβ1-4GlcNAc, Galβ1-4Fru, Galβ1-4Man, and by asialofetuin carrying a triantennary oligosaccharide with three Galβ1-4GlcNAc sequences and terminal non-reducing Gal residues [26]. However cellobiose (Glcβ1-4Glc) with an equatorial orientation of the 4-hydroxyl group of the non-reducing Glc was without inhibitory activity on the lectin induced Ca^{2+} response in Jurkat T cells showing the strong structural requirement of the lectin-carbohydrate interaction. From these inhibition experiments, we concluded that the interaction of HPL14 with complementary cell surface galactoglycoconjugates is associated with the induction of cell calcium signalling. However, when we extended our experiments to HL-60 human promyeloleukaemic cells and to THP-1 human monocytic leukaemia cells no effect on $[Ca^{2+}]_i$ was recorded. Therefore, it is conceivable that in spite of lectin-cell interaction remarkable differences exist in the cell surface expression of β-galactosidic lectin binding sites between the cell lines.

Binding specificity of three soluble rat lung lectins to β-galactosides as determined in a quantitative assay indicated positions 4 and 6 of Gal and 3-hydroxyl of Glc in lactose as critical determinants [27]. In carbohydrate-protein interaction a network of hydrogen bonds is of importance for carbohydrate binding specificity [31]. Site-directed mutagenesis experiments revealed that six highly conserved hydrophilic amino acid residues His44, Arg48, Asn61 [28] and Asn46, Glu71 and Arg73 [24] of human 14 kDa β-galactoside-binding lectin (human galectin-1) [29] are essential for carbohydrate-binding activity. Substitution of any of them resulted in the complete loss of asialofetuin-binding activity. On the other hand, substitutions of less well conserved hydrophilic amino acid residues (Lys 63, Arg 111, Asp 125) did not change the affinity to lactose [28]. In addition aromatic ring-sugar interaction is generally observed in

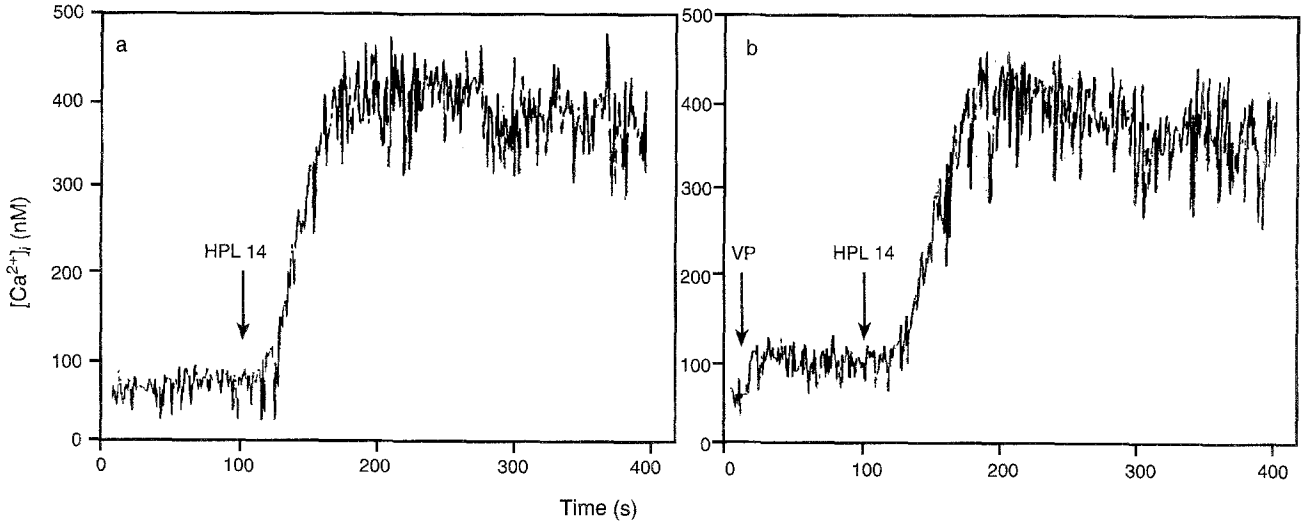

Figure 4. HPL14-induced rise in $[Ca^{2+}]_i$ in Jurkat T cells in the absence (a; control) and in the presence of 20 μM verapamil (VP; b).

carbohydrate-protein binding [32, 33]. Replacement of Trp62 in egg white lysozyme by site-directed mutagenesis with non-aromatic amino acids (Leu, Ile, Val, Ala and Gly) reduced the enzymatic interaction with non-charged substrates [34]. It was concluded that Trp62 interacts with oligosaccharides by van der Waals contact and maintains the local structural conformation required for lysozyme-oligosaccharide interaction. In human placental galectin-1 the tryptophan residue is not essential for the carbohydrate binding activity, but enhances the interaction. The mutant W68Y showed significant weaker asialofetuin-binding affinity than the wild-type lectin [28]. This might be a reason for the decreased calcium response in Jurkat T cells induced by W68Y compared to HPL14.

On the other hand, substitution of Cys2 with serine [24] or carboxyamidmethylation [22, 30] has been described as a procedure to protect asialofetuin-binding activity of some members of galectins in the absence of a thiol-reducing reagent. C2S and CM-HPL14 induced considerably lower $[Ca^{2+}]_i$-values in Jurkat cells than the wild-type lectin. Although six highly conserved hydrophilic amino acid residues form a carbohydrate-binding site of soluble human β-galactoside-binding lectins (galectin-1) [28], reduced cysteine residues are a requirement to retain a conformation essentially for carbohydrate recognition. The affinity of C2S was found to be slightly reduced when compared with the wild-type lectin as detected in a lectin-asialofetuin binding assay [24]. It may be that the lectin derivatives have different affinities to the glycopart of the functional cell surface receptor which is the reason for the differences in their potency when stimulating intracellular calcium.

The experiment in which the influx of extracellular calcium is blocked by calcium chelation to EGTA shows that the first rapid rise in the absence of extracellular calcium results from the release of calcium from intracellular stores (Fig. 3). Reconstitution of the medium with calcium results in a second rise in $[Ca^{2+}]_i$ caused by the influx from the extracellular space. The lectin induced increase in $[Ca^{2+}]_i$ in Jurkat T cells was not influenced by the potent calcium channel blocker verapamil (up to 100 μM) and by the partial calcium channel blocker prenylamine in spite of the fact that the elevation in $[Ca^{2+}]_i$ was represented by a phase of influx from the extracellular space (Fig. 3). We concluded that calcium channels normally blocked by the classical calcium channel inhibitor verapamil are not influenced in Jurkat T cells. Verapamil at low concentrations blocks voltage dependent channels and at high concentrations both voltage dependent and receptor operated calcium channels [35]. In OVCAR-3 cells prenylamine blocks an ATP receptor operated calcium channel in contrast to nifedipin and verapamil [36].

Often, the increase in $[Ca^{2+}]_i$ is required for the stimulation of cell growth [36, 37]. Our results clearly show that the non-mitogenic lectin induces an increase in $[Ca^{2+}]_i$ in Jurkat T cells caused by both the entry of extracellular calcium and by calcium mobilization from intracellular stores. It has been suggested that a rise in intracellular calcium may be involved in the induction of the apoptotic mechanism [38, 39]. Apoptosis is a physiological process by which cells are eliminated during normal development in fetal and adult life [40]. The pathways for activation of apoptosis are multiple and distinct in different cell types. It may be that HPL14 is involved in the induction of apoptosis as a local mechanism responsible for deletion of alloreactive T cells.

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