INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700  800/521-0600
MULTILINEAR ANALYSIS IN FLUORESCENCE & ION TRANSPORT BY IONOPHORES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By
Exing Wang, B.S., M.S.

The Ohio State University
1997

Dissertation Committee:
Professor Ruth A. Altschuld
Professor Elizabeth Gross
Professor Douglas R. Pfeiffer, Adviser

Approved by
Douglas R. Pfeiffer
Adviser
Biophysics Program
ABSTRACT

The distance between the location of the calmodulin-binding region on calmodulin kinase II relative to its nucleotide binding site has been determined to be ~ 50 Å using fluorescence resonance energy transfer method combined with multilinear analysis, a method that allows mathematical separation of spectra. The donor of the energy transfer pair was a nucleotide analog lin-benzoADP, whereas the acceptor was 7-diethyamino-3-(4'maleimidylphenyl)-4-methylcoumarin (CPM), which was labeled to the synthetic calmodulin. In addition, Ca^{2+} binding to calmodulin has been studied using fluorescence of CPM. The Hill coefficient was determined to be ~2-3, indicating Ca^{2+} binding to calmodulin is in a cooperative manner. The k_{1/2} of Ca^{2+} was ~ 2 \times 10^{-7} M.

Large unilamellar vesicles have been used as the model membrane system in the investigation of ion transport properties by ionophores. The phospholipid bilayer which defines the system is composed of 1-palmitoyl-2-oleoyl-sn-glycerophosphatidylcholine (POPC). Vesicles were loaded with Quin-2 or cations, as desired. Transport activities were monitored by difference absorbance measurements using an Aminco DW2a spectrophotometer operated in the dual wavelength mode. The results of lanthanide cations (Ln^{3+}) transport
by A23187, 4-BrA23187, and ionomycin indicate that the rate of transport is inversely dependent on the cation concentration. Accordingly, at low Ln³⁺ concentrations, the rates of Ln³⁺ and Ca²⁺ are comparable. Therefore, unrecognized Ln³⁺ transport could lead to misinterpretation of results when Ln³⁺ and the Ca²⁺ ionophores are used together to investigate cell regulation by Ca²⁺. A single transporting species was found for A23187 and 4-BrA23187, with a stoichiometry of 2:1 (ionophore: Ln³⁺) within the Ln³⁺ concentration range of 15-250 μM. Ionomycin can transport via two species, 2:1 and 1:1 stoichiometry, at low La³⁺ concentration, and via the 1:1 predominate species at high La³⁺ concentration. Further, co-transport of OH⁻ by all three ionophores has been found as a result of the net exchange of 3H⁺ : 1La³⁺. The faster transport seen when a membrane potential is established, especially for 4-BrA23187, could reflect an electrogenic mode of transport.

The neutral ionophore ETH 129 was found to transport Ca²⁺ electrogenically via a 3:1 (ETH 129 : Ca²⁺) complex. The logarithm of the initial rate has a linear relationship to the trans-membrane potential. The effect of the extravesicular Ca²⁺ concentration on Ca²⁺ transport by ETH 129 and the transport selectivity of the ionophore for divalent cations have also been investigated.
Dedicated to my parents
ACKNOWLEDGMENTS

I wish to thank my adviser, Dr. Douglas Pfeiffer, for his guidance and support and also his patience which made this thesis possible. His wide range of knowledge and willingness to teach have helped me overcome many difficult times.

My deep gratitude also goes to Dr. Elizabeth Gross for her long time support and encouragement.

I thank Dr. Robert Ross for his guidance and support when I was his student.

I am grateful to Jinkeun for his friendship and help.

I also wish to give my thanks to Erika for her collaboration in the energy transfer project.

My fellow group members, Warren, Kim, Cliff, Monica, Dennis, Pat and Ron have provided me with consistent support and a very friendly work environment. I cannot thank them enough for their generosity and friendship.

Finally, I offer my deepest appreciation to my wife, Yi, who has given me tremendous support and encouragement, and who has also shared frustration with me throughout these years.
VITA

October 3, 1962 .............. Born - Wuhan, China

1979-1983 .......... B.S. Beijing Normal University

1983-1987 .............. Teaching Assistant, Tongji University, Shanghai, China

1987-1990 .............. M.S. Beijing Agricultural University

1990-1991 .............. Researcher, Institute of Teaching Instrument, Beijing

1991-present ............ Graduate Teaching and Research Associate, The Ohio State University

PUBLICATIONS


FIELD OF STUDY

Major Field: Biophysics
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</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>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Introduction to CaM kinase II structural study.</td>
<td>1</td>
</tr>
<tr>
<td>CaM kinase II</td>
<td>1</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>4</td>
</tr>
<tr>
<td>2. Methods and materials</td>
<td>7</td>
</tr>
<tr>
<td>Monomeric form of α subunit of CaM kinase II</td>
<td>7</td>
</tr>
<tr>
<td>CPM-labeled calmodulin</td>
<td>8</td>
</tr>
<tr>
<td>lin-benzoADP</td>
<td>10</td>
</tr>
<tr>
<td>Data collection</td>
<td>10</td>
</tr>
<tr>
<td>Multilinear analysis</td>
<td>12</td>
</tr>
<tr>
<td>Fluorescence polarization</td>
<td>14</td>
</tr>
<tr>
<td>Energy transfer theory</td>
<td>15</td>
</tr>
<tr>
<td>3. Results</td>
<td>17</td>
</tr>
<tr>
<td>The effect of Ca(^{2+}) binding on CaM-CPM</td>
<td>17</td>
</tr>
<tr>
<td>The ternary complex: CPM-CaM : α315 : lin-benzoADP</td>
<td>22</td>
</tr>
<tr>
<td>Determination of the distance between CaM-CPM and lin-benzoADP on α315</td>
<td>23</td>
</tr>
<tr>
<td>4. Discussion</td>
<td>33</td>
</tr>
<tr>
<td>Cooperativity of Ca(^{2+}) binding to CaM</td>
<td>33</td>
</tr>
<tr>
<td>After thought for the distance calibration</td>
<td>35</td>
</tr>
<tr>
<td>Section</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td></td>
</tr>
<tr>
<td><strong>Introduction to Ion transport by Ionophores</strong></td>
<td>36</td>
</tr>
<tr>
<td>Model membrane system</td>
<td>36</td>
</tr>
<tr>
<td>Cation transport by ionophores</td>
<td>40</td>
</tr>
<tr>
<td><strong>Materials and Methods</strong></td>
<td>51</td>
</tr>
<tr>
<td>Reagents</td>
<td>51</td>
</tr>
<tr>
<td>Preparation of phospholipid vesicles</td>
<td>52</td>
</tr>
<tr>
<td>The determination of cation transport</td>
<td>54</td>
</tr>
<tr>
<td>The determination of H⁺:La³⁺ exchange ratio</td>
<td>59</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>60</td>
</tr>
<tr>
<td><strong>Results of Lanthanide Ion transport</strong></td>
<td>62</td>
</tr>
<tr>
<td>Comparative aspects of lanthanide cation transport by Ca²⁺ ionophores</td>
<td>62</td>
</tr>
<tr>
<td>Stoichiometry of the transporting species</td>
<td>66</td>
</tr>
<tr>
<td>La³⁺ release from La³⁺-loaded vesicles and the transport mode</td>
<td>70</td>
</tr>
<tr>
<td>H⁺:La³⁺ exchange ratio</td>
<td>72</td>
</tr>
<tr>
<td>Influence of membrane potential on La³⁺ transport</td>
<td>74</td>
</tr>
<tr>
<td>Comparison of La³⁺ and Lu³⁺</td>
<td>77</td>
</tr>
<tr>
<td><strong>Discussion and Conclusion</strong></td>
<td>82</td>
</tr>
<tr>
<td>The mechanism of lanthanide transport</td>
<td>82</td>
</tr>
<tr>
<td>The selectivity of A23187</td>
<td>84</td>
</tr>
<tr>
<td>Conclusion</td>
<td>84</td>
</tr>
<tr>
<td><strong>Ca²⁺ transport by ETH 129</strong></td>
<td>86</td>
</tr>
<tr>
<td>The effect of trans-membrane potential</td>
<td>86</td>
</tr>
<tr>
<td>Stoichiometry of the transporting species and the effect of external Ca²⁺ concentration</td>
<td>89</td>
</tr>
<tr>
<td>Selectivity and pH effect</td>
<td>92</td>
</tr>
<tr>
<td>Discussion</td>
<td>92</td>
</tr>
<tr>
<td>Conclusion</td>
<td>95</td>
</tr>
</tbody>
</table>

**Bibliography** | 97 |
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Energy transfer from lin-benzoADP to CaM26-CPM</td>
</tr>
<tr>
<td>2</td>
<td>Energy transfer from lin-benzoADP to CaM75-CPM</td>
</tr>
<tr>
<td>3</td>
<td>Energy transfer from lin-benzoADP to CaM115-CPM</td>
</tr>
<tr>
<td>4</td>
<td>Calculated values for distance measurements</td>
</tr>
<tr>
<td>5</td>
<td>Cooperativity of Ca(^{2+}) binding to CaM</td>
</tr>
<tr>
<td>6</td>
<td>Physical properties of POPC vesicles</td>
</tr>
<tr>
<td>7</td>
<td>Relative selectivities of Ca(^{2+}) ionophores</td>
</tr>
<tr>
<td>8</td>
<td>Apparent stoichiometry of the transporting species</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Domain structure of a CaM kinase II subunit</td>
</tr>
<tr>
<td>2</td>
<td>Structure of calmodulin from drosophila melanogaster</td>
</tr>
<tr>
<td>3</td>
<td>Amino acid sequence of the synthetic calmodulin UV1</td>
</tr>
<tr>
<td>4</td>
<td>Structures of CPM and lin-benzoADP</td>
</tr>
<tr>
<td>5</td>
<td>The effect of Ca(^{2+}) binding on CaM26-CPM fluorescence</td>
</tr>
<tr>
<td>6</td>
<td>The effect of Ca(^{2+}) binding on CaM75-CPM fluorescence</td>
</tr>
<tr>
<td>7</td>
<td>The effect of Ca(^{2+}) binding on CaM115-CPM fluorescence</td>
</tr>
<tr>
<td>8</td>
<td>The resolved spectra of lin-benzoADP and CaM26-CPM</td>
</tr>
<tr>
<td>9</td>
<td>The resolved spectra of lin-benzoADP and CaM75-CPM</td>
</tr>
<tr>
<td>10</td>
<td>The resolved spectra of lin-benzoADP and CaM115-CPM</td>
</tr>
<tr>
<td>11</td>
<td>Cooperativity of Ca(^{2+}) binding to CaM</td>
</tr>
<tr>
<td>12</td>
<td>Model membrane system</td>
</tr>
<tr>
<td>13</td>
<td>Structures of ionophores</td>
</tr>
<tr>
<td>14</td>
<td>Structures of ionophore-Ca(^{2+}) complexes</td>
</tr>
<tr>
<td>15</td>
<td>The sequential component reactions in the classical neutral</td>
</tr>
<tr>
<td></td>
<td>transport mode for A23187 and ionomycin</td>
</tr>
<tr>
<td>16</td>
<td>Spectra of Quin-La complex</td>
</tr>
<tr>
<td>17</td>
<td>Calibration of the vesicle transport system</td>
</tr>
</tbody>
</table>
18 Selectivity of Ca^{2+} ionophores on lanthanide ions 63
19 The effect of the external La^{3+} concentration on the initial rate of transport 65
20 Relationship between the initial rate of La^{3+} uptake and ionophore concentration 67
21 La^{3+} released by Ca^{2+} ionophores from LaCl$_3$ loaded vesicles 71
22 H$^+$ and La$^{3+}$ exchange ratio 73
23 The influence of membrane potential on La$^{3+}$ transport rate 75
24 The effect of membrane potential on H$^+$ and La$^{3+}$ exchange 79
25 Fluorescence titration of A23187 with La$^{3+}$ and Lu$^{3+}$ 80
26 Fractional fluorescence change as a function of La$^{3+}$ 81
27 The effect of membrane potential on Ca$^{2+}$ transport 88
28 Stoichiometry of the transporting species 90
29 The effect of Ca$^{2+}$ concentration on the initial rate of transport 91
30 Selectivity of ETH 129 93
31 pH insensibility of the transport 94
32 The effect of uncatalyzed diffusion 96
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α315</td>
<td>the truncated form of calmodulin kinase II α subunit (amino acids 1-315)</td>
</tr>
<tr>
<td>BCECF</td>
<td>2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CCP</td>
<td>carbonyl cyanide m - chlorophenylhydrazone</td>
</tr>
<tr>
<td>CPM</td>
<td>7-diethyamino-3-(4'maleimidylphenyl)-4- methylcoumarin</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>Mes</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycerophosphatidyl-choline</td>
</tr>
<tr>
<td>TPP⁺</td>
<td>tetraphenylphosphonium cation</td>
</tr>
<tr>
<td>VAL</td>
<td>valinomycin</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction to CaM Kinase II Structural Study

The goal of this project was to determine the location of the calmodulin (CaM)-binding region on CaM kinase II relative to its nucleotide binding site using fluorescence resonance energy transfer method combined with multilinear analysis, a method that allows mathematical separation of spectra. This was a joint project between the laboratories of Drs. Marita King (Department of Chemistry) and Robert Ross (Department of Biochemistry). Chemical reagents and biochemical samples were prepared by Dr. Erika Takeuchi-Suzuki from Dr. King's group, whereas as a member of Dr. Ross's research group, I was responsible for data collection and most data analysis tasks.

CaM kinase II

CaM kinase II, also known as multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase, calmodulin-dependent multiprotein kinase, or type II CaM kinase, is widespread in many tissues and phosphorylates a large diversity of proteins in response to Ca\(^{2+}\) signals (Hanson & Schulman, 1992). Although CaM-kinase II can be found in most tissues, it is particularly abundant in neural tissues, constituting one percent of the total brain proteins. Brain CaM-kinase II is an
oligomeric enzyme of 650 kDa, containing α (50-54 kDa) and β (58-60 kDa) subunits in a ratio of 3-4:1 (Hanson & Schulman, 1992). Typically, a holoenzyme consists of 10-12 subunits, which are arranged like flower petals (Soderling, 1996; Kanaseki et al. 1991; Hanson & Schulman, 1992). Like other members in the CaM kinase family, CaM kinase II has a CaM-binding and an autoinhibitory domain (AID). One-to-one stoichiometry was seen when using polarized fluorescence to directly measure calmodulin binding to CaM kinase II subunits (Hanson & Schulman, 1992). However, what makes this kinase unique is its autophosphorylation feature.

Fig. 1 illustrates the domain organization of a CaM kinase II subunit (Schulman, 1993; Soderling, 1996). The catalytic domain, which is most homologous to other CaM protein kinases, spans from the N-terminal to the vicinity of residue 270. The adjacent regulatory domain, covering approximately residues 275 to 314, contains the autoinhibitory and CaM-binding sequences and the regulatory autophosphorylation sites. The CaM-binding domain is basic and hydrophobic. The core calmodulin-binding domain is composed of residues 296-309 of the α subunit (Hanson & Schulman, 1992). The remaining C-terminal is an oligomerization domain which is responsible for subunit association. The greatest diversity among the isoformic subunits is seen in this region. A holoenzyme will not be formed without the association domain.

CaM kinase II has attracted attention in neurophysiology due to its abundance in the brain and its involvement in spacial memory (Silva et al. 1992)
Fig. 1  Domain structure of a CaM kinase II subunit.
and long term potentiation, (Stanton & Gage, 1996; Brown et al. 1988). Up to forty proteins have been reported as substrates of this protein kinase. As a result of its having many substrates, CaM kinase II is sometimes referred to as multifunctional. Autophosphorylation, a prominent characteristic of CaM kinase II, is initiated upon binding of calmodulin, which in turn, is activated by Ca^{2+}. Thr286 on the α subunit and Thr287 on the β subunit are identified as the critical sites for autophosphorylation, although additional sites have been found to be involved. The process of autophosphorylation is considered to be an intramolecular reaction within holoenzymes because the rate of the reaction is independent of the kinase concentration. After autophosphorylation the kinase is able to phosphorylate its substrates in a Ca^{2+}-independent manner (Hanson & Schulman, 1992).

**Calmodulin**

Calmodulin is a ubiquitous calcium-dependent protein of 148 residues (M, 16,700) that is involved in regulating the activity of a wide variety of enzymes and other proteins. Calmodulin, found in all eukaryotic cells, binds one Ca^{2+} at each of its four EF-hand calcium-binding sites, for a total of four Ca^{2+} per protein. The crystal structure of calmodulin (Fig. 2) shows that this protein has a dumbbell shape consisting of a long central helix and two globular homologous domains, each containing two helix-loop-helix calcium-binding motifs. There are two hydrophobic patches, one in each domain, which presumably form the binding site for the target protein and for the interaction with calmodulin.
antagonist drugs which inhibit calmodulin-target protein interaction. The dimensions of the molecule are approximately $65 \times 30 \times 30\ \AA^3$. The unusually high $\alpha$-helix content constitutes approximately 63% of the whole structure (Babu et al. 1985). The four calcium-binding sites are named from N to C terminal, i.e. sites I and II are in the amino terminal, sites III and IV are in the carboxyl terminal. Each of these sites contains 12 amino acid residues: 20-31, 56-67, 93-104, 129-140; the first 9 form the loop and the last three begin another helix in the helix-loop-helix motif (Babu et al. 1985). The Ca$^{2+}$ ion at each of the four EF-hand binding sites is coordinated to seven oxygen ligands from six of the 12 amino acids. Sites III and IV have a higher binding affinity for calcium compared to sites I and II. Conformational changes, which include exposure of hydrophobic sites, are induced by calcium binding. These are necessary for interaction of calmodulin with its target proteins or peptides.
Fig. 2  Structure of calmodulin from drosophila melanogaster (down loaded from Brookhaven Protein Data Bank).
CHAPTER 2

METHODS AND MATERIALS

Monomeric form of α subunit of CaM kinase II

As described in the previous Chapter (illustrated by Fig. 1) the association sequence for oligomerization on CaM kinase II is at the C-terminal, starting from the vicinity of residue 310 on an α subunit. Potential interference from the adjacent subunits in an oligomer would make it difficult to study the relationship between the calmodulin binding site and the nucleotide binding site on an individual subunit. Thus, in order to avoid oligomerization, a truncated form of CaM kinase II α subunit, α315 (corresponding to amino acid 1 through 315) was expressed and purified using method described by Takeuchi-Suzuki (1993). The purified α315 was determined to be homogeneous since it migrated as a single band on SDS-polyacrylamide gels by both protein-staining with Coomassie blue and immunoblotting Ca²⁺/CaM-dependent kinase activity (Takeuchi-Suzuki, 1993). The average molecular weight of the protein obtained by SDS-PAGE and size exclusion chromatography is 32 kDa, which is close to the value predicted by amino acid sequence analysis, indicating that the protein was in monomeric form.
Nonphosphorylated α315 was activated by calmodulin, exhibiting a $K_{1/2}$ for calmodulin of 120 nM, similar to the value obtained for a holoenzyme. The Michaelis-Menten constant for ATP of the fully activated kinase was also similar to the previously reported values. These similarities, together with other characteristics that are shared by α315 and the holoenzyme (Takeuchi-Suzuki, 1993), suggest that α315 is a good model for the holoenzyme.

**CPM-labeled Calmodulin**

A synthetic calmodulin (VU1) containing a consensus sequence of several known CaM was chosen for use in this work. The sequence (shown in Fig. 3) was based on higher plant calmodulins. Since calmodulin is highly conserved, the resulting protein still exhibits extensive homology to mammalian calmodulin. Furthermore, VU1 is capable of activating rat brain CaM kinase II effectively (Takeuchi-Suzuki, 1993). Mutations at selected sites (one at a time at residues 26, 75, 115) on calmodulin do not lower calmodulin's ability to regulate its target proteins (Kwiatkowski, 1988; Mills et al, 1988). As indicated in Fig. 3, residues 26, 75 and 115 are located respectively at Ca$^{2+}$-binding site I, the central helix, and the helix connecting Ca$^{2+}$-binding sites III and IV. In order to attach a fluorescent label [CPM (7-diethylamino-3-(4'maleimidylphenyl)-4-methycoumarin) from Molecular Probes] to a selected site, the original amino acid at that position was first replaced by a cysteine residue in the new sequence. The structure of CPM is shown in Fig. 4a. This fluorescent molecule has a quantum yield of 0.44. Its excitation and emission peaks appear at 387
Fig. 3 Amino acid sequence of the synthetic Calmodulin UV1. The residues underlined are in the Ca\textsuperscript{2+}-binding loops. The sites are numbered I through IV starting from the N-terminal.
and 485 nm respectively. Detailed information about VU1 mutagenesis, purification and modification with CPM was described by Takeuchi-Suzuki (1993).

**Lin-benzoADP**

The fluorescent nucleotide analog lin-benzoADP (structure shown in Fig. 4b) was obtained from Dr. Nelson Leonard of University of Illinois, Urbana. This molecule has been used successfully in the study of nucleotide binding to protein kinases because of its relatively high affinity to these enzymes (Cheng et al. 1997; Hartl et al. 1983). Throughout this work, lin-benzoADP was used in the presence of Mg$^{2+}$ to stabilize α315 and enhance the affinity of the nucleotide analog for the kinase. In addition, divalent cations significantly change the fluorescence spectrum of the probe. Such an effect is minimized by using a constant level of Mg$^{2+}$. The excitation and emission peaks of lin-benzoADP are at 330 and 370 respectively.

The significant overlap between the excitation spectrum of lin-benzoADP and the emission spectrum of CPM (Fig. 8-10) makes them a good donor and acceptor pair for resonance energy transfer.

**Data Collection**

All fluorescence measurements were made on an SLM 500C fluorometer along with a SPF-500C accessory for polarized light when applicable. All data were collected at 8 °C unless otherwise stated. The data collection process was controlled by a personal computer through a C program written by Dr. Bilal M.
Fig. 4 Structures of A) CPM and B) lin-benzoADP
Ezzeddine. The temperature was controlled by the same computer via a semiconductor cooling/heating device. The collected data were uploaded via the internet to both the University main frame computer system and the Ohio Super Computer Center, where the raw data were processed using the multilinear analysis program written by Dr. Robert Ross, Biochemistry Department, Ohio State University. The results of the calculations from the program were then downloaded back to the lab PCs.

**Multilinear Analysis**

When spectroscopic methods are used in investigating biological systems, mathematical decomposition of spectra from different chromophores becomes necessary since physical separation of each individual chromophore, without altering some important features of the system being studied, is not only difficult but impossible in many cases. By applying a multilinear Model (Kruskal, 1983; Leurgans S. & Ross, 1992; Ross et al. 1985), one of many mathematical decomposition methods, a mixed spectra can be resolved without prior information about the properties of the components (Ross & Leurgans, 1995). Multilinear analysis methods have been applied successfully in resolving different properties of tryptophans in a protein, in chlorophyll-protein complexes in thylakoids (Ross et al. 1991), and in the study of specific and nonspecific interactions between tyrosine and its environment (Lee et al. 1992).

Given a dilute specimen containing multiple fluorophores, the intensity (in steady state measurement) can be written as (Ross & Leurgans, 1995):
\[
\mu[i, j, k] = \sum_{f=1}^{F} \varepsilon_f[i] \pi_f[j] c_f[k]
\]  
(1)

where \( F \) is the number of fluorophores, \( \varepsilon_f[i] \) is the extinction coefficient of fluorophore \( f \) at wavelength \( \lambda[i] \), \( \pi_f[j] \) is the relative emission of the fluorophore at wavelength \( \lambda[j] \), and \( c_f[k] \) is the concentration of fluorophore \( f \) in circumstance \( k \).

In cases when the relative concentrations of individual fluorophores are fixed an additional independent variable is needed, and a quencher will usually be used. Eq. (1) can be modified as:

\[
\mu[i, j, k] = \sum_{f=1}^{F} \varepsilon_f[i] \pi_f[j] c_f[k] \phi_f[i]
\]  
(2)

where \( \phi_f[i] \) is the quantum yield of fluorophore \( f \) in correspondence to the treatment \( k \).

In a case where the energy transfer occurs between fluorophores \( f_1 \) (donor) and \( f_2 \) (acceptor) with probability \( p_{f_1, f_2} \), the intensity can be expressed as:

\[
\mu[i, j, k] = \sum_{f=1}^{F} \varepsilon_f[i] c_{f_1}[i] \pi_{f_1}[j] p_{f_1, f_2} \pi_{f_2}[k]
\]  
(3)

The experimental data \( y_n \) are fit to the above models (Eqs. 1-3) by weighted least-squares, minimizing the \( r \) value in the following function (Ross & Leurgans, 1995):

\[
r = \sum_{n=1}^{N} w_n (y_n - \mu_n)^2
\]  
(4)
where \( w_a \) are the weights.

**Fluorescence Polarization**

The fluorescence polarization method is often used in determination of ligand binding to proteins because of the differences in the depolarization properties of the free and bound ligand. Depolarization is usually caused by rotational diffusion of fluorophores. Given a linear polarized excitation, the corresponding emission will have an angular displacement (average of all the fluorophore molecules), which is determined by the rate and extent of rotational diffusion during the lifetime of the fluorophore.

Fluorescence polarization is usually measured by polarization (P) or anisotropy (A), which are given below:

\[
P = \frac{I_{x-x} - I_x}{I_x + I_y}
\]

\[
A = \frac{I_{x-x} - I_y}{I_x + 2I_y}
\]

where \( I_x \) is the measured fluorescence intensity when the observing polarizer is parallel to the excitation polarizer, and \( I_y \) is the intensity when the orientation of the two polarizers are perpendicular.

Anisotropies were measured in our work by the single-channel method. The polarization device, SPF-500C was an accessory to an SLM-500C spectrofluorometer on which all the fluorescence measurement were conducted. It is important that in actual measurement, a correction be made to eliminate the effect caused by differences in sensitivities for horizontally and vertically
polarized light in the detection system. The correction can be made through a simple measurement to obtain the G factor, the ratio of the instrumental sensitivities for horizontally and vertically polarized light (Lakowicz, 1983).

Energy Transfer Theory

Energy transfer is the process by which the energy of an excited state fluorophore (the donor) is transferred to another chromophore (the acceptor) through dipole-dipole coupling. The technique has been applied widely in determination of the distance between two points in biological systems. For example, when energy transfer occurs between a tyrosine (the donor) and a tryptophan (the acceptor) residues on a protein, the distance between the two amino acids can thus be measured by applying this method. Extrinsic fluorescence probes, CPM and lin-benzoADP, were used in this work to measure the distance between the calmodulin binding site and the nucleotide binding site on CaM kinase II.

Energy transfer efficiency derived from the Förster formulation is given by:

\[ E = \frac{R_0}{R_0^* R} \]  \hspace{1cm} (7)

where \( R_0 \) is the distance between the donor and the acceptor when 50% of the excitation energy is transferred to the acceptor. The \( R_0 \) value can be obtained from Eq. (8) without conducting energy transfer:

\[ R_0 = 9.78 \times 10^3 (\kappa^{-4} \kappa^2 q_e J)^{1/6} \text{Å} \]  \hspace{1cm} (8)
where \( n \) is the refractive index of the medium, \( \kappa^2 \) is the orientation factor of the dipole-dipole interaction, \( q_d \) is the fluorescence quantum yield of the donor in the absence of the acceptor, and \( J \) is the normalized spectral overlap integral. \( J \) is defined by Eq. 9:

\[
J = \frac{\int I_d^*(\lambda) e_a(\lambda) d\lambda}{\int I_d^*(\lambda) d\lambda}
\]  

(9)

where \( I_d^*(\lambda) \) is the fluorescence intensity (emission) of the donor in the absence of the acceptor, \( e_a(\lambda) \) (in \( M^{-1} cm^{-1} \)), is the extinction coefficient of the acceptor at wavelength \( \lambda \) (in cm), and \( J \) is in \( M^{-1} cm^3 \). The energy transfer efficiency \( E \) is obtained experimentally by using Eq. 10:

\[
E = \frac{I_{d\alpha} / q_\alpha}{I_d / q_d + I_a / q_a}
\]

(10)

where \( I_d \) is the fluorescence intensity of the donor in the presence of the acceptor, \( I_{d\alpha} \) is the energy transfer from the donor to the acceptor, and \( q_d \) and \( q_a \) are the quantum yields for the donor and the acceptor respectively.

The distance between the donor and the acceptor can therefore be calculated using Eq. 7.
CHAPTER 3

RESULTS

The effect of Ca\(^{2+}\) binding on CaM-CPM

Ca\(^{2+}\) titration of CPM-labeled CaM induced dramatic changes in fluorescence spectra for all three forms of CPM-CaM. As described in the previous chapter, residue 26 is located in the middle of the sequence of Ca\(^{2+}\) binding site I, whereas residue 75 is located at the central \(\alpha\) helix which joins the two domains, and residue 115 is in the segment connecting sites III and IV (Fig. 3). As shown in Figs. 5-7, each CPM-labeled form of CaM has distinct spectral properties, which could be interpreted as the influence of the different conformational changes in the local environment of each of the fluorescence probes, in addition to the influence of the overall conformational changes of the protein. This also suggests that CPM is a very sensitive indicator of the conformational changes of calmodulin.

All measurements in this part were made in the normal sized quartz cuvette, 10X10X40 mm, containing 100 mM Hepes, pH 7.5, 5 mM EGTA, 0.1 mM EDTA, and 50 nM CaM. The free Ca\(^{2+}\) concentration was calculated by GENDIS, a program written by Dr. Daniel Leussing, Department of Chemistry.
Fig. 5 The effect of Ca\(^{2+}\) binding on CaM-26-CPM fluorescence. Three components have been resolved. The medium contained 100 mM Hepes, pH 7.5, 5 mM EGTA, 0.5 mM EDTA, and 50 nM Cam-26-CPM. The data were collected at 8 °C. Panel A, the spectra of CaM-26-CPM; The spectra have been normalized to the highest intensity of each individual peak. Panel B, shows the relative fluorescence intensities as a function of \(pCa^{2+}\).
Fig. 6 The effect of Ca\(^{2+}\) binding on CaM-75-CPM fluorescence. Two components have been resolved. The conditions were the same as described in the legend to Fig. 5, except that a different form of CaM-CPM was used.

Panel A, the spectra of CaM-75-CPM;
Panel B, shows the relative fluorescence intensities as a function of -pCa\(^{2+}\).
Fig. 7  The effect of Ca\(^{2+}\) binding on CaM-115-CPM fluorescence. Two components have been resolved. The conditions were the same as described in the legend to Fig. 5, except that a different form of CaM-CPM was used.
Panel A, the spectra of CaM-115-CPM;
Panel B, shows the relative fluorescence intensities as a function of -pCa\(^{2+}\).
When no Ca\(^{2+}\) was added, the free Ca\(^{2+}\) concentration was calculated as 1 pM, based on the presence of 5 mM EGTA and estimated contaminating Ca\(^{2+}\) concentration of 1 nM (Takeuchi-Suzuki, 1993).

The three-component model yields the best fit for CaM26-CPM (Fig. 5), namely, a low Ca\(^{2+}\) form, a Ca\(^{2+}\) bound form, and an intermediate form. The content of the low Ca\(^{2+}\) component falls as the Ca\(^{2+}\) concentration increases, however, it still remains at a significant level, even at the highest Ca\(^{2+}\) concentration applied. While the intermediate component exists at a marked level in the absence of Ca\(^{2+}\), it then reaches its maximum at around 500 nM Ca\(^{2+}\) and gradually disappears as the Ca\(^{2+}\) concentration is increased further. The Ca\(^{2+}\) bound component rises from a very low level in the absence of Ca\(^{2+}\) and becomes significant at high Ca\(^{2+}\) concentration. The result may reflect a rapid equilibrium between two conformations of CaM, or simply an equilibrium between two forms of CPM relative to CaM, such that neither form exists as a predominant species, but one is more probable than the other at a certain Ca\(^{2+}\) concentration. The intermediate component may reflect the existence of an intermediate conformation of CaM when two of the four Ca\(^{2+}\)-binding sites are occupied, or it might simply indicate that the program failed to resolve the components of the system.

Only two components were resolved for CaM75-CPM and CaM115-CPM, since the third component was not only insignificant but also meaningless in
terms of the fluorescence spectral information and the value of the sum of the square residues (data not shown). In the case of CaM75-CPM, the low Ca\(^{2+}\) form and Ca\(^{2+}\) bound form appear to be similar to that observed for CaM26-CPM. The interpretation for the coexistence of two forms of CPM that was applied to CaM26-CPM may also be applied to CaM75-CPM. A rather clear transition from one form to the other is seen in the case of CaM115-CPM as the concentration of Ca\(^{2+}\) increases. This indicates that CPM labeled at this position better reflects the overall conformational change of its target, calmodulin.

In addition, as will be further discussed later, fitting the intensity curves in Figs. 5-7 to the Hill equation yields a midpoint of Ca\(^{2+}\) binding as approximately 200 nM, which generally agrees with the values reported from other sources (Linse et al. 1991; Forsen et al. 1986). This result suggests that CPM labeling does not change the overall behavior of calmodulin in response to Ca\(^{2+}\) binding. This measurement, together with the study on the activation of \(\alpha315\) by the CPM-labeled CaMs (Takeuchi-Suzuki, 1993), supports the use of the modified proteins as valid substitutes for the naturally occurring form in our study.

The ternary complex: CPM-CaM : \(\alpha315\) : lin-benzoADP

Fluorescence polarization was measured to determine the binding of CaM-CPM or lin-benzoADP to \(\alpha315\) (Takeuchi-Suzuki, 1993). In case of CaM-CPM the \(K_d\) values were determined to be 70 nM, 200 nM, 500 nM for CaM26-CPM, CaM75-CPM, and CaM115-CPM respectively. Whereas, the \(K_d\) for lin-benzoADP was obtained as 23 \(\mu\)M from a Scatchard plot. The binding of CaM-
CPM was found to have no significant effect on the binding of lin-benzoADP to the kinase (Takeuchi-Suzuki, 1993). These $K_d$ values will be used for the determination of the amount of lin-benzoADP that actually participated in the ternary complex.

**Determination of the distance between CaM-CPM and lin-benzoADP on $\alpha_{315}$**

As described in the preceding chapter, the multilinear analysis method allows one to obtain the energy transfer efficiency by exploiting the properties of full spectra, instead of the single wavelength used in the conventional method. Figs. 8-10 show the normalized fluorescence spectra resolved by the multilinear analysis program.

Smaller sized cuvettes, 5X10X30 mm, were used for the measurements presented in this section. The buffer contained 20 mM Hepes, pH 7.5, 10 mM MgCl$_2$, 0.1 mM EDTA, 0.01% Tween 40, and 5% glycerol. As the reference, the signal from the buffer was subtracted when the data were processed. All measurements were made at wavelength ranges 310-430 and 350-600 nm, for excitation and emission, respectively. For each CaM-CPM, in addition to the reference, eight samples of different contents were measured in the order: 1) 1 $\mu$M CaM-CPM alone, 2) 1 $\mu$M CaM-CPM and 0.1 mM CaCl$_2$, 3) 1 $\mu$M CaM-CPM and 0.3 mM CaCl$_2$, 4) 1 $\mu$M CaM-CPM, 0.3 mM CaCl$_2$ and 1.5 $\mu$M $\alpha_{315}$, 5) 1 $\mu$M CaM-CPM, 0.3 mM CaCl$_2$ and 1.5 $\mu$M $\alpha_{315}$ and 5 $\mu$M lin-benzoADP, 6) 1 $\mu$M CaM-CPM, 0.3 mM CaCl$_2$ and 1.5 $\mu$M $\alpha_{315}$ and 5 $\mu$M ADP, 7) 5 $\mu$M lin-
benzoADP only. Samples containing only CaM-CPM with different Ca\(^{2+}\) concentrations (samples 1-3) were included to allow better resolution of the spectra of CaM-CPM. Non-labeled ADP, employed in sample 6, was a control for lin-benzoADP. In addition to the normalized spectra, the output from the multilinear analysis program also contains the values of the relative fluorescence intensity and the amount of energy transfer. For samples 3-7 these values are shown in Tables 1-3.

Since energy transfer only occurs when both lin-benzoADP (the donor) and CPM-CaM (the acceptor) bind to the kinase, the percentage of lin-benzoADP that participates in energy transfer is dependent on the affinity of CPM-CaM to the kinase. The amounts of lin-benzoADP that were expected to appear in the ternary complex are estimated as 4.6%, 4.01%, 3.25% for CaM26-CPM, CaM75-CPM, and CaM115-CPM, respectively, based on the binding constants shown in the previous section and the conditions shown above. The fluorescence intensities of the donor, rather than the total fluorescence intensities of lin-benzoADP, were used in the calculation (see Tables 1-3). The relative fluorescence intensities of all species and the amount of energy transfer are shown in Tables 1-3. These values are normalized to the highest intensity in each case.

\(J\) values (Eq. (9)) were calculated using a program written by Dr. Jinkeun Lee. As shown in Figs. 8-10, two components were resolved for CaM-CPM. As a result, the excitation spectra, instead of the absorbance spectrum, were used.
for Eq. 9, with the maximum extinction coefficient of $2.9135 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Takeuchi-Suzuki, 1993). The parameters that were used for calculating $R_0$ values (Eq. 8) were $q_d = 0.44$, $n=1.33$, and $\kappa^2=2/3$. The $J$ and $R_0$ values are given in Table 4.

The average distance between the donor (lin-benzoADP) and the acceptor (CPM) obtained with CaM26-CPM, CaM75-CPM, and CaM115-CPM is $\sim 51 \text{ Å}$. This is the approximate distance that separates the CaM binding site and the nucleotide binding site on the CaM kinase II subunit being studied. The distance value for each individual form of CaM-CPM is shown in Table 4.
Fig. 8  The resolved spectra of lin-benzoADP and CPM. The medium contained 20 mM Hepes, pH 7.5, 10 mM MgCl₂, 0.3 mM CaCl₂, 0.1 mM EDTA, 1.5 μM α315, 1 μM CaM26-CPM, and 5 μM lin-benzoADP.
Fig. 9 The resolved spectra of lin-benzoADP and CPM. The medium contained 20 mM Hepes, pH 7.5, 10 mM MgCl₂, 0.3 mM CaCl₂, 0.1 mM EDTA, 1.5 μM α315, 1 μM CaM75-CPM, and 5 μM lin-benzoADP.
Fig. 10 The resolved spectra of lin-benzoADP and CPM. The medium contained 20mM Hepes, pH 7.5, 10mM MgCl₂, 0.3 mM CaCl₂, 0.1 mM EDTA, 1.5 μM α315, 1μM CaM115-CPM, and 5 μM lin-benzoADP.
Table 1  Energy transfer from lin-benzoADP to CaM26-CPM

The sample numbers are assigned in the order of measurement (see the descriptions of sample composition in this section). The designated numbers for CaM-CPM species were assigned randomly by the program, which allows energy transfer to occur to both species. The number in the bracket is the corrected lin-benzoADP intensity that was contributed by those lin-benzoADP molecules that were expected to participate in energy transfer.
Table 2  Energy transfer from lin-benzoADP to CaM75-CPM

The sample numbers are assigned in the order of measurement (see the descriptions of sample composition in this section). The designated numbers for CaM-CPM species were assigned randomly by the program, which allows energy transfer to occur to both species. The number in the bracket is the corrected lin-benzoADP intensity that was contributed by those lin-benzoADP molecules that were expected to participate in energy transfer.
<table>
<thead>
<tr>
<th>sample</th>
<th>CaM-CPM1</th>
<th>CaM-CPM2</th>
<th>lin-benzoADP to CaM-CPM1</th>
<th>lin-benzoADP to CaM-CPM2</th>
<th>lin-benzoADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.20313</td>
<td>1.00000</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.00000</td>
</tr>
<tr>
<td>4</td>
<td>0.20263</td>
<td>0.82097</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.00000</td>
</tr>
<tr>
<td>5</td>
<td>0.20457</td>
<td>0.80536</td>
<td>0.01446</td>
<td>0.00000</td>
<td>0.58492 (0.019)</td>
</tr>
<tr>
<td>6</td>
<td>0.19573</td>
<td>0.77141</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.00000</td>
</tr>
<tr>
<td>7</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.73696</td>
</tr>
</tbody>
</table>

Table 3  Energy transfer from lin-benzoADP to CaM115-CPM

The sample numbers are assigned in the order of measurement (see the descriptions of sample composition in this section). The designated numbers for CaM-CPM species were assigned randomly by the program, which allows energy transfer to occur to both species. The number in the bracket is the corrected lin-benzoADP intensity that was contributed by those lin-benzoADP molecules that were expected to participate in energy transfer.
Table 4 Calculated values for distance measurements

$R_0$, $J$, and $E$ were obtained using Eqs. 8, 9, and 10 respectively. The quantum yield of the donor $q_d$ is 0.44, refractive index $n$ is 1.33, and the orientation factor $\kappa^2$ is 2/3.
Cooperativity of Ca\(^2+\) binding to CaM

Data from experimental binding studies under various conditions strongly suggests that binding of Ca\(^2+\) to calmodulin occurs in a cooperative manner, at least for the first two Ca\(^2+\) ions, although arguments can also be found to support the hypothesis that the protein has four identical Ca\(^2+\)-binding sites (Burger et al. 1984).

To investigate the cooperativity of Ca\(^2+\)-binding to CaM-CPM the relative intensity data in Figs. 6 and 7 were fit to the Hill equation. The results are presented in Table 5 and Fig. 11. Both CaM75-CPM and CaM115-CPM exhibit

<table>
<thead>
<tr>
<th></th>
<th>Hill Coefficient (n)</th>
<th>(K_{1/2} \text{ (M} \times 10^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM75-CPM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.91</td>
<td>2.13</td>
</tr>
<tr>
<td>2</td>
<td>2.00</td>
<td>2.19</td>
</tr>
<tr>
<td>CPM115-CPM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.09</td>
<td>1.98</td>
</tr>
<tr>
<td>2</td>
<td>3.17</td>
<td>2.16</td>
</tr>
</tbody>
</table>

Table 5. Cooperativity of Ca\(^2+\) binding to CaM
Fig. 11  Experimental data points (in symbols) from Figs 6 and 7 have been fit to the Hill equation (lines). CaM-CPM species 1 and 2 are in the open and closed circles respectively.
positive cooperativity which appears to be greater for CaM115-CPM. A possible reason could be that residue 115 is located in the segment between the two Ca^{2+}-binding sites, in the C-terminal, where the first two Ca^{2+} ions bind. Being labeled at such a position, CPM might experience a more dramatic conformational change of its target molecule than a label at residue 75. The $K_{1/2}$ values shown in Table 5 are comparable to the published values for naturally occurring calmodulin (Beckingham, 1991; Johnson & Mills, 1986).

After thought for the distance calculation

Because of the relatively low affinity of lin-benzoADP, less than 5% of the total analog molecules were expected to participate in energy transfer. The small energy transfer was therefore masked by the strong fluorescence intensity from the majority of the molecules that were not found in the ternary complex. In such a case, given 1% of error in intensity, the error for the calculated distance could be as large as ~ 20%. If the project were to be continued, an improvement for the donor's affinity would be very critical. However, in spite of all this, we can still see the great potential of the multilinear analysis method in solving problems like this.

Epilogue

Due to the loss of data it becomes extremely difficult to present other work I did in Dr. Robert Ross's laboratory. That work includes the study of tyrosine only proteins (no tryptophan) and energy transfer from tyrosine to tryptophan. The study on tyrosine only proteins was an extension of the study on hydrogen
bonding of tyrosine residue using model tyrosine compounds (Lee et al. 1992). The proteins used in this work were plastocyanin from spinach, poplar and parsley (gift of Dr. Elizabeth Gross) and calmodulin (gift of Dr. Marita King). The goal of the tyrosine-tryptophan energy transfer project was to define the accuracy of the multilinear analysis method when used in distance estimation. Proteins and peptides with known structural information would be good subjects to carry out this work. I wrote a C program which allows one to find proteins from the Protein Data Bank (Brookhaven National Laboratory) that contain any given number of tyrosine and tryptophan residues. With the help of this program, CPI, a small protein inhibitor from potato was chosen. The sequence of this protein contains two tryptophan and one tyrosine residues. Unfortunately, the attempt to resolve the spectra from different tryptophans using a quencher (KI) failed because the two residues are both exposed to the aqueous phase due to the small size of the protein.
CHAPTER 5

INTRODUCTION TO ION TRANSPORT BY IONOPHORES

This part of my dissertation contains the work I have done with Dr. Douglas Pfeiffer. Synthetic vesicles have been used in the study of ionophore catalyzed ion transport properties. The transition to this new research phase began in September, 1994 after my first academic adviser Dr. Robert Ross decided to retire.

Model membrane system

Large unilamellar vesicles (LUV) have been used as the model membrane system throughout the work presented in this part of my dissertation. The model membrane system is sometimes referred to as 'liposomes', although this term originally referred to multilamellar vesicle (MLV) systems. LUV made by the method described later in Materials and Methods meet the criteria (Hope et al. 1985) for the so called 'faithful model membrane systems', which require that the vesicles be closed, unilamellar, and reasonably large, so as to maintain an acceptable level of encapsulation and to overcome the problems associated with small, highly curved vesicles.
The technique used for making such vesicles is a combination of freeze-thaw and extrusion, which has been widely applied for vesicle preparation (Chapman et al. 1991; Chapman et al. 1990; Chakrabarti et al. 1992; Veiro & Cullis, 1990) in recent years. It has been shown that the vesicles resulting from extrusion alone exhibit a very homogenous size distribution, with a mean diameter that approaches the size of the pores in the polycarbonate membrane through which the vesicles are extruded (Olson et al. 1979). Executing this procedure repeatedly leads to production of unilamellar vesicles (Nayar et al. 1989; Hope et al. 1985). Solute entrapment is enhanced by repeated freeze-thaw cycles (Chapman et al. 1990; Chapman et al. 1991) before and after the extrusion. Thus, to make homogenous unilamellar vesicles with high solute entrapment, our vesicle preparation protocol includes alternative cycles of freeze-thaw and extrusion. The phospholipid bilayer which defines the system is composed of 1-palmitoyl-2-oleoyl-sn-glycerophosphatidylcholine (POPC) which has a low phase transition temperature -7°C, making it possible to prepare, store, and use the vesicles at room temperature.

The model membrane system is illustrated in Fig. 12. Vesicles can be loaded with Quin-2 or cations, as desired. The average diameter and the bilayer thickness (drawn to scale) of the vesicles are around 70 and 2.9 nm respectively, as determined by freeze-fracture electron microscopy (Chapman et al. 1990). For comparison to the size of the vesicle, the relative dimensions of
Dimensions (to Scale) of the Transport System

Fig. 12 Model membrane system, adapted from Erdahl and Pfeiffer, 1996
the hydrated Ca\(^{2+}\) ion and the transporting complexes, \((A23187)_2\)Ca and ionomycin-Ca are also shown.

**Cation transport by ionophores**

There is a high activation energy for the movement of ions across a phospholipid bilayer which relates to the energy of hydration. To lower this barrier and thereby increase the rate of permeation, the charge on the ion needs to be either delocalized or shielded. This task can be accomplished by a variety of molecules, naturally occurring or synthetic, which are known as ionophores. In catalyzing ion transport across the membrane, ionophores can function as carriers or as channel formers. In the former case, ionophores exhibit high discrimination between different ions, whereas in the latter case, the discrimination is poor. However, ion transport via ionophore-derived channels is much more efficient. Ionophores have been very useful tools in cell biology because of their ability of selectively increasing the permeability of specific ions.

The ionophores being studied in my work include the carboxylic ionophores A23187, 4-BrA23187, ionomycin, and a neutral ionophore called ETH 129. The structures of these compounds are shown in Fig. 13. Properties of the carboxylic acid ionophores, including structure, ion complexation, and ion transport has been a long-standing interest in our laboratory. The applications of these ionophores, especially A23187, can be found in thousands of research papers. Compounds that are classified as carboxylic ionophores have an open chain-like structure, with a carboxyl group attached to one end and a hydroxyl or
Fig. 2 Structures of ionophores
ketone group to the other. The deprotonated form of carboxylic acid ionophores is negatively charged and forms complexes with cations via electrostatic and coordinate-covalent interactions. The resulting complexes are stabilized by hydrogen bonding (Dobler, 1981; Deber & Pfeiffer, 1976). The structures of Ca$^{2+}$ complexes of the two widely studied and utilized carboxylic ionophores, A23187 and ionomycin, are shown in Fig. 14. Although A23187 and ionomycin are known as Ca$^{2+}$ ionophores, they also form complexes with other divalent cations (Suzuki et al. 1988; Chapman et al. 1987; Chapman et al. 1990) and with monovalent and trivalent cations (Pfeiffer et al. 1978; Tissier et al. 1985; Divakar & Easwaran, 1987). A more comprehensive description of their cation complexation and transport properties can be found in literature review (Taylor et al. 1982). Based on the available data for A23187 and ionomycin, it seems unreasonable to conclude that these ionophores are Ca$^{2+}$ specific.

The sequential reactions of classical models depicting the electroneutral transport of the divalent cation by A23187 and ionomycin are shown in Fig. 15. In both cases, maintaining a zero net charge movement in a complete transport cycle, requires the movement of 2H$^+$ in the opposite direction of the divalent cation. Recent studies on Ca$^{2+}$ transport catalyzed by A23187, ionomycin and 4-BrA23187, using the model membrane system described above, indicate that within the detection limit of ~1 in 10$^4$ events, all three ionophores transport Ca$^{2+}$ by the electroneutral mode when there is no potential imposed across the membrane (Erdahl et al. 1994). An earlier report using electrical measurements
Fig. 14 Structures of Ionophore-Ca complexes
Fig. 15  The sequential component reactions in the classical neutral transport models for A) A23187 and B) ionomycin (adapted from Erdahl, et al., 1994)
on planar phospholipid membrane also indicates that A23187 transports divalent cations via the electroneutral mode (Morrone & Cohen, 1982). However, a dependence of divalent cation influx, catalyzed by A23187 and ionomycin, on the magnitude of membrane potential was later observed in both plasma and artificial membranes (Fasolato & Pozzan, 1989). These investigations suggested that both neutral and positively charged ionophore-cation complexes transport Ca²⁺ across the membrane.

Other studies on Ca²⁺ transport indicate that the stoichiometry for the transporting species is 2:1 for A23187 and 4-BrA23187 and 1:1 for ionomycin (Erdahl et al. 1994; Blau et al. 1984), with the net exchange of 2:1 between H⁺ and Ca²⁺ for all. These observations also support the idea that A23187, 4-BrA23187 and ionomycin transport Ca²⁺ via a predominantly electroneutral mode. A recent study reveals that 4-BrA23187, although a poor ionophore for Ca²⁺, exhibits an extremely efficient transport for Zn²⁺ and Mn²⁺. In contrast, a similarly high selectivity for Zn²⁺ and Mn²⁺ are not seen with A23187 and ionomycin (Erdahl et al. 1996). Although ionomycin has been found to transport Zn²⁺ and Mn²⁺ through a complex of 1:1 stoichiometry, which is consistent with the transport for Ca²⁺, the other two ionophores both transport the transition divalent cations via a mixture of 1:1 and 2:1 (ionophore : cation) structures. Apparently, the transport of Zn³⁺ and Mn²⁺ would be either through a partially electrogenic mode, such that species of MA⁺ are transported, or through a strict electroneutral mode that requires cotransport of an anion, such as OH⁻.
According to solution equilibrium studies, the ternary complex of A23187-Zn-OH should form when the ionophore is membrane associated (Chapman et al. 1987; Chapman et al. 1990), although there is no available transport data showing directly that the ternary complex can be transported. The decrease in Ca\(^{2+}\) transport activity for 4-BrA23187 was interpreted to indicate that the interligand hydrogen bonds which stabilize the (ionophore)\(_2\)Ca complex are weakened by introduction of bromine into the parent compound (Erdahl et al. 1996). The fact that 4-BrA23187 transports Zn\(^{2+}\) and Mn\(^{2+}\), but not Ca\(^{2+}\), through a mixture of 1:1 and 2:1 stoichiometries may be the reason why this ionophore displays the transport activities observed for these two metal ions (Erdahl et al. 1996).

Lanthanides transport by the above Ca\(^{2+}\) ionophores is a major research project that will be presented in this portion of my dissertation. The fact that lanthanide ions (Ln will be used as the generic symbol for lanthanides throughout this dissertation) have similar ionic radii and co-ordination properties to Ca\(^{2+}\) make them excellent substitutes for this biologically significant, yet spectroscopically "silent", metal ion. In forming complexes, both Ln\(^{3+}\) and Ca\(^{2+}\) prefer charged and uncharged oxygen groups to nitrogen donor atoms (Martin & Richardson, 1979). Some Ca\(^{2+}\)-dependent proteins are inhibited by the substitution of Ln\(^{3+}\) for Ca\(^{2+}\) (Furie et al. 1974; Meissner, 1973; Coopermann & Chiu, 1973), but in many other cases, function similar to the original form is maintained (Matthews & Weaver, 1974; Gomez et al. 1974; Furie et al. 1976; Smolka et al. 1971). On the other hand, because of their high charge density,
lanthanide ions have a much greater electrostatic attraction for any negative Ca²⁺ binding site, such as those located in the membrane, than Ca²⁺ ion per se. For this reason lanthanide ions are used, often in very small amounts, as inhibitors of Ca²⁺ transport through endogenous channels in cellular and subcellular membranes (Boucek & Snyderman, 1976; Moyle & Mitchell, 1977; Batra, 1973). As will be seen in Results, all three ionophores transport lanthanide ions efficiently at low concentrations of Ln³⁺. Therefore, caution must be taken when lanthanide ions and the Ca²⁺ ionophores are used simultaneously in investigating cell regulation.

The study of ionophore-mediated transport of lanthanide ions was initiated by Fernandez, et al (Fernandez et al. 1973) who reported that ionophore X-537A is capable of transporting Pr³⁺ across lecithin liposomes. Similar studies by Hunt (Hunt et al. 1978; Hunt, 1975) revealed that lanthanides Pr³⁺ and Eu³⁺ can also be transported by A23187. Their measurements were based on a ¹H NMR technique which distinguishes the inner and outer surfaces of phospholipid vesicles through binding of lanthanide ions to the head groups of the phospholipid molecules and the resultant chemical shifts. The transporting stoichiometry for A23187-Ln complex was reported to be 1:1. A subsequent report (Hunt & Jones, 1982), from the same group using the same technique, indicates a 2:1 (A23187:Pr) stoichiometry for the transporting species. Shastri and co-workers also found that A23187 can transport Pr³⁺, Nd³⁺ and Eu³⁺ through 2:1 stoichiometry across dimyristoylphosphatidylcholine (DMPC) and
dipalmitoylphosphatidylcholine (DPPC) vesicles. Furthermore, A23187 was found to induce histamine release from rat mast cells in the presence of La$^{3+}$ and Tb$^{3+}$ (Amellal & Landry, 1983), another indication, indirectly though, that A23187 is capable of catalyzing transport of lanthanide ions.

Due to the limitations of the methods used in existing lanthanide transport studies, the available data are not sufficient to identify the complexes that are responsible for transport of trivalent cations. Questions regarding the mechanism of transport and the rate of transport compared to Ca$^{2+}$, as well as whether electroneutrality is maintained during the process of transport are beyond the capability of the NMR technique.

Like other Ca$^{2+}$ chelators, Quin-2, introduced by Tsien (Tsien, 1980), tends to have much higher affinities to lanthanide ions than to Ca$^{2+}$. The complexes's stoichiometry of this chelator-indicator for a variety of metal ions, including lanthanides, is 1:1 (Jones, Jr. et al. 1992). Given the model system described above, the spectroscopic properties of Quin-2 were exploited in investigating lanthanides transport by A23187, 4-BrA23187 and ionomycin. Transport selectivities, mechanism, rate, and mode have been studied. Evidence for the co-transport of anion OH$^{-}$ has also been obtained for the first time.

Finally, it is important to understand the effect of a $\Delta$pH across the membrane on cation transport by the above carboxylic ionophores. As demonstrated in Fig. 15, only the free acid, not the ionized form of the
uncomplexed ionophores are membrane permeant. The distribution of the ionophores across the membrane is therefore ΔpH dependent. The ΔpH value for the regular Quin-2-loaded vesicles, the kind of vesicle used most often for the measurements that I present in this dissertation, is ~0.6 pH unit, basic inside. This relatively small pH difference has been shown to have a strong influence on the rate and extent of transport (Erdahl et al. 1995). When the ΔpH is collapsed by applying valinomycin plus the uncoupler CCP, an accelerated transport with an increased extent can be seen. The evidence indicates that in the presence of ΔpH we can not rely on ionophores to fully equilibrate the H⁺ and Ca²⁺ gradients across the membrane. In other words, in view of the above-mentioned facts, the validity of the so called null-point titration method for determining intracellular free cation concentrations, which has been used in investigating regulation of cell systems by Ca²⁺, is questionable.

In addition to the carboxylic ionophores, I also studied the transport properties of the neutral ionophore ETH 129. This synthetic compound, along with ETH 1001, was originally introduced for making Ca²⁺ selective electrodes by the Swiss Federal Institute of Technology (ETH). ETH 129 has been reported to have an extremely high Ca²⁺ selectivity, ~10⁴ over Mg²⁺, and ~10⁸ over Na⁺ and K⁺ (Schefer et al. 1986). The ionophore ETH 129 can form a highly lipophilic 3:1 (ETH 129 :Ca²⁺) complex with Ca²⁺ and a 2:1 complex with Mg²⁺. The lack of an optimal octahedral array of ligands is considered to be the reason for the high rejection, relative to Ca²⁺, of the compound towards Mg²⁺ in forming the complex.
Unlike A23187 and ionomycin, which have been widely studied and used, ETH 129 is relatively unknown. When transporting Ca\(^{2+}\), the charges on the cation are shielded by the lipophilic backbones of three ionophore molecules, and thus the Ca\(^{2+}\) ion is moved across the membrane via an electrogenic mode. An imposed membrane potential \(\Delta \psi\) or, alternatively, a system with collapsed \(\Delta \psi\) is required to allow the transmembrane movement of Ca\(^{2+}\). A recent report (Prestipino et al. 1993) on ETH 129 shows that this neutral ionophore can move Ca\(^{2+}\) into mitochondria in response to a negative membrane potential. It has also been reported from the same source that this ionophore is much more efficient than A23187 in promoting activation of sea urchin eggs.

In this dissertation, I report the results of my studies on ETH 129, including the effect of an imposed membrane potential on the transport of ETH 129, the selectivities of the ionophore on a variety of cations, the effect of Ca\(^{2+}\) concentration, and the stoichiometry of the transporting species.
CHAPTER 6

MATERIALS AND METHODS

**Reagents**  Synthetic 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids, Inc. Purity was confirmed by thin-layer chromatography before use. A23187, 4BrA23187, and ionomycin were obtained from Sigma or Calbiochem and were used without further purification. ETH 129 was purchased from Fluka AG (Buchs, Switzerland). Stock solutions in ethanol were standardized spectrophotometrically using the following extinction coefficients: 4-BrA23187 ($\Sigma_{280} = 15,600$); A23187 ($\Sigma_{278} = 21,040$); ionomycin ($\Sigma_{278} = 13,560$). The concentration of ETH 129 stock solution was determined gravimetrically. Quin-2 (K$^+$ salt) from Sigma was purified by passage over Chelex 100 resin (100-200 mesh) in the Cs$^+$ form as described by Erdahl et al. (1994). Stock solutions of CaCl$_2$ (ultrapure grade from Alfa Products) were standardized by titration with a primary standard EDTA solution (Vogel A.I. 1961). Lanthanide series metal oxides, also ultra pure, were obtained from Alfa products or Aldrich Chemical Co. Samples were reacted with an excess of 1 M perchloric acid, filtered, and then standardized by titration.
against primary standard EDTA. Excess hexamethylenetetraamine, pH 5.5-6.0, was employed to buffer the unknown samples, and the titration endpoints were indicated by xylenol orange (Lyle and Rahman, 1963).

**Preparation of phospholipid vesicles**

Freeze-thaw extruded POPC vesicles were prepared as described by Chapman *et al.* (Chapman et al. 1990; Chapman et al. 1991). Briefly, 300 mg of POPC in chloroform was dried by rotation under a nitrogen stream to produce a film on the wall of a 25 x 150 mm culture tube. Residual solvent was removed under high vacuum (4 h) and the film was subsequently hydrated in 6 ml of a solution containing 5 mM of purified Quin-2 (Cs⁺) and 10.0 mM Hepes buffer adjusted to pH 7.00 with Chelex -treated CsOH (Erdahl *et al.*, 1994). The mixture was vortexed and the resulting multilamellar vesicles were frozen in a dry ice-acetone bath, thawed in lukewarm water, and vortexed again. The freeze-thaw and vortexing procedures were repeated two times, after which the vesicles were extruded three times through two stacked 100 nm polycarbonate membrane filters. This step was followed by six additional freeze-thaw cycles coupled with eight additional extrusions. The resulting preparations were applied to Sephadex G-50 mini-columns (Fry *et al.* 1978) to remove extravesicular Quin-2. These columns were eluted by low speed centrifugation and had previously been equilibrated with a solution containing 10 mM Hepes buffer, pH 7.00. A single pass over such columns effectively removes the external Quin-2 (Erdahl *et al.* 1994; Chapman et al. 1990; Chapman et al. 1991).
The nominal concentration of POPC in the final preparations was determined by measurement of lipid phosphorus (Bartlett, 1959) and was near 80 mM. The average diameter of these vesicles is 71 nm as determined by freeze-fracture electron microscopy (Chapman et al. 1991), and they contain entrapped solutes at the following concentrations; Quin-2, 10.5 ± 0.8 mM; Hepes, 33.7 ± 7.6 mM (pH ≈ 7.4); and Cs⁺, 60 ± 5 mM. Specific values for Quin-2 and Cs⁺ were determined for each preparation by the methods described by Erdahl et al. (1994, 1995). Briefly, entrapped Quin-2 is determined by spectrophotometric titration with standard CaCl₂ following dispersion of the vesicles in deoxycholate. Entrapped Cs⁺ is determined by atomic absorption spectroscopy, following replacement of the external medium with one not containing Cs⁺, and dispersion of the vesicles in 0.1 N HCl. Buffer entrapment is determined from the other values by calculation, using the Henderson-Hasselbach equation, the buffer pKᵢ, and the internal pH. When buffer entrapment is to be determined, the vesicles also contain the fluorescent pH indicator BCECF, so that the internal pH can be ascertained. The internal pH and solute concentrations differ from those of the vesicle formation medium because of a freeze-thaw driven solute concentrating effect which operates during preparation of the vesicles (Chapman et al. 1990; Chapman et al. 1991).

Vesicles loaded with La³⁺ were prepared and purified in an analogous way except that 5 mM LaCl₃ was present in the formation medium rather than Quin-2. In addition, the Hepes buffer used in the Quin-2 containing preparations
was replaced with Mes at pH 6.00. To determine entrapped La\(^{3+}\), an aliquot of the final preparation containing 1 mM POPC was lysed with DOC and then titrated with a standard solution of Quin-2 while monitoring the titration spectrophotometrically (267 vs. 343 nm). La\(^{3+}\) is tightly bound by Quin-2, forming a 1:1 complex with a stability constant of \(\sim 10^{12}\) (Jones et al., 1992, Yuchi et al., 1993). A sharp end point is therefore obtained, which is seen as an inflection point in plots of Quin-2 difference absorbance vs. La\(^{3+}\) concentration. This method gave an internal La\(^{3+}\) concentration of 16.9 ± 0.5 mM, similar to the level of Ca\(^{2+}\) entrapped by an analogous procedure (Erdahl et al., 1994). Internal pH and buffer entrapment could not be determined with these preparations because of an interaction between BCECF and La\(^{3+}\).

Physical properties of POPC vesicles are summarized in Table 6.

**The Determination of cation transport**

The spectra of La-Quin-2 complex are shown Fig. 16. The data were obtained on a Beckman DU-8 spectrophotometer at room temperature. There was no obvious difference in spectroscopic features when the spectra of La-Quin-2 complex in solution and within intact vesicles are compared. Difference spectra were shown in Fig. 16B in order to get rid of the strong scattering signal from the vesicles. Further, as shown in Fig. 17, La\(^{3+}\) titration of Quin-2 in solution and of La\(^{3+}\)-loaded vesicles are in excellent agreement within the linear portion of the calibration curves. These results indicate that Quin-2 trapped in the vesicles has the same spectroscopic properties as in solution. Thus, the
Fig. 16  All data were obtained at room temperature. Buffer contained 10 mM Hepes, plus 60 mM CsCl, pH 7.0.
A) Spectra of Quin-2-La complexes in solution.
B) Difference spectra of Quin-2-La complex. Spectrum containing no La³⁺ was substracted from the rest of the spectra. The nominal POPC concentration was at 1.5 mM. The concentration of A23187 was 5 μM.
Fig. 17 Calibration of the vesicle transport system. The medium contained 10 mM Hepes, plus 60 mM CsCl, pH 7.0. Data were obtained at 25 °C. A23187 concentration was at 10 μM. Nominal POPC concentration for the intact vesicles was at 1.5 mM.
<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average diameter</td>
<td>70 nm</td>
</tr>
<tr>
<td>Bilayer thickness</td>
<td>2.9 nm</td>
</tr>
<tr>
<td>Entrapment volume (at 1.0 mM POPC)</td>
<td>2.02 µl/ml</td>
</tr>
<tr>
<td>POPC molecules/vesicle</td>
<td>41,400</td>
</tr>
<tr>
<td>Outer monolayer</td>
<td>22,500</td>
</tr>
<tr>
<td>Inner monolayer</td>
<td>18,900</td>
</tr>
<tr>
<td>Internal Quin-2 concentration</td>
<td>10.5 ± 0.8 mM</td>
</tr>
<tr>
<td>Internal La³⁺ concentration</td>
<td>16.9 ± 0.5 mM</td>
</tr>
<tr>
<td>Internal Cs⁺</td>
<td>60 ± 5 mM</td>
</tr>
</tbody>
</table>

* Values taken from Chapman et al. (1990)

For La³⁺-loaded vesicles only

Table 6  Physical properties of POPC vesicles
validity of our method of using Quin-2 as a La³⁺ transport indicator is ensured. The data shown in Fig. 17 were obtained on DW2a dual wavelength spectrophotometer.

The transport of lanthanide cations into Quin-2 loaded vesicles was determined by monitoring formation of the Quin-2 : cation complexes spectroscopically. Vesicles containing Quin-2 were present at a nominal POPC concentration of 1.0 mM in a medium which also contained 60 mM CsCl, and 10 mM each of Hepes and Mes. The medium pH ranged from 6.0 to 8.0 and was adjusted with CsOH which had been passed over Chelex 100 columns to remove contaminating divalent cations (Erdahl et al., 1994). To maintain internal pH at the external value, VAL, (0.5 μM) and carbonyl cyanide m-chlorophenylhydrazone (CCP), (5 μM) were also present (Erdahl et al., 1995). Specific concentrations of ionophores, metal cation chlorides, and pH values are given in the figure legends. Reactions were started by addition of the divalent cation ionophore, following an initial 2-3 min period which was allowed for the equilibration of internal and external pH.

Formation of the Quin-2 : cation complexes was monitored by difference absorbance measurements using an Aminco DW2a spectrophotometer operated in the dual wavelength mode. An Oriel No. 59800 band pass filter was used between the cuvette and the beam scrambler-photomultiplier assembly to prevent detection of the fluorescent light emitted by Quin-2 and A23187. The sample and reference wavelengths were 267 and 343 nm, respectively, with the latter
value representing an isosbestic point in the Quin-2/Quin-2: cation complex difference spectrum. At this wavelength pair, the difference extinction coefficient for the Quin-2/La\(^{3+}\) complex was 27,180 ± 275 (M\(^{-1}\) cm\(^{1}\)) (n = 5). Values for other lanthanide series cations were very similar but values specific to each cation were employed when determining relative rates of transport. Data were collected on disk through a computer that was interfaced to the spectrophotometer, using Unkel Scope software (Unkel Software, Inc., Lexington, MA).

**The Determination of H\(^+\) : La\(^{3+}\) exchange ratio**

The net mole exchange ratios between H\(^+\) and La\(^{3+}\) were determined by simultaneous measurement of the extravesicular pH change during La\(^{3+}\) transport. The experimental conditions were maintained the same as described above for determination of cation transport, except that the external buffer concentration was reduced to 3 mM to magnify the pH change, which was monitored using a Fisher Scientific AccupHast electrode, a Beckman model 4500 pH meter, and a Sargent-Welch model XKR recorder. The electrode was inserted into the cuvette which was in turn mounted in the cuvette holder of the dual-wavelength spectrophotometer. The measurement was carried out in a completely dark environment in order to allow the lid of cuvette holder compartment of the Aminco spectrophotometer to be open. To avoid disturbing the equilibrium of the electrode by manual mixing, a magnetic stirring accessory provided by Aminco was used throughout the measurement. The change of H\(^+\)
concentration was determined by calibrating the system using standard HCl. Points were taken from the continuous recordings of pH and absorbance change. The change of extravesicular concentrations of $H^+$ and $La^{3+}$ versus time were plotted in the same graph to show the exchange ratios of the ions.

Data Analysis

External and internal methods were compared when calibrating the transport data. For the external method, vesicles containing a known amount of Quin-2 were lysed with 0.33% (w/v) of Cs*-deoxycholate and titrated with a standard solution of the cation under the conditions of interest. For the internal method, Quin-2 was titrated without lysing the vesicles, by including an excess of an appropriate ionophore in the system. Data obtained by the two methods were coincident, indicating that entrapment of Quin-2 and its lanthanide complexes does not perturb their spectral properties.

To extract the initial rates of transport, an early portion of the progress curves was fit to Eq (11) using standard nonlinear least squared methods.

$$A_t = A_o + Bt + Ct^2$$

In Eq (11), $A_t$ and $A_o$ are the observed and the initial absorbance values, respectively, $B$ is the initial rate, $C$ is a correction factor for nonlinearity, and $t$ is time. Rates are expressed in the unit $\mu M/sec$ of external cation transported into.
the vesicles. Transport selectivities are expressed as $S$ values defined by Eq (12).

$$S_m = \frac{\text{Initial Rate of } M^{n+} \text{ Transport}}{\text{Initial Rate of } Ca^{2+} \text{ Transport}}$$

When determining $S$, an equal concentration of the cation in question is substituted for $Ca^{2+}$ with all other conditions held constant. Complexation and extraction selectivities are expressed in an analogous way. All data were obtained at 25.0° C.
CHAPTER 7

RESULTS OF LANTHANIDE ION TRANSPORT

The goal of this project was to characterize the transport properties of the "so-called" Ca$^{2+}$ ionophores, A23187, 4-BrA23187 and ionomycin for lanthanide series cations. Parameters to be determined were the transport rates of the lanthanides relative to Ca$^{2+}$, the mechanism of transport, including the stoichiometry of the transporting species, the exchange ratio of Ln$^{3+}$ to H$^+$. The mode of transport was also to be determined.

Comparative Aspects of Lanthanide Cation Transport by Ca$^{2+}$ Ionophores

Fig. 18 shows that lanthanide cations are transported by Ca$^{2+}$ ionophores with considerable selectivity and that among the three compounds investigated, marked differences exist in the rates of transport and the shapes of the progress curves. Considering first the selectivity aspects of the data, initial rates were extracted from each progress curve and used to generate the sequences which are shown in Table 7. Selectivity was calculated according to Eq. 12, using these initial rates and the initial rates of Ca$^{2+}$ transport by each compound, which were determined under identical conditions (Fig. 18). For 4-BrA23178 and
Fig. 18 Selectivity of Ca$^{2+}$ ionophores on Lanthanide ions. Data were obtained at 25°C using 10 mM Hepes (Cs*), 60 mM CsCl, pH 7.0, plus 0.5 μM VAL, 5 μM CCP. The nominal POPC concentration and the initial external concentration of lanthanide ion were 1 mM and 9 μM respectively. Ionophores A23187, 4-BrA23187, at 1.2 μM each and ionomycin at 1.5 μM, were injected into the solution at time 0. The transport of lanthanide ions was monitored spectrospecally at 267 vs 343 nm.
<table>
<thead>
<tr>
<th>Ionophore</th>
<th>S Values (Cation)</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Nd&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>La&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>Eu&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>Gd&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>Er&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>Yb&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>Lu&lt;sup&gt;3+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A23187</td>
<td></td>
<td>1.000</td>
<td>0.322</td>
<td>0.246</td>
<td>0.234</td>
<td>0.183</td>
<td>0.063</td>
<td>0.022</td>
<td>0.010</td>
</tr>
<tr>
<td>4-BrA23187</td>
<td></td>
<td>1.000</td>
<td>1.240</td>
<td>0.323</td>
<td>0.722</td>
<td>0.436</td>
<td>0.137</td>
<td>0.084</td>
<td>0.038</td>
</tr>
<tr>
<td>Ionomycin</td>
<td></td>
<td>1.000</td>
<td>0.789</td>
<td>1.390</td>
<td>0.393</td>
<td>0.345</td>
<td>0.419</td>
<td>0.980</td>
<td>0.571</td>
</tr>
</tbody>
</table>

Table 7: Relative Selectivities of Ca<sup>2+</sup> Ionophores
Fig. 19  The Effect of the External La$^{3+}$ Concentration on the Initial Rate of Transport. Data were obtained at 25 °C using 10 mM Hepes (Cs+), 60 mM CsCl, pH 7.0, plus 0.5 μM VAL, 5 μM CCP and 1 μM ionophore. The nominal concentration of POPC was at 1 mM. The initial rates were obtained by fitting the initial portion of progress curves to Eq. 11.
Ionomycin the selectivity ratios are near 1 when the lanthanides which are transported most rapidly are considered. With A23187, Ca\(^{2+}\) is transported more rapidly than all of the lanthanides, but only by a factor of ~3 compared to Nd\(^{3+}\). The absolute rates at which the lanthanides are transported span ~35 fold in the case of A23187 and 4-BrA23187 and ~4 fold in the case of Ionomycin (from Table 7). These findings indicate that lanthanide cation transport by Ca\(^{2+}\) ionophores is not a minor activity, but approaches the efficiency at which Ca\(^{2+}\) is transported under the conditions of Fig. 18.

With 4-BrA23187 and Ionomycin, the rates of transport for all lanthanides decrease progressively as the process proceeds, as would be expected if the absolute rate were limited by the cation concentration in the external aqueous phase, and in turn by formation of the transporting species at the outer membrane interface. A different behavior is seen with A23187, particularly when the lanthanides which are transported most slowly are considered. In these cases a relatively slow and linear period of transport is followed by an accelerating rate as the external lanthanide concentration falls below 3–4 µM (Fig. 18A). As will be further discussed below, this unusual behavior may reflect the relative magnitudes of stability constants for the several complexes which are formed between A23187 and the present set of lanthanide cations (Chapman et al. 1990), as well as the adsorption of lanthanide ions to phospholipid head groups.

**Stoichiometry of the Transporting Species**
Fig. 20  Relationship between the Initial Rate of La\(^{3+}\) Uptake and Ionophore Concentration. The data were obtained at 25 °C in aqueous solution containing 10 mM Hepes, 60 mM CsCl, 0.5 μM VAL, 5 μM CCP and A) 15 μM La\(^{3+}\) or B) 250 μM La\(^{3+}\). The nominal POPC concentration was at 1 mM. The initial portion of progress curves were fit to Eq. 11 to obtain initial rates of transport. The slope values of lines are presented in Table 8.
<table>
<thead>
<tr>
<th>Initial External La(^{3+}) Concentration, (\mu\text{M})</th>
<th>Ionophore</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A23187</td>
<td>4-BRA23187</td>
<td>Ionomycin</td>
</tr>
<tr>
<td>15 (^a)</td>
<td>2.24</td>
<td>2.24</td>
<td>1.35</td>
</tr>
<tr>
<td>250 (^b)</td>
<td>2.09</td>
<td>2.30</td>
<td>1.02</td>
</tr>
</tbody>
</table>

\(^a\) Values were obtained from the Data sets shown in Fig. 20A

\(^b\) Values were obtained from the data sets shown in Fig. 20B

Table 8: Apparent Stoichiometry of the Transporting Species: slope values of the log-log plots shown in Fig. 20.
Studies directed at revealing the mechanism of lanthanide cation transport were conducted using La³⁺. Fig. 19 shows that an inverse relationship exists between La³⁺ concentration and the initial rate of transport, at least above a La³⁺ concentration of 5 μM (near the lower limit of external cation concentration which can be utilized with the present system). This type of relationship would be expected if the ionophore: La³⁺ stoichiometry of the transporting species were greater than 1:1, and this species were subject to disproportionation in the presence of excess cation to form species of lower stoichiometry (Eq. 13) which transported poorly.

\[ A_2\text{LaOH} + \text{La}^{3+} + \text{H}_2\text{O} \rightarrow 2(\text{ALaOH})^\dagger + \text{H}^+ \] (13)

A further point of interest regarding Fig. 19 is the fact that with Ionomycin, a relatively high rate of transport persists at high La³⁺ concentration whereas with A23187 and 4-BrA23187 the rate approaches 0 as the La³⁺ concentration rises. Thus with Ionomycin, the disproportionation product(s) could retain some transport activity, whereas with the other compounds this would not be the case.

If it is assumed that transmembrane diffusion of the transporting species is the slowest step in the mechanism, the ionophore: cation stoichiometry of this species can be obtained as the slope from plots of log transport rate vs. log of the ionophore concentration. As seen in Fig. 20A and Table 8, these slopes are near 2 in the case of A23187 and 4-BrA23187 and near 1.5 with Ionomycin,
when determined at a La\(^{3+}\) concentration of 15\(\mu\)M (i.e., at a concentration which minimizes disproportionation). The former values indicate a stoichiometry of 2 : 1, ionophore to cation, whereas with Ionomycin, both the 2 : 1 and 1 : 1 could be contributing. Alternatively, 3 : 2 complex could be involved in the transport of La\(^{3+}\) by Ionomycin. At a La\(^{3+}\) concentration of 250 \(\mu\)M, the slopes obtained with A23187 and 4-BrA23187 were still near 2, however the value for Ionomycin became near 1 (Fig. 20B and Table 8), favoring the former interpretation. Thus considering Figs. 19 and 20 together, it appears that A23187 and 4-BrA23187 transport La\(^{3+}\) via 2 : 1 but not 1 : 1 complexes whereas species of both types contribute in the case of Ionomycin Eq. (14)

\[
\text{I}_2\text{HLa} + \text{La}^{3+} + 2\text{H}_2\text{O} \rightleftharpoons 2\text{ILaOH} + 3\text{H}^+ \quad (14)
\]

La\(^{3+}\) release from La\(^{3+}\)-loaded vesicles and the transport mode

As illustrated in Fig. 21, when the direction of La\(^{3+}\) transport was reversed the progress curves have different characteristics compared the uptake curves shown in Fig. 18. Sigmoid-shaped progress curves, similar to the uptake curves of Yb\(^{3+}\) and Lu\(^{3+}\) in Fig. 18, are seen with both A23187 and 4-BrA23187. Based on the inverse relationship between La\(^{3+}\) concentration and the initial rate of transport shown above, together with the fact that the internal La\(^{3+}\) concentration for the La\(^{3+}\)-loaded vesicles was \(\sim 16\) mM (see Materials and Methods), an extremely slow transport in La\(^{3+}\) release would be expected. Nevertheless,
Fig. 21  La$^{3+}$ Released by Ca$^{2+}$ Ionophores from LaCl$_3$ loaded vesicles. Vesicles loaded with 5 mM LaCl$_3$ and 10 mM Cs$^+$ salt of Mes (pH 6.0) as described in Materials and Methods. The data were obtained at 25 °C in a aqueous solution containing 10 mM Hepes, 10 mM CsCl, 0.5 μM VAL, 5 μM CCP, plus a) 2.0 μM A23187, or b) 5.0 μM 4-BrA23187, or c) 1.0 μM ionomycin. The nominal POPC concentration was 1 mM.
efficient transport was seen for all three compounds especially for ionomycin. Similar observations have been reported for Ca\(^{2+}\) transport (Erdahl et al., 1994). The membrane surface to aqueous phase ratio and the partition coefficient of transporting species were considered as possible factors that might make cation release faster than uptake. The sigmoid shape of the progress curves may be interpreted as a change of disproportionation during the process of La\(^{3+}\) release (see DISCUSSION).

When a tetraphenylphosphonium (TPP\(^{+}\)) electrode (prepared as described in Kamo et al. 1979) was applied to monitor the effect of La\(^{3+}\) release (in the absence of VAL and CCP) on membrane potential, negative results were obtained for all three ionophores (Data not shown). This indicates that at least within the detection limit of the TTP\(^{+}\) electrode (-50 mV) La\(^{3+}\) transport is electroneutral.

**H\(^{+}\):La\(^{3+}\) exchange ratio**

Mixed complexes containing A23187, Lanthanide ions and OH\(^{-}\) were detected during solution equilibrium studies (Chapman et al. 1990). The possibility of OH\(^{-}\) cotransport with Zn\(^{2+}\) by 4-BrA23187 was also reported (Erdahl et al., 1996). Studies on Ca\(^{2+}\) transport by A23187, 4-BrA23187 and ionomycin indicate that the exchange ratio between H\(^{+}\) and Ca\(^{2+}\) is 2:1 for all three ionophores (Erdhal et al., 1994). In the case of La\(^{3+}\), however, this ratio becomes 3 : 1 (H\(^{+}\): La\(^{3+}\)) (Fig. 22). Based on the stoichiometry studies shown above, it becomes evident that within the extravesicular La\(^{3+}\) concentration range
Fig. 22  

H⁺ and La³⁺ Exchange Ratio. The data were obtained at 25 °C in 3mM Hepes (Cs⁺ salt), pH 7.0, plus 21 μM La(ClO₄)₃. The nominal POPC concentration was 1.5 mM. The dark traces were La³⁺ transport progress curves, lines with open circle symbols were from pH measurement. Ionophore concentrations were 5 μM, 14 μM and, 5 μM for A23187, 4-BrA23187 and ionomycin respectively.
(10 - 250 μM) used in this work, the transporting species for La³⁺ uptake (A23187 and 4-BrA23187) was A₂LaOH because species such as A₂La⁺ would give a ratio of 2 : 1 (H⁺ : La³⁺) and the transport mode would be electrogenic. Ionomycin on the other hand, which transports La³⁺ via two species, also gives a 3 : 1 (H⁺ : La³⁺) exchange ratio, suggesting that this ionophore transports La³⁺ via I₂HLa and ILaOH. The H⁺ and La³⁺ exchange ratio can be readily explained by Eqs. 13 and 14.

Influence of membrane potential on La³⁺ Transport

Although in the absence of membrane potential La³⁺ transport by the Ca²⁺ ionophores is predominantly electroneutral, with a pre-existing membrane potential of ~160 mV transport of La³⁺ was considerably accelerated with all three ionophores (Fig. 23, See legend of the figure for the vesicle preparation and other conditions).

Analogous results were obtained with Ca²⁺ (Erdahl et al., 1994), however, the acceleration in terms of initial rate and extent of transport was less dramatic than in case of La³⁺. The faster transport seen in the presence of the membrane potential was attributed to an electrogenic mode and/or the effect of uncatalyzed H⁺ diffusion into the vesicles (Erdahl et al., 1994). The reason that H⁺ diffusion may account for the acceleration is that the more acidic internal environment would help the protonation of ionophores, thus shortening the transport cycle. The greater change seen in the case of 4-BrA23187 is probably due to the lower pKₐ value of the ionophore. However, a partially electrogenic transport mode is
Fig. 23  The Influence of Membrane Potential on La$^{3+}$ Transport Rate. Vesicle forming medium contained 10mM Hepes, 5 mM Quin-2 and 100 mM KCl at pH 7.0. The data were obtained at 25 °C in 10 mM Hepes, plus 100 mM NaCl, 15 μM La(ClO$_4$)$_3$. VAL concentration was 0.5 μM. The nominal concentration of POPC was 1.5 mM. Ionophores were added at time 0 at 1 μM, 3.2 μM and 1 μM respectively for A23187, 4-BrA23187 and ionomycin.
also a possibility. The same kind of reasoning may be applied for La$^{3+}$. The adsorption constants of Ca$^{2+}$ and La$^{3+}$ to bilayer membranes are 10-20 M$^{-1}$ and $4.1 \times 10^3$ M$^{-1}$ respectively (Lehrmann & Seelig, 1994). Binding of Cations to zwitterionic lipids brings positive charges in the interface which generates a membrane surface potential. In the case of PC, binding of Ca$^{2+}$ and Mg$^{2+}$ will generate a surface potential of 10-60 mV (Tocanne & Teissie, 1990). Due to the higher affinity for the bilayer, and higher charge density, La$^{3+}$ tends to generate a higher membrane surface potential than Ca$^{2+}$. In addition, binding of Ca$^{2+}$ and La$^{3+}$ involves changes in orientation and conformation of PC head-groups (Tocanne and Teissie, 1990). La$^{3+}$ would be expected to produce a greater effect on these changes, which in turn may speed up uncatalyzed H$^+$ diffusion. However, at this point we can not exclude the possibility that an electrogenic mode of transport may exist in the presence of pre-existing membrane potential, especially, in the case of 4-BrA23187.

Fig. 24 shows the effect of a pre-existing membrane potential of $\sim$150 mV, negative inside, on the exchange ratio of H$^+$:La$^{3+}$. The salt concentration in the vesicle forming medium was lowered from what is described in the legend to Fig. 23, so as to enhance the entrapment of Quin-2, because high salt content results in low entrapment of Quin-2 (see the legend to Fig. 24). In the presence of the membrane potential, the H : La$^{3+}$ exchange ratio is no longer 3:1. The H$^+$ output was apparently reduced as shown in panel A. However, in the absence of the membrane potential (no VAL) the net exchange ratio is 3:1, as shown in
panel B. The marked deviation from the 3:1 H⁺ to La³⁺ exchange ratio suggests that in the presence of membrane potential, charged species such as A₂La⁺ could be involved in the transport. In other words, transport of La³⁺ by 4-BrA₂3187 might be in a mixture of electroneutral and electrogenic mode. Analogous measurements were also conducted with A₂3187 and ionomycin; however, 3:1 ratio was seen regardless of membrane potential (data not shown).

Comparison of La³⁺ and Lu³⁺

Titration with La³⁺ and Lu³⁺ was conducted by using fluorescence, using 4 μM A₂3187, to further explore the differences of the two lanthanide cations in complexation with A₂3187 in the presence of POPC. The vesicles for this experiment were prepared without Quin-2 (see legend to this figure). The vesicle forming medium contained 5 mM Mes, 5 mM Hepes and 40 mM CsCl. The pH was adjusted to pH 7.00 using CsOH. The trapped Cs⁺ concentration was determined to be ~ 90 mM (see Materials and Methods). As shown in Fig. 25, when the La³⁺ concentration increases, the band at 380 nm drops until the accumulated La³⁺ concentration is ~10 μM. Therefore, the band starts to rise as the La³⁺ titration proceeds further. The falling and rising phases of the spectra are shown in panels A and B, respectively. Fractional fluorescence change at 380 nm during the falling phase was plotted against the accumulated La³⁺ concentration, as shown in Fig. 26. The dashed lines identify endpoint titration behavior for formation of strict 1:1 and 2:1 complexes. The result indicates that
2:1 stoichiometry (A23187 : La) was the predominant conformation when La^{3+} concentration was less than 2 μM. The rising phase of the spectra might indicate formation of the 1:1 complex. A much shorter falling phase at the same wavelength was seen for Lu^{3+}. The band starts to rise when Lu^{3+} concentration is only around 1 μM, suggesting that the 1:1 stoichiometry becomes the predominant species at a much lower Lu^{3+} concentration.
Fig. 24  The Effect of the Membrane Potential on H⁺ and La³⁺ Exchange. Vesicles forming medium contained 10 mM Hepes, 5 mM Quin-2 and 20 mM CsCl. The data were obtained at 25 °C in aqueous solution containing 3 mM Hepes (Na⁺ salt), pH 7.0, 21 μM La(ClO₄)₃. 0.5 μM VAL was applied to the data in the left panel. 4-BrA23187 (injected at time 0) and the nominal POPC concentrations were 14 μM and 1.5 mM respectively.
Fig. 25 Fluorescence Titration of A23187 with La$^{3+}$ and Lu$^{3+}$. The excitation spectra were obtained at the emission wavelength of 437 nm. Vesicles were made without Quin-2. The vesicle forming medium contained 10mM Hepes, 40 mM CsCl (pH 7.0). The data were obtained at room temperature in aqueous solution containing 5 mM Hepes, 5mM Mes (pH 7.0), 0.5 μM VAL, 5 μM CCP, plus 85 mM CsCl to make the Cs$^+$ content equal to the internal Cs$^+$ concentration. The concentration of A23187 was 4 μM. La(ClO$_4$)$_3$ and Lu(ClO$_4$)$_3$ were added in small volumes. The nominal concentration of POPC was 1 mM. The spectrum labeled “0” is in the absence of lanthanide ion, whereas the one labeled “m” is when the band at 380 nm stops falling. The rising band stops at spectrum labeled “f”. The cation concentration ranges covered were La$^{3+}$, A) 0-9.5 μM and B) 9.5-415 μM; Lu$^{3+}$, C) 0-1 μM, D) 1-242 μM.
FIG. 26  Fractional Fluorescence Change as a Function of $\text{La}^{3+}$ Concentration. Values were obtained from Fig. 25A at 380 nm. The maximum change was obtained by extrapolating the $1/\Delta F$ vs. $1/\text{La}^{3+}$ plot.
CHAPTER 8

DISCUSSION AND CONCLUSION

The Mechanism of Lanthanide transport

A fundamental question regarding the mechanism of cation transport by ionophores is raised by the data from La\(^{3+}\) release and Lu\(^{3+}\) uptake. When La\(^{3+}\) loaded vesicles were used in investigation of La\(^{3+}\) release, plots of the logarithm of the initial rate versus the logarithm of ionophore concentration yield slope values of 3.2, 3.0 and 1.0 for A23187, 4-BrA23187 and ionomycin respectively (data not shown), indicating the apparent stoichiometry of the transporting species are 3:1 for A23187 and 4-BrA23187 and 1:1 for ionomycin, ionophore : La\(^{3+}\). Although an expected value of 1:1 was obtained for ionomycin according to Eq. 14, the results for A23187 and 4-BrA23187 are surprising because at the internal La\(^{3+}\) concentration of ~ 16 mM (see Materials and Methods), a 1:1 stoichiometry would also be expected for both ionophores (Eq. 13).

Another observation that may be related to this finding is that the stoichiometry of the transporting species for Lu\(^{3+}\) uptake with A23187 and 4-BrA23187 is 3:1 when the initial external concentration of this lanthanide ion was at 15 µM (data not shown). Whereas, under the same conditions, as shown in
Fig. 20A, A23187 and 4-BrA23187 transport La\(^{3+}\) via a 2:1 stoichiometry. Solution studies indicate that Lu\(^{3+}\) and La\(^{3+}\) have the highest and the lowest stability constant, respectively, for 1:1 complexes between A23187 and the set of lanthanide ions chosen for selectivity study in Fig. 18. Thus, in both cases when a 3:1 transporting stoichiometry was seen, the equilibrium was shifted in the direction where the formation of 1:1 species was favored. In other words, the predominant number of the internal (La\(^{3+}\) release) or external (Lu\(^{3+}\) uptake) surface ionophore molecules exist as 1:1 complexes with lanthanide ions. As discussed in RESULTS (Fig. 25) that in the presence of POPC vesicles, Lu\(^{3+}\) tends to form a 1:1 species with A23187 at a much lower cation concentration.

Previously, in the study of Ca\(^{2+}\) transport by A23187, it was reported that the classical electroneutral model failed to explain the effect of Ca\(^{2+}\) concentration on the initial rate of transport (Erdhal et al., 1994). Two possible alternative formulations were given based on the assumption that the rate of transport is maximal when all the external ionophore exists as ACa\(^{+}\). Thus, the transporting species would be formed without equilibration with the Ca\(^{2+}\) and H\(^{+}\) in the external aqueous phase. Eqs 15 and 16 can be obtained by extrapolating the same assumption to lanthanide ions (Ln\(^{3+}\)).

\[
3ALn^{2+} \rightleftharpoons A, Ln + 2Ln^{3+} \tag{15}
\]

\[
ALn^{2+} + 2AH \rightleftharpoons A, Ln + 2H^{+} \tag{16}
\]
In Eq. 15, as described above, all three molecules of \( \text{ALn}^{2+} \) could be located at the internal (\( \text{La}^{3+} \) release) or external (\( \text{Lu}^{3+} \) uptake) interface. In Eq. 16, the ionophore molecule \( \text{AH} \) could be approaching the 1:1 species from the opposite side of the membrane.

**The selectivity of A23187**

Solution studies (Chapman et al, 1990) provide the relative stability constants for 1:1 complexes between A23187 and the lanthanide ions in order \( \text{Lu}^{3+} > \text{Yb}^{3+} > \text{Er}^{3+} > \text{Gd}^{3+} > \text{Eu}^{3+} > \text{Nd}^{3+} > \text{La}^{3+} \). This is the reversed sequence, except for \( \text{Nd}^{3+} \) and \( \text{La}^{3+} \), of A23187 transport selectivity for the same set of lanthanide ions (Table 7). This relationship suggests that higher stability for 1:1 complexes renders disproportionation, which in turn reduces the formation of the faster transporting species of 2:1 stoichiometry. As shown in Fig. 18A the disproportionation becomes less significant as the external lanthanide concentration drops below \(~ 3-4 \mu \text{M} \), where an apparent acceleration was seen in the transport of \( \text{Yb}^{3+} \) and \( \text{Lu}^{3+} \) by A23187.

**Conclusion**

The results indicate that all three \( \text{Ca}^{2+} \) ionophores transport lanthanide cations. The rate of transport is inversely dependent on the cation concentration. Accordingly, at low \( \text{Ln}^{3+} \) concentrations, the rates of \( \text{Ln}^{3+} \) and \( \text{Ca}^{2+} \) are comparable. Therefore, unrecognized \( \text{Ln}^{3+} \) transport could lead to misinterpretation of results when \( \text{Ln}^{3+} \) and \( \text{Ca}^{2+} \) ionophores are used together to investigate cell regulation by \( \text{Ca}^{2+} \). A single transporting species was found for
A23187 and 4-BrA23187, with a stoichiometry of 2:1 (ionophore : La\(^{3+}\)) at La\(^{3+}\) concentration range of 15-250 \(\mu\)M. The stoichiometry of 3:1 was obtained for Lu\(^{3+}\) at the cation concentration of 15 \(\mu\)M. Ionomycin can transport via two species, 2:1 and 1:1 stoichiometry, at low La\(^{3+}\) concentration, and via the 1:1 predominate species at high La\(^{3+}\) concentration.

Co-transport of OH\(^-\) by all three ionophores has been found as a result of the net exchange of 3H\(^+\) : 1La\(^{3+}\). The faster transport seen when a membrane potential is established, especially for 4-BrA23187, could reflect an electrogenic mode.
CHAPTER 9

Ca\textsuperscript{2+} TRANSPORT BY ETH 129

Although ETH 129 has been available for some time as the material for making Ca\textsuperscript{2+} selective electrode, this neutral ionophore has not been used as much in the investigation of cell regulation, due to the fact that many of its transport properties have not yet been reported. The goal of this project was to characterize its transport properties, which include the effect of membrane potential on the transport, the stoichiometry of the transporting species, the influence of Ca\textsuperscript{2+} concentration on the transport, the selectivity, and the influence of pH, etc.

The effect of trans-membrane potential

Since ETH 129 transports cations electrogenically, a potential imposed across the membrane (negative inside) should favor the translocation of ionophore-cation complex across the membrane. In fact, as will be seen soon, in the absence of such potential the transport is extremely slow, unless the $\Delta \psi$ is collapsed by valinomycin plus uncoupler (data not shown). In order to generate such a trans-membrane potential, the vesicles were prepared in a slightly
different way. The vesicle-forming medium contained 10 mM Hepes plus 100 mM KCl, and the pH was adjusted to 7.0 using ultra-pure KOH. The other conditions and procedures for vesicle preparations, described earlier, were still applied. Vesicles prepared in such a medium yielded only ~1/3 of Quin-2 entrapment of vesicles prepared in a regular way. The low Quin-2 entrapment was caused by high solute concentration in the vesicle-forming medium (Chapman et al. 1990). The internal K⁺ concentration was 125 mM, determined by atomic absorbance method (see Materials and Methods).

The influence of a pre-existing membrane potential on Ca²⁺ transport is shown in Fig. 27. The total concentration of K⁺ plus Na⁺ contained in the solution was 200 mM. Membrane potential of a varied magnitude was generated using different proportions of K⁺ and Na⁺ in the medium, plus 0.5 μM VAL. Experimental conditions for this set of data are given in the legend. The external K⁺ concentrations and the values of the corresponding membrane potential, which in turn was determined using TPP⁺ electrode, are also shown in the figure. Membrane potentials lower than 50 mV were not detectable. Ca²⁺ transport was measured on the DW2a dual beam spectrophotometer at wavelengths 264 vs 338. The relationship of the initial rate of transport and the membrane potential is shown in Fig. 27-b, which exhibits a linear relationship between Log initial rate and the membrane potential. The initial rate at 0 membrane potential was estimated as 0.411 nM/sec by extrapolating the straight line (obtained by linear regression). This value is surprisingly close to the initial rate of the slowest
Fig. 27 The Effect of Membrane Potential on Ca\textsuperscript{2+} Transport
Buffer contained 10 mM Hepes (pH 7.0), 200 mM (NaCl + KCl), 0.5 \( \mu \)M Valinomycin, 30 \( \mu \)M CaCl\textsubscript{2}. The membrane potential was measured using a TPP\textsuperscript{+} electrode. Ca\textsuperscript{2+} transport was monitored on a Aminco DW2a dual wavelength spectrophotometer. The concentrations of K\textsuperscript{+} in the medium are shown along with \( \Delta \psi \). Membrane potentials lower than 50 mV were unable to be detected.
curve in panel A, which is 0.416 nM/sec. This curve was obtained in the absence of Na⁺ and the external K⁺ was at 200 mM. Given such conditions no membrane potential was expected. The good agreement between the expected and experimental values suggests that from the initial rate value we may be able to identify small membrane potentials which are impossible to measure by TPP⁺ electrode.

Stoichiometry of the transporting species and the effect of external Ca²⁺ concentration

As elaborated in the Introduction, the theoretical model (Schefer et al. 1986) shows that a highly lipophilic Ca²⁺-ETH 129 complex is formed when Ca²⁺ is fitted inside the cavity formed by three ionophore molecules. By measuring membrane conductance of planar lipid bilayers Prestiino et al. (1993) has found that there is a 2.8-power dependency of membrane conductance on ETH 129 concentration. The log, log plot of initial rate against ETH 129 concentration shown in Fig. 28 has a slope value of 2.79, indicating that the stoichiometry of the transporting species is 3:1 (ETH 129 : Ca²⁺), which agrees with the theoretical model.

The influence of external Ca²⁺ concentration on the initial rate of transport has been also studied. The slope value from the log, log plot displayed in Fig. 29 is 0.71, which is lower than the predicted value 1. However, Prestipino et al. (1993) found in their work that the membrane conductance is proportional to the first power of Ca²⁺ concentration. One possible interpretation might be that fast
Fig. 28  Stoichiometry of the Transporting Species
The slope of the log-log plot is 2.8, indicating the stoichiometry of the transporting species is 3:1 (ionophore:Ca\(^{2+}\)).
Medium contained 10 mM Hepes (pH 7.0), 100 mM NaCl, 0.5 μM Valinomycin, 30 μM CaCl\(_2\). POPC concentration was 1.5 mM.
Fig. 29 The effect of Ca\(^{2+}\) Concentration on the Initial Rate of Transport. The medium contained 10 mM Hepes (pH 7.0), 100 mM NaCl, and 0.5 \(\mu\)M VAL. The ionophore concentration was 4.5 \(\mu\)M.
Ca\textsuperscript{2+} transport seen at high concentration caused the reduction of membrane potential, which in turn affected the transport. If this is correct, since at low Ca\textsuperscript{2+} concentration the effect was minimized, the slope of the log, log plot is therefore lowered as a result.

**Selectivity and pH effect**

Transport of seven different metal ions were measured and the result is displayed in Fig. 30 (Conditions are described in the legend). As shown in this figure, ETH 129 does not appear to have a strong discrimination or preference on any of the divalent cations selected. The seemingly fast transport in case of Pb\textsuperscript{2+} is actually from the contribution of both transport and leakage. Uncatalyzed diffusion through POPC vesicles can be ignored for most cations, however Pb\textsuperscript{2+} has been found to have substantial leakage. After removing the contribution from the leakage the actual Pb\textsuperscript{2+} transport also falls in the region of the other divalent cations. One the other hand, ETH 129 can transport La\textsuperscript{3+} faster than Ca\textsuperscript{2+} by more than two fold. The higher charge density on the trivalent cation and the electrogenic transport mode are responsible for the efficient transport of La\textsuperscript{3+}. As expected from the structure of the Ca\textsuperscript{2+}-ETH 129 complex, the transport of Ca\textsuperscript{2+} by ETH 129 is not affected substantially by the pH of the medium (Fig. 31). This property of ETH 129 differs from the carboxylic ionophores which have to undergo pH dependent procedures such as protonation and deprotonation in completing a transport cycle.

**Discussion**
Fig. 30  Selectivity of ETH 129
The medium contained 10 mM Hepes (pH 7.0), 100mM NaCl and 30 μM of each cation. The nominal POPC concentration was 1.5 mM.
Fig. 31 pH Insensitivity of the Transport. Buffer was composed of 5 mM Mes, 5 mM Hepes, 100 mM NaCl, 0.5 μM Valinomycin, 30 μM CaCl$_2$. The ionophore concentration was 4.5 μM. POPC concentration was 1.5 mM.
The effect of uncatalyzed \( H^+ \) diffusion has been mentioned previously in this dissertation. As shown in Fig. 32, this diffusion is accelerated in the presence of membrane potential and is pH dependent. The medium contained 100 mM NaCl and no ionophore was present. The membrane potential began to form at the point when VAL was added. The difference absorbance of Quin-2 started to change in response to a decreasing internal pH, which was in turn caused by uncatalyzed \( H^+ \) diffusion that was more rapid at low pH. Another finding regarding uncatalyzed \( H^+ \) diffusion is that in the presence of \( La^{3+} \) the process is accelerated substantially (data not shown). The interpretation for this phenomenon has been given previously in the Result for lanthanide transport.

**Conclusion**

The linear relationship was found between the logarithm of the initial rate and the membrane potential, which could be used to identify small membrane potentials that are not detectable using TPP\(^+\) electrode. The stoichiometry of the transporting species was found to be 3:1, which agrees with the theoretical prediction. However, the rate of transport does not exhibit a first order relation to \( Ca^{2+} \) concentration. In addition, the initial rate of \( Ca^{2+} \) transport does not exceed other divalent cations selected in this work by more than two fold. The transport of \( La^{3+} \) was found to be more efficient than the divalent cations.
Fig. 32  The pH effect of uncatalyzed H⁺ diffusion. POPC concentration was at 1.5 mM. The buffer contained 5 mM Hepes, 5 mM Mes, 100 mM NaCl, 30 μM CaCl₂.


99


65. Pfeiffer, D.R. and Lardy, H.A. Ionophore A23187. The Effect of H+ Concentration on Complex Formation with Divalent and Monovalent Cations and


