Studies of calmodulin from herpesvirus infected cell cultures.

David Timothy Millinoff

University of Windsor

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STUDIES OF CALMODULIN FROM HERPESVIRUS INFECTED CELL CULTURES

by

David Timothy Millinoff

A Thesis
Submitted to the Faculty of Graduate Studies Through the Department of Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

WINDSOR, ONTARIO, CANADA
1987
DEDICATION

To my dad S.P. Millinoff
ABSTRACT

STUDIES OF CALMODULIN FROM HERPESVIRUS INFECTED CELL CULTURES

by

David Timothy Millinoff

This study appears to be the first report of the effect of productive herpesvirus infection upon the structure of calmodulin. Bioactivities of calmodulin from HSV-1-infected Vero cells are reduced almost 50% after 2 hr and by 8 hr postinfection, calmodulin activities had increased 35% over that detectable in the control uninfected cells. Proteases selected for limited proteolysis of porcine brain and Vero cell calmodulins were nagarse, thermolysin, and proteinase K. Cleavage of calmodulin by test proteases produced fragments of different molecular size, however, elution profiles of brain calmodulin suggested the presence of a fragment peak eluting at about 9.0 min which was absent in profiles of hydrolysed Vero cell preparations. Differences in cleavage profiles of 3 and 8 hr HSV-1 infected cell calmodulin were suggested by the absence of a fragment peak eluting 8.8 min in nagarse treated 8 hr HSV-1 infected sample. When the HPLC profiles of calmodulin derived from HSV-1 and HSV-2-infected cells were compared, the latter preparation lacked a 11.5 min peak. Such results of limited proteolysis of the virus infected preparations may indicate
alterations in the structure of calmodulin.
ACKNOWLEDGEMENTS

I am indebted to Dr. L.R. Sabina, Department of Biological Sciences, University of Windsor for the inspiration and guidance he provided during the course of this research. His advice is greatly appreciated.

For critically reviewing this thesis, I am grateful to Dr. D. Thomas, Department of Biological Sciences and Dr. B. Mutus, Department of Chemistry and Biochemistry, both of the University of Windsor.

For technical advice and materials, I wish to extend thanks to Dr. B. Mutus, N. Karuppiah and B. Palmer of the Department of Chemistry and Biochemistry, University of Windsor. A special thanks is also extended to Dr. K. Suryanarayana of the Department of Medical Microbiology, University of Alberta, Edmonton, Alberta for his assistance in the RIA studies and the data collected in Table 1.

To my wife, Loretta, a very special thanks for both the typing of this manuscript and her constant encouragement during this research.

This investigation was supported in part by a grant from the Natural Sciences and Engineering Research Council of Canada.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>5</td>
</tr>
<tr>
<td>RESULTS</td>
<td>11</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>40</td>
</tr>
<tr>
<td>APPENDIX A</td>
<td>47</td>
</tr>
<tr>
<td>APPENDIX B</td>
<td>48</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>49</td>
</tr>
<tr>
<td>VITA AUCTORIS</td>
<td>54</td>
</tr>
</tbody>
</table>

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## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Comparative amounts of calmodulin in extracts from primary and established cell cultures...</td>
<td>12</td>
</tr>
<tr>
<td>2.</td>
<td>Activation of phosphodiesterase by Vero cell extracts exposed to HSV-1</td>
<td>15</td>
</tr>
<tr>
<td>3.</td>
<td>Summary of purification scheme for Vero cell calmodulin</td>
<td>20</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Dose-response curves for the activation of calmodulin-deficient bovine heart phosphodiesterase</td>
<td>13</td>
</tr>
<tr>
<td>2.</td>
<td>Multiplication of HSV-1 in Vero cells</td>
<td>16</td>
</tr>
<tr>
<td>3.</td>
<td>DEAE cellulose chromatography of Vero cell calmodulin</td>
<td>18</td>
</tr>
<tr>
<td>4.</td>
<td>Elution profile of calmodulin by affinity chromatography on melittin Affigel-10</td>
<td>19</td>
</tr>
<tr>
<td>5.</td>
<td>SDS polyacrylamide gel analysis of porcine brain and Vero cell calmodulins.</td>
<td>22</td>
</tr>
<tr>
<td>6.</td>
<td>Elution profile of porcine brain calmodulin</td>
<td>23</td>
</tr>
<tr>
<td>7.</td>
<td>Enzymatic hydrolysis of porcine brain calmodulin</td>
<td>24</td>
</tr>
<tr>
<td>8.</td>
<td>Enzymatic hydrolysis of porcine brain calmodulin with trypsin</td>
<td>25</td>
</tr>
<tr>
<td>9.</td>
<td>Elution profile of uninfected Vero cell calmodulin</td>
<td>27</td>
</tr>
<tr>
<td>10.</td>
<td>Enzymatic hydrolysis of uninfected Vero cell calmodulin</td>
<td>28</td>
</tr>
<tr>
<td>11.</td>
<td>Elution profile of 3 hr HSV-1 infected Vero cell calmodulin</td>
<td>30</td>
</tr>
<tr>
<td>12.</td>
<td>Enzymatic hydrolysis of 3 hr HSV-1 infected Vero cell calmodulin</td>
<td>31</td>
</tr>
<tr>
<td>13.</td>
<td>Elution profile of 8 hr HSV-1 infected Vero cell calmodulin</td>
<td>32</td>
</tr>
<tr>
<td>14.</td>
<td>Enzymatic hydrolysis of 8 hr HSV-1 infected Vero cell calmodulin</td>
<td>33</td>
</tr>
<tr>
<td>15.</td>
<td>Elution profile of 8 hr HSV-2 infected Vero cell calmodulin</td>
<td>35</td>
</tr>
<tr>
<td>16.</td>
<td>Enzymatic hydrolysis of 8 hr HSV-2 infected Vero cell calmodulin</td>
<td>36</td>
</tr>
</tbody>
</table>
Figure 17. Urea SDS-PAGE analysis of porcine brain calmodulin hydrolyzed by different proteases......................... 38
LIST OF ABBREVIATIONS

CaCl₂ - calcium chloride
CaM - calmodulin
cAMP - cyclic adenosine monophosphate
DEAE - diethylaminoethyl
EGTA - ethyleneglycol bis (B-aminoethyl ether) N,N,N',N' tetraacetic acid
HEPES - N-2-hydroxyethylpiperazine N'-2-ethane sulfonic acid
HSV-1 - herpes simplex virus type 1
HSV-2 - herpes simplex virus type 2
KCl - potassium chloride
MEM - minimum essential medium
kd - kilodaltons
NaCl - sodium chloride
NAGARSE- subtilisin BPN'
(NH₄)₂HCO₃ - ammonium bicarbonate
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate buffered saline
PDE - phosphodiesterase
SDS - sodium dodecyl sulfate
TCID₅₀ - tissue culture 50% infective dose
TRIS - (hydroxymethyl) methyamine
INTRODUCTION

The role of calcium in the physiological activity of mammalian tissues has been well documented in the mechanism of blood clotting (Davie et al., 1977) and muscle contraction (Bagshaw, 1982; Harrington, 1972; Weber, 1973). Its involvement in the regulation of intracellular enzymatic activity was not understood until the discovery of calmodulin by Cheung, (1967) and Kakiuchi et al., (1970). The protein proved to be a modulator of many Ca\textsuperscript{2+}-dependent enzyme systems. This modulating effect of calmodulin has attracted much attention because of its presence in nearly all eukaryotic life forms tested.

Calmodulin was first purified from mammalian tissue extracts and has been extensively characterized from bovine brain (Lin et al., 1974; Watterson et al., 1976) and heart (Teo et al., 1973) tissues. The modulator has also been purified in large quantities from bovine testis (Chafouleas et al., 1979), pancreas (Sugden et al., 1979) and kidney (Morgan et al., 1980; Klee et al., 1982). Studies indicate that measurable amounts of calmodulin can be isolated from other eukaryotic systems such as barley seeds (Grand et al., 1980), the invertebrate Renilla reniformis (Jones et al., 1979), protozoans such as Tetrahymena pyriformis (Jamieson et al., 1979), and the mold Achyla ambisexualis (Suryanarayana et al., 1985).

According to Schutt (1985), the three dimensional...
structure of calmodulin is comprised of two pairs of Ca\(^{2+}\)-
binding domains interconnected by a large exposed helix.

In tissue culture, the modulator has been detected in a
number of primary and established cell lines. Selected cell
lines shown to contain calmodulin include C-6 glioma cells
(Bromstrom et al., 1974), chicken embryo fibroblasts
(Watterson et al., 1976; Van Eldik et al., 1979; Klee et
al., 1982), Chinese hamster ovary cells (Evain et al., 1979)
and baby hamster kidney cells (Yerna et al., 1979). Calmodulin
was also evident in cell lines derived from malignant tumors as in the case of Morris hepatoma tissue reported by Criss et al., (1982). Besides tissues of malignant origin, calmodulin has been demonstrated in virus transformed cells. Both Van Eldik et al., (1979) and Connor et al., (1983) reported elevated levels (2-3 fold greater than normal) of calmodulin in chicken embryo and rat kidney fibroblasts transformed by Rous sarcoma virus. Similar elevations in calmodulin content of Swiss mouse 3T3 cells and normal rat kidney cells transformed by SV40 and Rous sarcoma virus, respectively, were demonstrated by Chafouleas et al., (1981). Thus, viruses which induce an oncogenic transformation of cells rather than a productive virus infection can affect calmodulin activity.

When quantitating calmodulin content in any type of
tissue, one must realize that levels detected can also vary
on the basis of subcellular localization. Smoake et al.,
(1974) demonstrated that calmodulin is distributed
intracellularly in two forms, the first being soluble in the cytoplasm and the other form particulate in nature being associated with the cytoskeleton and organelles. According to Vanaman, (1976), partitioning of the two forms depends on the amount of calcium present during the purification procedure. For example, a majority of calmodulin in cell homogenates remains in the particulate form when higher levels of calcium are used. When an efficient chelator of calcium ions such as EGTA is added to the homogenization buffer, up to 90% of calmodulin in the particulate form is released. Therefore, it can be understood that levels of divalent cations in both assay and purification procedures of calmodulin are critical in determining which conformational state the protein assumes.

A survey of the literature has revealed no report of any in vitro investigations of the effects of productive virus infection on the conformational structure of calmodulin. To examine this question, a model system comprised of herpes simplex viruses and monkey kidney cells was employed. Herpes virus types 1 and 2 were specifically chosen for this study, since they differ considerably (i) in the time of shutdown of host cell protein synthesis after infection and (ii) in the molecular events necessary for inhibiting host protein synthesis (Hill et al., 1983; Fenwick et al., 1982).

The shutdown of protein synthesis seems to be intimately related with the regulation of herpesvirus gene expression.
A cascade system is apparently operative, initially alpha genes are produced and their products induce beta genes. Their products allow for the expression of gamma genes resulting in gamma polypeptides (Roizman and Batterson, 1985).

Preliminary experiments indicate that Vero monkey cells had appreciable quantities of calmodulin and both types of herpesviruses gave rise to productive infections in Vero cultures. Purified virus infected and uninfected monkey kidney cell calmodulin were subjected to protease treatment (Vanaman, 1983). The presence or absence of resulting proteolytic fragments in infected cell calmodulin preparations were compared to that of uninfected cell preparations and construed to be conformational changes in calmodulin.
MATERIALS AND METHODS

Cell Line

The African green monkey cell line designated Vero used throughout this study was obtained from American Type Tissue Collection, Rockville, Md., U.S.A.. Cells were grown in minimum essential medium (MEM) (Eagle, 1959) supplemented with 10% fetal calf serum, 0.07% sodium bicarbonate and antibiotics (penicillin, 50 IU/mL; streptomycin, 100 mcg/mL). Vero cells were utilized between passage 20 and 40 for all experiments. Cells were grown to confluency in Corning roller bottles at a speed of 1/4 bottle rotation/min and in Corning stationary flasks with growing areas of 850 and 75 cm², respectively.

Virus Stocks

Herpes simplex virus type 1 (HSV-1) (Mayo 1814 strain) and herpes simplex virus type 2 (HSV-2) (MS strain) were supplied by Dr. D.A. Kennedy, Laboratory Control Disease Centre, Ottawa, Ontario. Virus pools were prepared by infecting confluent cultures at a multiplicity of 0.05-0.2 TCID₅₀/cell. Infectious fluids were harvested at 48 hr postinfection, pooled and stored at -80°C. The tissue culture 50% infective dose (TCID₅₀) of infectious fluids were titrated on Vero cells according to Spearman-Karber (Finney, 1964). Pools of HSV-1 and HSV-2 had TCID₅₀ titers of about $10^{7.2}$/mL and $10^{6.8}$/mL, respectively.
Chemicals

Ammonium bicarbonate, sodium bicarbonate, potassium chloride, magnesium acetate and tris (hydroxymethyl) methylamine and trichloroacetic acid were obtained from BDH Chemicals, Toronto, Ontario. Imidazole, Fiske and Subbarow reducer, cAMP, EGTA, (ethylene glycol bis(B-aminoethyl ether) N,N,N',N' tetraacetic acid), melittin, 5'-nucleotidase, nagarse (subtilisin BPN') and trypsin were purchased from Sigma Chemical Co., St. Louis, Mo.. Calcium chloride was obtained from Merck Co., Darmstadt, West Germany and HEPES (N-2-hydroxyethylpiperazine N'-2-ethane sulfonic acid) was purchased from Gibco, Grand Island, N.Y.. Proteinase K, thermolysin, bovine heart phosphodiesterase (CaM deficient), porcine brain CaM were obtained from Boehringer Mannheim, West Germany. Bovine brain CaM and bovine heart phosphodiesterase were kindly supplied by Dr. B. Mutus, Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario.

Phosphodiesterase Assay

The assay for measuring phosphodiesterase (PDE) activity described by Sharma and Wang (1979) was used to detect the presence of CaM in uninfected and virus infected crude extracts or purified preparations. One activation unit is the amount of calmodulin required to raise the activity of Ca+2-dependent calmodulin-free bovine heart PDE to 50% of its maximal activation at calmodulin saturation.
Calmodulin Time Course Experiments

The procedure for preparing CaM extracts of virus-infected cells up to at least 16 hr postinfection initially involved infecting replicate Vero cultures with HSV-1 at a multiplicity of 5. Residual inoculum was removed after incubation at 37°C for 1 hr and cultures were covered with MEM lacking serum. Thereafter, duplicate cultures were harvested at varying intervals as follows. Culture medium was removed from monolayers, and cells mopped into phosphate buffered saline (PBS) (Dulbecco, 1954). Cells from replicate flasks were pooled, washed twice in PBS and resuspended in Buffer A (20 mM Tris, 1 mM magnesium acetate, 1 mM imidazole, pH 7.5) supplemented with 0.1 mM EGTA. The cell suspension was homogenized by 3 pulses of 5 sec at 45% power with a Tekmar Tissumizer and microprobe (model SDT 080EN). This homogenate was centrifuged at 20,000 X g for 45 min and the harvested supernatant was recentrifuged at 100,000 X g for 1 hr. The supernatant was heated at 95°C for 5 min, cooled on ice and centrifuged at 20,000 X g for 15 min to remove heat-precipitated protein. Following dialysis with Buffer A, the sample was centrifuged at 3,000 X g for 10 min. Total protein content was estimated by using the Biorad protein assay (Bradford, 1976). The bioactivity of crude CaM extract was tested by its ability to stimulate calcium-dependent bovine heart PDE activity in vitro as described under section Phosphodiesterase Assay. Replicate uninfected Vero cultures were treated similarly.
Purification of CaM from Infected and Uninfected Cell Culture

Cells from roller bottle cultures were infected with HSV-1 or HSV-2 and harvested as outlined under the section entitled Calmodulin Time Course Experiments. Thereafter, each cell homogenate was centrifuged at 5,000 X g for 20 min. The resulting supernatant (S2) from the pellet extraction was heat-treated and dialysed as above. Each supernatant was purified separately. Samples S1 and S2 were applied to DEAE cellulose (Cellex D, Biorad) columns (1.7 X 10 cm) which had been preequilibrated with 100 mL of Buffer A containing 1.0 mM EGTA and 0.1 M NaCl, pH 7.0. Absorbed proteins were eluted by the same buffer using stepwise changes in NaCl concentration of 0.1 M, 0.25 M, 0.35 M and 0.45 M. Column fractions (3 mL each) were measured at 230 nm and then assayed for PDE activation as outlined under section Phosphodiesterase Assay. The fractions containing high PDE activation were pooled, dialysed against distilled water and lyophilized. Samples were reconstituted in Buffer A, pH 7.5, supplemented with 1.0 mM CaCl₂ for affinity chromatography.

Melittin columns were prepared by linking 3 mg melittin with 1.0 mL of Affigel-10 (Biorad Laboratories, Richmond, California) in 0.1 M HEPES, pH 7.5, according to the manufacturer's procedure (Kincaid et al., 1985; Cox et al., 1985; Wallace et al., 1979). Calmodulin samples were applied

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to melittin columns (0.7 X 1.0 cm), preequilibrated with Buffer A, pH 7.5, containing CaCl$_2$. Columns were washed with equilibrating buffer and CaM eluted with Buffer A containing 1.0 mM EGTA and 0.6 M KCl, pH 7.5. The fractions having PDE activator activity were pooled and concentrated with a collodion bag apparatus (Schleicher and Schuell Ltd.). A small aliquot of the sample was tested for protein content.

Enzymatic Hydrolysis of CaM Sample

For the hydrolysis of calmodulin, the reaction mixture contained 10 ug CaM sample, 0.5 mM CaCl$_2$, 5 mM NaCl and 20 mM (NH$_4$)$_2$HCO$_3$, pH 7.0, and the specific protease in a total volume of 60 uL. An enzyme:substrate ratio of 1:50 was used unless otherwise stated. Reaction mixtures were incubated for 2 hr at 30 C and centrifuged at 16,000 X g for 10 min to remove particulates before high pressure liquid chromatography (HPLC) analysis.

HPLC of Hydrolyzed CaM Samples

Hydrolyzed CaM samples were subjected to HPLC using an isocratic Quick Check analyzer system (Biorad Laboratories) coupled to a Reporting Integrator (model 3392A). Samples were injected into a 50 uL loop and passaged through Biosil TSK 125 guard and Biosil TSK 125 gel filtration columns to separate calmodulin fragments. The mobile phase consisted of degassed and filtered Buffer A, pH 6.8, containing 1.0 mM CaCl$_2$ maintained at a flow rate of 1.0 mL/min. Absorbances of sample runs were monitored at a
detector wavelength of 230 nm with the sensitivity range switch set at 0.04.

Gel Electrophoresis

Calmodulin samples were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) as described by Laemmli, (1970) except the sample buffer was modified by adding 0.5 mM CaCl₂ and omitting 2-mercaptoethanol. The stacking and separating gels contained 3.3 and 12.5% acrylamide, respectively. The protease digested samples were analyzed by urea SDS-PAGE gels as described by Burr et al., (1983). The inclusion of 8 M urea in acrylamide gels indicated above, permitted resolution of proteolytic fragments having at least a molecular weight of 4 kd. All chemicals used for electrophoresis were purchased from Biorad Laboratories, Richmond, California, except for molecular weight standards SDS-PAGE and urea SDS-PAGE which were obtained from Sigma Chemical Co., St. Louis, Mo., and BDH Chemicals, Toronto, Ont., respectively.
RESULTS

Examination of Select Cell Cultures for Calmodulin

Culture extracts of primary chick embryo fibroblasts (CEF) and two established cell lines, HEL and Vero were investigated for calmodulin content by phosphodiesterase (PDE) activation and radioimmunoassay. The comparative amounts of calmodulin found in heat-treated cell extracts by enzyme assay are shown in Table 1. Calmodulin activation of PDE shows the specific activity of the Vero cell extract was approximately 2-fold higher than that of the CEF preparation. When extracts were examined by radioimmunoassay, much higher specific activities of calmodulin were detected (data not shown). However, the sensitivity of the enzyme assay was adequate for detecting levels of CaM under the experimental conditions used. Because of these results and the expense of the immunological assay, quantitation of modulator protein in Vero extracts by phosphodiesterase assay was chosen as the test system in subsequent experiments. Figure 1 illustrates dose-response curves by porcine brain CaM and a heat-treated Vero cell extract. Although both preparations activated PDE at comparable rates, the dose response curve produced with the Vero extract leveled off earlier. It is possible that the presence of Vero cell protein contaminants such as membrane phospholipids and heat stable proteins are responsible for the lower level of PDE activation.

11
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Total Protein (mg)</th>
<th>Total CaM (Units)</th>
<th>Specific Activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>** Vero</td>
<td>0.83</td>
<td>154.64</td>
<td>186.31</td>
</tr>
<tr>
<td>** HEL</td>
<td>0.52</td>
<td>78.00</td>
<td>150.00</td>
</tr>
<tr>
<td>*** CEF</td>
<td>4.44</td>
<td>363.40</td>
<td>81.85</td>
</tr>
</tbody>
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* Heat-treated cell extracts prepared from replicate confluent cultures were measured for their ability to activate phosphodiesterase.

** Established cell lines

*** Primary cell line
Fig. 1  Dose-response curves for the activation of calmodulin-dependent bovine heart phosphodiesterase. The phosphodiesterase activity was determined in the presence of varying quantities of porcine brain calmodulin (•—•) and heat-treated extract of uninfected Vero cells (○—○).
Fig. 1

Enzyme Activity $A_{660}^{nm}$

Calmodulin (ug/mL) 0.05 0.10 0.15 0.20

Cell Extract (ug/mL) 5 10 15 20

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Calmodulin Bioactivities During Replication of HSV-1 in Vero Cells

The time course of calmodulin bioactivities in crude extracts of Vero cell cultures infected at high multiplicity with HSV-1 and control uninfected cultures are compared in Table 2. The specific activity of virus-infected extract was reduced by 50% when compared with the uninfected extract at 2 hr postinfection. By extending the time of replication of herpesvirus, calmodulin activities increased until 8 hr when the specific activity was 35% higher than that of control uninfected extract. To establish the time course of HSV-1 replication in Vero cultures, a one-step multiplication experiment was performed. A logarithmic increase in virus yield occurred between 4 and 8 hr postinfection and maximum yield of virus was produced by 12 hr (Fig. 2). The calmodulin bioactivity data and the high yields of infectious virus which was obtained over a 12 hr period, suggest that Vero cell CaM is affected by an early event(s) in the replicative cycle of HSV-1.

Purification of Calmodulin from Virus-infected and Non-infected Vero Cell Culture

Since calmodulin bioactivities differ during the course of virus infection it was of interest to know if the earlier findings reflect (i) qualitative changes in CaM molecules or (ii) the presence of other competitor CaM-binding enzymes as a consequence of virus infection. The purification procedure
Table 2. Activation of Phosphodiesterase by Vero Cell Extracts Exposed to HSV-1 *

<table>
<thead>
<tr>
<th>Time Postinfection (hr)</th>
<th>Specific Activity (U/mg protein)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Uninfected</td>
</tr>
<tr>
<td>0</td>
<td>1229</td>
</tr>
<tr>
<td>2</td>
<td>1318</td>
</tr>
<tr>
<td>4</td>
<td>1409</td>
</tr>
<tr>
<td>8</td>
<td>1453</td>
</tr>
<tr>
<td>12</td>
<td>1274</td>
</tr>
<tr>
<td>16</td>
<td>1248</td>
</tr>
<tr>
<td>24</td>
<td>1074</td>
</tr>
</tbody>
</table>

* Replicate Vero Cultures were infected at multiplicity of 5, overlaid with serum-free solution EMEM and harvested at the times indicated for the preparation of crude cell extracts as described under Materials and Methods. Uninfected Vero cells were treated similarly. Bioactivities of calmodulin were monitored by using calcium-dependent phosphodiesterase.
Fig. 2  Multiplication of HSV-1 in Vero cells. Replicate infected cultures were sampled at indicated times. Infectious virus yields are expressed as tissue culture 50% infective dose (TCID₅₀/mL)
for Vero CaM involved the use of both anion exchange and affinity chromatography of heat-treated cell extracts. The DEAE cellulose column profile of the cell extracts (Fig. 3) demonstrated that four protein peaks were eluted in steps with increasing concentrations of NaCl (0.1 to 0.45 M) contained in Buffer A with 1mM EGTA (pH 7.0). Fractions which significantly activated PDE were eluted beginning at the top of the protein peak obtained with 0.35 M NaCl. Further purification of PDE activator-protein by affinity chromatography on a melittin Affigel-10 column resulted in the recovery of a single peak when monitored at 230 nm (Fig. 4). This peak had high activator activity and was eluted with 5 column volumes using Buffer A containing 1 mM EGTA and 0.6 M KCl (pH 7.5).

The overall purification procedure for Vero cell calmodulin is summarized in Table 3. A substantial increase in specific activity of calmodulin was not evident until the use of melittin affinity chromatography. Concentrated samples of affinity purified PDE activator material were then examined for contaminating protein using SDS polyacrylamide gel electrophoresis (PAGE) in the presence of calcium. Figure 5 compared the electrophoretic migration rates of porcine brain CaM (lane b), and calmodulins from uninfected (lane c) and 8 hr (lane d) and 3hr (lane e) HSV-1 infected Vero cells. All four protein samples had relatively similar electrophoretic mobilities and appear as single bands in the gel except for the doublet obtained with
Fig. 3  DEAE cellulose chromatography of Vero cell calmodulin. Protein obtained from heat treated Vero cell extract was applied to a 1.7 X 10 cm column. Proteins were eluted with Buffer A, pH 7.0 and 1 mM EGTA containing 0.1, 0.25, 0.35 and 0.45 M NaCl concentrations. Three mL fractions were collected and monitored for absorbance at 230 nm. Fractions were screened for phosphodiesterase activity. The bulk of PDE activator protein began to elute at the fraction indicated by the arrow (↓).
Fig. 3

Protein Eluted

\[ A_{230\text{nm}} \]

Fraction No.
Fig. 4. Elution profile of calmodulin by affinity chromatography on melittin Affigel-10. One mL fractions were eluted with Buffer A containing 1 mM EGTA and 0.6 M KCl. Each fraction eluted was tested for PDE activation. PDE activator protein began to elute at the fraction indicated by the arrow (↓).
Table 3. Summary of Purification Scheme for Vero Cell Calmodulin *

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (mg)</th>
<th>Total CaM (Units)</th>
<th>Specific Activity (U/mg Protein)</th>
<th>Purification (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Homogenate</td>
<td>86.88</td>
<td>596</td>
<td>6.86</td>
<td>1.0</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>77.08</td>
<td>604</td>
<td>7.83</td>
<td>1.1</td>
</tr>
<tr>
<td>DEAE Column</td>
<td>13.92</td>
<td>268</td>
<td>19.25</td>
<td>2.8</td>
</tr>
<tr>
<td>Melittin Column</td>
<td>1.77</td>
<td>300</td>
<td>169.50</td>
<td>24.7</td>
</tr>
<tr>
<td>Conc. Sample</td>
<td>0.15</td>
<td>201</td>
<td>1809.00</td>
<td>263.7</td>
</tr>
</tbody>
</table>

* Data from purification of approximately $1.3 \times 10^9$ Vero cells grown in roller bottles (culture area, 850 cm$^2$).
the 3 hr HSV-1 Vero cell calmodulin (Fig. 5, lane e). With soyabean trypsin inhibitor (20.1 kd) and lysozyme (14.3 kd) as markers, it can be seen that all four calmodulin samples migrated to positions approximately between 20.1 and 14.3 kd.

Enzymatic Hydrolysis of Calmodulin

A. Hydrolysis of porcine brain calmodulin

Porcine brain calmodulin as a reference standard was used to establish optimal conditions for limited proteolysis studies. After a number of trials, it was decided to perform enzyme hydrolyses in the presence of calcium at a fixed enzyme:substrate ratio of 1:50. In the absence of enzyme, brain calmodulin eluted from the column just after 8 min (Fig. 6). It was noted that ammonium bicarbonate buffer eluted at about 18 min (Appendix B). Hydrolysis of calmodulin by nagarse (subtilisin BPN') separated 3 fragments eluting at 9.10, 11.26 and 11.95 min (Fig 7 (a)). The profile of thermolysin treated sample (Fig. 7 (b)) demonstrated only 2 fragments with elution times of 9.02 and 11.11 min. Proteinase K digestion (Fig. 7(c)) gave rise to 3 fragments similar in elution time to Fig. 7 (a); the 9.0 min peak appears to predominate in this hydrolysis. When brain calmodulin treated with trypsin (Fig. 8) was chromatographically fractionated, only 1 fragment eluting at 8.87 min was clearly resolved. This finding is in agreement with the report of Walsh et al., (1977).
Fig. 5. SDS polyacrylamide gel analysis of porcine brain and Vero cell calmodulins. Samples in the presence of 0.5 mM CaCl$_2$ were electrophoresed on a 3.3% stacking and 12.5% separating acrylamide slab. The gel was stained with Coomassie brilliant blue. Lanes: (a) molecular weight markers, 10 ug each of soyabean trypsin inhibitor, 20.1 kd and lysozyme, 14.3 kd (b) 10 ug porcine brain CaM (c) 10 ug uninfected Vero cell CaM (d) 10 ug 8 hr HSV-1 infected Vero cell CaM (e) 3 hr HSV-1 infected Vero cell CaM.
Fig. 5

MW x 10^{-3}

20.1 -
14.3 -
Fig. 6. Elution profile of porcine brain calmodulin. A sample of 10 ug porcine brain CaM resuspended in buffer with 5 mM NaCl, 0.5 mM CaCl$_2$, and 20 mM ammonium bicarbonate, pH 7.0 was subjected to HPLC fractionation as described in Materials and Methods. Absorbance was monitored at 230 nm.
Fig. 6

ELUTION TIME min →

A_230 nm →
Fig. 7. Enzymatic hydrolysis of porcine brain CaM. Samples were incubated in 20 mM ammonium bicarbonate, pH 7.0 at an enzyme:substrate ratio of 1:50 in the presence of 0.5 mM CaCl₂ and 5 mM NaCl for 2 hr at 30 °C. HPLC fractionation was monitored at 230 nm. Treatment of brain CaM with (a) nagarse (b) thermolysin (c) proteinase K.
Fig. 8    Enzymatic hydrolysis of porcine brain CaM with trypsin. Experimental conditions were the same as for Fig. 7, except that trypsin was used in place of nagarse, thermolysin or proteinase K.
B. Hydrolysis of uninfected Vero cell calmodulin

Calmodulin derived from the purification of uninfected Vero cell extracts was examined first for the purpose of comparison with the results from porcine brain and virus-infected cellular preparations. Uninfected cell calmodulin in the absence of enzyme eluted after 8.2 min (Fig. 9). A peak eluting at 10.05 min was probably a breakdown product during the purification process. Treatment of Vero cell calmodulin with nagarse (Fig. 10 (a)) indicated that native protein eluted at 8.4 min and the accompanying increase in the size of the 10 min fragment peak substantiated that the presence of the breakdown product observed in Fig. 9 was a proteolytic fragment. Minimal hydrolysis of uninfected cell calmodulin by thermolysin was apparent (Fig. 10 (b)) when compared to the untreated preparation (Fig. 9). This is suggested by the predominance of the native protein peak at 8.31 min elution time. The elution profile generated by the addition of proteinase K to the calmodulin preparation (Fig. 10 (c)) indicates complete hydrolysis of native calmodulin and the appearance of a new fragment eluting at 11.9 min. This new fragment was not observed among the proteinase K fragments of porcine brain calmodulin (Fig. 7).

C. Hydrolysis of 3 hr HSV-1 infected Vero cell calmodulin

Calmodulin from 3 hr HSV-1 infected Vero cell extracts was subjected to hydrolysis by enzymes with different specificities to attempt to detect differences in cleavage
Fig. 9. Elution profile of uninfected Vero cell CaM. A sample of 10 ug Vero cell CaM resuspended in buffer with 5 mM NaCl, 0.5 mM CaCl₂, and 20 mM ammonium bicarbonate, pH 7.0 was subjected to HPLC fractionation as described in Materials and Methods. Absorbance was monitored at 230 nm.
Fig. 9

ELUTION TIME min →

A_{230} \text{ nm} → 

5.67 7.25 8.28 11.16 13.63 17.24
Fig. 10. Enzymatic hydrolysis of uninfected Vero cell CaM. Experimental conditions were the same as for Fig. 7. Absorbance monitored at 230 nm. Treatment of Vero cell CaM with (a) nagarse (b) thermolysin (c) proteinase K.
Fig. 10

ELUTION TIME min →

a.

b.

c.

A₂₃₀ nm →

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fragments from those of uninfected Vero CaM. Such results would indicate that conformational changes in calmodulin occur early in the replicative cycle of HSV-1. In the absence of enzyme (Fig. 11), the chromatographic profile obtained for virus-infected cell calmodulin resembles that of the uninfected preparation although the profile was not fully resolved. When 3 hr HSV-1 infected preparations were treated with nagarse or thermolysin, their elution patterns were similar to that of uninfected Vero calmodulin except for the appearance of a novel fragment eluting at 8.7 min (Fig. 12 (a)(b); Fig. 11). Hydrolysis of the calmodulin preparation with proteinase K (Fig. 12 (c)) yielded essentially the same profile as that obtained with the other two enzymes but the 8.7 min fragment peak was absent.

D. Hydrolysis of 8 hr HSV-1 infected Vero cell calmodulin

Vero cell calmodulin was then examined at a later stage in the replicative cycle of HSV-1, 8 hr postinfection, to ascertain whether any changes took place in protease cleavage patterns as compared to 3 hr HSV-1 infected and uninfected preparations. Examination of the profile generated by the infected sample in the absence of enzyme showed a distinct similarity to that of 3 hr virus-infected sample (Fig. 13) except for the appearance of a novel fragment eluting at 11.57 min. This fragment possibly represents a breakdown product from purification. Hydrolysis of the 8 hr HSV-1 infected preparation with nagarse (Fig. 14
Fig. 11. Elution profile of 3 hr HSV-1 infected Vero cell CaM. Absorbance was monitored at 230 nm. A sample of 10 ug 3 hr HSV-1 infected Vero cell CaM was resuspended in buffer with 5 mM NaCl, 0.5 mM CaCl₂, and 20 mM ammonium bicarbonate, pH 7.0 was subjected to HPLC fractionation as described in Materials and Methods.
Fig. 12. Enzymatic hydrolysis of 3 hr HSV-1 infected Vero cell CaM. Experimental conditions were the same as for Fig. 7. Absorbance monitored at 230 nm. Treatment of virus infected cell CaM with (a) nagarse (b) thermolysin (c) proteinase K.
Fig. 13. Elution profile of 8 hr HSV-1 infected Vero cell CaM. Absorbance was monitored at 230 nm. A sample of 10 ug 8 hr HSV-1 infected Vero cell CaM was resuspended in buffer with 5 mM NaCl, 0.5 mM CaCl2, and 20 mM ammonium bicarbonate, pH 7.0 was subjected to HPLC fractionation as described in Materials and Methods.
Fig. 13

ELUTION TIME min →

A$_{230\text{nm}}$
Fig. 14. Enzymatic hydrolysis of 8 hr HSV-1 infected Vero cell CaM. Experimental conditions were the same as for Fig. 7. Absorbance monitored at 230 nm. Treatment of virus infected cell CaM with (a) nagarse (b) thermolysin (c) proteinase K.
Fig. 14

ELUTION TIME min

A$_{230}$ nm

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(a)) indicated an increase in the proportion of fragment peaks eluting at 10.3, 11.1 and 11.5 min as compared to Fig. 13. Calmodulin preparation hydrolysed with thermolysin generated a chromatographic profile demonstrating the presence of a novel fragment eluting at 8.8 min (Fig. 14 (b)). Fig. 14 (c) illustrated that proteinase K treatment of 8 hr HSV-1 infected cell calmodulin displayed a profile identical to that of Fig. 14 (a).

E. Hydrolysis of 8 hr HSV-2 infected Vero cell calmodulin

Since changes in enzymatic cleavage patterns were occurring at different times in the replicative cycle of HSV-1, it was probable that differences could occur in a closely related herpes virus, HSV-2. For comparison with data derived from HSV-1, Vero cell calmodulin was purified from a 8 hr HSV-2 infected cell extract which represents the late stage in the replicative cycle. As shown in Fig. 15, PDE activatable protein from HSV-2 infected cells in the absence of enzyme eluted at 7.93 min. Whereas the time of appearance of native calmodulin of 3 hr HSV-1 infected-, 8 hr HSV-1 infected-, and uninfected Vero cell extracts from the column ranged between 8.28 and 8.43 min under similar conditions. Hydrolysis of the HSV-2 infected preparation with nagarse (Fig. 16 (a)) demonstrated the separation of 2 fragments, eluting at 10.3 min and 11.1 min. Also it is apparent that the 11.1 min peak appears to be split which may be indicative of an additional fragment peak. Evidence of a
Fig. 15. Elution profile of 8 hr HSV-2 Vero cell CaM. Absorbance was monitored at 230 nm. A sample of 10 ug 8 hr HSV-2 infected Vero cell CaM was resuspended in buffer with 5 mM NaCl, 0.5 mM CaCl₂, and 20 mM ammonium bicarbonate, pH 7.0 was subjected to HPLC fractionation as described in Materials and Methods.
Fig. 15

ELUTION TIME min →

A230 nm

5.58 5.88 7.93 10.86 13.42 17.58
Fig. 16  Enzymatic hydrolysis of 8 hr HSV-2 infected Vero cell CaM. Experimental conditions were the same as for Fig. 7. Absorbance monitored at 230 nm. Treatment of virus infected CaM with (a) nagarse (b) proteinase K
Fig. 16

ELUTION TIME min →

a.

A_{230 \text{ nm}}

b.

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novel fragment eluting at 13.9 min was suggested. When the proteinase K fragments of the preparation were fractionated by HPLC (Fig. 16 (b)), the elution profile was almost identical to that obtained with nagarse. Even the split peak appeared at the fragment eluting at 11.08 min.

Urea SDS–PAGE Analysis of Brain Calmodulin Proteolytic Fragments

Urea SDS–PAGE gels were used to obtain profiles of proteolytic fragments of porcine brain calmodulin for comparison with profiles generated by HPLC analysis. Because of the time, cost and quantities of Vero cell calmodulin required for this technique, limited proteolysis experiments were performed with brain calmodulin from a commercial source.

Untreated calmodulin in the absence and presence of calcium was seen as a single band of protein with a molecular weight greater than 14 kd (Fig. 17, lanes b and f). Proteinase K treatment of calmodulin in the presence of calcium produced hydrolysis fragments having approximate molecular weights of 12 and 7 kd (Fig. 17, lane c). Only one very lightly stained band appeared in the gel when CaM was hydrolysed with nagarse at an enzyme:substrate ratio of 1:50 (lane d). This fragment migrates to a position that corresponds to that of the 12 kd fragment generated by proteinase K. With an enzyme:substrate ratio of 1:200, the gel profile had two distinct bands presumably due to
Fig. 17  Urea SDS-PAGE analysis of porcine brain CaM hydrolysed by different proteases. Reaction mixtures had enzyme:substrate ratios of 1:50 and hydrolysis was carried out in the absence and presence of 0.5 mM CaCl₂ as described under Materials and Methods. To separate fragments, samples were subjected to electrophoresis on a 3.3% stacking and 12.5% running acrylamide/8M urea slab gel. Lanes: (a) molecular weight markers (myoglobin (16.9 kd), Myoglobin I & II (14.4 kd), myoglobin I (8.1 kd), myoglobin II (6.2 kd), myoglobin III (2.5 kd), (b) control CaM with CaCl₂, (c) proteinase K with CaCl₂, (d) nagarse with CaCl₂, (e) thermolysin with CaCl₂, (f) control CaM with EGTA (g) proteinase K with EGTA, (h) nagarse with EGTA, and (i) thermolysin with EGTA.
incomplete hydrolysis by the nagarse concentration used (data not shown). The profile of hydrolysis fragments of calmodulin obtained with thermolysin treatment differed from profiles produced with nagarse and proteinase K. In the presence of calcium, incomplete hydrolysis of CaM and a novel fragment with an approximate molecular weight of 9 kd were noted (Fig. 17, lane e).

Calmodulin samples were hydrolysed with the same test enzymes in the presence of EGTA to investigate whether additional cleavage sites in the modulator molecule become available for attack by proteases. Both proteinase K and nagarse hydrolysed calmodulin into smaller undetectable components as evidenced by the absence of protein bands in the gel system (Fig. 17, lanes g and h). Although the majority of the calmodulin remained unhydrolysed when treated with thermolysin, the 9 kd hydrolysis fragment was detected irrespective of the absence or presence of calcium during enzyme hydrolysis (Fig. 17, lanes i and e).

The results indicate that limited proteolysis of CaM by select endolytic serine or metalloproteases in the presence of calcium may yield peptide fragments which are measurable by HPLC.
DISCUSSION

The data presented appears to be the first report of the effects of productive virus infection on the structure of calmodulin. An apparent reduction in calmodulin bioactivity at 2 hr postinfection was observed. This early reduction during virus replication may be due to limited turnover of protein although recovery of protein synthesis occurred by 4 hr postinfection. Also, the findings correlate well with a report by Hill et al., (1983) that HSV-1 induced shutdown of host cell protein synthesis was not detectable in Friend erythroleukemia cells until 2 hr postinfection. This reduction in calmodulin activity also appears to coincide with peak synthesis of HSV-1 alpha-type polypeptides (Honess et al., 1974). Thus, synthesis of calmodulin could have been curtailed while the virus initiated its own direction of Vero cell macromolecular synthesis. Conversely, the increase in the bioactivities of calmodulin after 8 hr postinfection coincides with two major events in HSV-1 polypeptide synthesis. The first is the production of beta polypeptides that represent the virus encoded enzymes such as thymidine kinase, DNA polymerase, and ribonucleotide reductase. Secondly, the infection of cells with HSV-1 for 8 hr is about the time at which the synthesis and posttranslational modification of the structural gamma-type polypeptides (Honess et al., 1974) occurs. It should be noted that many of these proteins are
phosphorylated by HSV-1 associated protein kinase (Lemaster et al., 1980, Rubenstein et al., 1972). An early report by Epstein et al., (1963), noted the presence of herpesvirus-associated ATPase activity. Therefore, the increase in calmodulin levels after 8 hr postinfection might represent a possible role of calmodulin in modulating the activity of virus-encoded protein kinase and ATPase during the synthesis and modification HSV-1 capsid (structural) proteins.

Based on the reports of Cox et al., (1985) and Kincaid and Coulson., (1985), high affinity binding between calmodulin and melittin can occur. When melittin is immobilized to Affigel-10, the hydrophobic interaction between melittin and CaM allows the formation of tight complexes in the presence of calcium. Using this system, purification was achieved for Vero cell CaM preparations.

The subsequent purification of Vero cells infected at 3 and 8 hr postinfection was hampered by a number of problems. For example, yields were poorer in virus-infected cultures than the uninfected cultures. This, however, may have been simply because lytic infection by HSV-1 could release the cytoplasmic content of cells into the tissue culture fluid. Heat treatment of Vero cell extracts appeared to reduce yields (unpublished data), however, this technique appears to facilitate the removal of extraneous protein and inactivates calmodulin binding proteins (Klee et al., 1982). SDS-PAGE analysis of purified virus-infected and
uninfected extracts indicated relative homogeneity by the presence of 1 band of native protein > 14 K M.W..

Enzymatic hydrolysis of calmodulin has been utilized by a number of groups for the purpose of structural studies (Manalan et al., 1984). Apparently, a majority of these studies focused on bovine brain calmodulin treated with trypsin (Drabikowski et al., 1977; Walsh et al., 1977; Newton et al., 1984). In studying the tryptic digestion of calmodulin by urea SDS-PAGE analysis, both Walsh et al., (1977) and Drabikowski et al., (1977) reported that hydrolysis of CaM proceeded much faster in the absence of calcium. Their data, however, differ in the number of hydrolysis fragments observed at an early stage of digestion when low trypsin to substrate (1:500) was used. This result would indicate that there is difficulty in comparing enzymatic hydrolyses of calmodulin between other laboratories because of different sources of enzyme and methodologies available for isolating the protein modulator. Other studies on limited proteolysis of brain calmodulin indicate that the sensitive HPLC technique can be conveniently used in separating hydrolysis fragments (Klee et al., Newton et al., 1984).

During the standardization of conditions for hydrolysing porcine brain calmodulin at a fixed enzyme to substrate ratio of 1:50 and the analysis of fragments by HPLC, it was found that calcium in the digestion mixture was essential to obtain cleavage fragments with nagarse and proteinase K.
Treatment of porcine brain calmodulin with nagarse, thermolysin, and proteinase K (Fig. 7) displayed one feature in common, a fragment peak eluting after 9.0 min. Comparison of hydrolyses performed on uninfected Vero calmodulin showed the absence of the 9.0 min peak (Fig. 9), thus an enzymatic cleavage site accessible in brain calmodulin was not available in Vero calmodulin. Nagarse hydrolysis of brain calmodulin demonstrated a fragment peak eluting at 11.9 min which was absent in the Vero cell preparation, indicating the loss of a second cleavage site (Fig. 10(a)). It should be noted that while Vero calmodulin appears to be resistant to nagarse and thermolysin hydrolysis, it was quite susceptible to proteinase K digestion.

When profiles of uninfected Vero cell (Fig. 10) and 3 hr HSV-1-infected Vero cell calmodulin treated with nagarse, thermolysin and proteinase K (Fig. 12) were compared, the apparent resistance of uninfected cell calmodulin with nagarse and thermolysin was not evident in the virus-infected preparation. However, an apparently novel fragment eluted at 8.7 min (Fig. 12 (a)(b)), suggesting the presence of calmodulin with a small terminal fragment removed enzymatically.

The profiles visualized by treatment of 8 hr HSV-1 infected Vero cell calmodulin with nagarse, thermolysin, and proteinase K were essentially the same as the untreated control except for two major differences (Fig. 14). The first being the absence of a 8.7 min fragment peak as in the 3 hr
HSV-1 infected Vero calmodulin and the second being an appreciable increase in the proportion of a fragment eluting at approximately 14 min. This fragment may represent the terminally cleaved peptide from the 8.7 min peak. The same fragment may be represented by a peak eluted at 13.6 min in the protease treated 3 hr HSV-1 calmodulin sample (Fig. 12). Another important distinction between 8 hr HSV-1 infected cell calmodulin and other preparations is the appearance of a second breakdown product eluted at 11.5 min. The identity of the peak as a fragment was substantiated by its increased magnitude following protease treatment. The presence of this second fragment in addition to the first breakdown product for the other preparations may represent the instability of calmodulin conformation in Vero cells infected with HSV-1 until late in the replicative cycle. Thus, a new enzymatic cleavage site may have been exposed because of progressive structural alteration in calmodulin.

Comparison of 8 hr HSV-2 infected Vero cell calmodulin treated with nagarse and proteinase K (Fig. 16) demonstrated the absence of the second breakdown product in the 8 hr HSV-1 infected preparation (Fig. 14(a)(c)). This indicates that the apparent instability of calmodulin in 8 hr HSV-1 infected cells may be virus-specific and not a function of time of infection. Treatment of the 8 hr HSV-1 infected calmodulin preparation with proteinase K resulted in the apparent absence of the peak eluting at 14 min, indicating the loss of a potential cleavage site.
In summary, the appearance and disappearance of proteolytic fragments in these preparations leads one to a number of inferences. First of all, there are drastic changes in the conformation between uninfected and virus-infected preparations of Vero cell calmodulin as evidenced by the absence of the 9.0 min fragment peak in the Vero cell preparations. This conclusion is also supported by the study of Yerna et al., (1979) in the characterization of calmodulin from baby hamster kidney cells. Yerna's report indicated that significant differences exist in the amino acid composition of BHK-21 cell calmodulin and bovine brain calmodulin. Differences between uninfected and 3 hr HSV-1 infected Vero cell calmodulin was provided by the finding of a novel fragment eluting after 8.7 min using either nagarse or thermolysin hydrolysis. Further conformational changes were indicated between 3 hr and 8 hr HSV-1 infected CaM preparations. This conclusion is based on the absence of a 8.7 min fragment peak in the 8 hr sample along with the appearance of a novel fragment at 14 min. Moreover, conformational changes in calmodulin derived from 2 different herpesviruses, HSV-1 and HSV-2 are plausible by the absence of the 11.5 min fragment peak of 8 hr virus-infected samples. Thus, one may conclude that limited proteolysis of uninfected and virus-infected preparations of Vero cell calmodulin as shown by specific enzyme cleavage patterns in the presence of calcium is a useful tool for determining whether structural differences in the protein...
are occurring. Therefore, preliminary studies can be performed before more extensive sequencing and spectroscopic analyses are required.
Lack of detection of proteases used in enzyme hydrolysis experiments. Approximately 10 ug of (a) nagarse (b) thermolysin and (c) proteinase K were suspended in 50 uL of 20 mM (NH₄)₂HCO₃ (elutes at about 18 min).
APPENDIX B

Profiles of Additional Components in the Enzyme Reaction Mixture and Molecular Weight Standards

a.

b.

c.

Samples include (a) 50 uL of 20 mM (NH₄)₂HCO₃ buffer (b) 10 uL of 10 mM EGTA and 40 uL of 20 mM (NH₄)₂HCO₃ and (c) molecular weight standards consisting of (i) 20 ug of beta-lactoglobulin (M.W. 18.4 kd), (ii) 20 ug of alpha-lactalbumin (M.W. 14.2 kd) and (iii) 20 ug of ribonuclease A (M.W. 13.7 kd).
REFERENCES


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