



1993

## Roles of Ion Channels in Nervous System Tumor Cell Growth

Yong Soo Lee  
*Loyola University Chicago*

Follow this and additional works at: [https://ecommons.luc.edu/luc\\_diss](https://ecommons.luc.edu/luc_diss)



Part of the [Physiology Commons](#)

---

### Recommended Citation

Lee, Yong Soo, "Roles of Ion Channels in Nervous System Tumor Cell Growth" (1993). *Dissertations*. 3119.  
[https://ecommons.luc.edu/luc\\_diss/3119](https://ecommons.luc.edu/luc_diss/3119)

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact [ecommons@luc.edu](mailto:ecommons@luc.edu).



This work is licensed under a [Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License](#).  
Copyright © 1993 Yong Soo Lee

**ROLES OF ION CHANNELS IN NERVOUS SYSTEM  
TUMOR CELL GROWTH**

by

**YONG SOO LEE**

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University  
of Chicago in Partial Fulfillment of the Requirements for  
the Degree of Doctor of Philosophy

May

1993

Copyright by Yong Soo Lee, 1993

All rights reserved.

## ACKNOWLEDGEMENTS

I would like to express sincere gratitude to my advisor, Dr. Robert D. Wurster for his invaluable advise, encouragement and patience. Without his knowledgeable insight and guidance this dissertation would not have been possible. His philosophy and dedication to the principles of scientific training and enthusiasm have been an inspiration to me and will surely remain so in the future.

I would also like to thank my committee members, Drs. Mohammed M. Sayeed, Thomas C. Origitano, Russell Pieper and Ronald R. Fiscus for their selfless time and effort, expert advise and valuable criticism. The technique guidance for intracellular  $Ca^{2+}$  measurements by Dr. Sayeed and for cell cultures by Ms. Maria Weber is greatly appreciated.

My enormous gratitude goes to all the faculty members, staff and graduate students in the Department of Physiology for their innumerable help and support during the course of my training.

## VITA

The author, Yong Soo Lee, is the son of Bok Yul Choi and Ki Sup Lee. He was born on June 14, 1962 in Sangju, Republic of Korea.

He attended elementary and middle schools in Sangju, and graduated from Kyung Nam High School in Pusan in February, 1980. In March, 1980, he entered Pusan National University in Pusan, Korea. He received a Bachelor of Science in Pharmacy degree in February, 1984. In March, he entered a master degree program in Physical Pharmaceutics at Pusan National University and earned a Master of Science degree in February, 1986. After completing 6 months of military service, he entered a doctoral degree program and completed two years of the three year program.

In August, 1989, Yong Soo entered the graduate program in the Department of Physiology at Loyola University of Chicago Stritch School of Medicine. His dissertation work was completed under the direction of Dr. Robert D. Wurster.

The author married Jung Ae Kim and they have a daughter, Joo Yun. Jung Ae is a graduate student in the Neuroscience program at Loyola.

## PUBLICATIONS

1. Suk Kyu Han, Nam Hong Kim and **YONG SOO LEE**. 1986. Effect of phenothiazine derivatives on the thermotropic phase transition of liposomal phospholipid membrane. *Arc. Pharma. Res.*9:75-79.
2. Suk Kyu Han and **YONG SOO LEE**. 1986. Fluorescence probe study on the solubilization sites of aniline derivatives in Triton X-100 and Zephiramine micelles. *Arc. Pharma. Res.* 9:139-144.
3. Suk Kyu Han, Jin Suk Kim, **YONG SOO LEE** and Min Kim. 1990. Effect of drug substances on the microviscosity of lipid bilayer of liposomal membrane. *Arc. Pharma. Res.* 13:192-197.
4. **LEE, Y.S.**, Weber, M. and Wurster, R.D. 1992. Roles of  $Ca^{2+}$  and  $K^{+}$  in nervous system tumor cell growth. *Society of Neuroscience*. 18:1360 (Abstract).
5. **YONG SOO LEE** and Robert D. Wurster. 1993. Differential effects of methionine enkephalin on the growth of brain tumor cells. (Submitted).
6. **YONG SOO LEE**, Mohammed M. Sayeed and Robert D. Wurster. 1993. A receptor-operated  $Ca^{2+}$  channel blocker inhibits brain tumor cell growth by affecting intracellular  $Ca^{2+}$  mobilization. (Submitted).
7. **YONG SOO LEE**, Mohammed M. Sayeed and Robert D. Wurster. 1993. Inhibition of cell growth by  $K^{+}$  channel modulators is due to interference with agonist-induced  $Ca^{2+}$  release. (Submitted).
8. **YONG SOO LEE**, Mohammed M. Sayeed and Robert D. Wurster. 1993. Alteration of brain tumor cell growth and agonist-induced intracellular  $Ca^{2+}$  liberation by  $Ca^{2+}$  channel antagonists. (In preparation).

## TABLE OF CONTENTS

|   | <u>Page</u> |
|---|-------------|
| ACKNOWLEDGEMENT   | iii         |
| VITA  | iv          |
| PUBLICATIONS  | v           |
| TABLE OF CONTENTS   | vi          |
| LIST OF FIGURES   | x           |
| LIST OF TABLE   | xiv         |
| LIST OF ABBREVIATIONS   | xv          |
| <u>CHAPTER</u>  |             |
| I. INTRODUCTION   | 1           |
| II. LITERATURE REVIEW   | 3           |
| A. Roles of Cell Proliferation in Carcinogenesis                      | 3           |
| B. Signal Transduction Mechanisms in Cell Proliferation               | 6           |
| Receptor Tyrosine Kinases   | 7           |
| Phosphatidylinositol Turnover   | 8           |
| IP <sub>3</sub> and Calcium   | 10          |
| Diacylglycerol and Protein Kinase C                                   | 11          |
| Cyclic AMP  | 13          |
| Na <sup>+</sup> -H <sup>+</sup> Antiport and Cytoplasmic Alkalization | 14          |
| Expression of <i>c-fos</i> and <i>c-myc</i> Proto-Oncogenes           | 16          |
| Polyamines and Ornithine Decarboxylase                                | 18          |
| C. Role of Calcium in Cell Proliferation                              | 20          |
| Regulation of Intracellular Calcium                                   | 20          |
| a) Calcium Channels   | 20          |

|   |    |
|---|----|
| b) Release from Intracellular Calcium Stores  | 21 |
| c) Extrusion Calcium from the Cytoplasm   | 23 |
| Effector Systems Regulated by Intracellular Calcium   | 23 |
| Role of Calcium in Normal Cell Proliferation  | 25 |
| Calcium Messenger Systems in Tumor Cell Proliferation   | 27 |
| <br>  |    |
| III. MATERIALS AND METHODS  | 31 |
| <br>  |    |
| A. Cell Culture   | 31 |
| Cell Line Characteristics   | 31 |
| a) U-373 MG human astrocytoma   | 31 |
| b) SK-N-MC human neuroblastoma  | 31 |
| Cell Growth   | 32 |
| a) Culture Medium   | 32 |
| b) Initial Culture  | 33 |
| c) Culture Maintainance   | 34 |
| <br>  |    |
| B. Cell Growth and Cell Cytotoxicity Assay  | 34 |
| Cell Growth Assay   | 34 |
| a) Initial Seeding  | 34 |
| b) Drug Treatment   | 35 |
| c) Cell Counting  | 36 |
| d) Effect of Carbachol on Astrocytoma   |    |
| Cell Growth   | 37 |
| Cell Cytotoxicity Assay   | 38 |
| Data Analysis   | 39 |
| <br>  |    |
| C. Measurement of Intracellular Calcium Concentration   | 39 |
| Solutions   | 39 |
| a) Fura-2-AM  | 39 |
| b) Krebs-Ringer Buffer  | 40 |
| c) Triton X-100   | 40 |
| d) EGTA Solution  | 41 |
| Procedure   | 41 |
| a) Preparation of Aliquot of Tumor Cells  | 41 |
| b) Loading Fura-2-AM  | 41 |
| c) Removing Unloaded Fura-2-AM  | 42 |
| d) Measurement of Intracellular Calcium   | 42 |
| Data Analysis   | 43 |
| <br>  |    |
| IV. MECHANISM OF Ca <sup>2+</sup> CHANNEL ANTAGONIST-INDUCED<br>INHIBITION OF TUMOR CELL GROWTH | 45 |



|   | <u>Page</u> |
|---|-------------|
| A. Introduction   | 45          |
| B. Results  | 48          |
| Extracellular Calcium Dependency of Tumor<br>Cell Growth  | 48          |
| Calcium Channel Antagonist-Induced Inhibition<br>of Tumor Cell Growth   | 52          |
| A Receptor-Operated Calcium Channel Blocker,<br>SK&F 96365 Is a Potent Inhibitor of<br>Tumor Cell Growth  | 60          |
| The Relationship Between Carbachol-Induced Increased<br>Intracellular Calcium and Tumor Cell Growth in<br>U-373 MG Human Astrocytoma Cell Line  | 61          |
| a) Carbachol-Induced Intracellular Calcium<br>Increase Is not Dependent upon<br>Extracellular Calcium Concentration   | 65          |
| b) Carbachol-Induced Increased Intracellular<br>Calcium Concentration Is<br>Dose-Dependent and Muscarinic<br>Receptor-Mediated  | 67          |
| c) Carbachol-Induced Increased Tumor Cell<br>Growth Is Dose-Dependent   | 67          |
| Serum-Induced Intracellular Calcium Increase Is<br>Dose-Dependent and not Dependent upon<br>Extracellular Calcium Levels in<br>SK-N-MC Human Neuroblastoma Cell Line                    | 72          |
| Inhibition of an Agonist-Induced Increased Intracellular<br>Calcium Concentration by Calcium Channel<br>Antagonists   | 75          |
| The Inhibition of Agonist-Induced Intracellular Calcium<br>Responses by Calcium Channel Antagonists Is<br>not Related to Their Effects on Basal Levels<br>of Free Intracellular Calcium | 81          |
| C. Discussion   | 82          |
| Signal Transductional Role of Intracellular Calcium<br>Concentration from Internal Store Release<br>in Tumor Cell Proliferation   | 86          |
| Proposed Mechanism of Inhibitory Actions of Calcium<br>Channel Antagonists in Tumor Cell Growth   | 87          |

|     |   |     |
|-----|---|-----|
| V.  | MECHANISM OF K <sup>+</sup> CHANNEL MODULATOR-INDUCED INHIBITION OF TUMOR CELL GROWTH                                     | 93  |
|     | A. Introduction   | 93  |
|     | B. Results  | 95  |
|     | Inhibition of Tumor Cell Growth by High Extracellular Potassium   | 95  |
|     | Potassium Channel Modulator-Induced Inhibition of Tumor Cell Growth   | 97  |
|     | No Voltage-Dependent Calcium Channel Are Present in U-373 MG Human Astrocytoma and SK-N-MC Human Neuroblastoma Cell Lines | 106 |
|     | Inhibition of Agonist-Induced Release of Calcium from the Internal Stores by Potassium Channel Modulators                 | 106 |
|     | Potassium Channel Modulators do not Affect on the Basal, Free Cytosolic Calcium Concentrations in Tumor Cells             | 107 |
|     | C. Discussion   | 112 |
|     | Proposed Mechanism of Inhibitory Effects of Potassium Channel Modulators on Tumor Cell Growth                             | 112 |
| VI. | SUMMARY AND CONCLUSIONS   | 119 |
|     | REFERENCES  | 124 |

## LIST OF FIGURES

|  | <u>Page</u> |
|--|-------------|
| Figure 4.1: Effect of EGTA on the growth of U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines                                | 49          |
| Figure 4.2: Effect of high extracellular $\text{Ca}^{2+}$ on the growth of U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines | 51          |
| Figure 4.3: Effect of $\text{Ca}^{2+}$ channel antagonists on the growth of U-373 MG human astrocytoma cell lines                                | 53          |
| Figure 4.4: Effect of $\text{Ca}^{2+}$ channel antagonists on the growth of SK-N-MC human neuroblastoma cell lines                               | 54          |
| Figure 4.5: Effect of inorganic $\text{Ca}^{2+}$ channel antagonists on the growth of U-373 MG human astrocytoma cell lines                      | 57          |
| Figure 4.6: Effect of inorganic $\text{Ca}^{2+}$ channel antagonists on the growth of SK-N-MC human neuroblastoma cell lines                     | 58          |
| Figure 4.7: Effect of Bay K-8644 on the growth of U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines                          | 59          |
| Figure 4.8: Effect of SK&F 96365 on the growth of U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines                          | 62          |
| Figure 4.9: Cytotoxic effect of EGTA or $\text{Ca}^{2+}$ channel antagonists on U-373 MG human astrocytoma cell line                             | 63          |
| Figure 4.10: Cytotoxic effect of EGTA or $\text{Ca}^{2+}$ channel antagonists on SK-N-MC human neuroblastoma cell line                           | 64          |
| Figure 4.11: Extracellular $\text{Ca}^{2+}$ concentration independency of  |             |

|   |    |
|---|----|
| carbachol (0.8mM)-induced increased intracellular Ca <sup>2+</sup> concentration in U-373 human astrocytoma cell line   | 66 |
| Figure 4.12: Dose-dependent increase of intracellular Ca <sup>2+</sup> concentration by carbachol in U-373 MG human astrocytoma cell line   | 68 |
| Figure 4.13: Effect of carbachol on intracellular Ca <sup>2+</sup> concentration in U-373 MG human astrocytoma cell line  | 69 |
| Figure 4.14: Abolishment of carbachol (0.8mM)-induced increased intracellular Ca <sup>2+</sup> concentration by the pretreatment of atropine (100M) in U-373 MG human astrocytoma cell line | 70 |
| Figure 4.15: Effect of carbachol on the growth of U-373 MG human astrocytoma cell lines   | 71 |
| Figure 4.16: Dose-dependent increase of intracellular Ca <sup>2+</sup> concentration by serum in SK-N-MC human neuroblastoma cell line  | 73 |
| Figure 4.17: Effect of EGTA (1mM) on serum (2%)-induced increased intracellular Ca <sup>2+</sup> concentration in SK-N-MC human neuroblastoma cell line                                     | 74 |
| Figure 4.18: Effect of Ca <sup>2+</sup> channel antagonists on carbachol (0.8mM)-induced intracellular Ca <sup>2+</sup> mobilization in U-373 MG human astrocytoma cell line                | 77 |
| Figure 4.19: Quantitative changes of carbachol (0.8mM)-induced intracellular Ca <sup>2+</sup> mobilization by Ca <sup>2+</sup> channel antagonists in U-373 MG astrocytoma cell line        | 78 |
| Figure 4.20: Effect of Ca <sup>2+</sup> channel antagonists on serum (2%)-induced intracellular Ca <sup>2+</sup> mobilization in SK-N-MC human neuroblastoma cell line                      | 79 |
| Figure 4.21: Quantitative changes of serum (2%)-induced intracellular Ca <sup>2+</sup> mobilization by Ca <sup>2+</sup> channel antagonists in SK-N-MC human neuroblastoma cell line        | 80 |

|              |   |     |
|--------------|---|-----|
| Figure 4.22: | No effect of Ca <sup>2+</sup> channel antagonists on basal, free cytosolic Ca <sup>2+</sup> concentration in U-373 MG human astrocytoma cell line   | 83  |
| Figure 4.23: | No effect of Ca <sup>2+</sup> channel antagonists on basal, free cytosolic Ca <sup>2+</sup> concentration in SK-N-MC human neuroblastoma cell line  | 84  |
| Figure 4.24: | Proposed inhibitory mechanisms of Ca <sup>2+</sup> channel antagonists in tumor cell growth   | 92  |
| Figure 5.1:  | Effect of high extracellular K <sup>+</sup> on the growth of U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines  | 96  |
| Figure 5.2:  | Effect of K <sup>+</sup> channel modulators on the growth of U-373 MG human astrocytoma cell lines  | 98  |
| Figure 5.3:  | Effect of K <sup>+</sup> channel modulators on the growth of SK-N-MC human neuroblastoma cell lines   | 99  |
| Figure 5.4:  | No cytotoxic effect of K <sup>+</sup> channel modulators on U-373 MG human astrocytoma cell line  | 101 |
| Figure 5.5:  | No cytotoxic effect of K <sup>+</sup> channel modulators on SK-N-MC human neuroblastoma cell line   | 102 |
| Figure 5.6:  | Effect of veratridine and TTX on the growth of U-373 MG human astrocytoma cell lines  | 104 |
| Figure 5.7:  | Effect of veratridine and TTX on the growth of SK-N-MC human neuroblastoma cell lines   | 105 |
| Figure 5.8:  | Effect of high extracellular K <sup>+</sup> or K <sup>+</sup> channel modulators on carbachol (0.8mM)-induced intracellular Ca <sup>2+</sup> mobilization in U-373 MG human astrocytoma cell line | 108 |
| Figure 5.9:  | Effect of high extracellular K <sup>+</sup> or K <sup>+</sup> channel modulators on serum (2%)-induced intracellular Ca <sup>2+</sup> mobilization in SK-N-MC human neuroblastoma cell line       | 109 |

|  |     |
|--|-----|
| Figure 5.10: Quantitative changes of carbachol (0.8mM)-induced intracellular Ca <sup>2+</sup> mobilization by high extracellular K <sup>+</sup> or K <sup>+</sup> channel modulators in U-373 MG human astrocytoma cell line | 110 |
| Figure 5.11: Quantitative changes of serum (2%)-induced intracellular Ca <sup>2+</sup> mobilization by high extracellular K <sup>+</sup> or K <sup>+</sup> channel modulators in SK-N-MC human neuroblastoma cell line       | 111 |
| Figure 5.12: No effect of high extracellular K <sup>+</sup> or K <sup>+</sup> channel modulators on basal, free cytosolic Ca <sup>2+</sup> concentration in U-373 MG human astrocytoma cell line                             | 113 |
| Figure 5.13: No effect of high extracellular K <sup>+</sup> or K <sup>+</sup> channel modulators on basal, free cytosolic Ca <sup>2+</sup> concentration in SK-N-MC human neuroblastoma cell line                            | 114 |
| Figure 5.14: Proposed inhibitory mechanisms of high extracellular K <sup>+</sup> or K <sup>+</sup> channel modulators in tumor cell growth   | 118 |

## LIST OF TABLE

|   | <u>Page</u> |
|---|-------------|
| Table 4.1: Basal, free intracellular $\text{Ca}^{2+}$ concentrations of tumor cells | 85          |

## LIST OF ABBREVIATIONS

|               |                                       |
|---------------|---------------------------------------|
| AM            | acetoxymethyl esters                  |
| AMP           | adenosine 5'-monophosphate            |
| ANOVA         | one way analysis of variance          |
| 4-AP          | 4-aminopyridine                       |
| ATCC          | American Type Culture Collection      |
| ATP           | adenosine 5'-triphosphate             |
| ATPase        | adenosine 5'-triphosphatase           |
| $Ca^{2+}_i$   | intracellular $Ca^{2+}$               |
| $[Ca^{2+}]_i$ | intracellular $Ca^{2+}$ concentration |
| CaM           | calmodulin                            |
| Carb          | carbachol                             |
| CHO           | Chinese hamster ovary                 |
| ConA          | concanavalin A                        |
| CSF-1         | colony stimulating factor-1           |
| cyclic AMP    | cyclic adenosine 3',5'-monophosphate  |
| DAG           | diacylglycerol                        |
| DMFO          | $\alpha$ -difluoromethylornithine     |
| DMSO          | dimethyl sulfoxide                    |
| DNA           | deoxyribonucleic acid                 |



|                  |   |
|------------------|---|
| EBSS             | Earle's basal salt solution                                     |
| EBV              | Epstein-Barr virus  |
| EC <sub>50</sub> | half-maximal effective concentration                            |
| EGF              | epidermal growth factor   |
| EGTA             | ethylene glycol-bis-(aminoethylether)N,N,N',N'-tetraacetic acid |
| E <sub>m</sub>   | membrane potential  |
| ER               | endoplasmic reticulum   |
| FBS              | fetal bovine serum  |
| FGF              | fibroblast growth factor  |
| GFAP             | glial fibrillary acidic protein                                 |
| GM-CSF           | granulocyte-macrophage colony-stimulating factor                |
| GMP              | guanosine 5'-monophosphate                                      |
| GTP              | guanosine 5'-triphosphate                                       |
| GTPase           | guanosine 5'-triphosphatase                                     |
| G protein        | GTP binding protein   |
| HBV              | hepatitis B virus   |
| HEPES            | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid              |
| HPV              | human papilloma virus   |
| IGF-II           | insulin-like growth factor-II                                   |
| IL 2             | interleukin 2   |
| IP <sub>3</sub>  | inositol 1,4,5-triphosphate                                     |
| IP <sub>4</sub>  | inositol 1,3,4,5-tetrakisphosphate                              |

|                  |  |
|------------------|--|
| KRB              | Krebs-Ringer buffer                      |
| MDR              | multidrug resistance                     |
| MEM              | Eagle's minimum essential medium         |
| $\mu$ l          | microliter                               |
| ml               | milliliter                               |
| mm               | millimeter                               |
| $M_r$            | molecular weight                         |
| nm               | nanometer                                |
| NMDA             | N-methyl-D-aspartate                     |
| OAG              | 1-oleyl-2-acetylglycol                   |
| ODC              | ornithine decarboxylase                  |
| PDGF             | platelet derived growth factor           |
| PI3-kinase       | phosphatidylinositol 3-kinase            |
| PIP <sub>2</sub> | phosphatidylinositol 4,5-bisphosphate    |
| PKC              | protein kinase C                         |
| PLC              | phospholipase C                          |
| PMA              | phorbol-12,13-myristate acetate          |
| PPI              | polyphosphoinositide                     |
| PRG              | primary response gene                    |
| RMCE             | receptor-mediated Ca <sup>2+</sup> entry |
| RNA              | ribonucleic acid                         |
| RTK              | receptor tyrosine kinase                 |

|      |                                       |
|------|---------------------------------------|
| SEM  | standard error of the mean            |
| SH   | <i>src</i> homology                   |
| TEA  | tetraethylammonium chloride           |
| TPA  | 12-O-tetradecanoyl phorbol-13-acetate |
| TSH  | thyroid stimulating hormone           |
| TRE  | TPA response element                  |
| TTX  | tetradotoxin                          |
| VSCC | voltage sensitive calcium channel     |

# CHAPTER I

## INTRODUCTION

Cancer is one of the leading causes of death in the United States, in spite of advances in cancer prevention and therapy. Recently, ion channels have been shown to be involved in the proliferative signalling mechanisms in various cell types. This dissertation is designed to investigate the roles of  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channels in tumor cell growth. Two frequently studied brain tumor cells were selected in this study: U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines.

In order to study the roles of  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channels in the growth of tumor cells, a pharmacological approach was taken, i.e. the effects of various these ion channel agonists and antagonists on tumor cell growth were examined. Assessment of tumor cell growth was made by counting the number of cells using a hemacytometer. For clarifying the mechanism of action of these drugs, their cytotoxicity was assayed by the trypan blue exclusion method.

Intracellular  $\text{Ca}^{2+}$  is known to modulate a variety of cellular functions including cell proliferation. The effects of growth-promoting agents on intracellular  $\text{Ca}^{2+}$  concentrations were studied to ascertain the role of intracellular  $\text{Ca}^{2+}$  in the proliferative signalling mechanisms in the tumor cell lines. Moreover, since  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channel activities may regulate directly or indirectly intracellular  $\text{Ca}^{2+}$ , the effects of these ion channel modulators on the basal and agonist-induced elevations in intracellular  $\text{Ca}^{2+}$  concentrations were investigated in order to elucidate the mechanism of action of these drugs in the tumor cell growth. Intracellular  $\text{Ca}^{2+}$  concentrations were measured by

fluorophotometric methods using Fura-2 dye.

The results of this dissertation show that growth-promoting agents caused elevations in intracellular  $\text{Ca}^{2+}$  concentration in a dose-dependent manner and that these increases were due to release of  $\text{Ca}^{2+}$  from intracellular stores. Further, the results illustrate that  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channel modulators inhibited tumor cell growth in a dose-related fashion and reduced agonist-induced increased intracellular  $\text{Ca}^{2+}$  concentrations. These drugs did not induce significant alterations in basal intracellular  $\text{Ca}^{2+}$  concentration. The results suggest that an increase in intracellular  $\text{Ca}^{2+}$  concentration is an important proliferative signalling mechanisms and that the growth-inhibitory effects of ion channel modulators are at least partly due to the blockade of agonist-induced increase in intracellular  $\text{Ca}^{2+}$  concentration in the tumor cell lines used in this study.

This dissertation contributes to the understanding of the roles of ion channels as targets of modulation of tumor cell growth. This information may be valuable to the field of cancer therapy. The role of agonist-induced  $\text{Ca}^{2+}$  mobilization from intracellular stores without  $\text{Ca}^{2+}$  influx in tumor cell proliferation supports the notion of the existence of a diversity of cellular responses that are mediated by intracellular  $\text{Ca}^{2+}$  signalling.

## CHAPTER II

### LITERATURE REVIEW

#### A. Role of Cell Proliferation in Carcinogenesis

Mounting evidence strongly supports the contention developed in 1914 that cancer results from genetic alterations (Boveri, 1914). Utilizing molecular biologic techniques, distinct genetic alterations have been identified in several types of cancers. These changes involve the activation of cellular oncogenes and the inactivation of tumor suppressor genes (Bishop, 1991). However, many etiologic agents (e.g. peroxisome proliferators, dioxin, estrogens, cyproterone and phenobarbital) do not directly cause genetic damage (Green, 1992). Similarly, some environmental agents associated with cancers do not directly damage DNA. Thus, although genetic damage is most likely an eventual common pathway to the development of cancer, other pivotal mechanisms contribute to carcinogenesis (Aaronson, 1991; Ames and Gold, 1990; Goustin *et al.*, 1986). Moreover, various genetic changes can occur only during cell division (Preston-Martin *et al.*, 1990).

Although cancer arises from defective control of cell proliferation, the etiologic and pathogenetic role of cell proliferation has received relatively little attention. Nevertheless, as early as 1953, Nordling (1953) stated that the likelihood that certain types of cancers would develop could be greatly augmented by sustaining cell proliferation of the target tissue. A decade ago, a specific role for cell proliferation was integrated into a carcinogenesis model developed by Moolgavkar and coworkers (Moolgavkar and

Knudson, 1981; Moolgavkar *et al.*, 1990), which was derived from epidemiologic data. Greenfield *et al.* (1984) and Cohen and Ellwein (1990) formulated a biologically similar model, using data from animal experiments. Both models quantify genetic and proliferative events and thus, offer insight into assessments dealing with the risk of developing cancer. In the context of these models, an agent can alter the likelihood of developing a cancer in only two ways: it can increase the probability of irreversible genetic damage occurring during cell division; and/or it can increase cell proliferation, usually accompanied by an increased cell number, and consequently increased number of opportunities for spontaneous genetic damage.

Increased cell proliferation appears to be necessary for hormonally related tumors (Henderson *et al.*, 1982). Hormones govern a number of cellular functions including cell proliferation and growth. Clinical and epidemiologic studies demonstrate that sustained hormonal stimulation and consequent enhanced cell proliferation result in estrogen-dependent endometrial (Ziel and Finkle, 1975) and breast carcinomas (Russo *et al.*, 1990), thyroid stimulating hormone (TSH)-dependent thyroid tumors (Hill *et al.*, 1989) and androgen and estrogen interactions in the development of prostatic cancer (Bosland, 1988). Chronic increased cell proliferation induced by estrogen also increases the appearance of benign and malignant hepatocellular tumors in experimental animals and humans (Barrows *et al.*, 1988).

Most virally related human tumors are also a result of sustained increased proliferation. Some RNA retroviruses can produce cancer without carrying a specific oncogenes as part of their RNA (Varmus, 1988) by increasing the proliferation of the

target tissue. Transmission of virus occurs from cell to cell, eventually resulting in the interposition of virally generated DNA next to a cellular oncogene. Thus, sustained cell proliferation and, ultimately, tumors can arise. Several DNA viruses, including hepatitis B virus (HBV) (Beasley, 1988; Dunsford, 1990), human papilloma virus (HPV) (zur Hausen, 1989) and Epstein-Barr virus (EBV) (Henderson, 1989) are associated with certain type of cancers in humans. In each instance the development of the malignancies results from a sustained proliferation of the target cells. For example, HBV-related hepatocarcinogenesis is probably not related directly to a specific oncogenic DNA alteration induced by the virus itself. Transgenic mice that overproduce the large envelope polypeptide of HBV, accumulate hepatitis B surface antigen and develop chronically active hepatitis, regenerative nodules and ultimately hepatomas (Dunsford, 1990). This protein has none of the characteristics of oncogenes or tumor suppressor genes, but rather appears to be involved in the development of hepatocellular necrosis, chronic active hepatitis and sustained, increased hepatocyte proliferation.

Increased cell proliferation also appears to contribute to the development of tumors secondary to various chemical exposures in humans, including cigarette smoking, snuff use, betel-quid chewing, aromatic amines, polycyclic aromatic hydrocarbons and nitrosamines (IARC monograph, 1975). Cigarette smoking is known to cause bladder cancer in humans, perhaps due to a hyperplastic effect on the urothelium of many cigarette smokers, in addition to the probable genotoxic damage that occurs (Auerbach and Garfinkel, 1989).



## **B. Signal Transduction Mechanisms in Cell Proliferation**

Extracellular factors such as hormones, growth factors, cytokines and the extracellular matrix appear to play a decisive role in determinations whether the cell will proliferate or differentiate (Rozenfurt, 1989; Rozenfurt, 1986; Rozenfurt and Ober, 1990). In order to understand how cell proliferation is controlled, it is necessary to identify the key pathways that convey extracellular signals to the nuclei, where repression of the differentiation program and induction of essential genes needed for cell cycle progression and replication are executed. The components of the pathways include the signalling network, from growth factors, growth factor-receptor tyrosine kinases, cytoplasmic protein kinases, GTP-binding proteins, to nuclear transcription factors (Karin, 1992). The signalling pathways show multiplicity as is illustrated by the cooperation between oncogenes (Hunter, 1991) or by the synergy observed between growth promoting agents (Rozenfurt and Mendoza, 1985; Whitfield *et al.*, 1987). Although cooperation between distinct classes of growth factors appears to be the rule, some mitogens are so effective that they can act alone (Pouyssegur and Seuwen, 1992).

One of the major incentives for studying factors that regulate the processes of proliferation and differentiation is the recognition of their potential contribution to tumorigenesis - a premise that has been reinforced by the detection of growth factor secretion by many types of tumor cell (Gross *et al.*, 1990; Herlyn and Malkowicz, 1991).

Growth factors initiate their action by binding to specific cell surface receptors. These activated receptors mediate a cascade of rapid biochemical and physiological

changes in the cell, which ultimately lead to DNA synthesis and cell proliferation (Reviewed in Rozengurt, 1986; Rozengurt, 1989). One of the immediate consequences of growth factor-receptor interaction is protein phosphorylation. The receptors for growth factors, like epidermal growth factor (EGF) and platelet derived growth factor (PDGF), are transmembrane glycoproteins that possess intrinsic and ligand-stimulated protein tyrosine kinase activity. This intrinsic tyrosine-specific kinase activity is shared with several viral oncogene products, such as the transforming protein of Rous sarcoma virus. Other immediate consequences of receptor activation include cytoplasmic alkalization via activation of the  $\text{Na}^+/\text{H}^+$  antiport and increases in intracellular cyclic AMP levels, and the degradation of phosphoinositides to produce inositol triphosphates ( $\text{IP}_3$ ) and diacylglycerol (DAG). These degradation products, in turn, affect the release of  $\text{Ca}^{2+}$  ions from intracellular stores and the activation of protein kinase C (PKC), respectively.

### ***Receptor Tyrosine Kinases***

A large number of serum growth factors stimulate cell proliferation by interaction with a family of cell-surface receptors that possess an intrinsic, ligand-sensitive, protein tyrosine kinase activity (Carpenter, 1992; Yarden, 1988). These receptor tyrosine kinases (RTKs) comprise an extracellular ligand-binding domain that is linked directly to a cytoplasmically oriented, catalytic domain, which not only transduces a hormonal signal but also generates a biochemical message. The RTKs remain active following incorporation of growth factor-receptor complexes into endocytic vesicles, suggesting that

internalization of activated kinase may allow its interaction with cytoplasmic substrates (Cohen and Fava, 1985).

Three proteins that serve as tyrosine kinase substrates are related by the presence of sequence motifs termed SH, or *src* homology, domains (Koch *et al.*, 1991). Receptor tyrosine kinase substrates that contain SH regions do require localization at the cytoplasmic face of the membrane for activity. Two of these are enzymes that modulate the metabolism of phosphoinositides: phospholipase-C- $\gamma$ 1 (PLC- $\gamma$ 1) and phosphatidylinositol 3-kinase (PI3-kinase). Third substrate, termed *ras*GAP, is a molecule that modulates the GTPase activity of *ras*.

Tyrosine kinase substrates seem likely to have key roles in the signal transduction pathway that regulates cell proliferation. For example, by means of removing effectively tyrosine phosphorylation sites in PLC- $\gamma$ 1, this site-directed mutagenesis abrogated growth factor-induced inositol phosphate formation and reduced the growth factor response by 50% (Kim *et al.*, 1991). In addition, an involvement of tyrosine phosphorylation in proliferation is supported by data showing that vanadate, an inhibitor of tyrosine phosphatases (Swarup *et al.*, 1982), can enhance growth factor-induced DNA synthesis (Smith, 1983).

### ***Phosphatidylinositol Turnover***

Some growth factors also stimulate intramembrane lipid metabolism (Berridge, 1987). The activation of two distinct classes of receptors (G protein-coupled and intrinsic tyrosine kinase receptors) evokes the apparently identical response (i.e.

polyphosphoinositide (PPI) hydrolysis with marked preference for phosphatidylinositol 4,5-bisphosphate,  $\text{PIP}_2$ ) through the activation of PLC.

Four distinct families of PLC are known, classified as  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  by Rhee and associates (Rhee *et al.*, 1989). Of these,  $\beta 1$  has recently been identified as the isoform activated by hormones and neurotransmitters via the pertussis toxin-insensitive G proteins of the  $G_q$  family (Sternweis and Smrcka, 1992). Other PPI hydrolysis-coupled receptors operate via pertussis toxin-sensitive G proteins, however, and in this case the specific PLC involved has not been identified. In contrast to the others, the PLC- $\gamma 1$  isoform is unable to interact with G proteins but rather binds to and is tyrosine phosphorylated by both the epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptors (Carpenter, 1992). Tyrosine phosphorylation of the PLC- $\gamma 1$  isoform appears to permit the interaction of the enzyme with its substrates.

Functional significance of this signalling pathway can arise from the fact that drugs which act as tumor promoters, such as phorbol esters and thapsigargin, are known to work by mimicking the effects of second messengers generated by PPI hydrolysis. Phorbol esters and thapsigargin can exert their action by stimulation of PKC and discharge of the  $\text{Ca}^{2+}$  stores sensitive to  $\text{IP}_3$  via blockade of the store-specific  $\text{Ca}^{2+}$ -ATPase, respectively (Nishizuka, 1986; Thastrup *et al.*, 1990)). Moreover, in cultures of rodent fibroblasts, growth is stimulated by overexpression of PKC (Housey *et al.*, 1988) or 5-HT $_1c$  receptor coupled to PPI hydrolysis via G proteins (Julius *et al.*, 1989). The cell growth is also increased by overstimulation of a G protein-coupled receptor (e.g. the bombesin receptor (Rozenfurt and Sinnett-Smith, 1983)). Finally, microinjection of

either PLC ( $\beta$  or  $\gamma 1$ ) or antibodies against PLC- $\gamma 1$  in NIH 3T3 cells induced bursts of cell division or blocked cell growth, respectively (Meldolesi and Magni, 1991).

### *IP<sub>3</sub> and Calcium*

IP<sub>3</sub> induces the release of Ca<sup>2+</sup><sub>i</sub> from the Ca<sup>2+</sup> storing pool, i.e., the endoplasmic reticulum (Berridge, 1987). Addition of PDGF to cells induces a rapid and transient rise in Ca<sup>2+</sup><sub>i</sub> (Moolenaar *et al.*, 1986; Villereal and Byron, 1992). This response does not require external Ca<sup>2+</sup> and, thus, presumably is mediated by IP<sub>3</sub> which is also increased in PDGF-treated cells. Elevations in cytosolic Ca<sup>2+</sup> in response to EGF have been detected in several cell lines (Moolenaar *et al.*, 1986; Villereal and Byron, 1992) in most cases, removal of Ca<sup>2+</sup> from extracellular medium abolished this effect, suggesting that EGF increases cellular Ca<sup>2+</sup> by enhancing Ca<sup>2+</sup> uptake. Thus, PDGF and EGF may modulate Ca<sup>2+</sup><sub>i</sub> levels via different mechanisms.

An optimal level of extracellular Ca<sup>2+</sup> is required for the proliferation of cells *in vivo* (Rixon and Whitfield, 1976) and *in vitro* (Boynton *et al.*, 1984). The level of Ca<sup>2+</sup> appears to vary depending on the origin of the cells. The extracellular Ca<sup>2+</sup> is required at two brief and distinct points through the cell cycle (Boynton, 1988). The first Ca<sup>2+</sup>-dependent state occurs immediately after stimulation of quiescent cells with serum and is referred to as G<sub>0</sub>-G<sub>1</sub> transition, while the second Ca<sup>2+</sup>-dependent state is late in the prereplicative period immediately before DNA synthesis, and is referred to as the G<sub>1</sub>-S transition.

The mechanism of calcium action in cell proliferation may be related to at least four

cellular constituents (Villereal and Byron, 1992). The first is calmodulin, a  $\text{Ca}^{2+}$ -binding protein which upon binding  $\text{Ca}^{2+}$  undergoes a conformational change that enables the protein to activate numerous enzymes (Carafoli, 1985). The second is cyclic AMP which, although not directly affected by  $\text{Ca}^{2+}$  ion, can indirectly be affected either through calmodulin activation of adenylate cyclase or cyclic AMP phosphodiesterase. The third is PKC which is activated by the joint effects of  $\text{Ca}^{2+}$ , phosphatidylserine and DAG. Lastly, there is an ornithine decarboxylase (ODC) which is the rate-limiting enzyme in polyamine biosynthesis and whose increased activity may be a necessary requirement for normal trophic response. The  $\text{Ca}^{2+}$  seems to be involved in the induction of ODC (Ginty and Seidel, 1989).

### ***Diacylglycerol and Protein Kinase C***

Diacylglycerol activates the enzyme protein kinase C by increasing the affinity of the kinase for  $\text{Ca}^{2+}$  and phosphatidylserine (Nishizuka, 1984). PKC, a serine and threonine kinase, is directly activated by the tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA) and presumably acts as a binding site and cellular mediator of TPA (Kikkawa *et al.*, 1984). Activation of PKC by TPA or DAG requires translocation of the kinase from the cytosol to the plasma membrane; recruitment is potentiated by  $\text{Ca}^{2+}$  (Wolf *et al.*, 1985). Membrane associated PKC is rapidly degraded and is not detectable in cells for several hours following treatment with TPA (Ballester *et al.*, 1985). PKC is preferentially associated with membranes in exponentially growing cultures, whereas in quiescent cells, it is mainly cytosolic (Adamo *et al.*, 1986). The evidence implicating

PKC in the mediation of cell proliferation is that direct activation of PKC by TPA has been shown to inhibit differentiation of cultured cell lines (Hollstein and Kamasaki, 1986) and enhance proto-oncogene expression (Cross and Dexter, 1991). TPA has also known to induce many characteristics associated with the transformed phenotype including morphological transformation (Gainer and Murray, 1986), enhanced DNA, RNA, and protein synthesis (Hollstein and Kamasaki, 1986), increased secretion of plasminogen activator (Wigler and Weinstein, 1976) and increased polyamine synthesis (O'Brien and Diamond, 1977).

Two major lines of evidence indicate that PKC activation constitutes a mitogenic signal-transduction pathway at least in Swiss 3T3 cells. First, the mitogens, PDGF, bombesin, vasopressin, and serum activate PKC as measured by a rapid increased phosphorylation state of an acidic 80 KD cellular protein. This protein provides a specific signal for PKC stimulation in intact and quiescent cells (Erusalimsky *et al.*, 1988). A second approach to investigate the role of PKC in the cellular proliferative response, is to exploit the selective loss of this enzyme caused by prolonged treatment of cells with phorbol esters. Chronic exposure of Swiss 3T3 cells to phorbol esters leads to a marked decreased number of specific phorbol ester-binding sites and to the disappearance of measurable PKC activity in cell-free preparations (Rozenfurt, 1986). Such treatment prevents the increased 80KD protein phosphorylation and the stimulation of DNA synthesis on subsequent additions of either phorbol esters, 1-oleyl-2-acetylglycerol (OAG; synthetic DAG) or bombesin.

### *Cyclic AMP*

The actions of cyclic AMP are mediated by cyclic AMP-dependent protein kinase, which exists as two isozymes differing in their regulatory but not in their catalytic subunits (Flockhart and Corbin, 1982). Binding of cyclic AMP to the regulatory subunits releases and, thus, activates the catalytic subunits. Cyclic AMP has been reported to both stimulate and inhibit the growth of cultured cells (Dumont *et al.*, 1989; Rozengurt and Ober, 1990). In an efforts to reconcile conflicting data, several investigators systematically analyzed the effects of elevated levels of cyclic AMP on synchronously cycling cells and found that the response elicited by cyclic AMP reflected the position of the cells in the cell cycle, the extent to which cyclic AMP was increased and the duration of the increase (Dunmont *et al.*, 1989).

A positive mitogenic effect of cyclic AMP is missing in many conventional cell culture systems and negative effects are common. Such opposite effects of the cyclic AMP cascade in different systems might perhaps be explained by a variation in the optimal concentration of cyclic AMP required, which would confer a biphasic feature to cyclic AMP action (Whitfield *et al.*, 1987). Alternatively, the effect of cyclic AMP may depend on the differentiation state of the cell. Positive and negative regulatory elements have been demonstrated in the promoter of a growth-related gene and could depend on the differentiation state of the cell. It is interesting in this regard that the growth of many cancer cells is often inhibited by increasing cyclic AMP, whereas the proliferation of normal cells is often stimulated (Olashaw and Pledger, 1988).

The regulatory subunit of cyclic AMP has been shown to possess topoisomerase



activity (Constantinou *et al.*, 1985) and, thus, may play a role in gene transcription. In support, recent studies have demonstrated homology between the regulatory subunit of type II cyclic AMP-dependent protein kinase and the procaryotic cyclic AMP receptor, a protein that specifically binds DNA when complexed to cyclic AMP (Nagamine and Reich, 1985). Cyclic AMP has been shown to affect the expression of several genes, including the *ras* oncogene (Dere *et al.*, 1986); whether these changes result from interaction of the regulatory subunit with DNA or from phosphorylation of DNA binding proteins by the catalytic subunit is not clear.

### ***Na<sup>+</sup>-H<sup>+</sup> Antiport and Cytoplasmic Alkalinization***

The term antiport is used to describe a transport mechanism which facilitates the movement of two substances in opposite directions across a membrane. The Na<sup>+</sup>-H<sup>+</sup> antiport regulates the electrically neutral exchange of one Na<sup>+</sup> for one H<sup>+</sup> across the plasma membrane. The exchange is driven by the electrochemical gradients for Na<sup>+</sup> and H<sup>+</sup> and does not utilize energy directly. Under physiological conditions the intracellular concentration of Na<sup>+</sup> is kept low by the Na<sup>+</sup>-K<sup>+</sup> pump, and the antiport mediates the entry of Na<sup>+</sup> into the cell and the efflux of H<sup>+</sup>. If the gradient for Na<sup>+</sup> and H<sup>+</sup> are reversed, however, the exchange can occur in the opposite direction. Intracellular pH also affects a modifier site which alters the Na<sup>+</sup>-H<sup>+</sup> antiport allosterically. Antiport activity is inhibited by the potassium sparing diuretic amiloride and its analoges, which compete with Na<sup>+</sup> for its binding site (Reviewed in Grinstein *et al.*, 1989; Moolenaar, 1986).

The addition of mitogens to quiescent cells results in stimulation of the  $\text{Na}^+\text{-H}^+$  antiport and an increased intracellular pH. In Swiss 3T3 cells intracellular alkalization occurs after the addition of a variety of mitogens. Thus, serum, the tumor promoter phorbol dibutyrate, vasopressin, PDGF, insulin, EGF and the neuropeptide bombesin increase intracellular pH by 0.1-0.3 units (Hesketh *et al.*, 1988). Similar activation of the antiport have been reported by endothelin in vascular smooth muscle cells (Lonchampt *et al.*, 1991), growth factors in a pancreatic cell line, AR 4-2J (Delvaux *et al.*, 1990) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in normal murine bone marrow macrophages (Vallance *et al.*, 1990).

The importance of the  $\text{Na}^+\text{-H}^+$  antiport in the proliferative response has been emphasized many reports from several laboratories. For example, in hamster fibroblasts the half maximal concentrations for inhibition of the  $\text{Na}^+\text{-H}^+$  antiport by amiloride analogues correlated well with their half maximal concentrations for the inhibition of DNA synthesis (L'Allemain *et al.*, 1984).

Several lines of evidence strongly suggest that activation of  $\text{Na}^+\text{-H}^+$  antiport by external stimuli is mediated by PKC. First, biologically active phorbol esters like TPA stimulate  $\text{Na}^+\text{-H}^+$  antiport and, thereby, raise intracellular pH in a wide variety of cell types (Grinstein *et al.*, 1989). Second, the addition of a synthetic DAG can mimic phorbol esters in raising intracellular pH (Nishizuka, 1984). Furthermore, trifluoperazine, an inhibitor of PKC *in vitro*, can block the activation of  $\text{Na}^+\text{-H}^+$  antiport by external stimuli (Moolenaar, 1986).

### ***Expression of c-fos And c-myc Proto-Oncogenes***

In addition to the events in the plasma membrane and cytosol, growth factors induce the expression of a set of cellular genes. These genes have been referred to as cellular immediate early genes or primary response genes (PRGs) (McMahon and Monroe, 1992). One common characteristics of PRGs is that their transcriptional induction does not require de novo protein synthesis. This suggests that stimulus-induced posttranslational modification of preexisting regulatory proteins is sufficient for coupling of receptor-linked second messenger pathways to PRG induction (Herschman, 1991). A second characteristic of this class of genes is that their rapid but transient induction occurs in a wide variety of cell types in response to growth or differentiation factors (Herschman, 1991).

Proteins encoded by *c-fos* function with those encoded by *c-jun* by forming a dimer, generally referred to as the AP-1 transcription factor (Karin, 1992). Dimer formation occurs via an amino acid domain referred to as a leucine zipper, which is composed of an  $\alpha$  helix containing four leucine residues spaced at every seventh amino acid (Karin, 1992). DNA binding by dimers occurs by means of a region rich in basic amino acids that lies adjacent to the leucine zipper domain (Karin, 1992). One mechanism by which AP-1 dimers affect transcription is dependent upon binding of their basic domains to a short DNA sequence (TGACTCA), referred to as the TRE (TPA response element) (McMahon and Monroe, 1992).

Chronology of activation and deactivation differs among these genes, with *c-fos* being the earliest detectable (within 5 minutes), followed by *c-myc* (1-2 hours). Other proto-

oncogenes are induced as much as 6 hours later. These observations and the ample evidence implicating *c-fos* and *c-myc* in the control of cell proliferation led to a focused attention on these two genes. Whereas PDGF, fibroblast growth factor (FGF) and PMA induce *c-myc* in all cell types, the effect of EGF is strain-dependent for mouse fibroblasts (Muller *et al.*, 1984). PDGF is also a much more potent inducer of *c-fos* than EGF (Muller *et al.*, 1984), and insulin is not effective at all. However, both EGF and insulin induce *c-fos* transcripts in cells bearing high numbers of the corresponding receptor, suggesting that *c-myc* and *c-fos* are not strictly "competence" genes whose expression is required for leading cells from the quiescent state (G0) to competent state (G1) in which cells are considered to respond to the continuous action of progression factors and enter the S phase (DNA synthesis) (Pledger *et al.*, 1977; Stiles, 1985).

Expression of *c-fos* and *c-myc* is correlated with growth stimulation in several respects. For example, *c-myc* mRNA is induced by agents that initiate the proliferation of quiescent cells (Kelly *et al.*, 1983). Microinjection of *c-myc* protein into the nuclei of Swiss 3T3 cells stimulated DNA synthesis in cells (Kaczmarek *et al.*, 1985). BALB/c-3T3 cells transfected with *c-myc* recombinant plasmids responded mitogenically to EGF and insulin in the absence of PDGF (Armelin *et al.*, 1984). Levels of *c-fos* mRNA are higher in growing cells than in quiescent cells (Muller *et al.*, 1984) and increase during liver regeneration at a time corresponding to peak thymidine incorporation (Corral *et al.*, 1985). The correlation between *c-fos* expression and mitogenesis suggested by these findings is strongly supported by the data demonstrating reduced proliferative activity in BALB/c-3T3 cells transfected with an antisense *c-fos*

RNA (Holt *et al.*, 1986).

Multiple signal transduction pathways regulate the expression of these early genes (Chiarugi *et al.*, 1987; Nigg, 1990; Rozengurt and Sinnett-Smith, 1988). Activation of the PKC pathway induces both *c-fos* and *c-myc*, whereas agents that elevate cyclic AMP preferentially stimulate the expression of *c-myc* in Swiss 3T3 cells (Rozengurt and Ober, 1990). Colony stimulating factor-1 (CSF-1) induces both *c-fos* and *c-myc* in macrophages (Muller *et al.*, 1985), with an unusual biphasic induction response for *c-fos*. The later more stable phase was attributed to a mechanism that involves protein kinase A (Nigg, 1990). On the other hand, the early transient phase common to other mitogens is mediated by both a PKC-dependent pathway as well as other mechanisms (Chiarugi *et al.*, 1987) which involve  $\text{Ca}^{2+}$  ion (Sheng *et al.*, 1990) and possibly cyclic AMP (Nigg, 1990).

### ***Polyamines and Ornithine Decarboxylase***

Putrescine, spermidine and spermine, collectively known as the polyamines, are essential for the life of all eukaryotic cells, since mutants lacking the ability to synthesize them die unless the polyamine are provided in cell culture medium (Canellakis *et al.*, 1989). These metabolically related polyamines carry a positive charge at a physiological pH and are the most abundant endogenously produced cations, but this important function does not explain their essential role in cell survival. These low molecular weight, aliphatic amines have been shown to be elevated in association with increased cellular activity and to be implicated in a wide range of diverse cell functions (Janne *et al.*,

1991). Putrescine, the parent diamine, arise intracellularly by decarboxylation of the amino acid ornithine by the enzyme ornithine decarboxylase (ODC). Spermidine and spermine are formed from putrescine by the sequential addition of two aminopropyl moieties derived from methionine. Putrescine, spermidine and spermine are collectively referred to as the parent polyamines. Both acetylated and oxidized forms of these compounds are known to occur naturally.

In spite of their highly conserved and essential nature, the exact physiological functions of the polyamines are not clear. In addition to their role as intracellular cations, they are known to modulate the conformation and transcription of DNA (at least *in vitro*) and to participate in the post-translational modification of proteins (Canellakis *et al.*, 1989). Nevertheless, the involvement of polyamines in the cell growth has become evident (Janne *et al.*, 1991).

ODC, which has a turnover rate amongst the fastest of any known mammalian enzymes, is subject to a number of influences that increase its activity, including growth promoters or trophic influences, such as serum, growth factors and hormone (Tabor and Tabor, 1984). The increased activity of ODC is always associated with a rapid rise in cellular proliferation. Furthermore, the use of  $\alpha$ -difluoromethylornithine (DMFO), a specific and irreversible inhibitor of ODC, has been shown to abolish completely the growth factor-induced increased ODC activation as well as cell proliferation (Arlow *et al.*, 1990). Transformed cells usually have elevated baseline ODC activities (Chang, 1991). Expression of ODC becomes constitutively activated during cell transformation induced by carcinogens (Arlow *et al.*, 1989), viruses (Auvinen *et al.*, 1992) or oncogenes

(Auvinen *et al.*, 1992). In general, influences that cause rapid increased ODC activity do so by enhanced transcription and protein synthesis (Porter and Bergeron, 1988).  $\text{Ca}^{2+}$  ion has been shown to a requirement for the serum-mediated increased synthesis of ODC enzyme (Chang, 1991; Ginty and Siedel, 1989). Synthesis of ODC also proved to be increased by the pathways involving PKC or cyclic AMP (Canellakis *et al.*, 1989).

### **C. Role of Calcium in Cell Proliferation**

#### ***Regulation of Intracellular Calcium***

A low  $[\text{Ca}^{2+}]_i$  ( $\approx 10^{-7}$  M) is maintained by a complex interaction of several biochemical processes. These include the regulation of plasma membrane  $\text{Ca}^{2+}$  channel opening, the binding of  $\text{Ca}^{2+}$  to membrane surfaces and  $\text{Ca}^{2+}$ -binding proteins, the extrusion of  $\text{Ca}^{2+}$  from the cytosol either out of the cell across the plasma membrane or into multiple, intracellular storage sites and the regulation of release of  $\text{Ca}^{2+}$  from these intracellular compartments. In different cell types the  $\text{Ca}^{2+}_i$  can be regulated via different combinations of these regulatory pathways (Reviewed in Carafoli, 1987; Rasmussen and Rasmussen, 1990; Villereal and Byron, 1992).

#### ***a) Calcium Channels***

Since the  $\text{Ca}^{2+}$  concentration is  $10^4$  times higher outside the cell than in the cytosol, and since  $\text{Ca}^{2+}$  would move down an electrical gradient, the "opening" of the plasma membrane  $\text{Ca}^{2+}$  channels, even transiently, is a very effective mechanism for elevating intracellular  $\text{Ca}^{2+}$  concentration, as well as for depolarizing the cell. Depending on the

cell type, several mechanisms are involved in opening  $\text{Ca}^{2+}$  channels.

Excitable cells have voltage-sensitive  $\text{Ca}^{2+}$  channels (VSCCs) which open in response to a membrane depolarization. Based on biophysical and pharmacological criteria, the VSCCs have been characterized as either T, N or L types (Kostyuk, 1989; Bertolino, 1992). Recent studies suggest that VSCCs exist in nonexcitable cells, such as fibroblasts (Olsen et al., 1989) and glial cells (Barres *et al.*, 1990).

In addition to VSCCs,  $\text{Ca}^{2+}$  channels appear to be directly associated with membrane receptor proteins. The best example of a ligand-sensitive  $\text{Ca}^{2+}$  channel is the N-methyl-D-aspartate (NMDA) type of glutamate receptor, which appears to gate an ion channel that is highly permeable to  $\text{Ca}^{2+}$  as well as  $\text{Na}^+$  and  $\text{K}^+$  (McDonald and Johnston, 1990).

#### ***b) Release from Intracellular Calcium Stores***

In many cells the response to hormone stimulation is mediated by a release of  $\text{Ca}^{2+}$  from intracellular stores. Berridge and coworkers first described a new pool of phosphatidylinositol (i.e.  $\text{PIP}_2$ ), which, when acted on by PLC, could release  $\text{IP}_3$  and DAG (Berridge and Irvine, 1984). This work suggested that  $\text{IP}_3$  could serve as a type of second messenger. Subsequent studies in numerous other cell types have confirmed that the release of  $\text{IP}_3$  leads to the mobilization of  $\text{Ca}^{2+}$ ; in response to many hormones (Berridge, 1993; Berridge and Irvine, 1984; Fisher *et al.*, 1992; Henzi and MacDermott, 1992; Michell, 1992; Villereal and Byron, 1992).

Recently, a more highly phosphorylated metabolite of inositol, e.g. (1,3,4,5) $\text{IP}_4$ , is known to be produced when (1,4,5) $\text{IP}_3$  was phosphorylated by a (1,4,5) $\text{IP}_3$  kinase (Fisher



*et al.*, 1992). Current speculation is that  $IP_4$  may be involved in regulation of  $Ca^{2+}$  influx channels. This hypothesis arises from the observation that  $IP_4$  microinjection into sea urchin eggs caused the immediate elevation of the fertilization envelope for eggs in the presence, but not in absence, of external  $Ca^{2+}$  (Irvine and Moore, 1986). The synergism between the  $Ca^{2+}$  mobilizing agent  $IP_3$  and  $IP_4$  has been speculated to be related to an effect of  $IP_4$  on the reloading of  $IP_3$ -sensitive  $Ca^{2+}$  compartment via  $Ca^{2+}$  influx across the plasma membrane. In addition, an apparent positive feedback regulatory mechanism exist in which  $Ca^{2+}$  influx stimulates  $IP_4$  production and  $IP_4$  stimulates  $Ca^{2+}$  influx (DeLisle *et al.*, 1992). Moreover, for cells in whole cell-perfusion via patch clamp methods, both  $IP_3$  and  $IP_4$  are necessary for  $Ca^{2+}$ -activated  $K^+$  currents (Morris *et al.*, 1987).

The observation that (1,4,5) $IP_3$  mobilizes  $Ca^{2+}$  from intracellular stores whereas very similar inositol phosphates do not, argues for a high degree of specificity in the mechanism for  $Ca^{2+}$  release. This observation suggests the existence of specific receptors that recognize (1,4,5) $IP_3$  on the surface of  $Ca^{2+}$  store vesicles (Ferri and Snyder, 1992; Taylor and Marshall, 1992). Using binding of  $^3H$ - $IP_3$ , the  $IP_3$  receptor-channel complex was solubilized and purified to apparent homogeneity from rat cerebellum (Maeda *et al.*, 1991). The  $IP_3$  receptor is a very large homotetrameric protein with sequences near the carboxyl termini which are thought to form membrane-spanning helices that together comprise the  $Ca^{2+}$  channel (Ferris and Snyder, 1992). Opening of the channel is regulated largely by ligand binding to the large cytoplasmic amino termini of the receptors, which evokes a substantial conformational change in the

receptor and ultimately leads to channel opening (Taylor and Marshall, 1992).

### ***c) Extrusion Calcium from the Cytoplasm***

To maintain the resting  $[Ca^{2+}]_i$  at 100 nM, the cell must actively extrude  $Ca^{2+}$  from the cytoplasm. This  $Ca^{2+}$  is both extruded across the plasma membrane and sequestered into the intracellular  $Ca^{2+}$  compartments, from which it can be released upon receptor activation. The two major transport systems for moving  $Ca^{2+}$  are the  $Ca^{2+}$ -ATPase and the  $Na^+/Ca^{2+}$  exchanger. The  $Ca^{2+}$ -ATPase system couples the energy released in the hydrolysis of ATP to the movement of  $Ca^{2+}$  against large concentration gradients; the  $Na^+/Ca^{2+}$  exchanger utilizes the energy stored in the  $Na^+$  electrochemical gradient to move  $Ca^{2+}$  against a concentration gradient. Two distinct types of  $Ca^{2+}$ -ATPases exist: one present in the plasma membrane and the other present in the structures that sequester intracellular  $Ca^{2+}$  (Reviewed in Carafoli, 1987; Rasmussen and Rasmussen, 1990).

### ***Effector Systems Regulated by Intracellular Calcium***

The physiological effects of  $Ca^{2+}$  are mediated by a multiplicity of  $Ca^{2+}$ -binding proteins. In a few cases the binding protein is closely linked to or is a part of the effector molecule, e.g. this appears to be the case with  $Ca^{2+}$ -activated  $K^+$  channels. In many other instances the  $Ca^{2+}$ -binding protein has no biological activity of its own but must interact with other proteins to produce the final physiological effect. Thus,  $Ca^{2+}$ -binding proteins can be thought of as "third messengers" of the original extracellular signal (Reviewed in Villereal and Palfrey, 1989).

Calmodulin is one of the most important and best-characterized  $\text{Ca}^{2+}$ -binding proteins which include troponin C, calsequestrin, spasmoneme, colenterate photoproteins and parvalbumin. Calmodulin is a highly conserved, acidic, 16.7-kd protein that is ubiquitous in eukaryotic cells. The cytoplasmic concentration of calmodulin lies between 2 and 30  $\mu\text{M}$  in various mammalian tissues, making it an important  $\text{Ca}^{2+}$  buffer. Calmodulin has four binding sites for  $\text{Ca}^{2+}$  (two of high affinity and two of lower affinity) and two low-affinity metal-binding sites. The binding of each  $\text{Ca}^{2+}$  ion leads to stepwise conformational changes (the largest being with the binding of the third  $\text{Ca}^{2+}$ ) that result in the exposure of hydrophobic domains in the protein. These domains are thought to contribute to the interaction of calmodulin with various calmodulin-binding proteins and calmodulin antagonists such as trifluoperazine (Reviewed in Hait and DeRosa, 1988; Manalan and Klee, 1984).

The final effectors of many  $\text{Ca}^{2+}$ -regulated processes are the numerous calmodulin-binding proteins. The best studied of these proteins can be loosely grouped into four classes (Villereal and Palfrey, 1989): (i) protein kinases and phosphatases, (ii) phosphodiesterases and adenylate cyclases, (iii) cytoskeletal proteins and (iv)  $\text{Ca}^{2+}$ -ATPases. In an intact cell the effects of calmodulin are modulated not only by the affinity but also by the abundance of various calmodulin-binding proteins. This implies that differences in the kinetics and degree of occupancy of different calmodulin receptors will exist during the  $\text{Ca}^{2+}$  response (Villereal and Palfrey, 1989).

### *Role of Calcium in Normal Cell Proliferation*

Increased free  $\text{Ca}^{2+}_i$  concentration stimulated by growth factors are thought to play a key role in the initiation of a series of events that ultimately lead to cell proliferation (Reviewed in Villereal and Byron, 1992). Evidence for such a role of  $\text{Ca}^{2+}$  in growth factors action comes from the numerous findings: PDGF caused a transient rapid increased  $\text{Ca}^{2+}_i$  concentration in human fibroblasts (Moolenaar *et al.*, 1984), vascular smooth muscle cells (Roe *et al.*, 1989) and BALB/c-3T3 cells (Zagari *et al.*, 1989). Insulin-like growth factor II (IGF-II) also increased free  $\text{Ca}^{2+}_i$  in BALB/c-3T3 cells (Nishimoto *et al.*, 1987) as well as do various mitogens in human endothelial cells (Jacob *et al.*, 1988), human fibroblasts (Peres *et al.*, 1990), glomerular mesangial cells (Bonventre *et al.*, 1986), thyroid cells (Takada *et al.*, 1990), Swiss 3T3 cells (Takuwa *et al.*, 1991) and lymphocytes (Henry-Toulme *et al.*, 1990).

The importance of increased intracellular  $\text{Ca}^{2+}$  concentration in cell proliferation has been suggested from numerous studies which have used pharmacological agents to block growth factor-induced increased  $\text{Ca}^{2+}_i$  or used low amounts of  $\text{Ca}^{2+}$  in the culture medium. These studies also resulted in inhibition of growth factor-stimulated mitogenesis (Batra *et al.*, 1991; Block *et al.*, 1989; Cahalan and Lewis, 1990; Ogata *et al.*, 1991; Olsen *et al.*, 1989; Roe *et al.*, 1989; Shultz and Raij, 1990; Tucker *et al.*, 1989; Zagari *et al.*, 1989). Thus, in normal cell proliferation the increased  $\text{Ca}^{2+}_i$  appears to result from the influx of  $\text{Ca}^{2+}$  and the blockade of this source can apparently inhibit cell proliferation. Moreover, some electrophysiological studies showed that growth factors induce the activation of unusual, voltage-independent,  $\text{Ca}^{2+}$  permeable channels (Avdonin

*et al.*, 1991; Kuno *et al.*, 1986; Peppelenbosch *et al.*, 1992; Puro, 1991; Puro and Mano, 1991; Zschauer *et al.*, 1988).

Although these findings are suggestive, the major difficulty involved in establishing a direct cause-and-effect relationship between growth factor-induced alterations of  $\text{Ca}^{2+}_i$  and DNA synthesis arises from the lengthy period of time (24-36 hr) separating these two different phenomena. However, recent, notable reports demonstrated the requirement of early PDGF-stimulated alterations in  $\text{Ca}^{2+}_i$  in mitogenesis in two different PDGF-growth-dependent cellular systems, e.g. BALB/c-3T3 fibroblasts and porcine vascular smooth muscle cells (Diliberto *et al.*, 1991). The presence of pharmacological agents at a time after PDGF stimulation, is neither toxic nor inhibitory to subsequent cellular DNA synthesis, while these agents did, indeed, prevent alterations in  $\text{Ca}^{2+}_i$ . The critical time period for  $\text{Ca}^{2+}_i$  alterations in PDGF-induced mitogenesis has, thus, been narrowed down to the first few hours following growth factor exposure.

In serving as a  $\text{Ca}^{2+}$  receptor protein, calmodulin (CaM) appears to be critically involved in the regulation of mammalian cell proliferation, particularly at the G1/S boundary (Rasmussen and Means, 1988). CaM has also been implicated in growth factor-dependent proliferation of human hematopoietic progenitor cells, and the cell proliferation is highly sensitive to CaM antagonists (Katayama *et al.*, 1990). CaM levels are elevated two- to threefold during late G1 period, and a direct correlation was observed between intracellular CaM levels and the ability of mammalian cells to replicate DNA (Chafouleas *et al.*, 1982; Gruver *et al.*, 1992). Furthermore, the stimulatory effect of  $\text{Ca}^{2+}$  on DNA synthesis is blocked by CaM antagonists or antibody (Jones *et al.*,

1982). Although the exact mechanism of action of CaM in inducing cell growth remains obscure, some studies regarding to that issue have been reported. Proliferative stimulation of rat liver cells is observed to be accomplished by the nuclear rearrangement of CaM by its association with the nuclear matrix (Serratosse *et al.*, 1988). Specific CaM-binding proteins are found to be associated with DNA polymerase- $\alpha$  from a variety of mammalian cells (Hammond *et al.*, 1988) and with the multienzyme complex responsible for nuclear DNA replication in fibroblast cells (Subramanyam *et al.*, 1990). In addition, the nuclear localization of the 68-Kd CaM binding protein in Chinese hamster fibroblast cells is dependent on the induction of cell proliferation in these cells by exposure to insulin (Subramanyam *et al.*, 1990). These observations suggest that the induction and/or nuclear localization of specific CaM binding protein(s) may control a terminal event required for the onset of DNA replication and, therefore, for cell proliferation (Reddy *et al.*, 1992).

### ***Calcium Messenger Systems in Tumor Cell Proliferation***

Neoplastic transformation may be accompanied by various structural and behavioral changes in cultivated cells: (i) acquisition of unlimited growth and proliferative potential; (ii) production of plasminogen activator; (iii) loss of density-dependent inhibition of cell movement and proliferation; (iv) dispersal of the cytoskeleton of actin-containing microtubules (v) loss of a large surface glycoprotein; (vi) reduction of the serum requirement for proliferation (Reviewed in Calarkson and Baserga, 1974; Hynes, 1976; Poten, 1971). Neoplastic transformation of a wide variety of avian, human and rodent

cells of mesenchymal origin by chemicals (methyl-cholanthrene and nickel sulfide), viruses (adenoviruses, SV 40, and the Kirsten and Rous sarcoma viruses) and multiple passages *in vitro*, also eliminates or at least very greatly reduces the extracellular free  $\text{Ca}^{2+}$  requirement for the initiation of a later phase of prereplicative (G1) development (Balk *et al.*, 1973; Whitfield, 1990).

Since  $\text{Ca}^{2+}$  and CaM appear to play a critical role in normal cellular proliferation, alterations in this  $\text{Ca}^{2+}$  messenger system may occur in and be responsible for states of abnormal cellular proliferation in the cancer development.

This limb of the  $\text{Ca}^{2+}$  messenger could be possibly altered at six general sites: (i) the interaction of extracellular ligands with their receptors; (ii) the signal transduction between receptors and phospholipase C; (iii) the sensitivity of phospholipase C to activators; (iv) the release of  $\text{Ca}^{2+}$ ; through the interaction of  $\text{IP}_3$  with  $\text{Ca}^{2+}$ -storage sites; (v) the quantity or structure of calmodulin resulting in alterations in its sensitivity to  $\text{Ca}^{2+}$  and/or its interaction with target proteins; (vi) changes in calmodulin-binding proteins.

Several tumors are known to produce autocrine growth factors such as bombesin, PDGF and EGF which activate PLC through interaction with specific membrane receptors (Heldin *et al.*, 1987; Herlyn *et al.*, 1990; Herlyn and Malkowicz, 1991; Lang and Burgess, 1990). Thus, a fundamental step in initiation or propagation of malignancy might occur at the receptor-ligand locus.

Overexpression or altered expression of the growth factor receptors has been reported a variety of human carcinoma (Radinsky, 1991). For example, increased expression of

EGF receptor has been observed on metastatic human colon and renal carcinoma cells (Radinsky, 1991). The *v-erb B* oncogene of avian erythroblastosis virus encodes a truncated version of EGF receptor, which lacks the EGF-binding domain but contains a continuously-active tyrosine kinase domain which is required for the activation of PLC- $\gamma$  (Heldin *et al.*, 1987).

P21, the transforming protein encoded by the *ras* oncogene of the Kirsten and Harvey sarcoma viruses, resembles G proteins in its ability to bind GTP. Expression of this oncogene product in transformed cells has been shown to result in an exaggerated turnover of phosphoinositides in response to autocrine growth factors (Cantley *et al.*, 1991). Therefore, PLC can indirectly be sensitized to activation by oncogene products such as P21. PLC could also possibly be directly altered during malignant transformation.

Changes in  $\text{Ca}^{2+}_i$  due to altered responsiveness to  $\text{IP}_3$  have not been reported. However, differences in the intracellular concentration of  $\text{Ca}^{2+}$  between normal and malignant cells have been observed. For example, an increased free  $\text{Ca}^{2+}_i$  concentration was found in malignant compared with normal cells (Viegl *et al.*, 1982). Hickie and Kalant found a direct correlation between the rate of growth of Morris hepatomas and their intracellular  $\text{Ca}^{2+}$  concentration (Hickie and Kalant, 1967).

3T3 cells transformed by Rous sarcoma virus and normal rat kidney cells transformed by SV-40 virus demonstrated concentrations of calmodulin two to three times greater than those of untransformed cells (Criss and Kakiuchi, 1982). Qualitative changes in calmodulin have also been observed. For example, Isobe *et al.* purified from



porcine brain des-(alanine-lysine) calmodulin, a molecule that lacks alanine and lysine residues at positions 147 and 148, respectively (Isobe *et al.*, 1981). Qualitative abnormalities in calmodulin could alter its interaction with target proteins. For example, the biological activity of calmodulin based on the ability of the extractable calmodulin to activate a sensitive cyclic nucleotide phosphodiesterase were markedly increased in malignant tissue (Hait, 1987). Moreover, oncomodulin, a tumor-specific, calmodulin-like protein has been isolated from neoplastic cells (MacMaus *et al.*, 1984). Compared to calmodulin, oncomodulin is smaller (108 amino acids), more acidic and its spectrum of activity less broad than calmodulin.

Qualitative or quantitative changes could also occur in CaM-binding proteins. These changes might result in abnormal interaction between CaM and its target proteins, causing a selective increase or decrease in calcium-CaM-dependent processes. For example, differences in CaM-binding proteins have been found in rat kidney fibroblasts (NKK LA-23) transformed by a temperature-sensitive mutant of the Rous sarcoma virus or rat kidney cells (6M2) transformed by a temperature-sensitive mutant of the Moloney sarcoma virus (Connor *et al.*, 1983).

## CHAPTER III

### MATERIALS AND METHODS

#### A. Cell Culture

##### *Cell Line Characteristics*

##### *a) U-373 MG human astrocytoma*

Cultures of these cells were established by B. Westermark as explants which originated from 61-year old Caucasian male who had blood type-A<sup>+</sup> (Poten *et al.*, 1968). These cells have epithelial-like morphology. In addition, these cells have glial fibrillary acidic protein (GFAP), indicative of astrocytes, produce fibronectin and were tumorigenic in nude mice (Bigner *et al.*, 1981; Jones *et al.*, 1981). Some studies showed that these cells have functional substance-P receptors (Lee *et al.*, 1989). Trypsinization of the outgrowth or cells attached to the vessel floor with subsequent transfer to a growth medium permitted the cell line development. This cell line has been used very often in the studies of immunological characterization of glial cell origin (Boeckh *et al.*, 1991; Murphy and Hart, 1992). The cells (passage no. 181) were purchased in May of 1991 from American Type Culture Collection (Rockville, MA).

##### *b) SK-N-MC human neuroblastoma*

In 1971 this cell line was derived by J.L. Biedler and is of neurogenic origin from a 14-year old Caucasian female who had blood type-O<sup>+</sup> (Spengler *et al.*, 1973). The cells have an epithelial-like morphology. The cells showed tumorigenicity in nude mice and

in the hamster cheek pouch. The cell line was found to have moderate dopamine- $\beta$ -hydroxylase activity as well as formaldehyde-induced fluorescence indicative of intracellular catecholamines (Spengler *et al.*, 1973). NPY neurotransmitter was shown to bind to this cell line and induced intracellular  $\text{Ca}^{2+}$  mobilization (Michel *et al.*, 1992). This cell line was also used in the study of the signal transduction mechanism of neurotransmitters (Feth *et al.*, 1991; Hiley *et al.*, 1992). The cells (passage no. 41) were purchased in October of 1991 from ATCC (Rockville, MA).

### ***Cell Growth***

#### ***a) Culture Medium***

Eagle's minimum essential medium (MEM) (Sigma, St. Louis, MO) was used as a culture medium for the cell growth. The components (g/L) are: L-alanine, 0.0089; L-arginine.HCl, 0.126; L-asparagine.H<sub>2</sub>O, 0.015; L-aspartic acid, 0.0133; L-cystine.2HCl, 0.0133; L-glutamic acid, 0.0147; L-glutamine, 0.292; glycine, 0.0075; L-histidine.HCl.H<sub>2</sub>O, 0.042; L-isoleucine, 0.052; L-leucine, 0.052; L-lysine.HCl, 0.0725; L-methionine, 0.015; L-phenylalanine, 0.032; L-proline, 0.0115; L-serine, 0.0105; L-threonine, 0.048; L-tryptophan, 0.010; L-tyrosine.2Na, 0.0519; L-valine, 0.046; choline chloride, 0.001; folic acid, 0.001; myo-inositol, 0.002; niacinamide, 0.001; D-pantothenic acid.Ca, 0.001; pyridoxal.HCl, 0.001; riboflavin, 0.0001; thiamine.HCl, 0.001; calcium chloride.2H<sub>2</sub>O, 0.265; magnesium sulfate (anhydrous), 0.09767; potassium chloride, 0.400; sodium chloride, 6.800; sodium phosphate monobasic (anhydrous), 0.122; D-glucose, 1.000; phenol red.Na, 0.011. For washing the cells,

Earle's basal salt solution (EBSS) (Sigma, St. Louis, MO) was used. The components are the same as those of MEM without amino acids and vitamins. MEM or EBSS powders were dissolved in the sterilized water by gentle stirring. Supplements (i.e. 110 mg/L of sodium pyruvate (Sigma, St. Louis, MO) and 2.2 g/L of sodium bicarbonate (Sigma, St. Louis, MO)) were added prior to filtration. While stirring, the pH of the solution was adjusted by 1 N NaOH to 0.2 pH units below the desired pH (i.e. 7.4) since the pH usually goes up during filtration. The solution was sterilized immediately by filtration using a membrane with a porosity of 0.2 microns. The filtered medium was aseptically dispensed into sterile containers. The sterility of the medium was checked by overnight incubation of a few milliliters of the solution at 37°C. After checking for signs of contamination, fetal bovine serum (FBS) and antibiotics (penicilline and streptomycin mixtures) were added to the medium. The sterility of this final solution was also checked. All media and salt solutions were stored in a refrigerator at 4°C.

### ***b) Initial Culture***

Both astrocytoma and neuroblastoma cell lines were shipped in frozen medium containing DMSO. These were initiated culture as soon as possible upon receipt. The cells in a small ampule (volume:1.0 ml) were thawed by rapid agitation in 37°C water bath. The cells were diluted in serum-containing culture medium and centrifuged at 300 x g for 15 minutes in order to remove DMSO. The cell pellet was resuspended in culture medium and transferred to 75 cm<sup>2</sup> tissue culture flask. Cells were grown at 37°C in a humidified incubator under 5% CO<sub>2</sub>/95% air in an Eagle's minimum essential

medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 200 IU/ml of penicilline, 200  $\mu\text{g}/\text{ml}$  of streptomycin (Gibco BRL, Grand Island, NY) and 1 mM sodium pyruvate.

### *c) Culture Maintenance*

Culture medium was replaced twice weekly. After attaining confluency, the cells were subcultured. The old medium was removed and the cells were washed twice with fresh Eagle's basal salt solution (EBSS) . The cells were harvested by adding 0.25% trypsin (Sigma, St. Lous, MO) for 5 minutes. Serum-containing MEM was added and triturated well by suction in and out through pipette in order to break up large cell clumps. The cells were centrifuged at 300 x g for 15 minutes in order to prevent cell membrane damage possibly induced by residual trypsin. The cells were seeded at 1:5 (astrocytoma cell line) and 1:10 (neuroblastoma cell line) dilution into new 75 cm<sup>2</sup> flasks (Falcon, England).

## **B. Cell Growth and Cell Cytotoxicity Assay**

### *Cell Growth Assay*

#### *a) Initial Seeding*

Cells from four to five-day-old cultures were seeded in equivalent amounts in 35 x 10 mm culture dishes (Fisher Scientific, Pittsburgh, PA) at the density of  $10^5$  and  $2 \times 10^5$  cells/dish in neuroblastoma and astrocytoma cell lines, respectively. The volume of the medium in the dishes was 2 ml. Since the equivalence of the initial cell density is quite

important in cell growth assay, the clumps of cells were isolated into single cells by first trituration and, if necessary, passing through a 20 gauge needle. Particularly, the astrocytoma cell line was very resistant to the treatment of trypsin and, thus, needed more vigorous handling. However, this procedure did not affect the viability of cells as assessed by the trypan blue exclusion method.

### ***b) Drug Treatment***

After one day culture for the attachment of the cells, the cells were grown for two more days with or without various drugs.  $\text{Ca}^{2+}$  channel modulators such as verapamil, nifedipine, diltiazem and Bay K-8644 were used and purchased from ICN Chemicals (Cleveland, OH). As  $\text{K}^{+}$  channel modulators tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP) and diazoxide were used, and all were obtained from Sigma (St. Louis, MO). A receptor-operated calcium channel antagonist, SK&F 96365 was used and generously supplied by Dr. Hills from Smith Kline Beecham Pharmaceuticals (Welwyn, Hertfordshire AL6 9AR, England). Tetrodotoxin, a  $\text{Na}^{+}$  channel antagonist, and veratridine, a  $\text{Na}^{+}$  ionophore, were also used and obtained from Sigma. Cobalt chloride, nickel chloride, ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), methionine enkephalin and angiotensin II were also used, and all the drugs were obtained from Sigma. Concentrated stock solutions of the drugs were added directly to the culture dishes. The volume of the drug solutions did not exceed 20  $\mu\text{l}$ . Water-insoluble drugs were solubilized in ethanol, and the volume of these drug solutions was limited to 10  $\mu\text{l}$  in order to prevent the effect of ethanol itself. In fact, this

concentration, 0.5% of ethanol did not affect cell growth significantly. Drugs and culture medium were replaced every day.

### *c) Cell Counting*

Assessment of cell growth was made by counting the number of cells using a hemocytometer (Fisher Scientific, Pittsburg, PA). After three day culture the old medium was removed by aspiration and the cells were washed twice using EBSS. The cells were detached from the bottom of the dishes by incubation with 0.25% trypsin. 200  $\mu$ l of the trypsin solution was added to each dish. After complete detachment 1 ml of serum-containing MEM was added to each dish and transferred to a 12 x 75 mm test tubes (Baxter, McGaw, IL) by using a syringe attached to a 20 gauge needle (Becton Dickinson, Rutherford, NJ). The neuroblastoma cells were easily isolated into a single or countable small clumps of cells (less than 3 cells) by only this procedure. However, in the case of astrocytoma cells, a few more passing through the needle was needed for breaking the clumps of cells. The cell suspension was usually further diluted twice or three times using EBSS, depending upon final cell density. Practically, one hundred cells per 1 mm square of the hemocytometer are desirable cell density. With the cover-slip in place, a small amount of cell suspension was transferred to both chambers of the hemocytometer using a Pasteur pipette (Baxter, McGaw, IL) by carefully touching the edge of the cover-slip with the pipette tip, which allowed each chamber to fill by capillary action. In that procedure overfilling or underfilling of the chambers were prohibited. Starting with chamber 1 of the hemocytometer, the cells were counted in

four 1 mm corner squares. The cells on top and left touching middle line of the perimeter of each square were counted, but the cells touching the middle line at bottom and right sides were not counted. This procedure was repeated for chamber 2. Each square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm<sup>3</sup> or 10<sup>-4</sup> ml. The subsequent cell concentration per 1 ml (and the total number of cells) could be determined using the following calculations:

$$\text{Cells per ml} = \text{the average count per square} \times 10^4$$

$$\text{Total Cells} = \text{cells per ml} \times \text{the original volume of fluid (ml)}$$

The effects of the various drugs on the cell growth were observed as % of control condition in comparison with the number of cells using the following calculation:

$$\% \text{ of Control} = [\text{total cells (drug-treated)}/\text{total cells (control)}] \times 100$$

#### ***d) Effect of Carbachol on Astrocytoma Cell Growth***

In order to observe the effect of carbachol on astrocytoma cell growth, initial seeding procedure was the same as previously described. After one day culture in which 10% FBS was used, the cells were washed twice using EBSS for removing residual serum. For further cultures, 0.1% FBS-containing medium was used. This very low serum concentration prevented cell death but did not induce significant cell proliferation. 10 µl of the stock solutions of carbachol (Sigma, St. Louis, MO) dissolved in MEM were added at the desired concentrations. Cell counting was done as previously described. The effect of carbachol on the cell growth was shown as % of control condition in comparison with the number of cells using the same calculation as that in the case of



other drugs.

### ***Cell Cytotoxicity Assay***

Cell cytotoxicity was assayed by trypan blue exclusion method in order to observe the effect of drugs on cell viability. This method is based on the principle that live cells do not take up the dye, whereas dead cells do. Initial seeding and drug treatment were done by the same procedure as that of cell growth assay. However, after drug treatment, the medium and drugs were not replaced during subsequent 2 days cultures. The old medium was carefully collected in a 12 x 75 mm test tube (Baxter, McGaw, IL) using polyethylene transfer pipettes (Fisher Scientific, Pittsburgh, PA). The cells were washed twice and washing fluids were also collected. Remaining attached cells were harvested using 0.25% trypsin and also collected in the test tube. After mixing up well, the cell suspension was centrifuged at 300 x g for 15 minutes. Cell pellets were resuspended in 1 ml of EBSS, and 0.5 ml of trypan blue (0.4%) (Sigma, St. Louis, MO) was added. This solution was thoroughly mixed and was allowed to stand for 15 minutes. However, the cells were prohibited from prolong exposure to trypan blue because viable cells may begin to take up dye in this condition. The cells were counted by the same method which used in cell growth assay. With the only difference being that viable (unstained) and non-viable (stained) cells were separately counted. % cell death was determined using the following calculations:

$$\% \text{ Cell Death} = [\text{total dead cells (stained)}/\text{total cells (stained + unstained)}] \times 100$$

### ***Data Analysis***

For dose-response curves, at least four different experiments were done. The number of cells in a dish was derived from averaging the four different counts. All the data were displayed as % of control condition in comparison with the number of cells. Data were expressed as mean  $\pm$  standard error of the mean (SEM). The data were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for follow-up test. The data which have p value less than 0.05, were considered to be statistically significant. Plotting dose-response curves and bar graphs was made using the Sigma Plot program.

### **C. Measurement of Intracellular Calcium Concentration**

#### ***Solutions***

##### ***a) Fura-2-AM***

The AM (acetoxymethyl) ester derivative of fluorescent indicator (i.e. Fura-2) (Molecular Probes, Eugene, OR) was used in this study. Modification of carboxylic acid with an AM ester group results in an uncharged molecule which can permeate cell membranes. Once inside, the lipophilic blocking groups are cleaved by nonspecific esterase activity in the cell resulting in a charged, free acid form which leaks out of cells far more slowly than its parent compound. Hydrolysis of the esterified group is essential for binding of the target ion (i.e.  $\text{Ca}^{2+}$  ion). Since the AM esters are relatively insoluble in aqueous solutions, the low toxicity dispersing agent, pluronic acid was used. This nonionic detergent was dissolved in DMSO (Sigma, St. Louis, MO) at the final

concentration of 20% (w/v) by keeping in 37°C waterbath for 20 minutes, and this solution was used to make up the dye stock. This DMSO stock solution was stored at room temperature since refrigeration may result in separation of pluronic acid. The DMSO stock solution was added to a small vial which contains 50  $\mu\text{g}$  of Fura-2-AM (M.W. 1000) and vortexed for 15 minutes before making a 200  $\mu\text{M}$  Fura-2-AM stock solution. This stock solution was aliquoted out to 50  $\mu\text{l}$  of solution and kept in vials covered with aluminum foil and stored in the freezer (-70°C). Since the stability of AM ester of the dye may decrease in this condition, the stock solution was used as soon as possible (within a few days after being made).

***b) Krebs-Ringer Buffer (KRB)***

For washing and resuspending cells KRB solution was used. This solution contains 125mM NaCl, 5mM KCl (Mallinckrodt, Paris, KE), 1.2mM  $\text{KH}_2\text{PO}_4$ , 1.2mM  $\text{MgSO}_4$ , 5mM  $\text{NaHCO}_3$ , 1.3mM  $\text{CaCl}_2$  (Mallinckrodt, Paris, KE), 25mM HEPES and 6mM glucose. All salt powders were obtained from Sigma unless otherwise noted. The pH was adjusted to 7.4 with 1 N NaOH.  $\text{Ca}^{2+}$ -free KRB was made by just omitting  $\text{CaCl}_2$  in the solution.

***c) Triton X-100***

0.1% solution of Triton X-100 (Sigma, St. Louis, MO) was made by dissolving it in KRB solution.

#### ***d) EGTA Solution***

60mM EGTA solution was made by dissolving it in the solution of Tris base, at pH 8.2.

#### ***Procedure***

##### ***a) Preparation of Aliquot of Tumor Cells***

Tumor cells at about 70% confluency were harvested by trypsinization (trypsin concentration, 0.25 and 0.5% for neuroblastoma and astrocytoma cells, respectively). The cells were diluted in MEM solution and centrifuged at 400 x g for 5 minutes for removing residual trypsin. The pellet of the cells was resuspended in KRB solution at a density of  $5 \times 10^6$  cells/ml. The cell suspension was transferred to a 16 x 125 mm test tube.

##### ***b) Loading Fura-2-AM***

Fura-2-AM stock was added to the cell suspension at the final concentration of  $2 \mu\text{M}$ . In general, the concentration of the dye should be kept as low as possible to reduce potential artifacts from overloading including incomplete hydrolysis, compartmentalization and toxic effects from hydrolysis by-products such as formaldehyde. The test tubes containing the cell suspension and the dye were covered with aluminum foil during incubation time to prevent exposure of light. The cells were incubated for 60 minutes at room temperature in an incubator which alternatively moving at a speed of 120 rpm since some investigators have reported greater degrees of

compartmentalization at physiological temperatures than at room temperature.

***c) Removing Unloaded Fura-2-AM***

After incubation the cells were diluted ten times using KRB solution ( $\text{Ca}^{2+}$ -containing or  $\text{Ca}^{2+}$ -free, depending on the protocol) and centrifuged at 400 x g for 5 minutes. The pellet of the cells was resuspended in KRB solution. For neuroblastoma cells before second washing, the resuspended cells were allowed to sit for more than 20 minutes. This procedure allowed complete hydrolysis by nonspecific esterases of the loaded Fura-2-AM into the free acid form which does not readily leak out of cells. However, in the astrocytoma cells this additional incubation was not necessary. The cells were washed twice by repeating centrifugation and resuspension. This washing procedure did not affect cell viability and was enough to remove unloaded Fura-2-AM.

***d) Measurement of Intracellular Calcium Concentration,  $[\text{Ca}^{2+}]_i$***

Resuspended cells were transferred to a quartz cuvette at a volume of 500  $\mu\text{l}$  and stirred continuously. Fluorescence intensity was measured by HITACHI F-2000 fluorescence spectrophotometer. Fluorescence scanning time was usually 350 seconds which were enough to see the effects of ion channel modulators on basal levels and serum or carabchol-induced changes of intracellular  $\text{Ca}^{2+}$  concentrations. In order to see the effects of various drugs on basal, free intracellular  $\text{Ca}^{2+}$  concentrations, these drugs were added between 50 and 100 seconds after running experiments. In order to see the effects of ion channel modulators on agonist-induced increased intracellular  $\text{Ca}^{2+}$

concentrations, these drugs were injected 3 minutes prior to initiating experiments, and serum or carbachol was added between 50 and 100 seconds after running experiments. All the drugs were injected using a Hamilton syringe (Hamilton Company, Reno, NE). Volume of the drugs did not exceed 10  $\mu$ l. All experiments were done at 37°C. Autofluorescence of the cells was almost negligible and corrected when needed. Fluorescence emission (510 nm) was monitored with excitation wavelengths cycling between 340 and 380 nm. At the end of an experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by first lysing the cells with 0.1% Triton X-100 (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340:380 nm fluorescence ratios were converted into free  $\text{Ca}^{2+}$  concentrations using the formula and Fura-2  $\text{Ca}^{2+}$  binding constant (224 nM) described by Grynkiewicz *et al.* (Grynkiewicz *et al.*, 1985). The use of the 340/380 excitation ratio for Fura-2 results in measurements of the intracellular  $\text{Ca}^{2+}$  concentration that are essentially independent of the actual amount of dye in the cell. Ratioing considerably reduces problems associated with measuring  $\text{Ca}^{2+}$  in cells of unequal thickness and offsets the effects of uneven dye loading, leakage and photobleaching.

### *Data Analysis*

The qualitative data were shown as the relative changes of the free intracellular  $\text{Ca}^{2+}$  concentrations compared to those of basal levels. The quantitative data of the drug effects on the basal, free cytosolic  $\text{Ca}^{2+}$  concentrations were shown by % changes compared to control condition and determined using the following calculations:

$$\% \text{ Change} = ([\text{Ca}^{2+}]_{i,d} / [\text{Ca}^{2+}]_{i,b}) \times 100$$

where  $[\text{Ca}^{2+}]_{i,b}$  and  $[\text{Ca}^{2+}]_{i,d}$  represent the intracellular  $\text{Ca}^{2+}$  concentrations which were measured in the absence and in the presence of drugs, respectively. The quantitative data of the drug effects on agonist-induced increased intracellular  $\text{Ca}^{2+}$  concentrations were shown by % differences between agonists (serum or carbachol) alone and drug pretreatment groups, which calculated using the following equation:

$$\% \text{ Change} = (\Delta[\text{Ca}^{2+}]_{i,d+a} / \Delta[\text{Ca}^{2+}]_{i,a}) \times 100$$

where  $\Delta[\text{Ca}^{2+}]_{i,d+a}$  and  $\Delta[\text{Ca}^{2+}]_{i,a}$  represent agonist-induced increased intracellular  $\text{Ca}^{2+}$  concentrations in the presence and in the absence of drugs, respectively. The data were expressed as mean  $\pm$  standard error of the mean (SEM) and were compared using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparison. The data which have p value less than 0.05, were considered to be statistically significant.

**CHAPTER IV**

**MECHANISM OF CALCIUM CHANNEL ANTAGONIST-INDUCED  
INHIBITION OF TUMOR CELL GROWTH**

**A. Introduction**

Intracellular  $\text{Ca}^{2+}$  ions appear to play an important role in the regulation of a number of cellular processes including DNA synthesis and cell proliferation (Geck and Bereiter-Hahn, 1991; Mendoza, 1988; Metcalfe *et al.*, 1986; Whitfield, 1990; Whitfield *et al.*, 1987). Calmodulin (CaM) which may be activated by increased intracellular  $\text{Ca}^{2+}$  ions seems also to be involved in these processes (Rasmussen and Means, 1988). The intracellular  $\text{Ca}^{2+}$  ion is increased at anaphase initiation of the cell cycle (Boynton, 1988), and the level of CaM is also increased at the G1/S border (Rasmussen and Means, 1988). These  $\text{Ca}^{2+}$  or CaM increases have shown to be inhibited by their antagonists, causing a block in DNA synthesis (Katayama *et al.*, 1990; Ogata *et al.*, 1991). In addition, CaM levels are significantly elevated in exponentially growing transformed cells, as well as are total cellular  $\text{Ca}^{2+}$  levels in many tumor cells (Veigl *et al.*, 1982). The high  $\text{Ca}^{2+}$  level seems to be due to either the excessive influx of extracellular  $\text{Ca}^{2+}$  or the ability of neoplastic mitochondria to retain high concentrations of  $\text{Ca}^{2+}$  (Metcalfe *et al.*, 1980). Thus, prolonged, abnormally high levels of intracellular  $\text{Ca}^{2+}$  may lead to increased activation of the  $\text{Ca}^{2+}$  second messenger system resulting in the exaggerated growth rate of certain malignant cells.

The cell replication rate *in vitro* has been found to be positively correlated with  $\text{Ca}^{2+}$



concentration in medium (Swierenga *et al.*, 1978). The presence of  $\text{Ca}^{2+}$  in the culture medium is absolutely necessary for the stimulation of lymphocytes by lectin mitogens (Whitney and Sutherland, 1972), and calcium chloride stimulates *in vitro* cellular DNA synthesis (Maino *et al.*, 1974). The  $\text{Ca}^{2+}$  ionophore A23187 can mimic the action of mitogens on the lymphocyte DNA synthesis (Lichtman *et al.*, 1983). However, in cancer cells the dependence of cell proliferation upon extracellular  $\text{Ca}^{2+}$  concentration is controversial and appears to be cell type specific (Parsons *et al.*, 1983; Shirakawa *et al.*, 1986; Simpson and Arnold, 1986; Simpson and Taylor, 1988). Simpson and coworkers have shown that HT-39 and MCF-7 human breast cancer cell lines and the HL-60 human promyelocytic leukemia cell line exhibit  $\text{Ca}^{2+}$ -dependent proliferation, while the growth of the L1210 leukemia cell line is inhibited by an increased extracellular  $\text{Ca}^{2+}$  concentration (Simpson and Arnold, 1986; Simpson and Taylor, 1988). Others have shown that removal of  $\text{Ca}^{2+}$  from the culture medium did not inhibit cell growth in transformed fibroblasts (Balk *et al.*, 1979), liver hepatomas (Swierenga *et al.*, 1978), mouse embryo 3T3 cells (Paul and Ristow, 1979) and human ovarian cells (Chan, 1989), whereas, with their untransformed ancestors, removal of  $\text{Ca}^{2+}$  from the medium altered cell growth. The weight of the evidence favors the argument that the dependence of cancer cell growth on extracellular  $\text{Ca}^{2+}$  is not as great that of normal cells (Chan and Howell, 1986; Swierenga *et al.*, 1983).

The recognition of the importance of intracellular  $\text{Ca}^{2+}$  in cellular proliferation stimulated many researchers to test the effect of drugs which reduce the transmembrane  $\text{Ca}^{2+}$  influx on cell growth. Using typical  $\text{Ca}^{2+}$  channel antagonists, e.g. verapamil or

nifedipine, the investigators have shown that cellular proliferation was inhibited by those drugs in mouse spleen lymphocytes (Kunert-Radek *et al.*, 1990), human mesangial cells (Shultz and Raij, 1990), rat thyroid cells (Takada *et al.*, 1990), human retinal glial cells (Puro and Mano, 1991) and rat neuroblasts (Pincus *et al.*, 1991).  $\text{Ca}^{2+}$  channel antagonists also inhibit tumor cell growth as shown in HT-39 human breast cancer cell line (Taylor and Simpson, 1992), human glioma cells (Kunert-Radek *et al.*, 1989), human prostatic tumor cells (Batra *et al.*, 1991), human brain tumor cells (Schmidt *et al.*, 1988), human lung cancer cells (Schuller *et al.*, 1991) and human leukaemia cells (Brusterud, 1992). However, in these studies the cause-and-effect relationship has not been explored. Moreover,  $\text{Ca}^{2+}$  channel blockers have shown to bind to other sites other than  $\text{Ca}^{2+}$  channels and produce non- $\text{Ca}^{2+}$  channel actions (Zernig, 1990; Nandi *et al.*, 1990; Pancrazio *et al.*, 1991). Thus, the mechanisms of action of these drugs in tumor cell growth may involve  $\text{Ca}^{2+}$  channels as well as other effectors and are basically unknown.

In addition to the effect on tumor cell growth,  $\text{Ca}^{2+}$  channel blockers have also been known to reverse multidrug resistance (MDR) to chemotherapeutic agents (Helson, 1984). One basis for MDR within a given tumor cells resides with the ability of that cell to extrude cytotoxic compounds via active transport mechanism, thereby effectively reducing the available intracellular drug concentration (Helson, 1984). The P-glycoprotein, the product of MDR gene is responsible for this extrusion action. Acquired resistance to anticancer drugs is associated with increased expression of the MDR gene. Interestingly, adenylyl cyclase and various plasma membrane channels, including the  $\alpha 1$

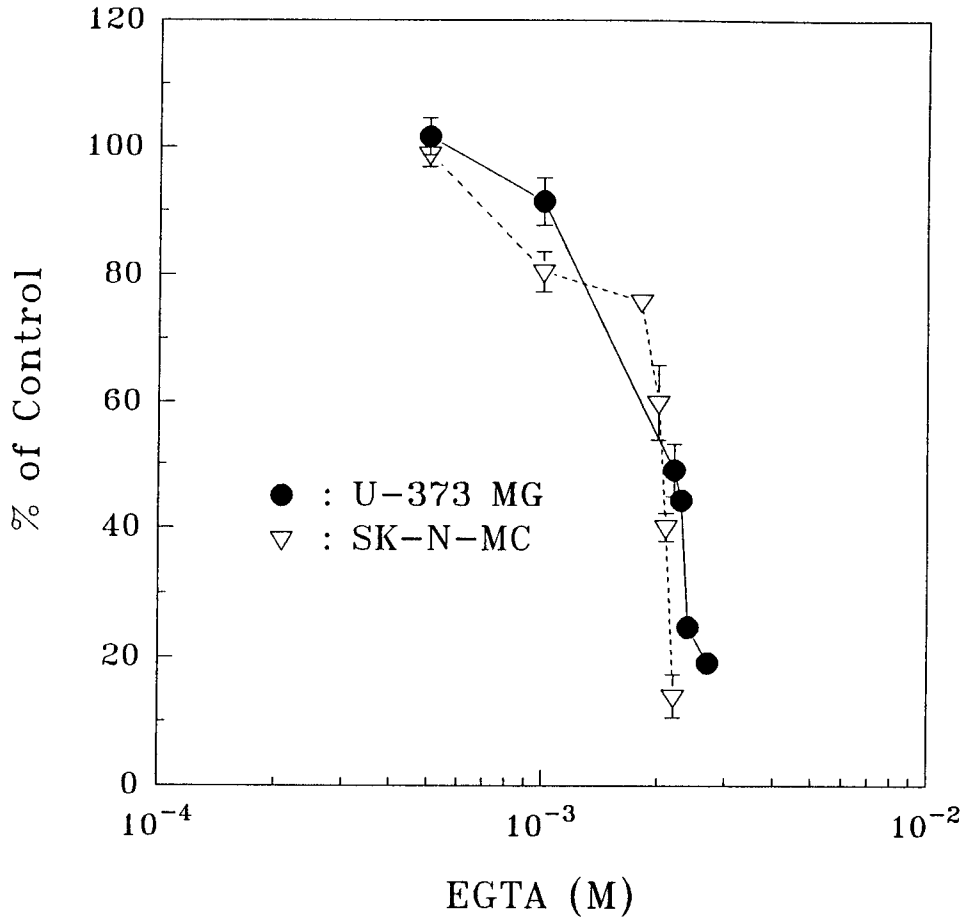
subunit of the L-type  $\text{Ca}^{2+}$  channel, possess a similar repeated transmembrane motif (Gottesman and Pastan, 1989). Although the  $\text{Ca}^{2+}$  regulatory effects of the  $\text{Ca}^{2+}$  channel antagonists may likely be a candidate, the mechanisms of action of these drugs in the reversal of MDR is unknown.

The data presented in this chapter describe the effects of some  $\text{Ca}^{2+}$  channel antagonists on both tumor cell growth and growth factor-induced increased intracellular  $\text{Ca}^{2+}$  using two nervous system tumor cell lines (astrocytoma, U-373 MG and neuroblastoma, SK-N-MC cell lines) as a model cellular system. Experiments were designed to elucidate the  $\text{Ca}^{2+}$ -related mechanisms responsible for the regulation of the tumor cell growth by these drugs. The data demonstrate that the increased intracellular  $\text{Ca}^{2+}$  concentration is an important signalling mechanism for growth factor-induced cell proliferation and that the most of the increased  $\text{Ca}^{2+}$  is from intracellular  $\text{Ca}^{2+}$  store release. Furthermore,  $\text{Ca}^{2+}$  channel blockers can effectively inhibit tumor cell growth and their mechanisms of action appear to be closely related to the inhibition of growth factor-induced increased intracellular  $\text{Ca}^{2+}$  concentration. Possible regulatory pathways regarding to this inhibition are discussed.

## **B. Results**

### ***Extracellular Calcium Dependency of Tumor Cell Growth***

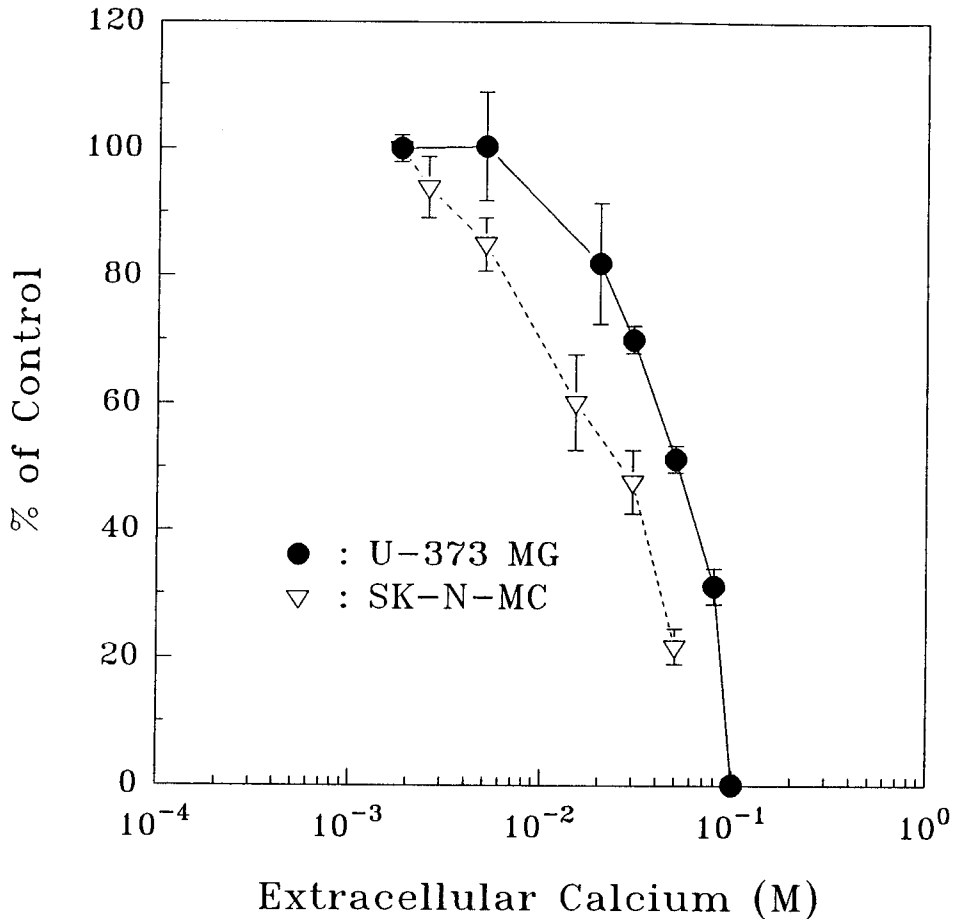
Fig. 4.1 shows the effect of EGTA on tumor cell growth. Chelation of extracellular  $\text{Ca}^{2+}$  with EGTA inhibited the tumor cell growth in both U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines. This inhibition was dose-dependent.



**Fig. 4.1.** Effect of EGTA on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of EGTA in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.

The inhibition of tumor cell growth by EGTA appeared not to be due to its cytotoxicity as shown in Figs. 4.9 and 4.10. These results can be interpreted to mean that during tumor cell proliferation, the influx of extracellular  $\text{Ca}^{2+}$  may occur and that EGTA will reduce the transmembrane concentration gradient for  $\text{Ca}^{2+}$  ion by lowering available extracellular  $\text{Ca}^{2+}$ . Thus, the inhibition of  $\text{Ca}^{2+}$  influx ultimately leads to growth inhibition. However, since  $\text{Ca}^{2+}$  mobilization from internal stores without  $\text{Ca}^{2+}$  influx seems to be involved in the proliferative signalling mechanisms in these cells, which will be described later, EGTA may indirectly affect growth factor-induced  $\text{Ca}^{2+}$  response. If extracellular  $\text{Ca}^{2+}$  is lowered, then the cell will be gradually depleted of  $\text{Ca}^{2+}$ , i.e. intracellular stores will be reduced (Jin *et al.*, 1992; Murphy and Miller, 1988). Thus, transmembrane  $\text{Ca}^{2+}$  concentration gradient is important for maintaining  $\text{Ca}^{2+}$  stores involving  $\text{Ca}^{2+}$  mobilization in response to agonist stimulation. Therefore, the EGTA action on tumor cell growth may be due to the reduction of available  $\text{Ca}^{2+}$  of internal stores. Alternatively, long term exposure of EGTA may enter into the cells and thus, chelate intracellular  $\text{Ca}^{2+}$ , resulting in blunting growth factor-induced  $\text{Ca}^{2+}$  signalling mechanisms. However, these possibilities are only speculative, and the actual mechanism of EGTA is unknown.

The increased extracellular  $\text{Ca}^{2+}$  concentration also led to growth-inhibitory effects on both tumor cell lines as shown in Fig. 4.2. Recent work has shown that a sustained increased cytosolic  $\text{Ca}^{2+}$  concentration is often linked to the onset of drug-induced cytotoxicity (Reviewed in Nicotera *et al.*, 1992; Orrenius *et al.*, 1989). The proposed cytotoxic mechanisms include the disruption of the cytoskeletal network and an

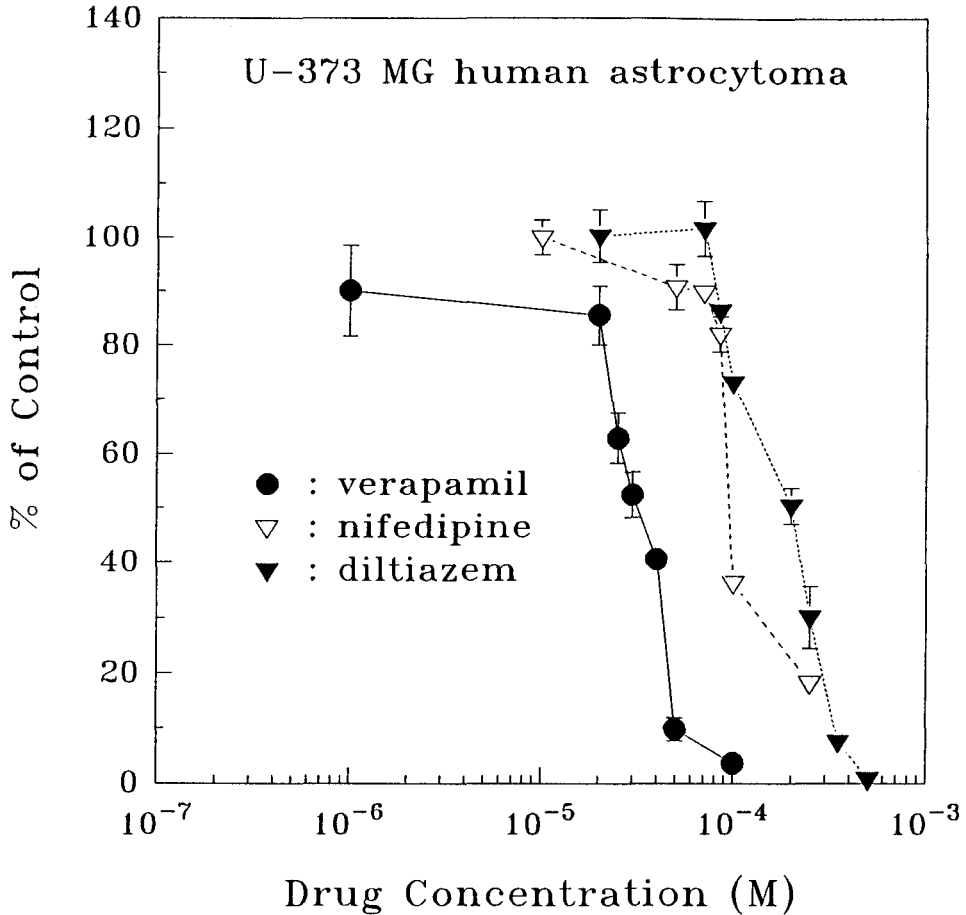


**Fig. 4.2.** Effect of high extracellular  $\text{Ca}^{2+}$  on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of  $\text{Ca}^{2+}$  in the culture medium. The cell number was counted 2 days after  $\text{Ca}^{2+}$  treatment. The results were expressed as a percent change of the number of the cells obtained in the culture medium containing normal  $\text{Ca}^{2+}$  concentration (1.8mM). The data points represent the mean value of at least four replicate dishes with bars indicating SEM.

uncontrolled activation of  $\text{Ca}^{2+}$ -stimulated catabolic enzymes, such as phospholipases, proteases and endonucleases. However, the effects of high extracellular  $\text{Ca}^{2+}$  concentration on the level of cytosolic  $\text{Ca}^{2+}$  has not been well described. If during cell proliferation  $\text{Ca}^{2+}$  influx occurs, then high extracellular  $\text{Ca}^{2+}$  concentration will increase the transmembrane concentration gradient for  $\text{Ca}^{2+}$  ions and, thus, possibly lead to a sustained increased intracellular  $\text{Ca}^{2+}$  concentration. However, the actual  $\text{Ca}^{2+}$  entry pathways are undefined, and how the intracellular  $\text{Ca}^{2+}$  regulatory mechanisms are disturbed is unknown.

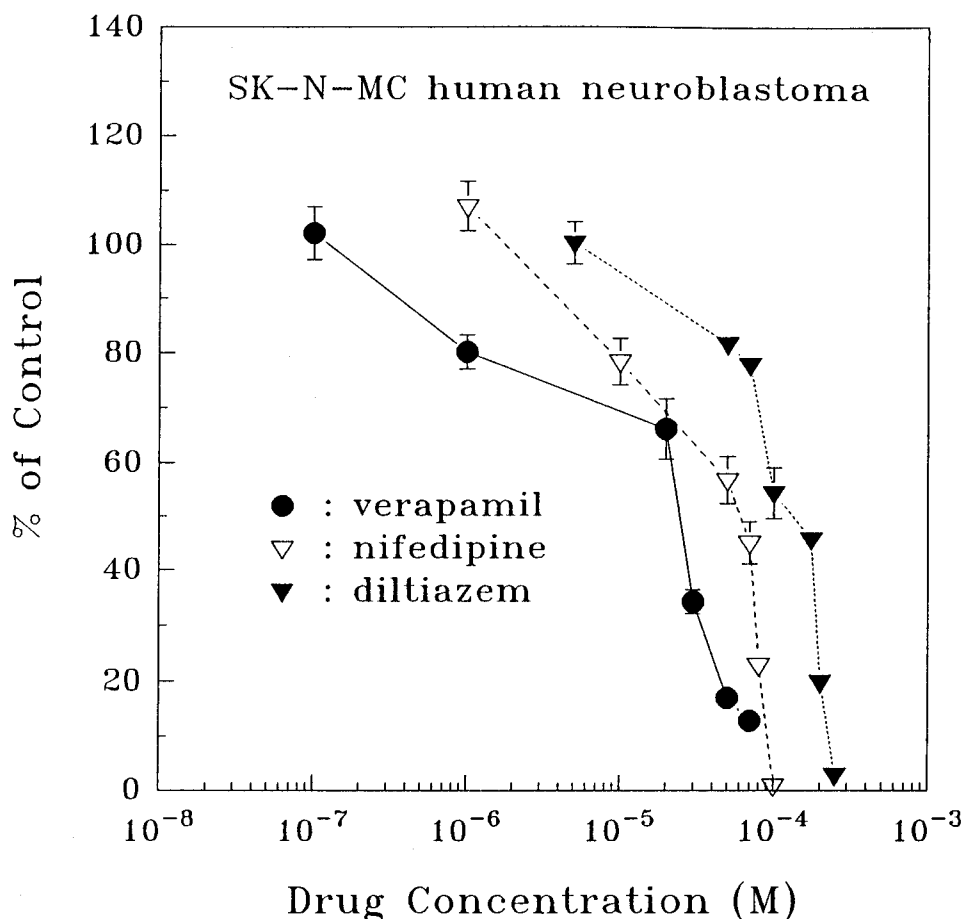
### ***Calcium Channel Antagonist-Induced Inhibition of Tumor Cell Growth***

Structurally different  $\text{Ca}^{2+}$  channel antagonists were used in order to see the possible involvement of  $\text{Ca}^{2+}$  channels in tumor cell growth. The culture medium and  $\text{Ca}^{2+}$  channel antagonists were replaced every day. The *in vivo* pharmacokinetic data show that the half-lives of these drugs in healthy volunteers is less than 10 hours, although the values are variable depending on the experimental conditions (Kates, 1987). However, no *in vitro* data on half-lives of these drugs are available but the half-lives would be expected to be much longer than the *in vivo* half-lives. Figs. 4.3 and 4.4 show the effects of  $\text{Ca}^{2+}$  channel antagonists on the growth of U-373 MG astrocytoma and SK-N-MC neuroblastoma tumor cells, respectively. In both cell lines these typical  $\text{Ca}^{2+}$  channel antagonists inhibited cell growth in a dose-dependent manner. Verapamil was the most effective in both cell lines. The order of the potency is verapamil > nifedipine > diltiazem in both cell lines. However, the concentrations of half-maximum effect



**Fig. 4.3. Effect of Ca<sup>2+</sup> channel antagonists on the growth of U-373 MG human astrocytoma cell lines.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of verapamil (closed circle), nifedipine (open inverted triangle) or diltiazem (closed inverted triangle) in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.





**Fig. 4.4. Effect of  $\text{Ca}^{2+}$  channel antagonists on the growth of SK-N-MC human neuroblastoma cell lines.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of verapamil (closed circle), nifedipine (open inverted triangle) or diltiazem (closed inverted triangle) in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.

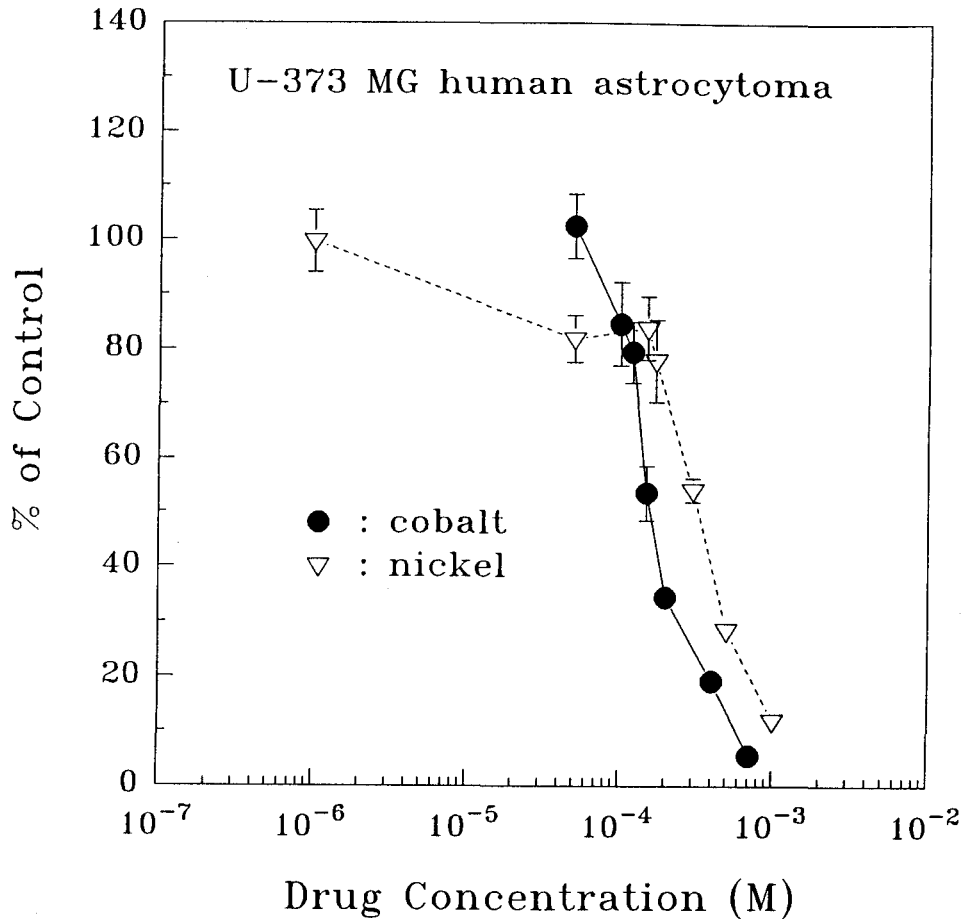
( $EC_{50}$ ) of these drugs are far greater than those in the inhibition of  $Ca^{2+}$  channels (far less than  $\mu M$ ). Thus, these results suggest that the inhibition of the tumor cell growth induced by these drugs may be less likely due to the inhibition of voltage-gated  $Ca^{2+}$  channels. Alternatively, in these tumor cells totally different type of  $Ca^{2+}$  channels may exist which require much higher concentrations of antagonists. Another possibility may exist that these  $Ca^{2+}$  channel antagonists may have other binding sites and produce effects other than the inhibition of  $Ca^{2+}$  channels (Zernig, 1990). For example, the functions of protein kinase C (PKC) and calmodulin (CaM) which are implicated in cell proliferation (Rasmussen and Rasmussen, 1986; Rozengurt, 1986), can be inhibited by these  $Ca^{2+}$  channel antagonists. Thus, the growth-inhibitory effects of these drugs may be due to their regulatory roles of PKC and/or CaM.

In this study the cell growth was assessed by counting the number of cells. Thus, the results might include two different phenomena: cell proliferation and cell death. However, the cytotoxic mechanism can be excluded in their growth inhibition of the tumor cells, since the cytotoxicity assay using the trypan blue exclusion method (Bowles *et al.*, 1990; Schanne *et al.*, 1979; Schmidt *et al.*, 1988) shown in Figs. 4.9 and 4.10 demonstrated that with the exception of  $Ni^{2+}$  no significant differences existed between  $Ca^{2+}$  channel antagonists and control in both tumor cell lines. Thus, their growth-inhibitory effects of tumor cells are most likely due to their effects on tumor cell proliferation. However, since this cytotoxicity assay using the trypan blue exclusion method may fail to detect disintegrated cells, this limitation of the method must be considered in the interpretation of the data.

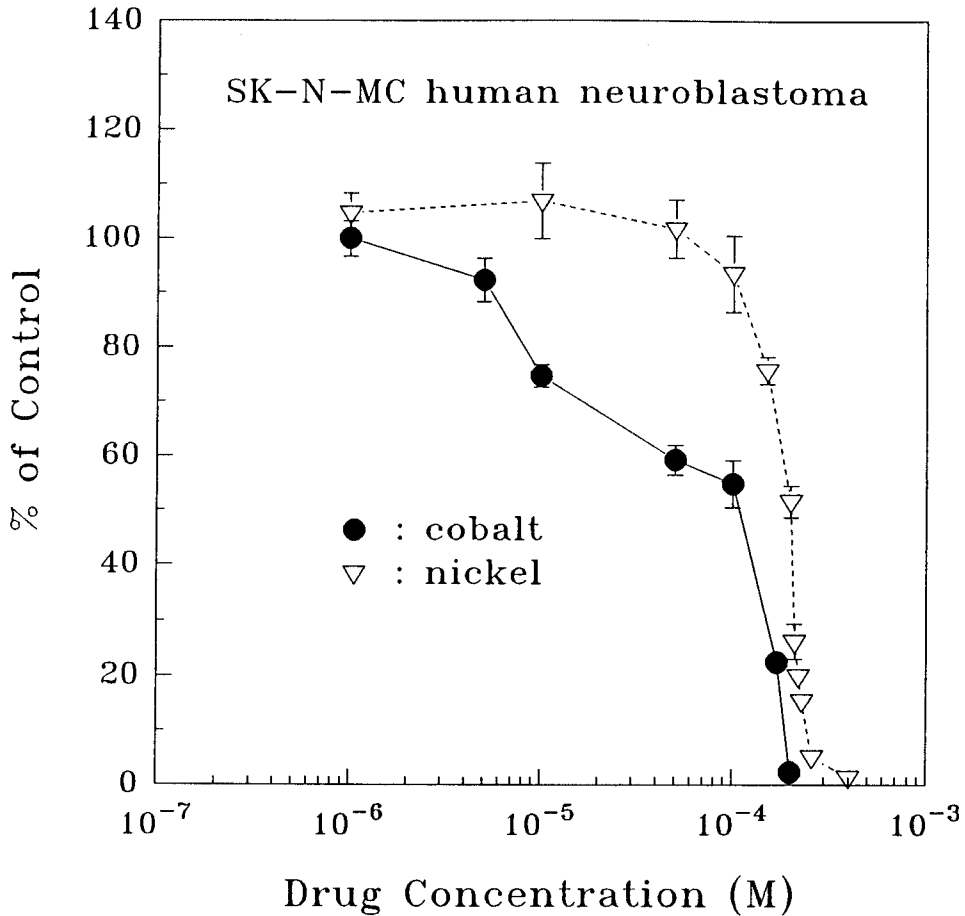
Inorganic  $\text{Ca}^{2+}$  channel antagonists, e.g.  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$ , also showed the inhibition of tumor cell growth in a dose-dependent manner illustrated in Figs. 4.5 and 4.6.  $\text{Co}^{2+}$  was more effective than  $\text{Ni}^{2+}$  in both cells. Since cytotoxicity assay (Figs. 4.9 and 4.10) showed that  $\text{Ni}^{2+}$  but not  $\text{Co}^{2+}$  was cytotoxic to both tumor cells, the growth-inhibitory action of  $\text{Ni}^{2+}$  may be due to also its cell-killing effect. In addition,  $\text{Ni}^{2+}$  has shown to be a potent carcinogen in experimental animals (Tveito *et al.*, 1989). This carcinogenic effect of  $\text{Ni}^{2+}$  may be related to its cytotoxicity since carcinogenicity induced by a certain chemical is often paradoxically caused by its cytotoxic effect (Ames and Gold, 1990). However, the actual mechanism of  $\text{Ni}^{2+}$  in carcinogenicity is not known (Tveito *et al.*, 1989). Interestingly,  $\text{Ni}^{2+}$  binds to CaM allosterically with  $\text{Ca}^{2+}$  ions and, thus, disturbs physiological functions of CaM (Raos and Kasprzak, 1989). Since CaM has numerous essential roles in cellular physiology including cell proliferation, the inhibition of the functions of CaM may induce cell cytotoxicity and/or tumorigenicity.

The inhibitory effects of various organic and inorganic  $\text{Ca}^{2+}$  channel antagonists on the tumor cell growth may be related to their interactions with  $\text{Ca}^{2+}$  channels. If  $\text{Ca}^{2+}$  influx is important in tumor cell growth, the blockade of the  $\text{Ca}^{2+}$  entry pathway could give rise to the inhibition of cell growth. However, since the role of  $\text{Ca}^{2+}$  influx in tumor cell growth is not known and these drugs have multiple actions, the experiments dealing with intracellular  $\text{Ca}^{2+}$  measurements were designed and pursued in order to elucidate the actual mechanism of action of these drugs in tumor cell growth.

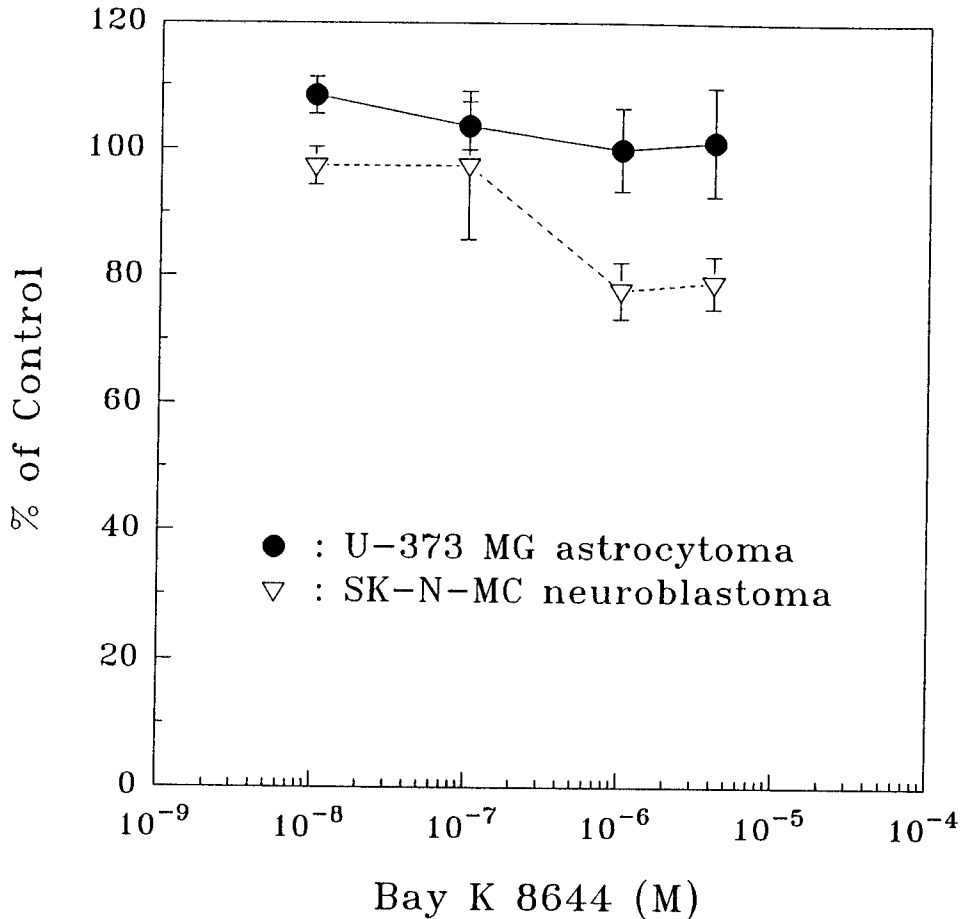
Moreover, as illustrated in Fig. 4.7, a  $\text{Ca}^{2+}$  channel agonist, Bay K-8644 did not induce a significant alteration of the growth of both tumor cells up to  $4\mu\text{M}$ . This result



**Fig. 4.5.** Effect of inorganic  $\text{Ca}^{2+}$  channel antagonists on the growth of U-373 MG human astrocytoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of cobalt (closed circle) or nickel (open inverted triangle) in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.



**Fig. 4.6.** Effect of inorganic  $\text{Ca}^{2+}$  channel antagonists on the growth of SK-N-MC human neuroblastoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of cobalt (closed circle) or nickel (open inverted triangle) in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.



**Fig. 4.7.** Effect of Bay K-8644 on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of Bay K-8644 in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.

further suggests that at least voltage-sensitive L-type  $\text{Ca}^{2+}$  channels are not involved in the tumor cell growth. However, this does not imply that  $\text{Ca}^{2+}$  signals are not important in the process of the cell proliferation. Internal  $\text{Ca}^{2+}$  release may be enough to trigger intracellular signalling mechanisms which ultimately lead to cell division. Thus, the intracellular  $\text{Ca}^{2+}$  measurements are required to determine the significance of  $\text{Ca}^{2+}$  signals as well as elucidating the targets for  $\text{Ca}^{2+}$  channel antagonists in terms of the regulation of the cell proliferation.

#### ***A Receptor-Operated $\text{Ca}^{2+}$ Channel Blocker, SK&F 96365 Is a Potent Inhibitor of Tumor Cell Growth***

Recently, the novel  $\text{Ca}^{2+}$  entry mechanism into the cells, e.g. receptor-mediated  $\text{Ca}^{2+}$  entry (RMCE) by occupation of membrane receptors has been described in many cellular systems which are mainly non-excitabile cells (Reviewed in Putney, Jr., 1990; Rink, 1990; Taylor, 1990). An operational definition of RMCE is "an influx of  $\text{Ca}^{2+}$  consequent to receptor occupation and not dependent on depolarization that generates a biologically, significantly increased intracellular  $\text{Ca}^{2+}$  concentration" (Rink, 1990). The function and mechanism of RMCE are less well understood compared to those of voltage-gated  $\text{Ca}^{2+}$  entry. However, possible important physiological functions of RMCE have been suggested: localized  $\text{Ca}^{2+}$  signalling, rapid signalling in non-excitabile cells, maintenance of  $\text{Ca}^{2+}$  signals, refilling of discharged internal  $\text{Ca}^{2+}$  pools and control of the intracellular  $\text{Ca}^{2+}$  spiking (Rink, 1990).

Merritt and associates developed a novel compound, SK&F 96365, which is structurally distinct from classic  $\text{Ca}^{2+}$  antagonists and which effectively blocks RMCE

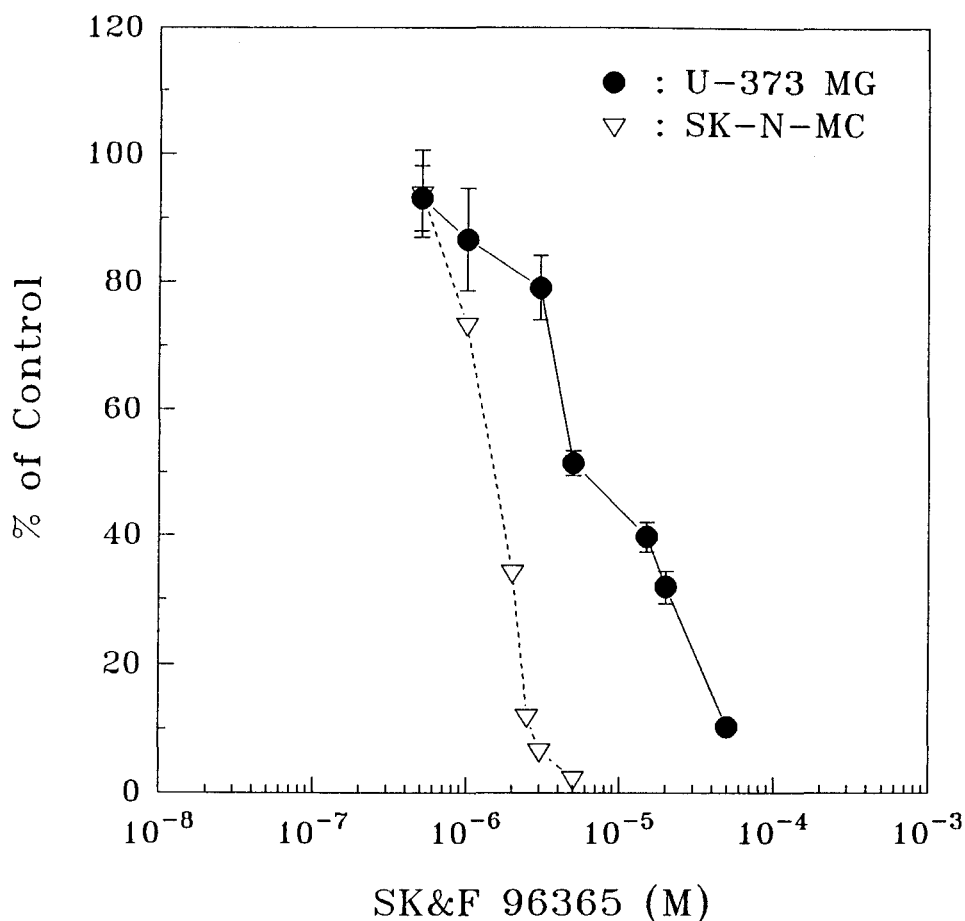
(Merritt *et al.*, 1990). This RMCE blocking action may result from either direct interaction with  $\text{Ca}^{2+}$  channels or indirect mechanisms including the inhibitory effect on cytochrome P-450 (Garcia-Sancho *et al.*, 1992). Since SK&F 96365 has such a regulatory role in the  $\text{Ca}^{2+}$  signalling mechanism which is important in cell growth, the effect of this drug on tumor cell growth would be very interesting.

The growth of the tumor cells was inhibited by SK&F 96365 in a dose-dependent fashion shown in Fig. 4.8. The  $\text{EC}_{50}$  of this inhibition by the drug is very comparable to that of RMCE in human platelets, human neutrophils and human umbilical vein endothelial cells (Rink, 1990). Although this RMCE inhibition may be suspected as a mechanism of inhibition of the tumor cell growth, the exact mechanism of this drug is not known. Thus, further investigations require observing its effect on the intracellular  $\text{Ca}^{2+}$  signals.

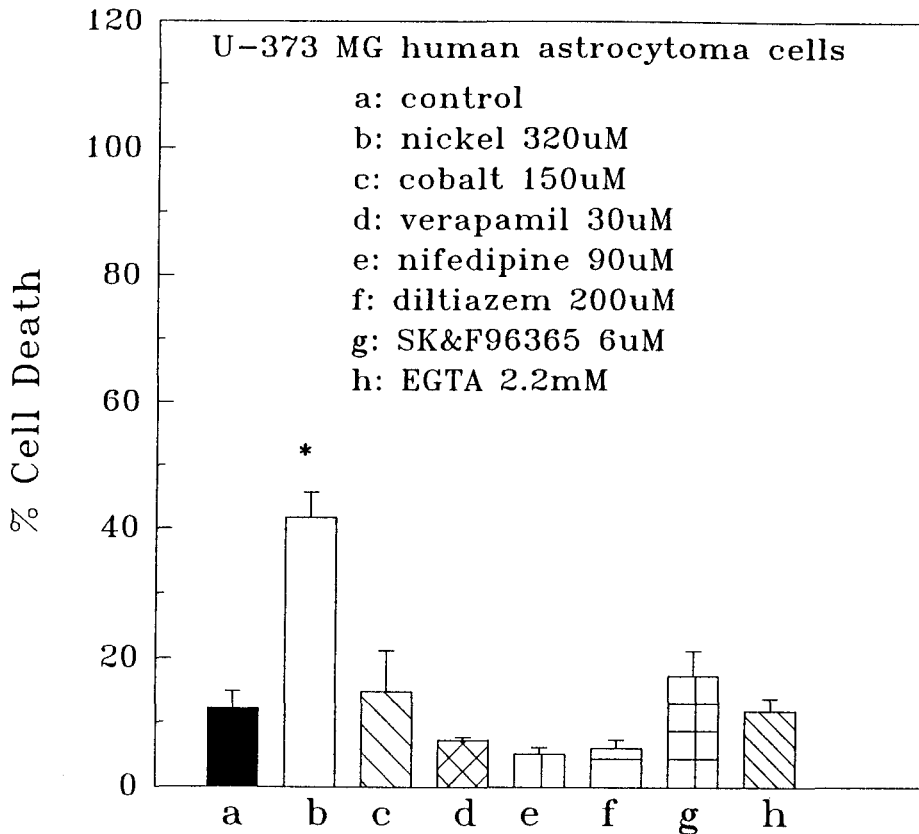
### ***The Relationship between Carbachol-Induced Increased Intracellular Calcium and Tumor Cell Growth in U-373 MG Human Astrocytoma Cell Line***

Carbachol (Carb), a cholinergic agonist, was chosen as an agonist to induce alteration of intracellular  $\text{Ca}^{2+}$  concentration in U-373 MG human astrocytoma cell line since many nonexcitable cells as well as neuronal cells respond to stimulation by this drug with a biphasic elevation of intracellular  $\text{Ca}^{2+}$  concentration (Felder *et al.*, 1992; Fischer *et al.*, 1992; Hiramatsu *et al.*, 1992; Lambert *et al.*, 1990; Shuttleworth and Thompson, 1992). The initial transient phase (peak) is due to the release of intracellular  $\text{Ca}^{2+}$  stores, whereas a secondary, sustained elevation of intracellular  $\text{Ca}^{2+}$  (plateau) is maintained by  $\text{Ca}^{2+}$  entry from the extracellular space into the cytosol (Felder *et al.*, 1992; Lambert *et*

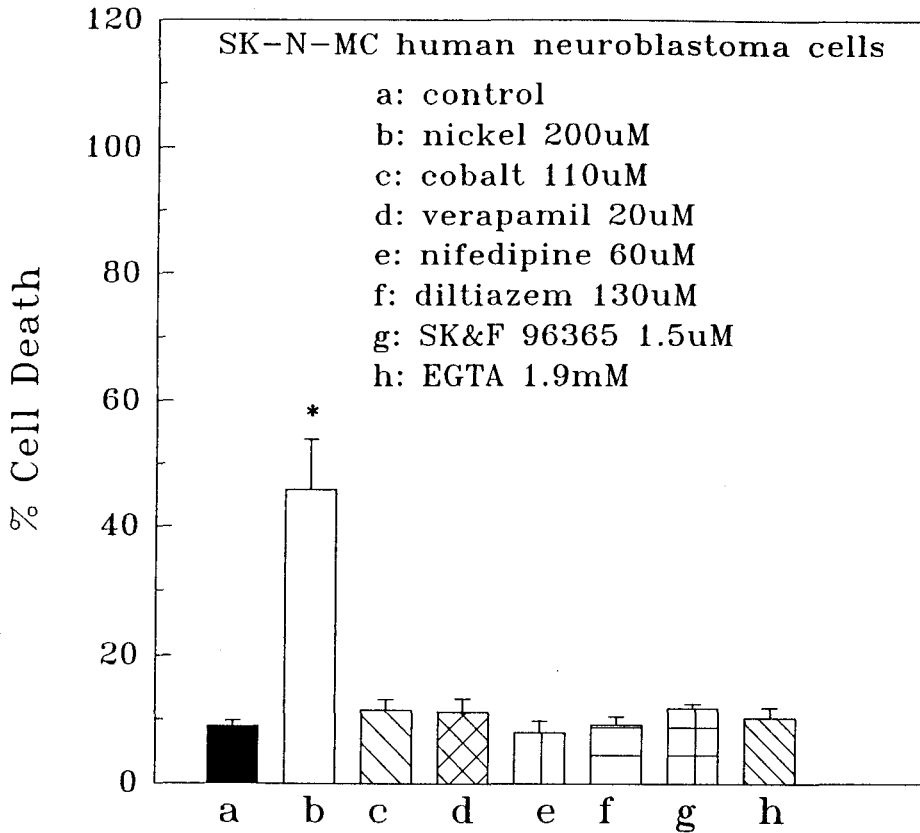




**Fig. 4.8.** Effect of SK&F 96365 on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of SK&F 96365, a receptor-operated  $\text{Ca}^{2+}$  channel blocker, in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.



**Fig. 4.9. Cytotoxic effect of EGTA or  $Ca^{2+}$  channel antagonists on U-373 MG human astrocytoma cell line.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of the drugs in the culture medium. Cell cytotoxicity was assayed by trypan blue extrusion method 2 days after drug treatment. The results were expressed as a percent change of the number of dead cells obtained in the absence of the drug. Each column represents the mean value of four replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison. (\* $p < 0.05$  compared to control condition).

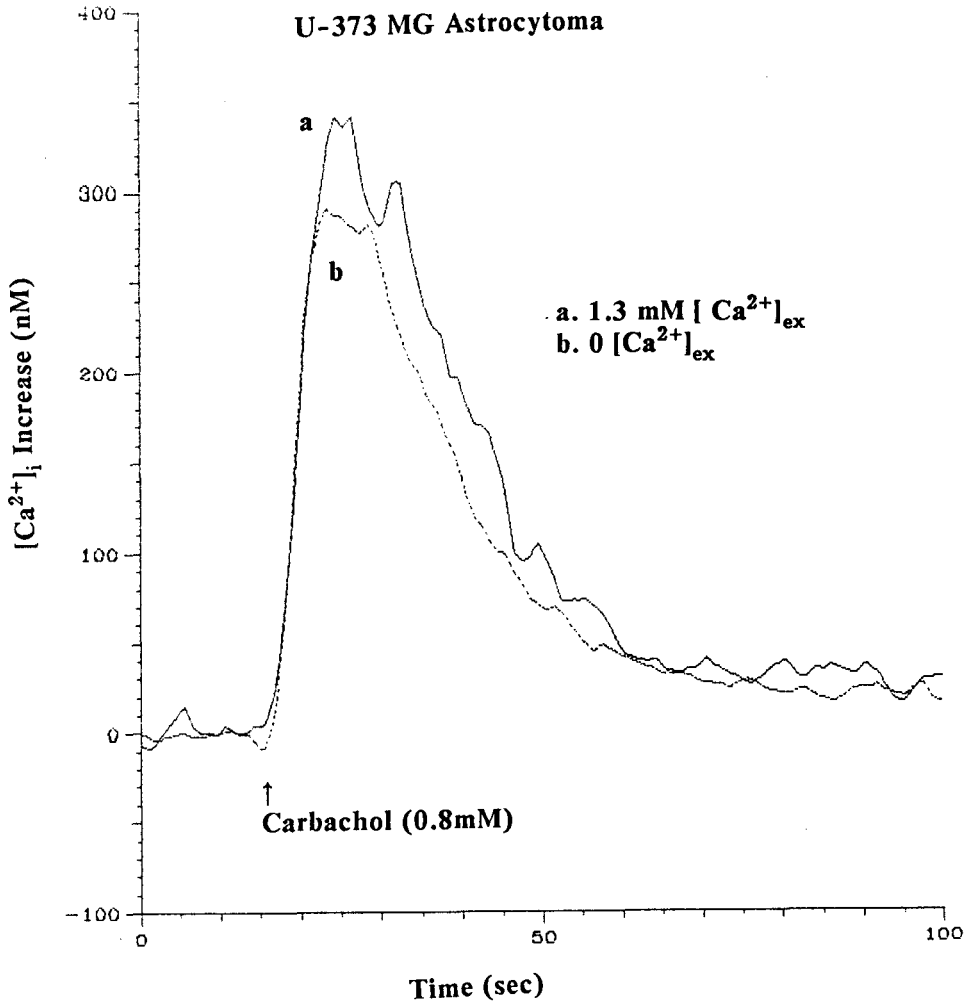


**Fig. 4.10. Cytotoxic effect of EGTA or  $\text{Ca}^{2+}$  channel antagonists on SK-N-MC human neuroblastoma cell line.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of the drugs in the culture medium. Cell cytotoxicity was assayed by trypan blue extrusion method 2 days after drug treatment. The results were expressed as a percent change of the number of dead cells obtained in the absence of the drug. Each column represents the mean value of four replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison. (\* $p < 0.05$  compared to control condition).

*al.*, 1992). This is referred to as a receptor-mediated  $\text{Ca}^{2+}$  entry (RMCE) (Putney, 1990; Sage *et al.*, 1992).

***a) Carbachol-Induced Intracellular Calcium Increase Is not Dependent upon Extracellular Calcium Concentration***

As shown in Fig. 4.11., carbachol rapidly increased intracellular  $\text{Ca}^{2+}$  concentration which was measured in Fura-2 loaded U-373 MG human astrocytoma cell line. In normal extracellular  $\text{Ca}^{2+}$  concentration (1.3mM), the response of these cells to Carb was transient without any significant, sustained increased intracellular  $\text{Ca}^{2+}$  concentration which was observed in many other cells (Felder *et al.*, 1992; Fischer *et al.*, 1992; Hiramatsu *et al.*, 1992; Lambert *et al.*, 1990; Shuttleworth and Thompson, 1992). This contradictory findings in our model cellular system suggest that this tumor cell may have a different response to carbachol stimulation resulting in no receptor-mediated  $\text{Ca}^{2+}$  influx. Since this  $\text{Ca}^{2+}$  entry has been suggested to contribute to the refilling of empty  $\text{IP}_3$ -sensitive stores, the absence of this pathway in this tumor cell further suggests that other mechanisms may exist for refilling the empty stores, for example, through "secret pathway" which allows  $\text{Ca}^{2+}$  to pass from the extracellular space to the stores without entering the cytoplasm (Taylor, 1990). In order to ascertain no contribution of  $\text{Ca}^{2+}$  influx from the extracellular space to Carb-induced increased intracellular  $\text{Ca}^{2+}$  concentration, the experiment was done with the resuspending medium containing 0  $\text{Ca}^{2+}$ . The results showed no differences of Carb-induced increased intracellular  $\text{Ca}^{2+}$  concentration between normal  $\text{Ca}^{2+}$ -containing and  $\text{Ca}^{2+}$ -free media (Fig. 4.11). Thus, the Carb-induced increased intracellular  $\text{Ca}^{2+}$  concentration is mainly due to internal store



**Fig. 4.11. Extracellular Ca<sup>2+</sup> concentration independency of carbachol (0.8mM)-induced increased intracellular Ca<sup>2+</sup> concentration in U-373 MG human astrocytoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca<sup>2+</sup> or 1.3mM Ca<sup>2+</sup> and transferred to a quartz cuvette for fluorescence measurements. The data represent net increases of intracellular Ca<sup>2+</sup> concentration with time. The arrow shows the time point for the addition of carbachol.

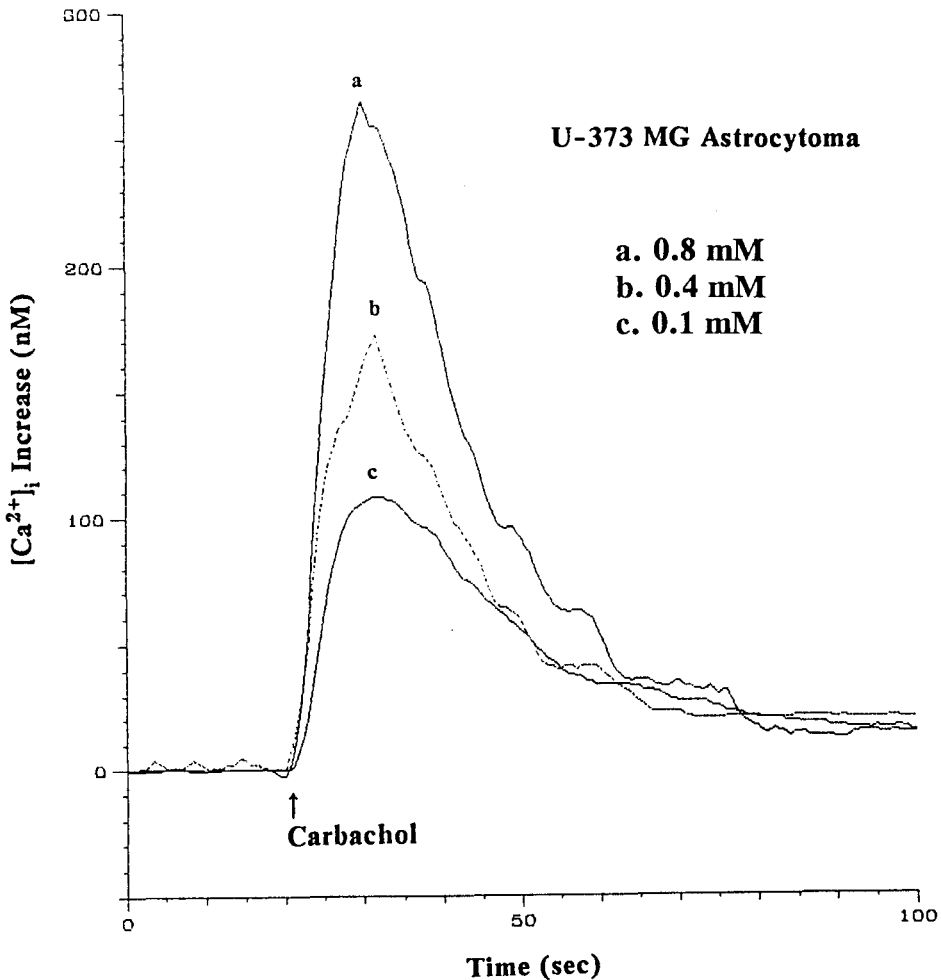
release.

***b) Carbachol-Induced Increased Intracellular Calcium Concentration Is Dose-Dependent and Muscarinic Receptor-Mediated***

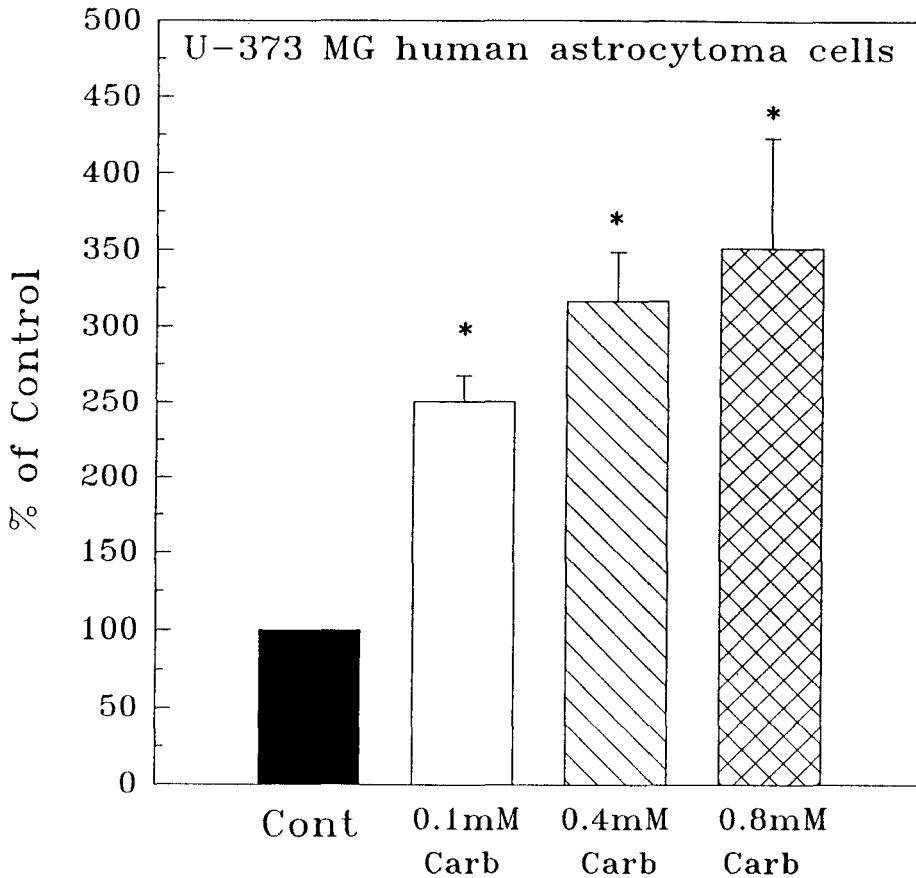
Figs. 4.12 and 4.13 show the dose-response relationships in Carb-induced increased intracellular  $\text{Ca}^{2+}$  concentration. The results clearly illustrate a dose-dependent increase of intracellular  $\text{Ca}^{2+}$  by Carb and suggest that this effect of Carb may be evoked by a receptor-mediated mechanism. Thus, a muscarinic receptor antagonist, atropine was applied prior to the addition of Carb. As shown in Fig. 4.14, the pretreatment of atropine completely abolished the effect of Carb, which demonstrates that the Carb-induced response results from the activation of muscarinic receptors. The stimulation of the muscarinic receptors probably generates  $\text{IP}_3$ , which can open  $\text{Ca}^{2+}$  channels of the endoplasmic reticulum and release  $\text{Ca}^{2+}$  from that store (Lambert and Nahorski, 1990). The concentrations of Carb up to a few mM range did not saturate the Carb-induced response.

***c) Carbachol-Induced Increased Tumor Cell Growth Is Dose-Dependent***

If the increased intracellular  $\text{Ca}^{2+}$  concentration is an important signalling mechanism in the tumor cell proliferation, Carb is expected to increase tumor cell growth, since Carb actually increased the intracellular  $\text{Ca}^{2+}$  concentration (Figs. 4.12 and 4.13). Thus, the growth response of the tumor cells to Carb was examined in order to see whether Carb has an effect on tumor cell growth. As illustrated in Fig. 4.15, Carb increased the tumor cell growth in a dose-dependent manner. Carb induced both the enhancement of

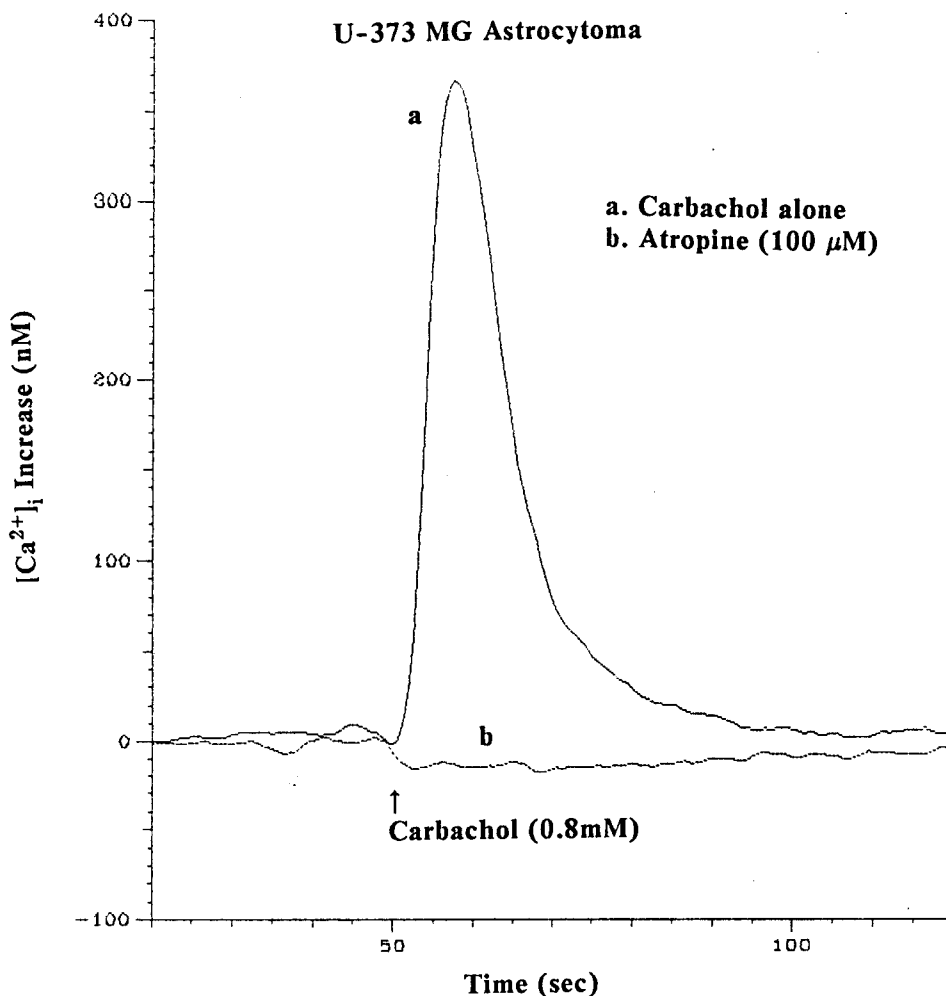


**Fig. 4.12. Dose-dependent increase of intracellular Ca<sup>2+</sup> concentration by carbachol in U-373 MG human astrocytoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AM for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The data represent net increases of intracellular Ca<sup>2+</sup> concentration with time. The arrow shows the time point for the addition of various concentrations of carbachol.

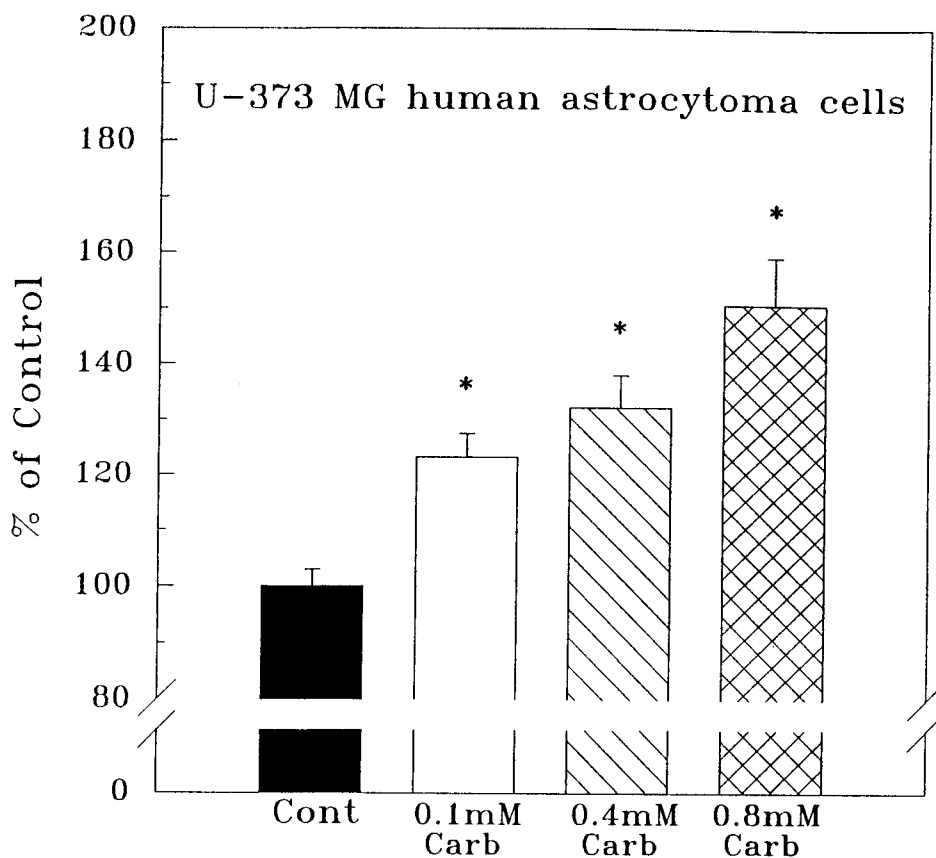


**Fig. 4.13. Effect of carbachol on intracellular  $\text{Ca}^{2+}$  concentration in U-373 MG human astrocytoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of the intracellular  $\text{Ca}^{2+}$  concentration induced by various concentrations of carbachol compared to basal level. Each column represents the mean value of four replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison. (\* $p < 0.05$  compared to control).





**Fig. 4.14. Abolishment of carbachol (0.8mM)-induced increased intracellular Ca<sup>2+</sup> concentration by the pretreatment of atropine (100μM) in U-373 MG human astrocytoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The data represent net increases of intracellular Ca<sup>2+</sup> concentration with time. The arrow shows the time point for the addition of carbachol. Atropine was treated for 3 minutes before starting the experiment.



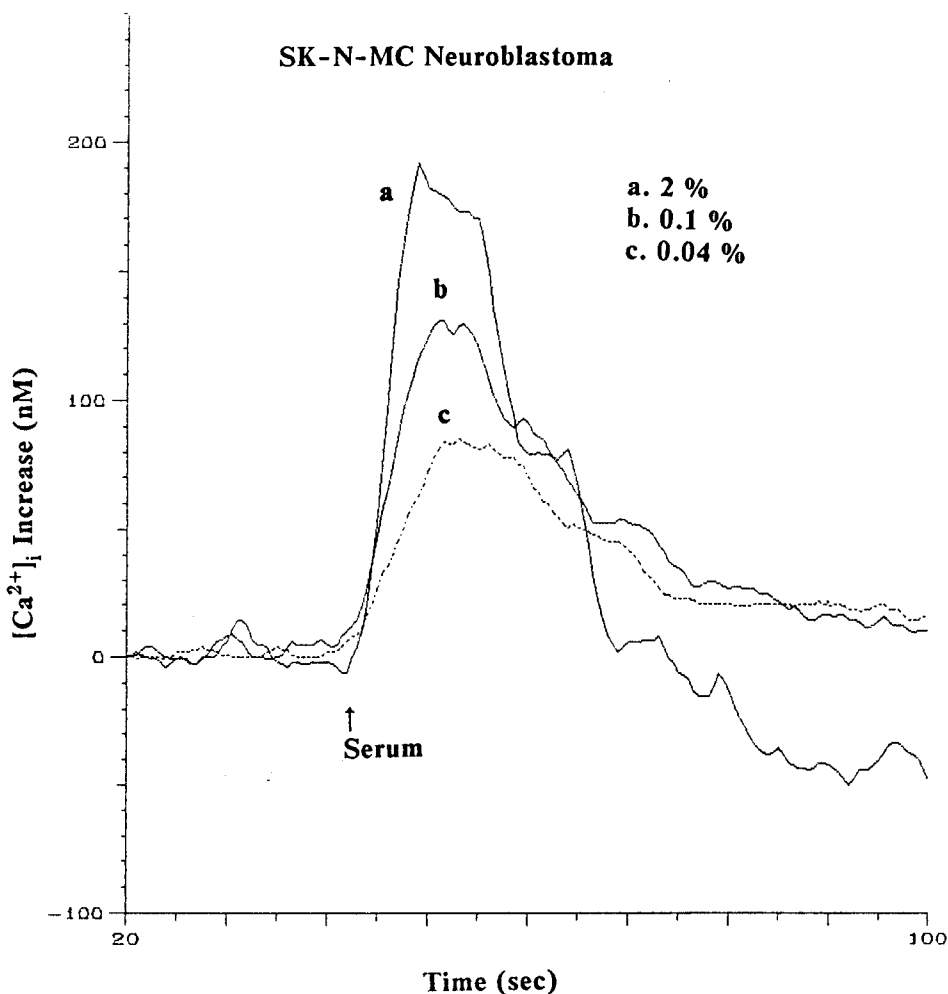
**Fig. 4.15. Effect of carbachol on the growth of U-373 MG human astrocytoma cell lines.** The cells were grown in MEM supplemented with 0.1% FBS in the absence or in the presence of various concentrations of carbachol in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. Each column represents the mean value of at least four replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison. (\* $p < 0.05$  compared to control).

the tumor cell growth and the increase of the intracellular  $\text{Ca}^{2+}$  concentration at a same concentration (Figs. 4.13 and 4.15). These results suggest that the increased intracellular  $\text{Ca}^{2+}$  concentration induced by growth factors (e.g. Carb) may be an important signalling mechanism in the proliferation of U-373 MG human astrocytoma cell line and that Carb is a very useful drug for investigating the role of  $\text{Ca}^{2+}$  signals in the cell proliferation. Therefore, Carb was used as an agonist for the elevation of intracellular  $\text{Ca}^{2+}$  concentration in further experiments where the effects of  $\text{Ca}^{2+}$  channel antagonists on the Carb-induced response were examined.

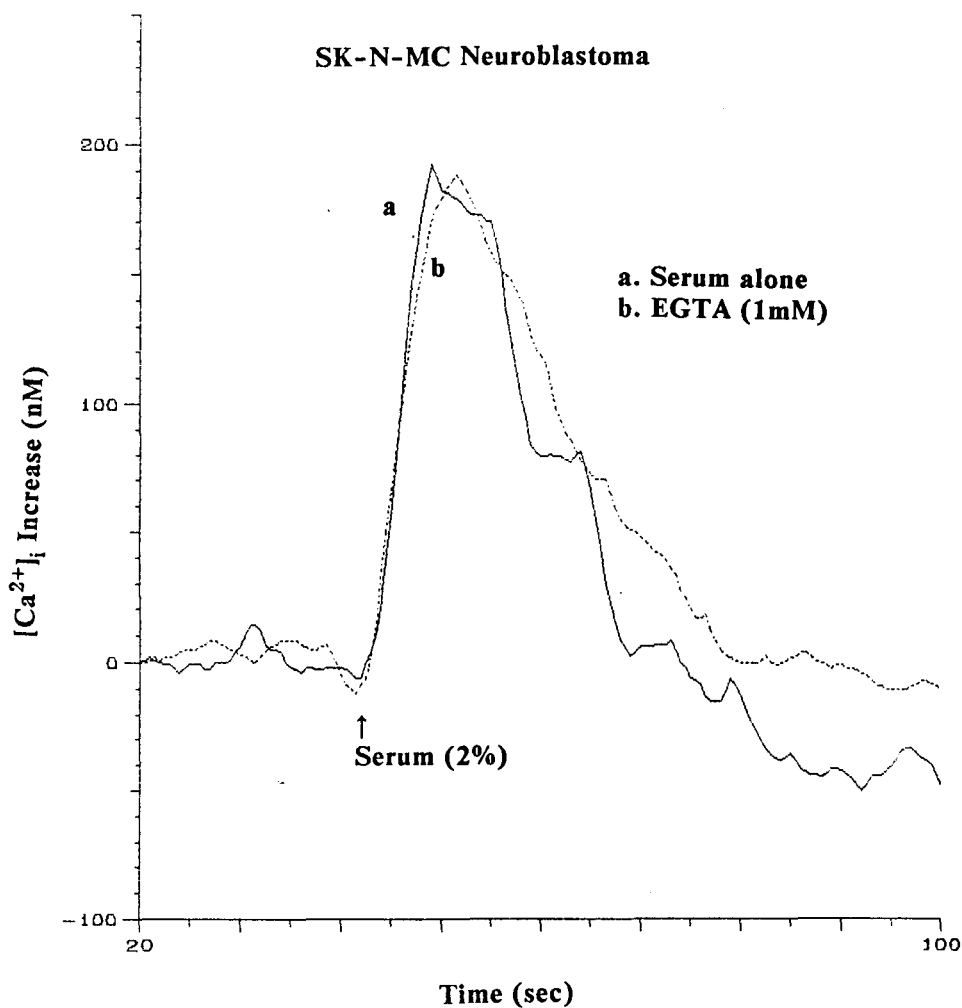
***Serum-Induced Intracellular Calcium Increase Is Dose-Dependent and not Dependent upon Extracellular Calcium Levels in SK-N-MC Human Neuroblastoma Cell Line***

Fetal bovine serum (FBS) increased intracellular  $\text{Ca}^{2+}$  concentration in a dose-dependent manner in SK-N-MC human neuroblastoma cell line as shown in Fig. 4.16. This increase appeared to be independent of the level of extracellular  $\text{Ca}^{2+}$  as illustrated in Fig. 4.17. These results suggest that serum component(s) can evoke intracellular  $\text{Ca}^{2+}$  increase through a receptor-mediated mechanism and that this increased intracellular  $\text{Ca}^{2+}$  is mainly due to internal  $\text{Ca}^{2+}$  release. These results further suggest that the increased intracellular  $\text{Ca}^{2+}$  concentration may be linked to the mechanism of the serum-induced proliferation of this cell line.

Since serum is composed of multiple components and heterogeneous from batch to batch, the identification of actual agonist(s) for elevating intracellular  $\text{Ca}^{2+}$  is very difficult. Thus, the usefulness of serum as an agonist may be criticized. Therefore, in order to find an agonist which increases both tumor cell growth and intracellular  $\text{Ca}^{2+}$



**Fig. 4.16. Dose-dependent increase of intracellular Ca<sup>2+</sup> concentration by serum in SK-N-MC human neuroblastoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The data represent net increases of intracellular Ca<sup>2+</sup> concentration with time. The arrow shows the time point for the addition of various concentrations of serum.



**Fig. 4.17.** Effect of EGTA (1mM) on serum (2%)-induced increased intracellular  $\text{Ca}^{2+}$  concentration in SK-N-MC human neuroblastoma cell line. Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $1.3 \text{mM}$   $\text{Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurement. The data represent net increases of intracellular  $\text{Ca}^{2+}$  concentration with time. The arrow shows the time point for the addition of serum. Cells were treated with EGTA for 3 minutes before starting the experiment.

concentration, the experiments were done using compounds which have shown such characteristics in other cellular systems, such as carbachol, serotonin, phenylephrine, bradykinin, angiotensin-II and Met-enkephalin. However, these compounds did not induce a significant alteration of intracellular  $\text{Ca}^{2+}$  concentration in SK-N-MC human neuroblastoma cell line (data not shown). Therefore, serum was continued to be used to stimulate proliferation of these cells. Thus, the homogeneous composition of the serum was required. To this end, the serum used in these experiments was prepared from a single batch, divided into small vials after well mixing and stored in a refrigerator. In addition, since the experiments using this serum actually showed a dose-related response (Fig. 4.16), the serum was used as an agonist for elevating intracellular  $\text{Ca}^{2+}$  for further experiments where the effect of various  $\text{Ca}^{2+}$  channel antagonists on an agonist-induced increased intracellular  $\text{Ca}^{2+}$  concentration was investigated.

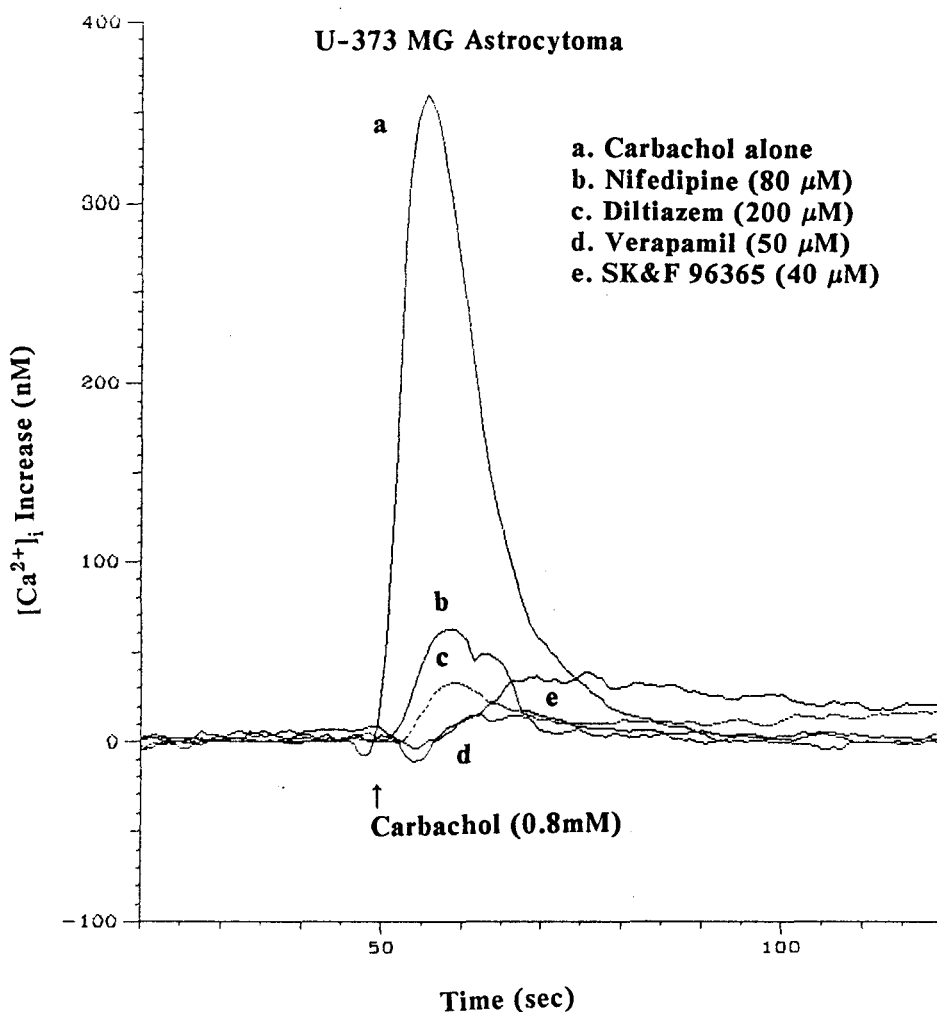
#### ***Inhibition of an Agonist-Induced Increased Intracellular Calcium Concentration by Calcium Channel Antagonists***

As previously shown in Figs. 4.11 and 4.17, a growth factor-induced release of  $\text{Ca}^{2+}$  from the internal stores seems to be an important signal transduction mechanism in the proliferation of tumor cells. Since the  $\text{Ca}^{2+}$  channel antagonists inhibited the growth of both tumor cell lines in a dose-dependent fashion (Figs. 4.3 - 4.6 and 4.8), their growth-inhibitory effects may be related to this signalling mechanism. Thus, the effects of these drugs on an agonist-induced release of  $\text{Ca}^{2+}$  from the internal stores were investigated in order to elucidate the mechanism of their actions. In these experiments carbachol and serum were used as agonists in U-373 MG human astrocytoma and SK-N-MC human

neuroblastoma cell line, respectively.

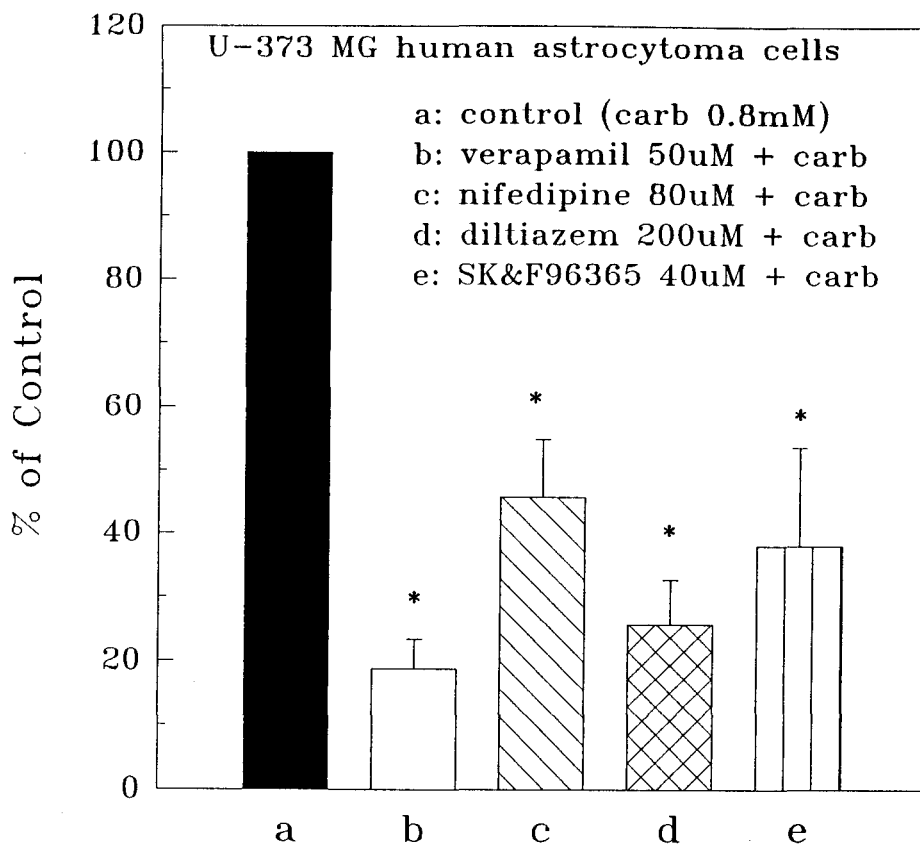
Fig. 4.18 shows the effects of the  $\text{Ca}^{2+}$  channel antagonists on Carb-induced release of  $\text{Ca}^{2+}$  from the internal stores in Fura-2 loaded U-373 MG human astrocytoma cell line. Since the resuspending buffer solution containing 0  $\text{Ca}^{2+}$  was used in these experiments, the Carb-induced increased intracellular  $\text{Ca}^{2+}$  concentration would result from the internal store release. The concentration of  $\text{Ca}^{2+}$  channel antagonists was chosen on the basis of their effects on the tumor cell growth. Fig. 4.19 shows the effects of  $\text{Ca}^{2+}$  channel antagonists on Carb-induced response, in which the data were expressed by % changes compared to the effect of Carb alone. These results (Figs. 4.18 and 4.19) clearly demonstrate that pretreatment with these drugs significantly reduced the Carb-induced release of  $\text{Ca}^{2+}$  from the internal stores. Verapamil was shown to be the most effective, which is well correlated with the results of the studies on the tumor cell growth. SK&F 96365 showed significant inhibition at a relatively high concentration compared to the growth study. These results suggest that SK&F 96365 may have other mechanisms in addition to the impairment of the intracellular  $\text{Ca}^{2+}$  signals in its inhibitory effect on tumor cell growth.

The effects of  $\text{Ca}^{2+}$  channel antagonists on a serum-induced release of  $\text{Ca}^{2+}$  from the internal stores in Fura-2 loaded SK-N-MC human neuroblastoma cell line were also investigated, and the results were shown in Figs. 4.20 and 4.21. These results show that these drugs also significantly inhibited the serum-induced response. In the growth studies of the tumor cells, all the  $\text{Ca}^{2+}$  channel antagonists were more effective in SK-N-MC human neuroblastoma than U-373 MG human astrocytoma cell line. In contrast to this

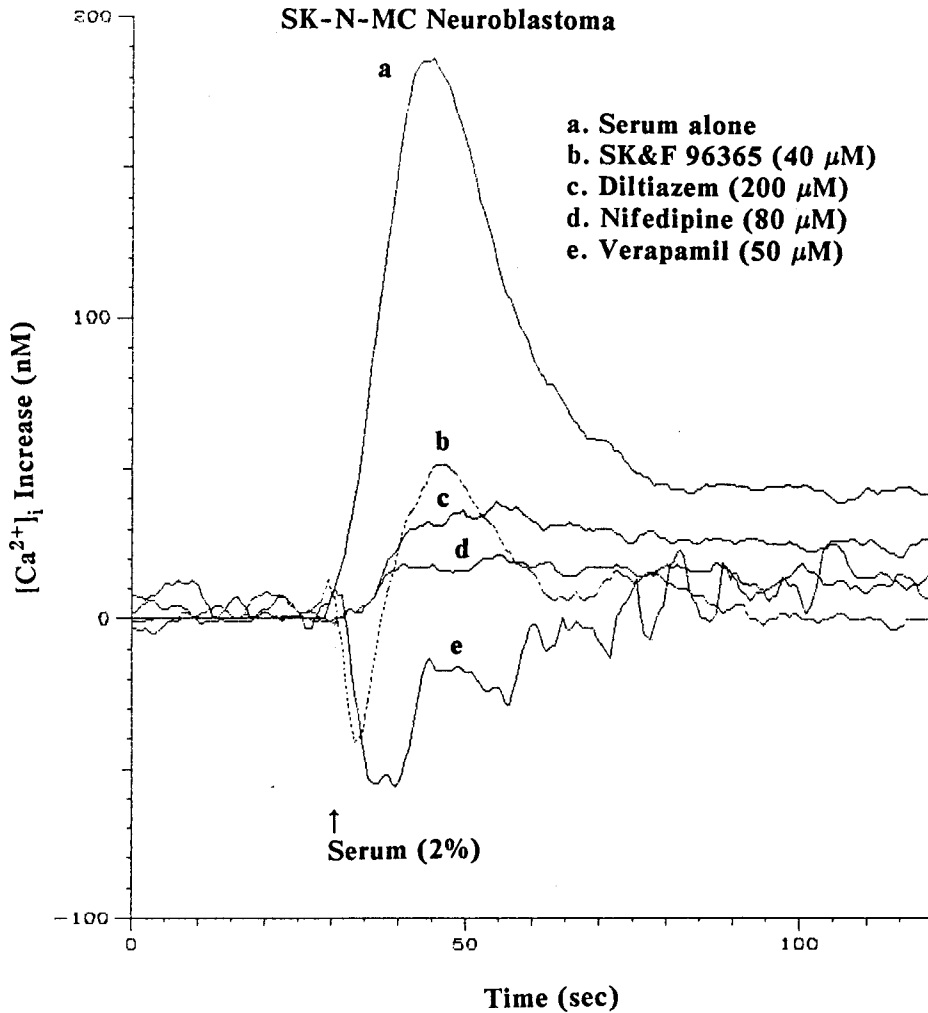


**Fig. 4.18.** Effect of Ca<sup>2+</sup> channel antagonists on carbachol (0.8mM)-induced intracellular Ca<sup>2+</sup> mobilization in U-373 MG human astrocytoma cell line. Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AM for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca<sup>2+</sup> and transferred to a quartz cuvette for fluorescence measurements. The data represent net increases of intracellular Ca<sup>2+</sup> concentration with time. The arrow shows the time point for the addition of carbachol. Ca<sup>2+</sup> channel antagonists were treated for 3 minutes before starting the experiment.

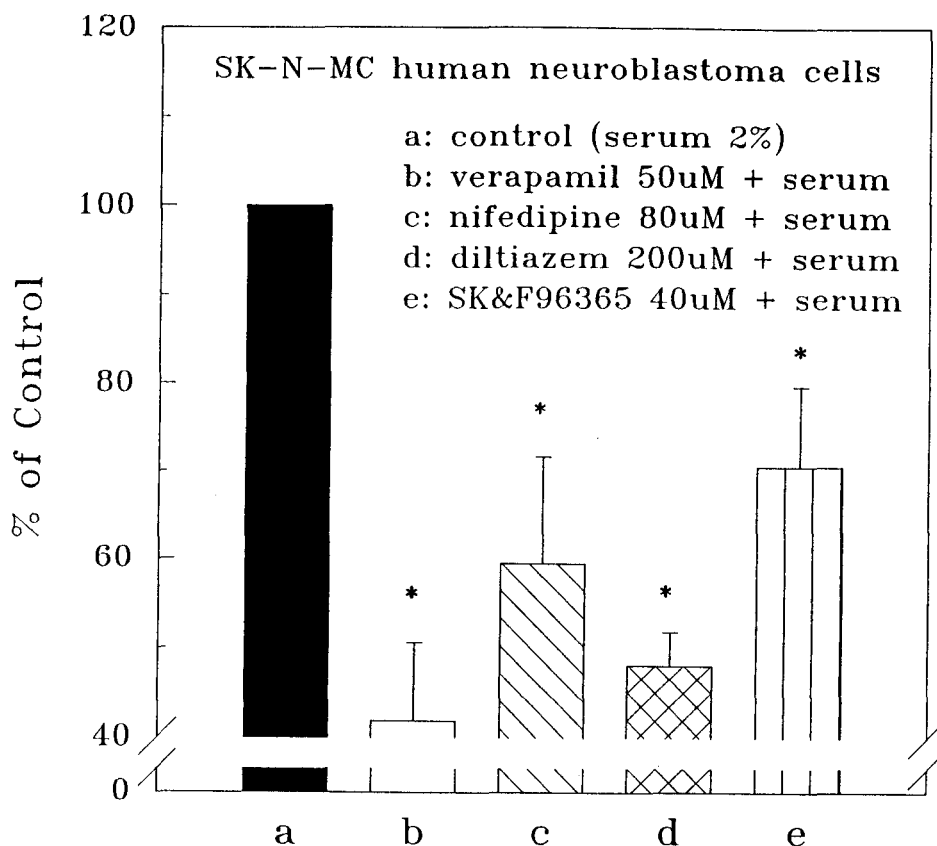




**Fig. 4.19. Quantitative changes of carbachol (0.8mM)-induced intracellular  $\text{Ca}^{2+}$  mobilization by  $\text{Ca}^{2+}$  channel antagonists in U-373 MG human astrocytoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AM for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of the increased intracellular  $\text{Ca}^{2+}$  concentration induced by the drugs compared to carbachol alone. Each column represents the mean value of at least five replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison. (\* $p < 0.05$  compared to carbachol alone).



**Fig. 4.20.** Effect of Ca<sup>2+</sup> channel antagonists on serum (2%)-induced intracellular Ca<sup>2+</sup> mobilization in SK-N-MC human neuroblastoma cell line. Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AM for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The data represent net increases of intracellular Ca<sup>2+</sup> concentration with time. The arrow shows the time point for the addition of serum. Ca<sup>2+</sup> channel antagonists were treated for 3 minutes before starting the experiment.



**Fig. 4.21. Quantitative changes of serum (2%)-induced intracellular  $\text{Ca}^{2+}$  mobilization by  $\text{Ca}^{2+}$  channel antagonists in SK-N-MC human neuroblastoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of the increased intracellular  $\text{Ca}^{2+}$  concentration induced by the drugs compared to serum alone. Each column represents the mean value of at least five replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison. (\* $p < 0.05$  compared to serum alone).

result, the inhibitory effects of  $\text{Ca}^{2+}$  channel antagonists on the serum-induced intracellular  $\text{Ca}^{2+}$  responses were much less prominent in SK-N-MC human neuroblastoma than U-373 MG human astrocytoma cell line. Thus, these results suggest that the mechanism of action of  $\text{Ca}^{2+}$  channel antagonists in tumor cell growth may differ in these tumor cell lines and that the signalling mechanism of increased intracellular  $\text{Ca}^{2+}$  concentration may have a greater contribution to the growth of the U-373 MG human astrocytoma than to that of the SK-N-MC human neuroblastoma cell line.

***The Inhibition of Agonist-Induced Intracellular Calcium Responses by Calcium Channel Antagonists is not Related to Their Effects on Basal Levels of Free Intracellular Calcium***

The intracellular free  $\text{Ca}^{2+}$  concentration appears to be tightly regulated via several different mechanisms (Carafoli, 1987; Rasmussen and Rasmussen, 1990). Thus, after the elevation of the cytosolic free  $\text{Ca}^{2+}$  concentration by a stimulus, the level is expected to be returned to a normal value. However, if these homeostatic regulatory mechanisms are impaired by a drug, the intracellular  $\text{Ca}^{2+}$  level will be remained elevated after an agonist stimulation. Interestingly, high micromolar concentrations of verapamil have been shown to lead to a rapid, sustained elevation of the intracellular  $\text{Ca}^{2+}$  concentration in the isolated rat osteoblast (Zaidi *et al.*, 1990). In addition, Taylor and Simpson found that the treatment of amlodipine, a  $\text{Ca}^{2+}$  channel antagonist, caused a rapid concentration-dependent decrease of the intracellular  $\text{Ca}^{2+}$  concentration in the HT-39 human breast cancer cell line (Taylor and Simpson, 1992). Thus, these alterations of the basal, cytosolic  $\text{Ca}^{2+}$  concentration may result in an altered response to an agonist stimulation. Therefore, the inhibitory effects of  $\text{Ca}^{2+}$  channel antagonists on agonist-induced

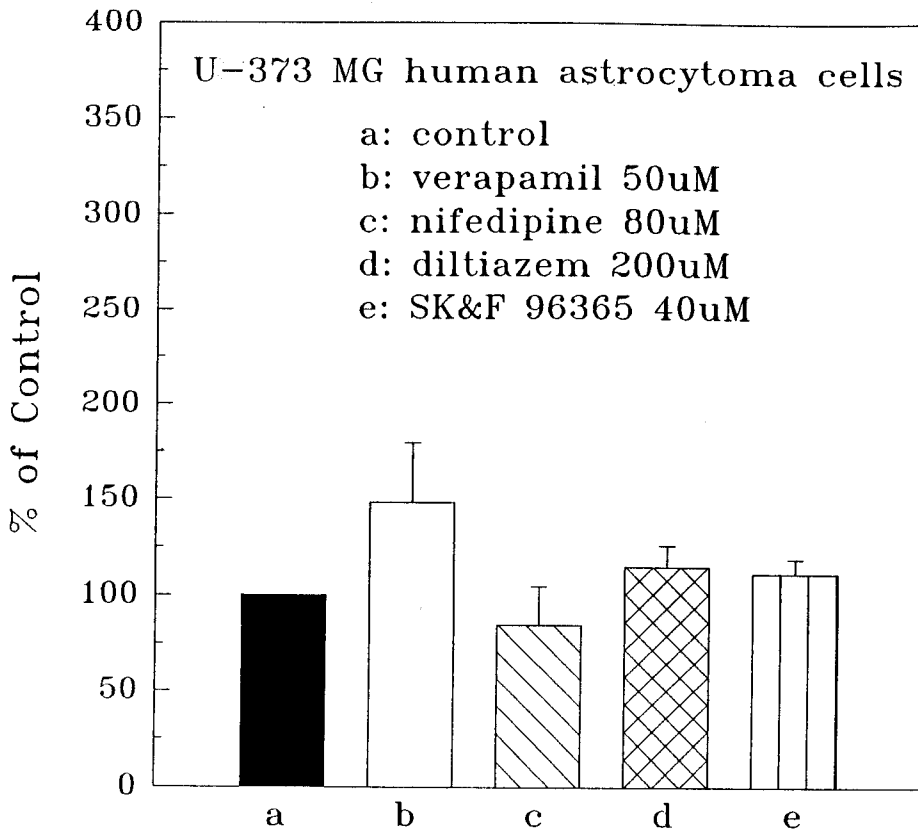
elevations of the intracellular  $\text{Ca}^{2+}$  concentration, shown in this study, may be due to their effects on the basal levels of cytosolic free  $\text{Ca}^{2+}$ .

In order to test this possibility, the effects of  $\text{Ca}^{2+}$  channel antagonists alone on the basal levels of free cytosolic  $\text{Ca}^{2+}$  in both Fura-2 loaded tumor cells were investigated. As depicted in Figs. 4.22 and 4.23, these drugs alone did not induce a significant alteration of the basal, intracellular  $\text{Ca}^{2+}$  concentration in either cell lines. Therefore, these results further suggest that the inhibition of agonist-induced intracellular  $\text{Ca}^{2+}$  responses evoked by  $\text{Ca}^{2+}$  channel antagonists may be not due to their effects on the basal levels of free cytosolic  $\text{Ca}^{2+}$ .

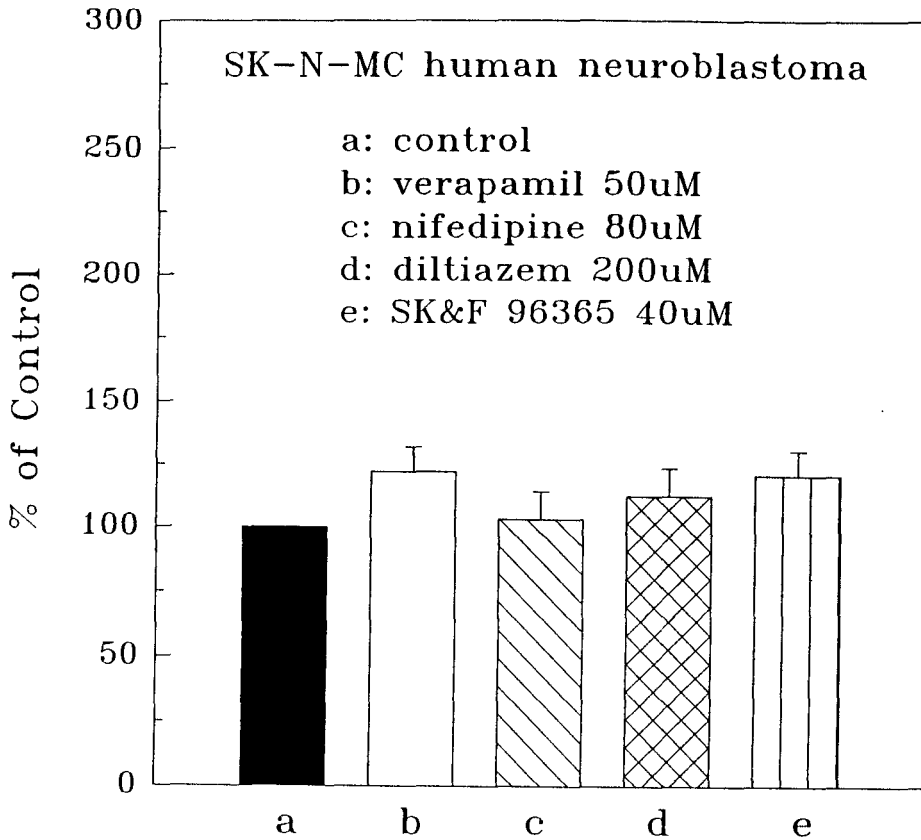
Throughout these experiments involving intracellular  $\text{Ca}^{2+}$  measurements, the basal, free intracellular  $\text{Ca}^{2+}$  concentrations of the cells were carefully monitored in the beginning in order to prevent possible contamination of these measurements by dead cells. Dead cells are likely to have increased intracellular  $\text{Ca}^{2+}$  levels. Thus, by monitoring basal intracellular  $\text{Ca}^{2+}$  levels, the existence of dead cells in the monitoring cell suspension can be indirectly checked. Variations of basal levels of intracellular  $\text{Ca}^{2+}$  concentration occurred in both types of cells as shown in Table 4.1, ranging between 100 and 150 nM and between 150 and 200 nM in SK-N-MC human neuroblastoma and U-373 MG human astrocytoma cell lines, respectively. These levels indicated relatively stable basal level of intracellular  $\text{Ca}^{2+}$  and minimal contamination by possible dead cells.

### **C. Discussion**

The major findings of this study are: 1) In tumor cell proliferation the increased



**Fig. 4.22.** No effect of  $\text{Ca}^{2+}$  channel antagonists on basal, free cytosolic  $\text{Ca}^{2+}$  concentration in U-373 MG human astrocytoma cell line. Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of intracellular  $\text{Ca}^{2+}$  concentration induced by the drugs compared to control. Each column represents the mean value of at least five replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison.



**Fig. 4.23. No effect of  $\text{Ca}^{2+}$  channel antagonists on basal, free cytosolic  $\text{Ca}^{2+}$  concentration in SK-N-MC human neuroblastoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AM for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of intracellular  $\text{Ca}^{2+}$  concentration induced by the drugs compared to control. Each column represents the mean value of at least five replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison.

**Table 4.1. Basal, free intracellular Ca<sup>2+</sup> concentrations of tumor cells**

| Cell Line | Intracellular Ca <sup>2+</sup> concentration [nM] |         |
|-----------|---|---------|
|           | U-373 MG  | SK-N-MC |
| Mean      | 173.1   | 131.8   |
| SEM       | 3.0   | 3.1     |
| Minimum   | 151.0   | 105.0   |
| Maximum   | 198.0   | 156.0   |
| Range     | 47.0  | 51.0    |
| n         | 20  | 20      |



intracellular  $\text{Ca}^{2+}$  concentration which results from the release of  $\text{Ca}^{2+}$  from the internal stores, is an essential signal transduction mechanism; 2) the blockade of this signalling mechanism by  $\text{Ca}^{2+}$  channel antagonists results in the inhibition of the tumor cell growth.

### ***Signal Transductional Role of Increased Intracellular Calcium Concentration from Internal Store Release in Tumor Cell Proliferation***

The importance of increased intracellular  $\text{Ca}^{2+}$  concentration in cell proliferation has been suggested from many studies which have used pharmacological agents including  $\text{Ca}^{2+}$  channel blockers to inhibit growth factor-induced increased cytosolic  $\text{Ca}^{2+}$  concentration or used low amount of  $\text{Ca}^{2+}$  in the culture medium (Batra *et al.*, 1991; Block *et al.*, 1989; Ogata *et al.*, 1991; Olsen *et al.*, 1991; Shultz and Rajj, 1990). Thus, these results suggest that during cell proliferation, the increased intracellular  $\text{Ca}^{2+}$  concentration occurs and that this  $\text{Ca}^{2+}$  increase is achieved through the influx of  $\text{Ca}^{2+}$  from the extracellular space. On the contrary the results of the present study, using brain tumor cells, showed that carbachol or serum which acted as growth factors, induced the release of  $\text{Ca}^{2+}$  from the internal stores without a significant  $\text{Ca}^{2+}$  influx from the extracellular space (Figs. 4.11 and 4.17). Thus, the results in these tumor cell systems suggest that the increased intracellular  $\text{Ca}^{2+}$  concentration due to the internal store release rather than  $\text{Ca}^{2+}$  influx from the extracellular space is involved in cell proliferation.

The establishment of a direct cause-and-effect relationship between growth factor-induced transiently increased intracellular  $\text{Ca}^{2+}$  concentrations and DNA synthesis is difficult since these two events occur separately over a lengthy period of time (24-36 hours). However, the recent observations of Diliberto *et al.* demonstrate that early

alterations in cytosolic free  $\text{Ca}^{2+}$  concentration (occurring within seconds to minutes) following platelet-derived growth factor (PDGF) stimulation are required for subsequent DNA synthesis in both BALB/c3T3 fibroblasts and vascular smooth muscle cells (Diliberto *et al.*, 1991). Thus, in the tumor cell lines, used in the present study, only a transiently increased intracellular  $\text{Ca}^{2+}$  concentration due to internal store release may be enough to evoke ultimate cell proliferation.

### ***Proposed Mechanism of Inhibitory Actions of Calcium Channel Antagonists in Tumor Cell Growth***

Although the mechanism of action of  $\text{Ca}^{2+}$  channel antagonists in the inhibition of tumor cell proliferation has been suggested to be due to their inhibitory effects on  $\text{Ca}^{2+}$  influx, the intracellular  $\text{Ca}^{2+}$  concentration has not been directly measured in these studies (Brusterud, 1992; Kunert-Radek *et al.*, 1989). Moreover, this notion about the mechanism of action of  $\text{Ca}^{2+}$  channel antagonists in the regulation of cell proliferation has been challenged. For example, in the  $^{45}\text{Ca}$  uptake studies, Schmidt and his associates suggested that the growth-inhibitory effects of verapamil appeared to be not due to  $\text{Ca}^{2+}$  influx or efflux in human medulloblastoma cells (Schmidt *et al.*, 1988). Thus, in order to elucidate the mechanism of action of  $\text{Ca}^{2+}$  channel antagonists in the present study, the effects of these drugs on both tumor cell growth and an agonist-induced increased intracellular  $\text{Ca}^{2+}$  concentration were investigated using human brain tumor cell lines.

The results of this study clearly show that  $\text{Ca}^{2+}$  channel antagonists inhibited both tumor cell growth (Figs. 4.3 - 4.6 and 4.8) and an agonist-induced release of  $\text{Ca}^{2+}$  from the internal stores (Figs. 4.18 - 4.21) in human brain tumor cell lines. Thus, these

results suggest that the inhibitory effects of  $\text{Ca}^{2+}$  channel antagonists on tumor cell growth may be due to their blocking actions on the release of  $\text{Ca}^{2+}$  from the internal stores during cell proliferation.

A remaining important question is how  $\text{Ca}^{2+}$  channel antagonists can block an agonist-induced release of  $\text{Ca}^{2+}$  from the internal stores. Although these drugs may have many sites of action, possible interactive sites can be divided into five different categories: (i) the plasma membrane receptors of growth factors (e.g. carbachol or serum factors); (ii) G-proteins, especially  $G_q$ , which couple the membrane receptors with phospholipase C; (iii) phospholipase C (PLC); (iv)  $\text{IP}_3$ -activated  $\text{Ca}^{2+}$  channels in the endoplasmic reticulum (ER); (v) indirect mechanism including the interaction with plasma membrane  $\text{K}^+$  channels.

The allosteric interaction of  $\text{Ca}^{2+}$  channel antagonists, particularly, verapamil and diltiazem, with the muscarinic receptors has been previously described in many different cellular systems (Arita *et al.*, 1991; Gerry *et al.*, 1987; Katayama *et al.*, 1987; Potter *et al.*, 1989; Waelbroeck *et al.*, 1984). The binding of platelet activating factor to human platelets (Valone, 1987) and human neutrophil granulocytes (Filep and Foldes-Filep, 1990) has also been shown to be inhibited by  $\text{Ca}^{2+}$  channel antagonists (verapamil and diltiazem). Thus, the blockade of carbachol-binding to its receptors by  $\text{Ca}^{2+}$  channel antagonists, at least verapamil and diltiazem, seems to be a likely mechanism of their inhibitory action on the carbachol-induced release of  $\text{Ca}^{2+}$  from internal stores shown in Figs. 4.18 and 4.19. However, the interaction of  $\text{Ca}^{2+}$  channel antagonists with the receptors of other serum growth factors has not been previously described. The

interaction of these antagonists with G-proteins and phospholipase C is also unknown. Homa *et al.* found that verapamil abolished a thrombin-induced increased phosphatidic acids in rabbit platelets, and proposed that this effect of verapamil may modify phosphoinositide metabolism. However, the exact mechanism of this action of verapamil has not been described.

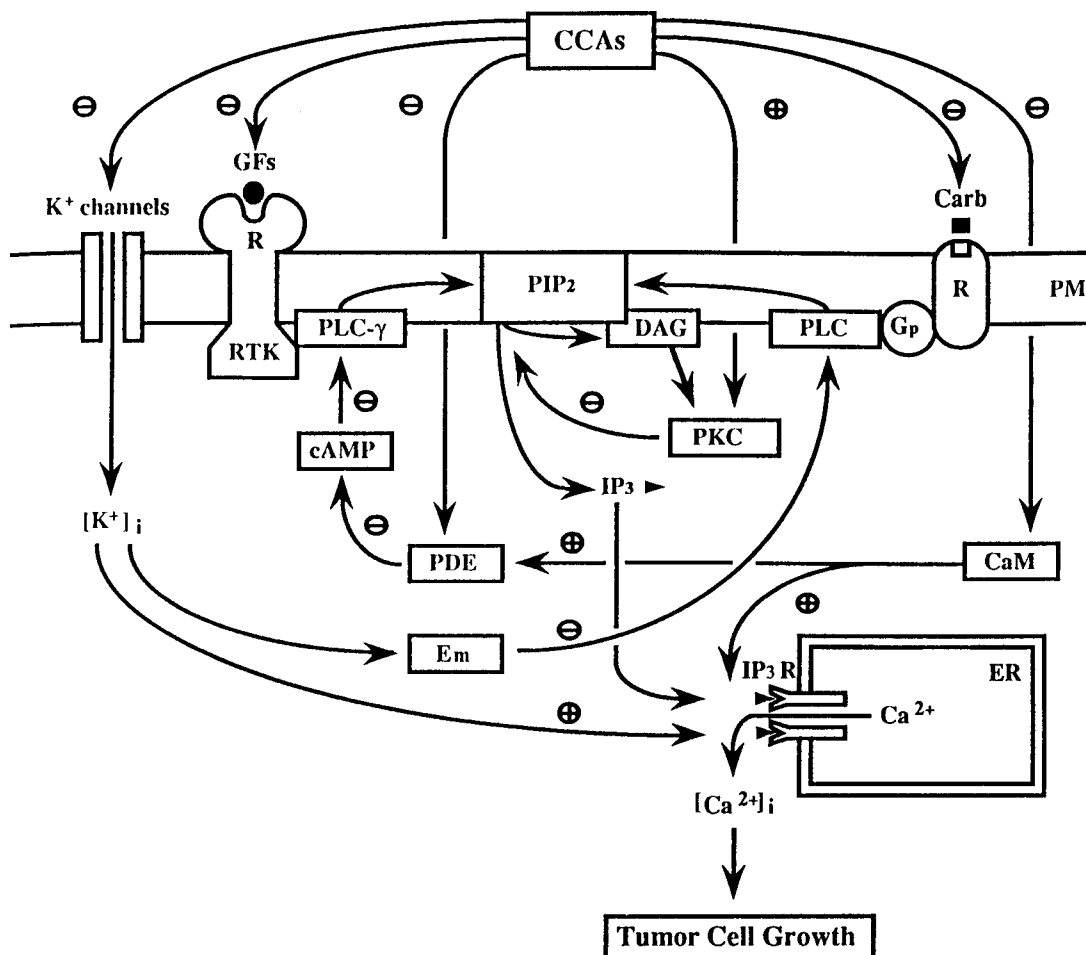
The possibility that  $\text{Ca}^{2+}$  channel antagonists may block  $\text{IP}_3$ -activated  $\text{Ca}^{2+}$  channels in the ER seems not to be a likely mechanism since Shah and Pant, using microsomes isolated from the rat brain, showed that the  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release was insensitive to a variety of  $\text{Ca}^{2+}$  channel blockers including verapamil and nifedipine (Shah and Pant, 1988). However,  $\text{Ca}^{2+}$  channel antagonists may affect indirectly  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release through their inhibition of calmodulin (CaM) action. Of particular interest, CaM antagonists have shown to inhibit  $\text{IP}_3$ -stimulated  $\text{Ca}^{2+}$  mobilization, and thus, CaM has been suggested to be tightly associated with the intracellular membrane mechanism coupling  $\text{IP}_3$  receptors to  $\text{Ca}^{2+}$  release channels (Hill *et al.*, 1988). Moreover, some  $\text{Ca}^{2+}$  channel antagonists are known to bind CaM and inhibit CaM functions (Walsh *et al.*, 1988; Zimmer and Hofmann, 1987).

Other indirect mechanisms may be possibly involved. The electrophysiological experiments have revealed that verapamil at concentration less than  $10 \mu\text{M}$ , can inhibit voltage-dependent  $\text{K}^+$  currents found in T lymphocytes (DeCoursey *et al.*, 1985), snail neurons (Kostyuk *et al.*, 1975) and small cell lung carcinoma cells (Pancrazio *et al.*, 1991). Since in most cells  $\text{K}^+$  channel activities have been shown to be directly involved in the regulation of the membrane potential (Amigorena *et al.*, 1990; Aronson, 1992;

Cahalan and Lewis, 1990; Curran *et al.*, 1992; Wu and Barish, 1992), the inhibition of  $K^+$  channels can induce an alteration of the membrane potential. Moreover, prior depolarization of plasma membrane potential has been demonstrated to reduce an agonist-induced  $Ca^{2+}$  release from intracellular stores in the promyelocytic cell line HL-60 (Pittet *et al.*, 1990) and human neutrophils (Di Virgilio *et al.*, 1987). In these results the inhibition of  $Ca^{2+}$  release by plasma membrane depolarization is paralleled and probably caused, by the inhibitory effect on  $IP_3$  formation (Pittet *et al.*, 1990). The mechanism by which depolarization inhibits  $IP_3$  formation and, thus,  $Ca^{2+}$  mobilization can only be speculated. Alteration of the plasma membrane potential may easily affect protein mobility, the lipid bilayer organization or the activity of various transmembrane enzymes (Pittet *et al.*, 1990). Thus, possible mechanisms may include changes in the accessibility of phosphatidylinositol (4,5)-bisphosphate to phospholipase C or uncoupling an important regulatory subunit, such as a G protein, from the phospholipase C.

Intracellular cyclic AMP may be considered to be a possible mediator of the action of  $Ca^{2+}$  channel blockers. Increased intracellular cyclic AMP has been shown to inhibit inositol phospholipid hydrolysis and this effect appeared to be associated with protein kinase A-mediated phosphorylation of phospholipase C- $\gamma$ 1 (Alava *et al.*, 1992). Some  $Ca^{2+}$  channel blockers are known to inhibit CaM-dependent enzymes, particularly, cyclic AMP phosphodiesterase which degrades cyclic AMP to adenosine nucleoside and inorganic phosphate (Walsh *et al.*, 1988; Zimmer and Hofmann, 1987). Thus,  $Ca^{2+}$  channel antagonists may increase intracellular cyclic AMP concentration and ultimately reduce  $IP_3$  generation through the inhibition of inositol phospholipid hydrolysis.

Finally, Protein kinase C (PKC) may be involved in  $\text{Ca}^{2+}$  channel antagonist-induced inhibition of  $\text{Ca}^{2+}$  release from internal stores. Felodipine, a dihydropyridine  $\text{Ca}^{2+}$  channel blocker, has been shown to enhance both the rate and extent of P47 phosphorylation by PKC (Sutherland and Walsh, 1989). This activation of PKC was dependent upon the presence of phospholipids but did not require diacylglycerol. In addition, phorbol esters, direct activators of PKC, have been shown to inhibit agonist-induced phosphoinositide hydrolysis in various cell types such as the rat hippocampal slices (Labarca *et al.*, 1984; Schoepp and Johnson, 1988), cultured vascular smooth muscle cells (Brock *et al.*, 1985), hamster vas deferens smooth muscle cells (Leeb-Lundberg *et al.*, 1985) and Chinese hamster ovary (CHO) cells (Aramori and Nakanishi, 1992). These effects of phorbol esters ultimately resulted in the inhibition of agonist-induced  $\text{Ca}^{2+}$  mobilization, and this effect appeared to occur via activation of PKC (Berridge, 1987; Drummond and Macintyre, 1985; He *et al.*, 1988; Nishizuka, 1986). Of particular interest, PKC has also been shown to inhibit insulin receptor-mediated tyrosine kinase activation (Bollag *et al.*, 1986). Thus,  $\text{Ca}^{2+}$  channel antagonist-induced blockade of intracellular  $\text{Ca}^{2+}$  mobilization may be possibly due to their modulatory effect on PKC. The whole cascades of these events which may be induced by  $\text{Ca}^{2+}$  channel antagonists are summarized diagrammatically in Fig. 4.24.



**Fig. 4.24. Proposed inhibitory mechanisms of  $\text{Ca}^{2+}$  channel antagonists in tumor cell growth.** This figure shows the signal transduction pathways of growth factors and carbachol-inducing tumor cell proliferation. In general, the stimulation of growth factor receptors can phosphorylate and then activate PLC- $\gamma$  by an intrinsic receptor tyrosine kinase. The stimulation of muscarinic receptors by carbachol can evoke PLC activation through the interaction with G-proteins (specifically,  $G_p$ ). PLC- $\gamma$  and PLC degrade  $\text{PIP}_2$  into DAG and  $\text{IP}_3$ .  $\text{IP}_3$  binding to the receptors on the ER membrane can open  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  channels, and DAG can activate PKC. An increased intracellular  $\text{Ca}^{2+}$  can ultimately lead to tumor cell proliferation with the cooperation of other signals. This figure also shows a possible intervening step in these pathways by  $\text{Ca}^{2+}$  channel antagonists. Abbreviations used: CCAs,  $\text{Ca}^{2+}$  channel antagonists; GFs, growth factors; Carb, carbachol; R, receptor; RTK, receptor tyrosine kinase; PLC, phospholipase C; PLC- $\gamma$ , phospholipase C- $\gamma$ ;  $G_p$ , GTP binding protein;  $\text{PIP}_2$ , phosphatidylinositol 4,5-bisphosphate;  $\text{IP}_3$ , inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; cAMP, cyclic adenosine 3',5'-monophosphate; PDE, cyclic AMP phosphodiesterase; CaM, calmodulin;  $E_m$ , membrane potential;  $[\text{K}^+]_i$ , intracellular  $\text{K}^+$  concentration;  $[\text{Ca}^{2+}]_i$ , intracellular  $\text{Ca}^{2+}$  concentration;  $\text{IP}_3$  R,  $\text{IP}_3$  receptor  $\text{Ca}^{2+}$  channel; ER, endoplasmic reticulum; PM, plasma membrane.

## CHAPTER V

### MECHANISM OF POTASSIUM CHANNEL MODULATOR-INDUCED INHIBITION OF TUMOR CELL GROWTH

#### A. Introduction

Cell proliferation induced by growth factors and mitogens, has been recognized to be initiated by transient changes in intracellular cation concentrations such as  $\text{Ca}^{2+}$ ,  $\text{H}^+$  and  $\text{Na}^+$  (Geck and Bereiter-Hahn, 1991; Mendoza, 1988; Moolenaar *et al.*, 1981; Prasad *et al.*, 1987). Moreover, several pieces of evidence suggest that  $\text{K}^+$  channels are directly or indirectly involved in the initiation of cell proliferation (DeCoursey *et al.*, 1984; Deutsch, 1990). Various mitogens have been shown to activate  $\text{K}^+$  channels. T cell mitogen, concanavalin A (ConA), increases the number of  $\text{K}^+$  channels in murine T lymphocytes (DeCoursey *et al.*, 1987). In a murine noncytolytic T lymphocyte clone, L2, voltage-sensitive  $\text{K}^+$  conductance is increased during interleukin 2 (IL2)-stimulated proliferation (Lee *et al.*, 1986).  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels seem also to be involved in the process of mitogen-induced cell proliferation as shown in mouse fibroblasts (Kusano and Gainer, 1991), rat thymic lymphocytes (Mahaut-Smith and Mason, 1991) and *ras*-transformed fibroblasts (Rane, 1991). Moreover, many studies with  $\text{K}^+$  channel blockers have demonstrated a reasonable quantitative agreement between block of  $\text{K}^+$  currents and inhibition of cell proliferation. Amigorena and coworkers have shown that  $\text{K}^+$  channel blockers such as TEA, quinine and verapamil suppressed both  $\text{K}^+$  current and DNA synthesis in murine B lymphocytes (Amigorena *et al.*, 1990). Similar



results have been found in neuroblastoma cells (Rouzair-Dubois and Dubois, 1990; Rouzair-Dubois and Dubois, 1991), rat malignant lymphocytes, Nb2 cells (Cukierman, 1992), human peripheral blood lymphocytes (Price *et al.*, 1989), rat astrocytes (Ohira *et al.*, 1991), retinal glial cells (Puro *et al.*, 1989) and Schwann cells (Chiu and Wilson, 1989).

However, the relationship between cell proliferation and  $K^+$  channel activity is not obvious (Deutsch, 1990; Gallin, 1991; Gardner, 1990), although their correlation has been successively attributed to a  $Ca^{2+}$  influx through  $K^+$  channels (Kuno *et al.*, 1986), the maintenance of a hyperpolarized resting membrane potential and, thus, probably enhancing  $Ca^{2+}$  influx by maintaining the electrical gradient for  $Ca^{2+}$  ions (Amigorena *et al.*, 1990; Cahalan and Lewis, 1990; Chiu and Wilson, 1989) or a regulation of cell volume and intracellular  $Na^+$  concentration (Rouzair-Dubois and Dubois, 1990).

Membrane potential ( $E_m$ ) changes induced by various mitogens have been detected, particularly in lymphocytes (Gelfand *et al.*, 1987; Kiefer *et al.*, 1980; Mahaut-Smith and Mason, 1991). In addition to these observed  $E_m$  changes, a concomitant rise in intracellular  $Ca^{2+}$  has been reported following mitogenic stimulation (Hesketh *et al.*, 1983; Tatham *et al.*, 1986; Tsien *et al.*, 1982). In general, membrane hyperpolarization followed by depolarization can be observed with the treatment of mitogens and these  $E_m$  changes are due to the activation of  $Ca^{2+}$ -dependent  $K^+$  channels and inward  $Na^+$  movement, respectively. Alteration of the  $K^+$  channel activity may change the  $E_m$  and, in turn, the altered  $E_m$  can affect other ion movements through the changes of transmembrane electrical gradients of these ions. Thus, interrelationship between the

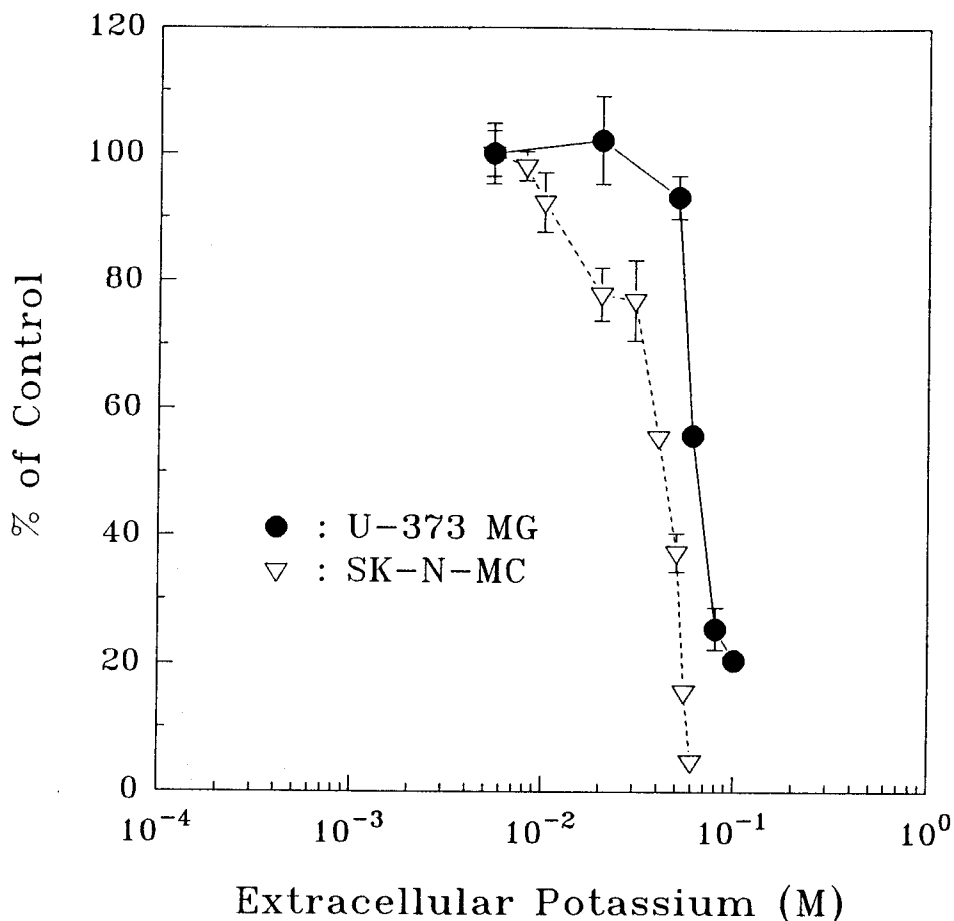
increased intracellular  $\text{Ca}^{2+}$  concentration and the  $\text{K}^+$  channel activities may occur, which would be an important cue for solving the mechanisms of action of  $\text{K}^+$  channel antagonists in the inhibition of cell proliferation. However, the exact role of the  $\text{K}^+$  channel activity in the intracellular  $\text{Ca}^{2+}$  regulation is essentially unknown.

The experiments in this chapter were designed to elucidate the mechanisms of action of  $\text{K}^+$  channel modulators in the inhibition of tumor cell growth. The data describe the effects of the  $\text{K}^+$  channel modulators on tumor cell growth and a growth factor-induced increased intracellular  $\text{Ca}^{2+}$  concentration. The  $\text{K}^+$  channel modulators effectively inhibited the tumor cell growth and blocked an agonist-induced increased intracellular  $\text{Ca}^{2+}$  concentration. The results suggest that inhibitory actions of the  $\text{K}^+$  channel modulators in tumor cell growth may be due to their modulatory effects on an agonist-induced alteration of intracellular  $\text{Ca}^{2+}$ .

## **B. Results**

### ***Inhibition of Tumor Cell Growth by High Extracellular Potassium***

Dependence of tumor cell growth on the extracellular  $\text{K}^+$  concentration was investigated. In these experiments the sum of NaCl and KCl concentrations in the growth medium was made 122 mM by substituting NaCl with an equivalent amount of KCl in order to maintain a constant osmolarity. Fig. 5.1 shows the effect of various concentrations of extracellular  $\text{K}^+$  on the growth of U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines. The results indicate that these tumor cell growth was inhibited by increasing the extracellular  $\text{K}^+$  in a dose-dependent manner.



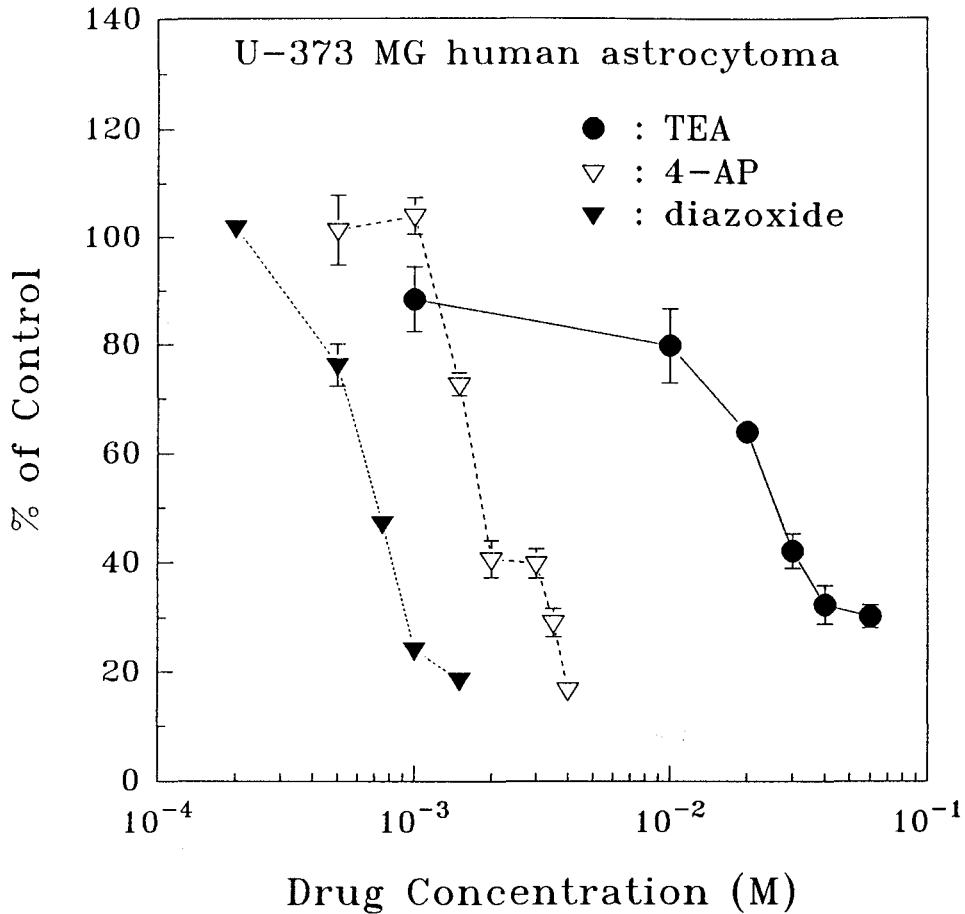
**Fig. 5.1.** Effect of high extracellular  $K^+$  on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of  $K^+$  in the culture medium. The cell number was counted 2 days after  $K^+$  treatment. The results were expressed as a percent change of the number of the cells obtained in the culture medium containing normal  $K^+$  concentration (5.4mM). The data points represent the mean value of at least four replicate dishes with bars indicating SEM.

These results suggest that tumor cell proliferation may be regulated by the alteration of the membrane potential since increased extracellular  $K^+$  concentration is known to depolarize plasma membrane potential (Pittet *et al.*, 1990).

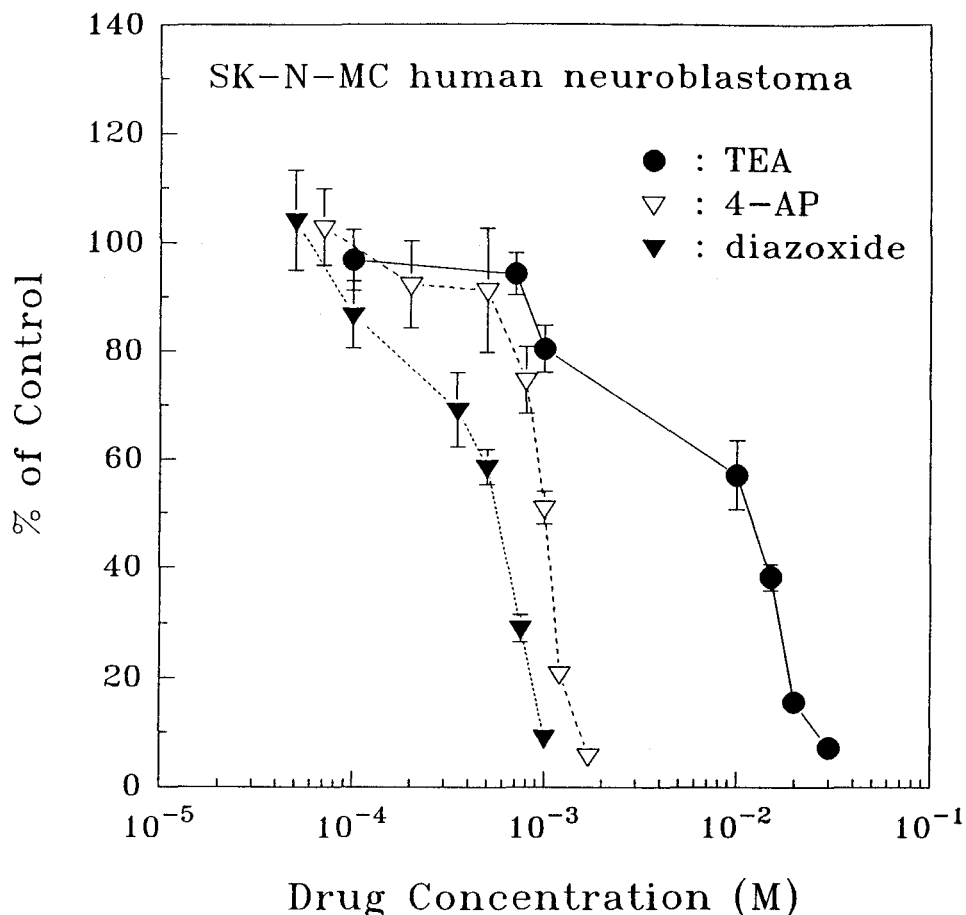
Membrane depolarization has been shown to reduce agonist-induced  $Ca^{2+}$  influx across the plasma membrane in nonexcitable cells from which voltage-dependent  $Ca^{2+}$  channels are absent (Di Virgilio *et al.*, 1987; Penner *et al.*, 1988; Pittet *et al.*, 1990). The inhibition of  $Ca^{2+}$  influx could be due, at least in part, to a decreased  $Ca^{2+}$  electrochemical gradient. However, since in the tumor cells which were used in this study,  $Ca^{2+}$  influx appeared not to be involved in the cell proliferation as described in Chapter 4, high extracellular  $K^+$ -induced inhibition of these tumor cell growth may be not due to its effect on  $Ca^{2+}$  influx. Recently, the alteration of plasma membrane potential has been shown to affect on agonist-induced second messenger generation including cytosolic free  $Ca^{2+}$  and  $IP_3$ , in HL-60, the promyelocytic cell line (Pittet *et al.*, 1990). Thus, the effects of high extracellular  $K^+$  on agonist-induced release of  $Ca^{2+}$  from the internal stores were investigated in order to elucidate the mechanism of its action on the regulation of tumor cell growth.

### ***Potassium Channel Modulator-Induced Inhibition of Tumor Cell Growth***

In order to see the involvement of  $K^+$  channels in tumor cell growth, typical  $K^+$  channel antagonists, tetraethylammonium chloride (TEA) and 4-aminopyridine (4-AP), and diazoxide, known as a  $K^+$  channel opener, were used in these experiments. Figs. 5.2 and 5.3 show the effects of these  $K^+$  channel modulators on the growth of U-373



**Fig. 5.2. Effect of  $K^+$  channel modulators on the growth of U-373 MG human astrocytoma cell lines.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of TEA (closed circle), 4-AP (open inverted triangle) or diazoxide (closed inverted triangle) in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.

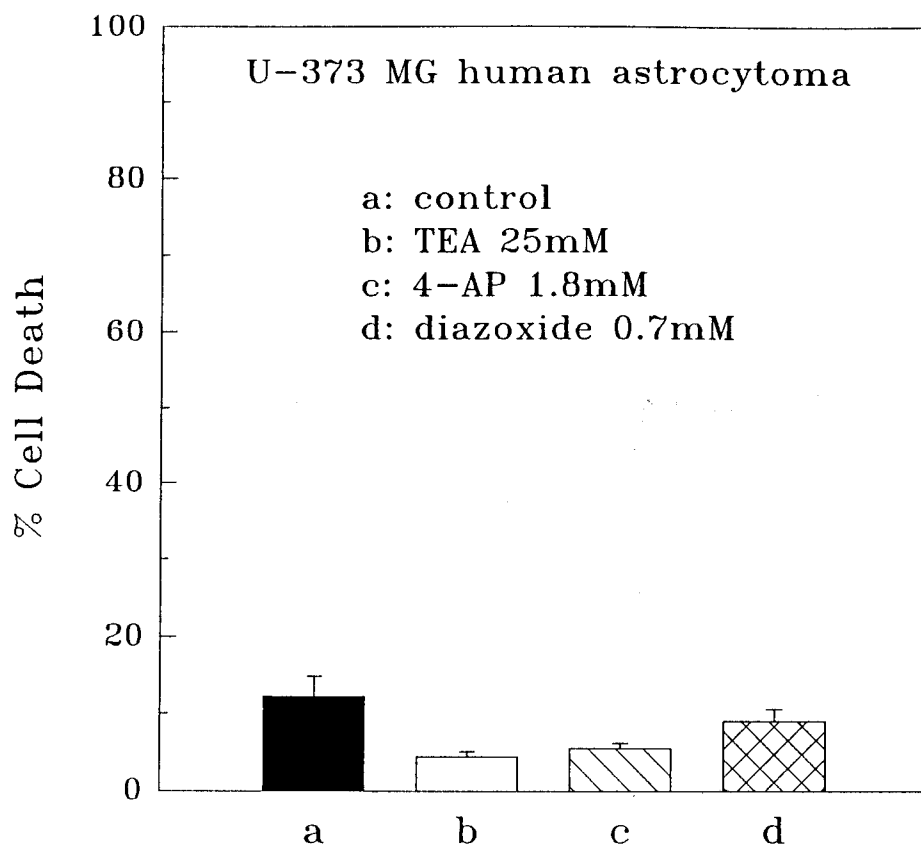


**Fig. 5.3. Effect of  $K^+$  channel modulators on the growth of SK-N-MC human neuroblastoma cell lines.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of TEA (closed circle), 4-AP (open inverted triangle) or diazoxide (closed inverted triangle) in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.

MG human astrocytoma and SK-N-MC human neuroblastoma cell lines, respectively. The results illustrate that these  $K^+$  channel modulators induced the inhibition of tumor cell growth in a dose-dependent fashion. Interestingly, diazoxide was the most effective, and followed by 4-AP and TEA in both tumor cell lines.

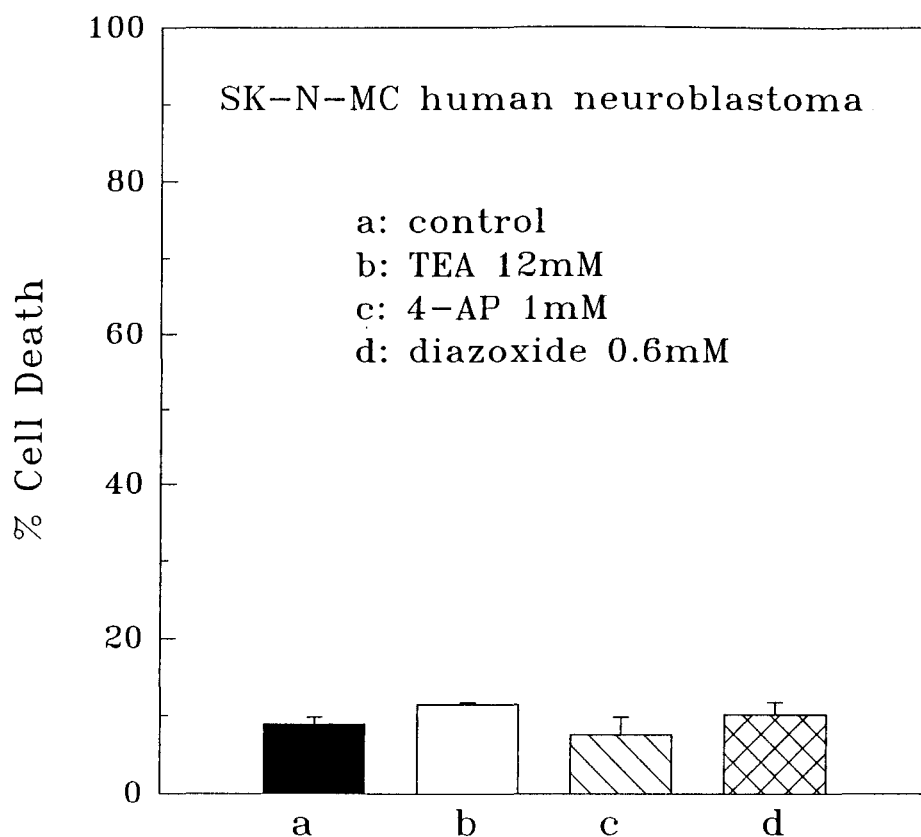
These growth-inhibitory effects of  $K^+$  channel modulators may be attributed either to the inhibition of cell proliferation or to cell cytotoxicity. Moreover, these two possibilities can not be differentiated by only the measurement of the number of cells as shown in Figs. 5.2 and 5.3. Thus, the cytotoxicity assay was made using trypan blue exclusion method (see Chapter 3 for the method description in detail). Figs. 5.4 and 5.5 show the effects of  $K^+$  channel modulators on the cell viability of U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines, respectively. The results indicate that these drugs did not induce cytotoxicity in both cell lines. Therefore, the growth-inhibitory effects of  $K^+$  channel modulators may be due to their suppression of tumor cell proliferation.

The inhibition of tumor cell proliferation by  $K^+$  channel modulators can be explained by several mechanisms. First, in lymphocytes an early mitogen-induced increased intracellular  $Na^+$  concentration has been shown to be a necessary and sufficient signal for the rejoining of DNA strand breaks, an event which must occur before the proliferating lymphocytes can replicate their DNA (Prasad *et al.*, 1987). The increased intracellular  $Na^+$  would only be effective if the  $Na^+$  influx is accompanied by an efflux of  $K^+$ . If the membrane pathway of  $K^+$  (e.g.  $K^+$  channels) is closed or blocked, the entry of  $Na^+$  into the cell would be accompanied by an influx of water and/or  $Cl^-$ ,



**Fig. 5.4.** No cytotoxic effect of  $K^+$  channel modulators on U-373 MG human astrocytoma cell line. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of the drugs in the culture medium. Cell cytotoxicity was assayed by trypan blue extrusion method 2 days after drug treatment. The results were expressed as a percent change of the number of dead cells obtained in the absence of the drug. Each column represents the mean value of four replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison.



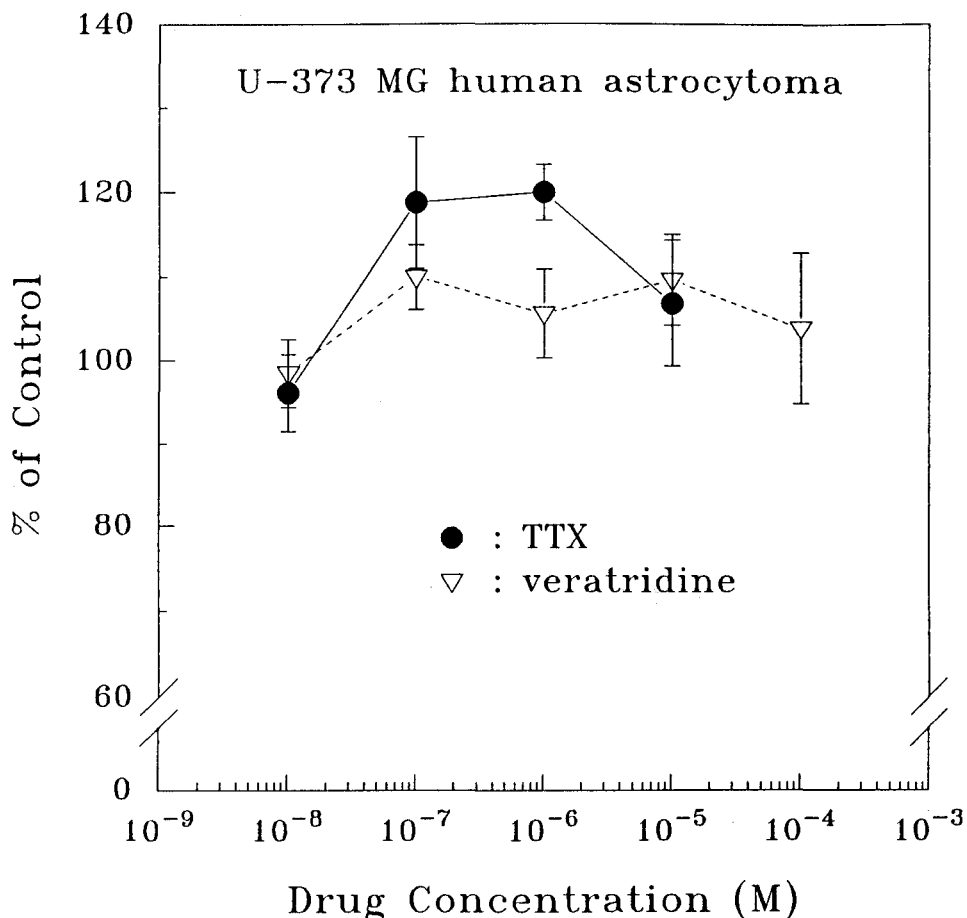


**Fig. 5.5 No cytotoxic effect of  $K^+$  channel modulators on SK-N-MC human neuroblastoma cell line.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of the drugs in the culture medium. Cell cytotoxicity was assayed by trypan blue extrusion method 2 days after drug treatment. The results were expressed as a percent change of the number of dead cells obtained in the absence of the drug. Each column represents the mean value of four replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison.

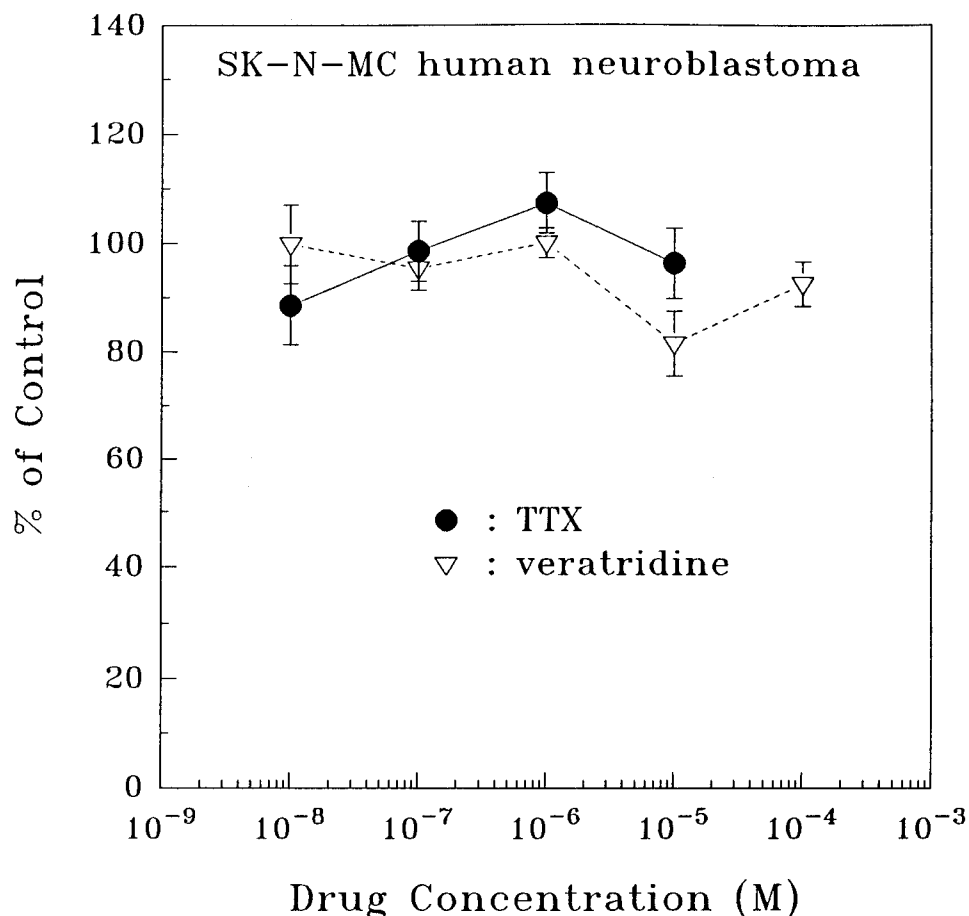
resulting in swelling of the cell and dilution of intracellular  $\text{Na}^+$ , whose concentration would remain more or less constant (Rouzair-Dubois and Dubois, 1990). Thus, the blockade of  $\text{K}^+$  channels can inhibit a mitogen-induced increased intracellular  $\text{Na}^+$  concentration, resulting in the inhibition of cell proliferation. However, the role of increased intracellular  $\text{Na}^+$  concentration has not been described in these tumor cells. Therefore, in order to test this possibility, the effects of veratridine, a  $\text{Na}^+$  ionophore, and tetrodotoxin (TTX), a voltage-sensitive  $\text{Na}^+$  channel blocker, on these tumor cell growth were investigated and the results are shown in Figs. 5.6. and 5.7. These results indicate that both veratridine and TTX did not induce a significant alteration of the growth of these tumor cells. Thus, these results suggest that increased intracellular  $\text{Na}^+$  concentration is not an important signal for cell proliferation in these tumor cells. These results further suggest that the growth-inhibitory effects of  $\text{K}^+$  channel modulators shown in this study, are not due to their inhibition of mitogen-induced increased intracellular  $\text{Na}^+$  concentration.

Second, Chandy *et al.* have suggested that in T lymphocytes  $\text{Ca}^{2+}$  entry is required for cell proliferation and that this  $\text{Ca}^{2+}$  entry may occur through the voltage-dependent  $\text{K}^+$  channels (Chandy et al., 1984). Thus, the blockade of these  $\text{K}^+$  channels may induce the inhibition of  $\text{Ca}^{2+}$  entry, resulting in the inhibition of cell proliferation. However, since in the tumor cells used in this study,  $\text{Ca}^{2+}$  influx appeared to be not necessary for their growth, this possibility may be excluded.

Lastly, since in these tumor cell lines the release of  $\text{Ca}^{2+}$  from the internal stores appeared to be an important signalling mechanism of growth factors in cell proliferation



**Fig. 5.6. Effect of veratridine and TTX on the growth of U-373 MG human astrocytoma cell lines.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of TTX (closed circle) and veratridine (open inverted triangle) in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.



**Fig. 5.7. Effect of veratridine and TTX on the growth of SK-N-MC human neuroblastoma cell lines.** The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of TTX (closed circle) and veratridine (open inverted triangle) in the culture medium. The cell number was counted 2 days after drug treatment. The results were expressed as a percent change of the number of the cells obtained in the absence of the drug. The data points represent the mean value of at least four replicate dishes with bars indicating SEM.

(described in Chapter 4), the growth-inhibitory effects of  $K^+$  channel modulators may result from the inhibition of this  $Ca^{2+}$  mobilization. In order to test this possibility, the effects of  $K^+$  channel modulators on agonist-induced intracellular  $Ca^{2+}$  mobilization were investigated.

### ***No Voltage-Dependent Calcium Channels Are Present in U-373 MG Human Astrocytoma and SK-N-MC Human Neuroblastoma Cell Lines***

In order to test the existence of voltage-dependent  $Ca^{2+}$  channels in these tumor cells, the intracellular  $Ca^{2+}$  concentration was monitored under the condition of the high extracellular  $K^+$ -induced membrane depolarization. This manipulation did not induce the increased intracellular  $Ca^{2+}$  concentration in both cell lines as shown in Figs. 5.12 and 5.13. The treatment of Bay K-8644, a  $Ca^{2+}$  channel agonist, and high extracellular  $K^+$  also did not induce the increased intracellular  $Ca^{2+}$  concentration (data not shown). These results suggest that these tumor cells do not have voltage-dependent  $Ca^{2+}$  channels.

### ***Inhibition of Agonist-Induced Release of Calcium from the Internal Stores by Potassium Channel Modulators***

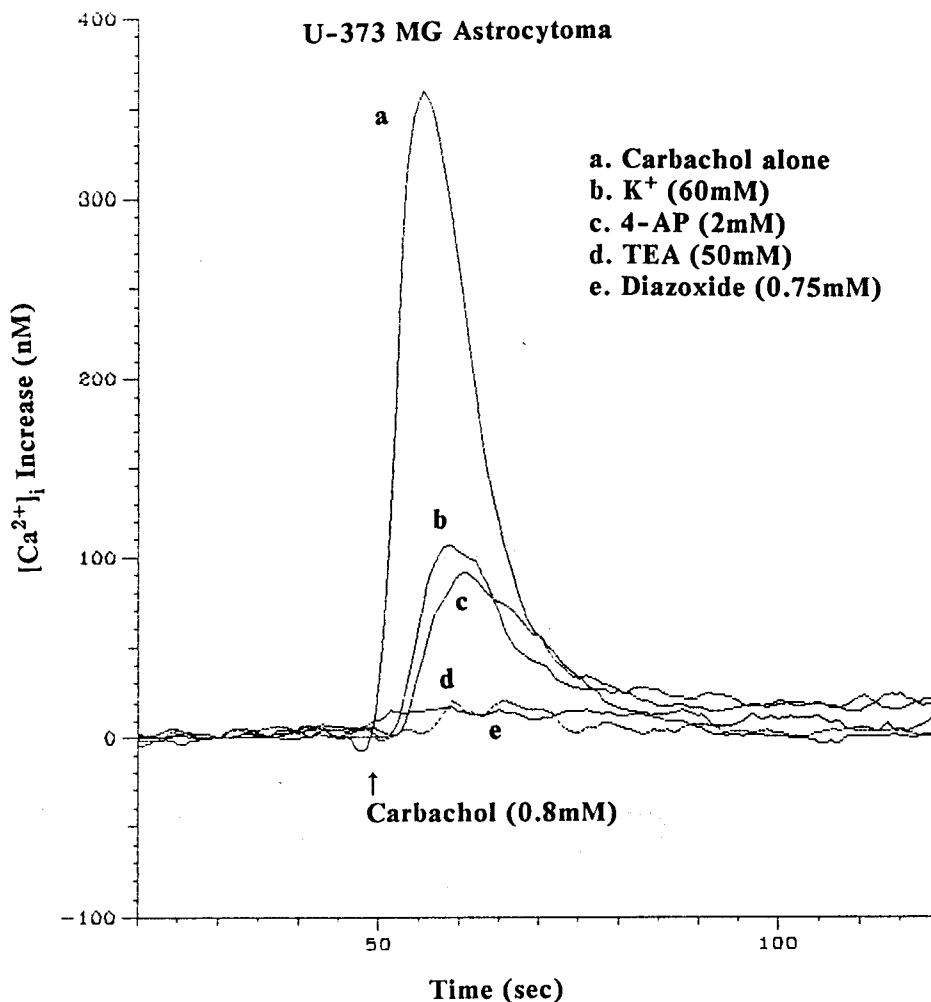
Since the intracellular  $Ca^{2+}$  mobilization from the internal stores appeared to be involved in these tumor cell proliferation as described in Chapter 4, the effects of  $K^+$  channel modulators including high extracellular  $K^+$  on an agonist-induced increased intracellular  $Ca^{2+}$  concentration were examined. In these experiments carbachol (Carb) and serum were used as  $Ca^{2+}$  mobilizing agents in U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines, respectively. Since the resuspending medium

containing 0  $\text{Ca}^{2+}$  was used, a agonist-induced increased intracellular  $\text{Ca}^{2+}$  concentration may result from the internal store release. The concentrations of  $\text{K}^+$  channel modulators were chosen on the basis of their effects on tumor cell growth.

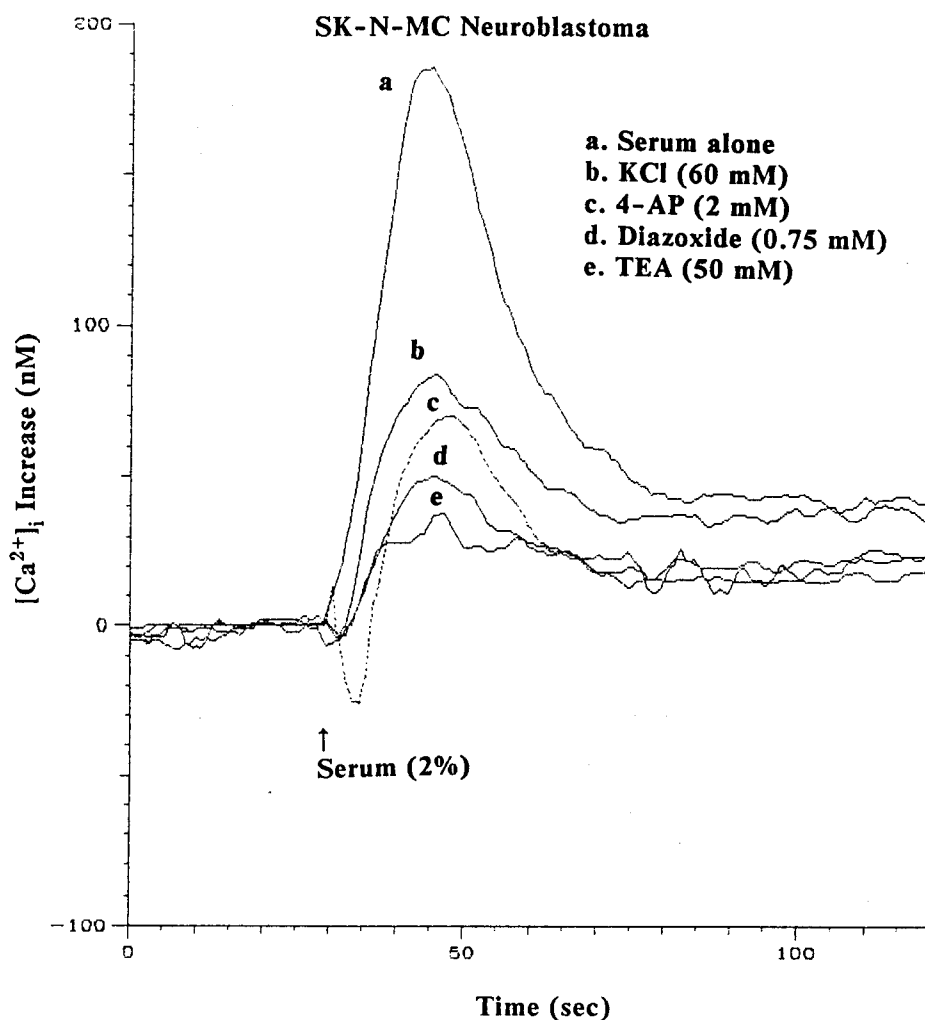
Figs. 5.8 shows single representative traces of net increases of intracellular  $\text{Ca}^{2+}$  concentrations induced by Carb and the effects of pretreatment of  $\text{K}^+$  channel modulators in U-373 MG human astrocytoma cell line. Fig. 5.9 depicts the effects of these drugs on serum-induced responses in SK-N-MC human neuroblastoma cell line. For quantitative comparison, percent changes of net increments of intracellular  $\text{Ca}^{2+}$  concentrations in the pretreatment of  $\text{K}^+$  channel modulators compared to Carb or serum-induced  $\text{Ca}^{2+}$  increases in the absence of these drugs were calculated and are displayed in Figs. 5.10 and 5.11. The bars in these figures represent the SEM obtained from at least four different measurements. These results demonstrate that  $\text{K}^+$  channel modulators induced statistically significant differences compared to the effect of an agonist alone in both cell lines. These results further suggest that their growth-inhibitory effects may be due to the blockade of agonist-induced intracellular  $\text{Ca}^{2+}$  mobilization in these tumor cells.

***Potassium Channel Modulators do not Affect the Basal, Free Cytosolic Calcium Concentrations in Tumor Cells***

If  $\text{K}^+$  channel modulators can alter the basal, free cytosolic  $\text{Ca}^{2+}$  concentrations, an agonist-induced intracellular  $\text{Ca}^{2+}$  mobilization may be affected by these changes which can result in the alteration of the electrochemical gradient for  $\text{Ca}^{2+}$  ion movements from the lumen of the ER to the cytosol. In order to test this possibility, the effects of  $\text{K}^+$

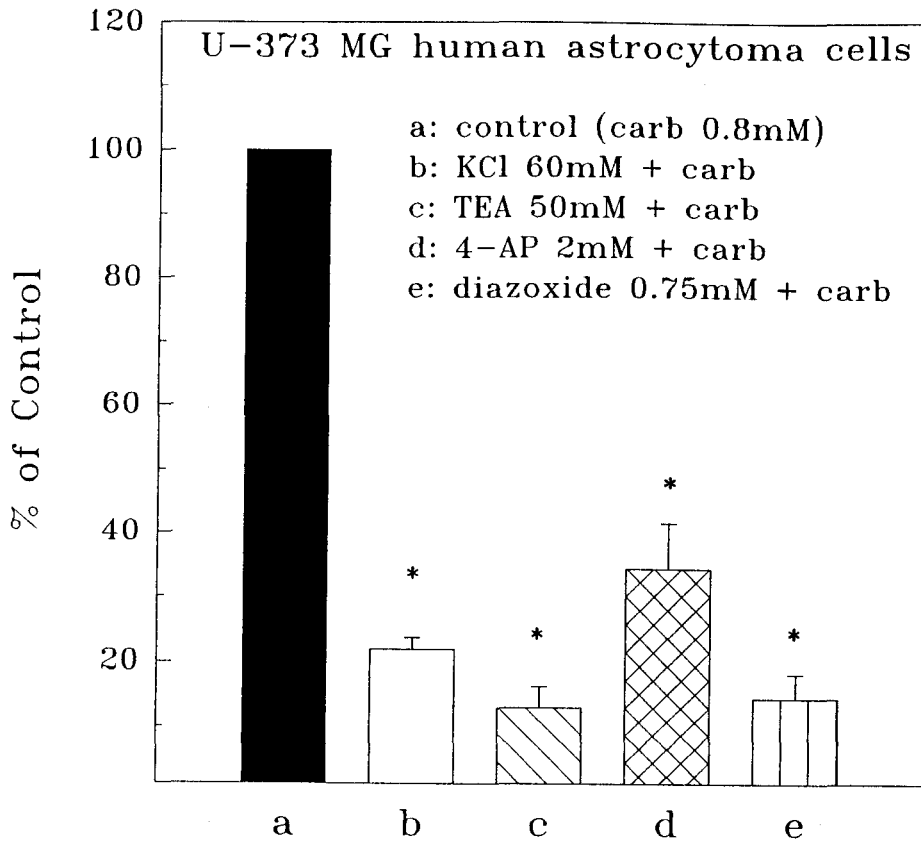


**Fig. 5.8.** Effect of high extracellular K<sup>+</sup> or K<sup>+</sup> channel modulators on carbachol (0.8mM)-induced intracellular Ca<sup>2+</sup> mobilization in U-373 MG human astrocytoma cell line. Aliquots of 5x10<sup>6</sup> cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca<sup>2+</sup> and transferred to a quartz cuvette for fluorescence measurements. The data represent net increases of intracellular Ca<sup>2+</sup> concentration with time. The arrow shows the time point for the addition of carbachol. KCl or K<sup>+</sup> channel modulators were treated for 3 minutes before starting the experiment.

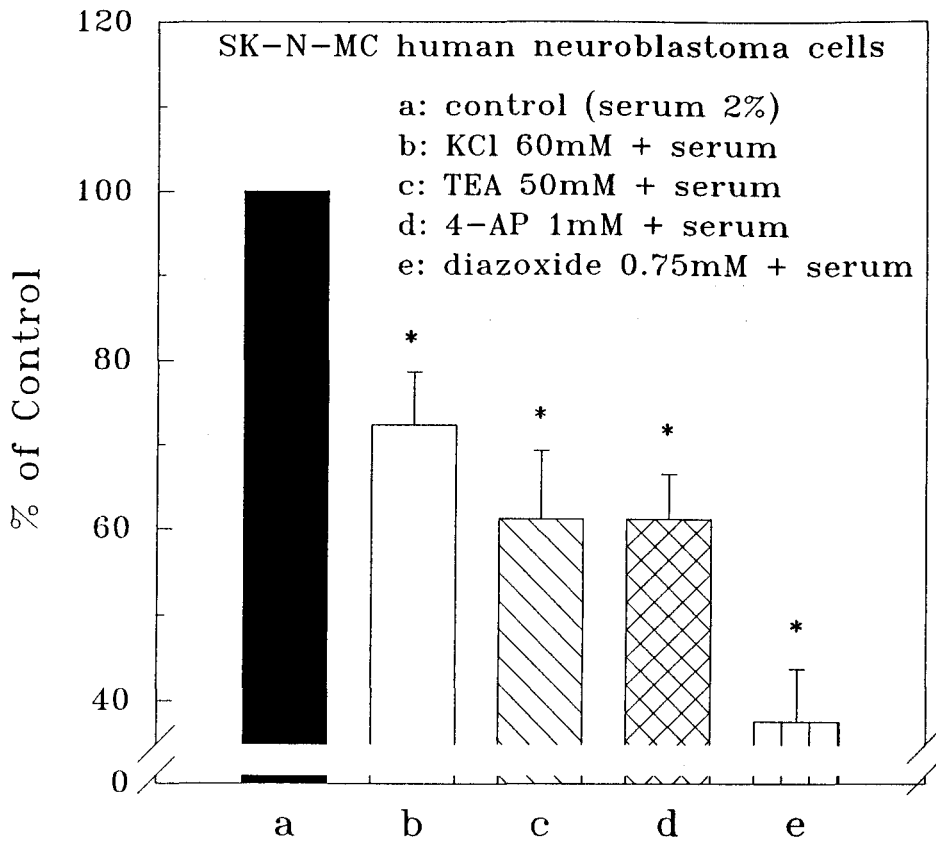


**Fig. 5.9.** Effect of high extracellular  $K^+$  or  $K^+$  channel modulators on serum (2%)-induced intracellular  $Ca^{2+}$  mobilization in SK-N-MC human neuroblastoma cell line. Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu M$  of Fura-2-AM for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 Ca^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The data represent net increases of intracellular  $Ca^{2+}$  concentration with time. The arrow shows the time point for the addition of serum. KCl or  $K^+$  channel modulators were treated for 3 minutes before starting the experiment.





**Fig. 5.10.** Quantitative changes of carbachol (0.8mM)-induced intracellular  $\text{Ca}^{2+}$  mobilization by high extracellular  $\text{K}^+$  or  $\text{K}^+$  channel modulators in U-373 MG human astrocytoma cell line. Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0  $\text{Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of the increased intracellular  $\text{Ca}^{2+}$  concentration induced by the drugs compared to carbachol alone. Each column represents the mean value of at least five replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison. (\* $p < 0.05$  compared to carbachol alone)



**Fig. 5.11.** Quantitative changes of serum (2%)-induced intracellular  $\text{Ca}^{2+}$  mobilization by high extracellular  $\text{K}^+$  or  $\text{K}^+$  channel modulators in SK-N-MC human neuroblastoma cell line. Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of the increased intracellular  $\text{Ca}^{2+}$  concentration induced by the drugs compared to serum alone. Each column represents the mean value of at least five replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison. (\* $p < 0.05$  compared to serum alone)

channel modulators on the basal levels of intracellular  $\text{Ca}^{2+}$  concentrations were investigated. The results are shown in Figs. 5.12 and 5.13.

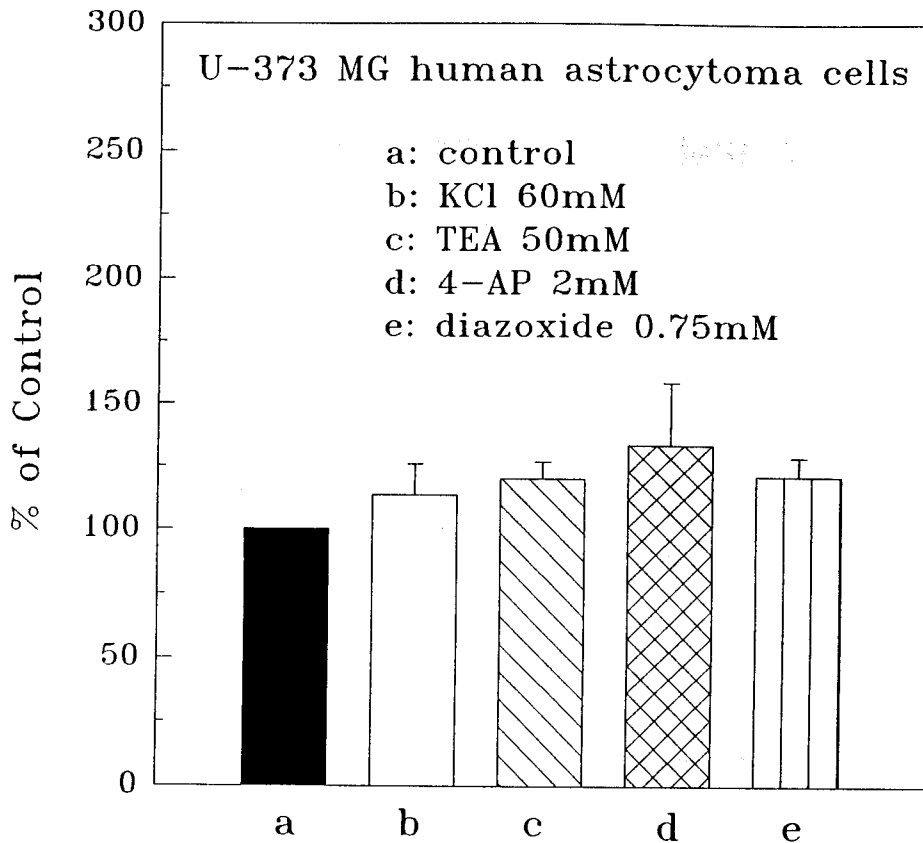
These results illustrate that  $\text{K}^+$  channel modulators did not induce the significant alteration of the basal levels of intracellular  $\text{Ca}^{2+}$  concentrations. Thus, the blockade of agonist-induced intracellular  $\text{Ca}^{2+}$  increases by these drugs may be not due to the alteration of basal, free cytosolic  $\text{Ca}^{2+}$  concentration. These results further suggest that  $\text{K}^+$  channel modulators may exert their effects only in the stimulatory state of the cells.

### **C. Discussion**

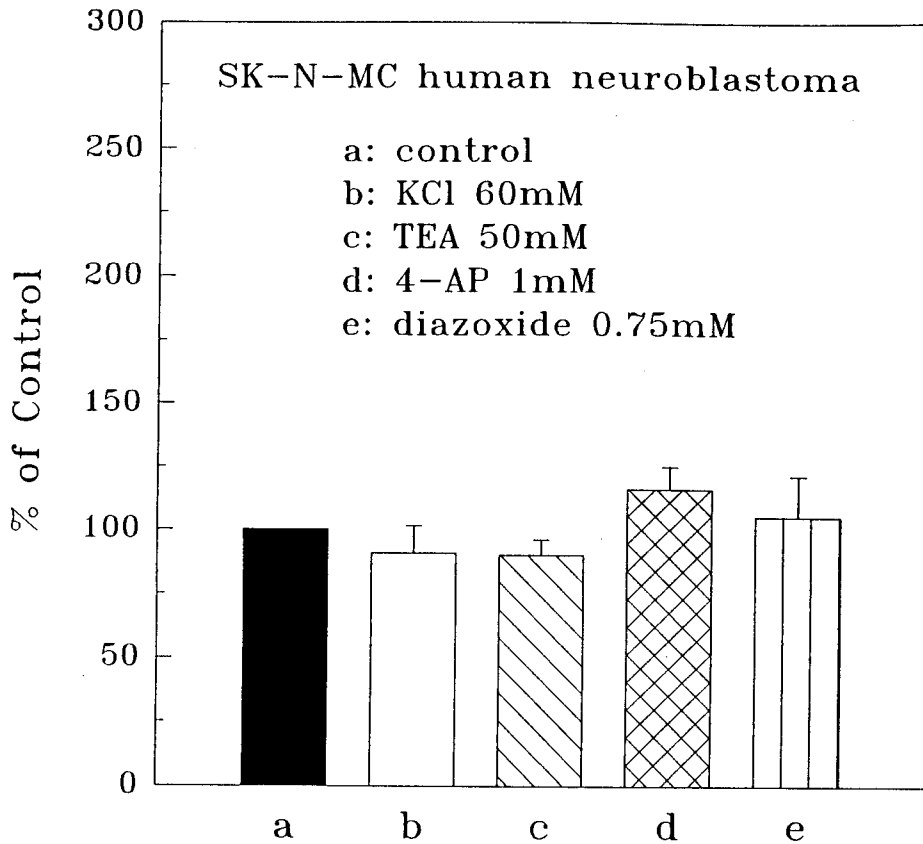
#### ***Proposed Mechanism of Inhibitory Effects of Potassium Channel Modulators on Tumor Cell Growth***

High extracellular  $\text{K}^+$  and some  $\text{K}^+$  channel modulators inhibited tumor cell growth as shown in Figs. 5.1 - 5.3. These drugs appeared to be non-cytotoxic to the tumor cells used in this study (Figs. 5.4 and 5.5) and, thus, their growth-inhibitory effects are due to the blockade of cell proliferation. In addition to their effects on the tumor cell growth,  $\text{K}^+$  channel modulators evoked the inhibition of growth factor-induced increased intracellular  $\text{Ca}^{2+}$  concentrations as shown in Figs 5.8 - 5.11. These  $\text{Ca}^{2+}$  increases resulted from the internal store release. Thus, apparent correlation between these two effects of  $\text{K}^+$  channel modulators suggests that the mechanism of their inhibitory action on tumor cell growth may be due to the blockade of growth factor-induced  $\text{Ca}^{2+}$  mobilization.

A remaining question is how  $\text{K}^+$  channel modulators can inhibit agonist-induced  $\text{Ca}^{2+}$  mobilization from internal stores. These  $\text{K}^+$  channel modulators are assumed to act only



**Fig. 5.12. No effect of high extracellular  $K^+$  or  $K^+$  channel modulators on basal, free cytosolic  $Ca^{2+}$  concentration in U-373 MG human astrocytoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu M$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 Ca^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of intracellular  $Ca^{2+}$  concentration induced by the drugs compared to control. Each column represents the mean value of at least five replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison.



**Fig. 5.13. No effect of high extracellular K<sup>+</sup> or K<sup>+</sup> channel modulators on basal, free cytosolic Ca<sup>2+</sup> concentration in SK-N-MC human neuroblastoma cell line.** Aliquots of  $5 \times 10^6$  cells/ml were incubated with  $2 \mu\text{M}$  of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing  $0 \text{ Ca}^{2+}$  and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of intracellular  $\text{Ca}^{2+}$  concentration induced by the drugs compared to control. Each column represents the mean value of at least five replicates with bars indicating SEM. The data were compared using one way ANOVA and Student-Newman-Keul's test for individual comparison.

on the extracellular sites of the plasma membrane since they do not readily penetrate the membrane due to their hydrophilic molecular structures (Palade *et al.*, 1989). Therefore, the main sites of action of these drugs are plasma membrane  $K^+$  channels and/or the receptors of  $Ca^{2+}$ -mobilizing agonists. Of particular interest, 4-AP appeared to bind the allosteric sites of muscarinic receptors in rabbit hippocampal membrane fractions (Potter *et al.*, 1989). Thus, the inhibition of the carbachol-induced intracellular  $Ca^{2+}$  mobilization by these  $K^+$  channel modulators at least 4-AP, may be due to the inhibition of the binding of carbachol to its receptors.

These drugs may also induce the alteration of membrane potential and intracellular  $K^+$  concentration through the modulation of plasma membrane  $K^+$  channel activities. Plasma membrane depolarization has been shown to reduce agonist-induced intracellular  $Ca^{2+}$  mobilization and in parallel  $IP_3$  generation in human neutrophils (Di Virgilio *et al.*, 1987) and HL-60 cells (Pittet *et al.*, 1990). Although in the present experiments the effects of these drugs on agonist-induced  $IP_3$  generation were not seen, the inhibition of agonist-induced  $Ca^{2+}$  mobilization by these drugs may be due to their effects on agonist-induced  $IP_3$  generation. However, the exact role of membrane potential changes in agonist-induced  $IP_3$  generation and ultimate  $Ca^{2+}$  mobilization is unknown, and can only be speculated. The alteration of the membrane potential may change the stability and organization of plasma membrane lipids and proteins (Matthew, 1985). Thus, these electrostatic effects may be expected to alter membrane enzyme activities, the interaction of these enzymes with their substrates and/or protein-protein interactions. Moreover, the activities of many enzymes associated with the plasma membrane, appeared to be

dependent on the surface membrane potential as shown in phosphocholine cytidyltransferase (Cornell, 1991), protein kinase C (Epanand and Lester, 1990; Lester *et al.*, 1990), apocytochrome c (Berkhout *et al.*, 1987) and synapsin (Benfenati *et al.*, 1989). Therefore, if membrane potential changes somehow induce the altered activities of phospholipase C (PLC), these changes can result in the altered IP<sub>3</sub> generation and, thus, the altered Ca<sup>2+</sup> mobilization during agonist stimulation. Other possibilities may include changes in the accessibility of substrate (e.g. PIP<sub>2</sub>), to the PLC, or uncoupling a regulatory subunit of G- protein from the PLC.

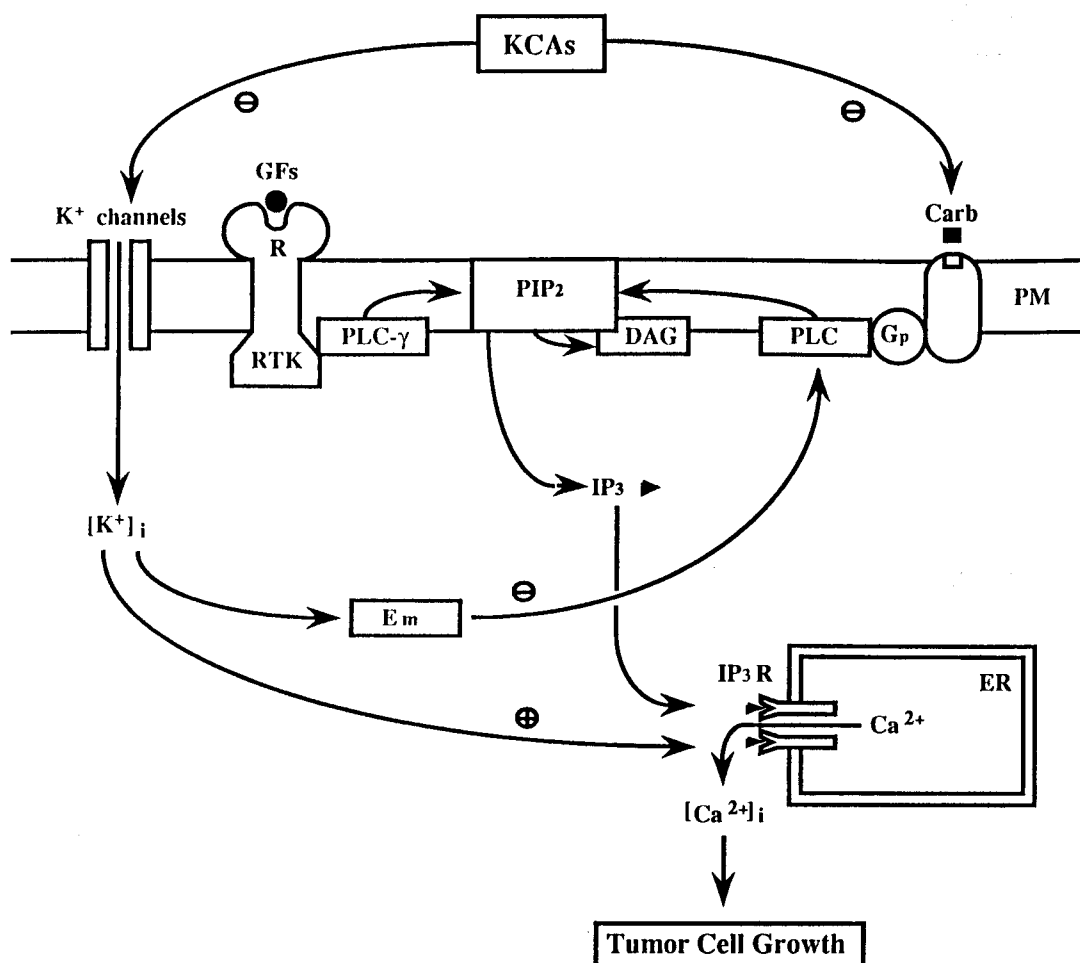
Interestingly, the same inhibitory effects of K<sup>+</sup> channel antagonists such as TEA and 4-AP, and K<sup>+</sup> channel activator such as diazoxide, on the tumor cell growth and agonist-induced Ca<sup>2+</sup> mobilization were observed in the present study. If these agents simply acted by varying intracellular K<sup>+</sup> concentrations, diazoxide would be expected to have the opposite effect of K<sup>+</sup> channel antagonists, i.e. increased tumor cell growth and agonist-induced Ca<sup>2+</sup> mobilization. Notable in this regard, Palade and his associates recently found that in canine microsomes, IP<sub>3</sub>-induced Ca<sup>2+</sup> release from the microsomes appeared to be dependent on the presence of K<sup>+</sup> in the medium (Palade *et al.*, 1989). Although a gradual reduction of K<sup>+</sup> concentration induced a parallel inhibition of IP<sub>3</sub>-induced Ca<sup>2+</sup> mobilization from the microsomes, the increase of K<sup>+</sup> permeability by the treatment of valinomycin, a K<sup>+</sup> ionophore, did not significantly affect on the IP<sub>3</sub>-induced Ca<sup>2+</sup> release. Thus, these results may suggest that K<sup>+</sup> channel blockers which can induce an increased intracellular K<sup>+</sup> concentration, would not have an effect on agonist-induced Ca<sup>2+</sup> mobilization. However, diazoxide-induced reduction of cytoplasmic K<sup>+</sup>

concentration through activation of plasma membrane  $K^+$  channels would result in the inhibition of agonist-induced  $Ca^{2+}$  mobilization.

Taken together,  $K^+$  channel modulators may affect an agonist-induced  $Ca^{2+}$  mobilization from the internal stores by acting on at least two different steps, e.g. generation of  $IP_3$  and  $Ca^{2+}$  release from internal stores. Interestingly, these two steps may be inversely regulated by a  $K^+$  channel modulator. However,  $K^+$  channel blockers seem to be more effective in the step of agonist-induced  $IP_3$  generation, on the other hand  $K^+$  channel opener may predominantly affect the  $Ca^{2+}$  releasing step. These speculations are based on the assumption that diazoxide may have a stimulatory effect on  $K^+$  channels in these tumor cells. However, the effect of diazoxide on  $K^+$  channels has not been described in these tumor cells and remains to be investigated.

In conclusion, the inhibition of tumor cell growth by high extracellular  $K^+$  and  $K^+$  channel modulators may be due to their blocking actions on agonist-induced  $Ca^{2+}$  mobilization from the internal stores through the alteration of membrane potential and intracellular  $K^+$  concentrations. These changes may alter agonist-induced  $IP_3$  generation and/or  $Ca^{2+}$  release from the stores. The summarized possible mechanisms of action of these drugs are shown diagrammatically in Fig. 5.13.





**Fig. 5.14. Proposed inhibitory mechanisms of high extracellular K<sup>+</sup> or K<sup>+</sup> channel modulators in tumor cell growth.** This figure shows the signal transduction pathways of growth factors and carbachol-inducing tumor cell proliferation. In general, the stimulation of growth factor receptors can phosphorylate and then activate PLC- $\gamma$  by an intrinsic receptor tyrosine kinase. The stimulation of muscarinic receptors by carbachol can evoke PLC activation through the interaction with G-proteins (specifically, G<sub>p</sub>). PLC- $\gamma$  and PLC degrade PIP<sub>2</sub> into DAG and IP<sub>3</sub>. IP<sub>3</sub> binding to the receptors on the ER membrane can open IP<sub>3</sub>-sensitive Ca<sup>2+</sup> channels, and DAG can activate PKC. An increased intracellular Ca<sup>2+</sup> can ultimately lead to tumor cell proliferation with the cooperation of other signals. This figure also shows a possible intervening step in these pathways by high extracellular K<sup>+</sup> or K<sup>+</sup> channel modulators. Abbreviations used: KCAs, K<sup>+</sup> channel antagonists; GFs, growth factors; Carb, carbachol; R, receptor; RTK, receptor tyrosine kinase; PLC, phospholipase C; PLC- $\gamma$ , phospholipase C- $\gamma$ ; G<sub>p</sub>, GTP binding protein; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; IP<sub>3</sub>, inositol 1,4,5-triphosphate; DAG, diacylglycerol; E<sub>m</sub>, membrane potential; [K<sup>+</sup>]<sub>i</sub>, intracellular K<sup>+</sup> concentration; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; IP<sub>3</sub> R, IP<sub>3</sub> receptor Ca<sup>2+</sup> channel; ER, endoplasmic reticulum; PM, plasma membrane.

## CHAPTER VI

### SUMMARY AND CONCLUSIONS

Many studies suggest that the increased intracellular  $\text{Ca}^{2+}$  concentration is a critical event in the proliferative signalling mechanism of growth factors. Moreover, plasma membrane  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channels are involved in the regulation of intracellular  $\text{Ca}^{2+}$  homeostasis. However, the mechanism of the involvement of these ion channels in tumor cell growth remains controversial. Thus, the major objective of this dissertation was to examine the roles of ion channels in nervous system tumor cell growth. Specifically, using U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines as model systems, three questions were addressed by this dissertation. 1) What are the effects of  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channels-regulating drugs on these tumor cell growth? 2) What are the characteristics and sources of increased intracellular  $\text{Ca}^{2+}$  concentration elicited by growth factors in these tumor cell lines? 3) Does a correlation exist between the effects of these drugs on tumor cell growth and intracellular  $\text{Ca}^{2+}$  concentration changes induced by growth factors?

In order to explore the first question, the effects of various  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channel agonists and antagonists on tumor cell growth were investigated. Tumor cell growth was assessed by counting the number of cells using a hemocytometer. The results were described in Chapter 4 and 5 in this dissertation. The findings are:

1) Prototypic voltage-sensitive  $\text{Ca}^{2+}$  channel antagonists, verapamil, nifedipine and

diltiazem, and inorganic  $\text{Ca}^{2+}$  channel blockers,  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$ , induced the inhibition of the growth of these tumor cells in a dose-dependent manner.

2) Bay K-8644, a  $\text{Ca}^{2+}$  channel agonist, did not induce a significant alteration of the growth of these tumor cells.

3) SK&F 96365 which is known as a receptor-operated  $\text{Ca}^{2+}$  channel antagonist, very effectively inhibited tumor cell growth.

4)  $\text{K}^+$  channel antagonists such as tetraethyl ammonium chloride and 4-aminopyridine, inhibited tumor cell growth in a dose-related fashion.

5) Diazoxide, a  $\text{K}^+$  channel activator, also induced the inhibition of tumor cell growth.

6) In addition to ion channel modulators, these tumor cells responded to changes of the extracellular concentrations of  $\text{Ca}^{2+}$  and  $\text{K}^+$ . Increased extracellular  $\text{Ca}^{2+}$  and  $\text{K}^+$  concentrations inhibited tumor cell growth, and decreased extracellular  $\text{Ca}^{2+}$  concentrations by chelation with EGTA also induced the inhibition of tumor cell growth.

7) These ion channel modulators except  $\text{Ni}^{2+}$  did not elicit a significant cytotoxicity which was assessed by trypan blue exclusion method.

These results indicate that a variety of ion channel modulators can effectively inhibit tumor cell growth and that this growth inhibition is due not to cytotoxicity, but to interference of cell proliferation. These results further suggest the potential therapeutic applicability of  $\text{Ca}^{2+}$  and  $\text{K}^+$  channel-regulating drugs on these tumor cell proliferation.

The intracellular  $\text{Ca}^{2+}$  concentrations were measured in a variety of conditions in order to answer the second question. The levels of intracellular  $\text{Ca}^{2+}$  were monitored

by fluorophotometry using Fura-2 dye. The major findings are:

- 1) Carbachol, a muscarinic agonist, increased both cell growth and intracellular  $\text{Ca}^{2+}$  concentration in U-373 MG human astrocytoma cells.
- 2) Carbachol induced a monophasic increase of intracellular  $\text{Ca}^{2+}$  concentration, and under the condition of 0 extracellular  $\text{Ca}^{2+}$  concentration, carbachol elicited a very similar response as that in a normal extracellular  $\text{Ca}^{2+}$  concentration.
- 3) Serum monophasically increased intracellular  $\text{Ca}^{2+}$  concentration in SK-N-MC human neuroblastoma cells, and this increase was insensitive to the alteration of extracellular  $\text{Ca}^{2+}$  concentration.
- 4) High extracellular  $\text{K}^+$  or/and Bay K 8644 did not induce the significant alteration of intracellular  $\text{Ca}^{2+}$  concentration.

These results indicate that agents which act as growth factors, can induce the increased intracellular  $\text{Ca}^{2+}$  concentration and that this  $\text{Ca}^{2+}$  increase comes not from extracellular space but from intracellular stores. These results further suggest that no voltage-dependent  $\text{Ca}^{2+}$  channels are present and that intracellular  $\text{Ca}^{2+}$  mobilization from intracellular stores is an important proliferative signalling mechanism of growth factors in both model tumor cells.

In relation to third question, the effects of various  $\text{Ca}^{2+}$  and  $\text{K}^+$  channel modulators on agonist-induced intracellular  $\text{Ca}^{2+}$  mobilization were investigated in order to elucidate the mechanism of their inhibitory actions in tumor cell growth. Carbachol and serum were used as agonist for the mobilization of intracellular  $\text{Ca}^{2+}$  in U-373 MG human

astrocytoma and SK-N-MC human neuroblastoma cell lines, respectively. The results are:

- 1) The effective growth-inhibitory concentrations of  $\text{Ca}^{2+}$  channel antagonists such as verapamil, nifedipine, diltiazem and SK&F 96365 inhibited significantly agonist-induced intracellular  $\text{Ca}^{2+}$  mobilization from intracellular stores in both model tumor cells.
- 2) The effective growth-inhibitory concentrations of  $\text{K}^{+}$  channel modulators such as TEA, 4-AP and diazoxide induced significant reduction of increased intracellular  $\text{Ca}^{2+}$  concentrations induced by agonists in these tumor cells.
- 3) These ion channel modulators did not elicit a significant alteration of basal, free intracellular  $\text{Ca}^{2+}$  concentrations in these tumor cells.

These results suggest that the growth-inhibitory effects of these ion channel modulators may be attributed to their abilities to intervene agonist-induced increased intracellular  $\text{Ca}^{2+}$  concentration mobilized from internal stores.

The mechanism of these ion channel modulators in the inhibition of agonist-induced  $\text{Ca}^{2+}$  mobilization from internal stores was not addressed in this dissertation and remains to be determined. However, since membrane potential changes have been demonstrated to induce alteration of agonist-induced second messenger generations, these effects of at least  $\text{K}^{+}$  channel modulators may be explained by their modulation of membrane potential.

In conclusion, intracellular  $\text{Ca}^{2+}$  mobilization from internal stores is an essential

signalling event in the proliferation of the tumor cells used in this dissertation.  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channel blockers effectively inhibit agonist-induced  $\text{Ca}^{2+}$  mobilization and, thus, tumor cell growth. These findings may contribute to the understanding of cancer cell biology and provide insight into the role of ion channels as therapeutic targets for disease management.

## REFERENCES

- Aaronson S.A. 1991. Growth factors and cancer. *Science*. 254:1146-1153.
- Adamo, S., Caporale, C., Aguanno, S., Lazdis, J., Faggioni, A., Belli, L., Cortesi, E., Nervi, C., Gastali, R., and Molinaro, M. 1986. Proliferating and quiescent cells exhibit different subcellular distribution of protein kinase C activity. *FEBS Lett.* 195:352-356.
- Alava, M.A., DeBell, K.E., Conti, A., Hoffman, T. and Bonvini, E. 1992. Increased intracellular cyclic AMP inhibits inositol phospholipid hydrolysis induced by perturbation of the T cell receptor/CD3 complex but not by G-protein stimulation. Association with protein kinase A-mediated phosphorylation of phospholipase C- $\gamma$ 1. *Biochem. J.* 284:189-199.
- Ames, B.N. and Gold, L.S. 1990. Chemical carcinogenesis: Too many rodent carcinogens. *Proc. Natl. Acad. Sci. USA.* 87:7772-7776.
- Amigorena, S., Choquet, D., Teillaud, J.L., Korn, H. and Fridman, W.H. 1990. Ion channel blockers inhibit B cell activation at a precise stage of the G1 phase of the cell cycle. *J. Immunol.* 144:2038-2045.
- Amigorena, S., Choquet, D., Teillaud, J.L., Korn, H., and Fridman, W.H. 1990. Ion channels and B cell mitogenesis. *Mol. Immunol.* 27:1259-1268.
- Aramori, I. and Nakanishi, S. 1992. Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron.* 8:757-765.
- Arita, Y., Kimura, T., Yazu, T., Ogami, Y. and Nawata, H. 1991. Effects of calcium-channel blockers on cytosolic free calcium and amylase secretion in rat pancreatic acini. *Pharmacol. Toxicol.* 68:83-87.
- Arlow, F.L., Walczak, S.M., Luk, G.D. and Majumdar, A.P.N. 1989. Attenuation of azoxymethane-induced colonic mucosal ornithine decarboxylase and tyrosine kinase activity by calcium in rats. *Cancer Res.* 49:5884-5888.
- Arlow, F.L., Walczak, S.M., Moshier, J.A., Pietruk, T and Majumdar, A.P.N. 1990. Gastrin and epidermal growth factor induction of ornithine decarboxylase in rat colonic explants. *Life Sci.* 46:777-784.
- Ashkenazi A., Ramachandran J and Capon, D.J. 1989. Acetylcholine analoge stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes. *Nature.* 340:146-150.

- Auerbach, O. and Garfinkel, L. 1989. Histologic changes in the urinary bladder in relation to cigarette smoking and use of artificial sweeteners. *Cancer*. 64:983-987.
- Auvinen, M., Paasinen, A. Anderson, L.C. and Holtta, E. 1992. Ornithine decarboxylase activity is critical for cell transformation. *Nature*. 360:355-358.
- Avdonin, P.V., Cheglakov, I.B., and Tkachuk, V.A. 1991. Stimulation of non-selective cation channels providing  $\text{Ca}^{2+}$  influx into platelets by platelet activating factor and other aggregation inducers. *Eur. J. Biochem*. 198:267-273.
- Balk, S.D., Polimeni, P.I., Hoon, B.S., Lestourgeon, D.N. and Mitchell, R.S. 1979. Proliferation of Rous sarcoma virus-infected, but not of normal, chicken fibroblasts in medium of reduced calcium and magnesium concentration. *Proc. Natl. Acad. Sci.* 76:3913-3916.
- Balk, S.D., Whitfield, J.F., Youdale, T. and Braun, A.C. 1973. Roles of calcium, serum, plasma and folic acid in the control of proliferation of normal and Rous sarcoma virus-infected chicken fibroblasts. *Proc. Natl. Acad. Sci. USA*. 70:675-679.
- Ballester, R. and Rosen, O.M. 1985. Fate of immunoprecipitable protein kinase C in GH3 cells treated with phorbol 12-myristate 13-acetate. *J. Biol. Chem*. 260:15194-15199.
- Barres, B.A., Chun, L.L.Y. and Corey, D.P. 1990. Ion channels in vertebrate glia. *Ann. Rev. Neurosci*. 13:441-474.
- Barrows, G.H., Mays, E.T. and Christopherson, W.M. 1988. Steroid related neoplasia in human liver. In *Proceedings of the 18th International Symposium of the Princess Takamatsu Cancer Research Fund*, p 47. Tokyo, Japan Scientific Societies Press.
- Batra, S., Popper, L.D., and Hartley-Asp, B. 1991. Effect of calcium and calcium antagonists on  $^{45}\text{Ca}$  influx and cellular growth of human prostatic tumor cells. *Prostate*. 19:299-311.
- Beasley, R.P. 1988. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer*. 61:1942-1956.
- Benfenati, F., Greengard, P., Brunner, J. and Bahler, M. 1989. Electrostatic and hydrophobic interactions of synapsin I and synapsin I fragments with phospholipid bilayers. *J. Cell Biol*. 108:1851-1862.
- Berkhout, T.A., Rietveld, A. and de Kruijff, B. 1987. Preferential lipid association and mode of penetration of apocytochrome c in mixed model membrane as monitored by tryptophanyl fluorescence quenching using brominated phospholipid. *Biochim. Biophys.*



*Acta.* 897:1-4.

Berridge, M.J. 1993. Inositol triphosphate and calcium signalling. *Nature.* 361:315-325.

Berridge, M.J. 1987. Inositol lipids and cell proliferation. *Biochim. Biophys. Acta.* 907:33-45.

Berridge, M.J. 1987. Inositol triphosphate and diacylglycerol: Two interacting second messengers. *Annu. Rev. Biochem.* 56:159-193.

Berridge, M.J. and Irvine, R.F. 1984. Inositol phosphate, a novel second messenger in cellular signal transduction. *Nature.* 312:315-321.

Bertolino, M. 1992. The central role of voltage-activated and receptor-operated calcium channels in neuronal cells. *Annu. Rev. Pharmacol. Toxicol.* 32:399-421.

Bigner, S.H., Bullard, D.E., Pegram, C.N., Wikstrand, C.J. and Bigner, D.D. 1981. Relationship of *in vitro* morphologic and growth characteristics of established human glioma-derived cell lines to their tumorigenicity in athymic nude mice. *J. Neuropathol. Exp. Neurol.* 40:390-409.

Bishop, J.M. 1991. Molecular themes in oncogenesis. *Cell.* 64:235-248.

Block, L.H., Emmons, L.R., Vogt, E., Sachinidis, A., Vetter, W. and Hoppe, J. 1989. Ca<sup>2+</sup>-channel blockers inhibit the action of recombinant platelet-derived growth factor in vascular smooth muscle cells. *Proc. Natl. Acad. Sci.* 86:2388-2392.

Boeckh, M., Gleaves, C.A., Bindra, R. and Meyers, J.D. 1991. Comparison of MRC-5 and U-373MG astrocytoma cells for detection of cytomegalovirus in shell vial centrifugation cultures. *Eur. J. Clin. Microbiol. Infect. Dis.* 10:569-572.

Bollag, G.E., Roth, R.A., Beaudoin, J., Mochly-Rosen, D. and Koshland, Jr., D.E. 1986. Protein kinase C directly phosphorylates the insulin receptor *in vitro* and reduces its protein-tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA.* 83:5822-5824.

Bonventre, J.V., Skorecki, K.L., Kreisberg, J.I. and Cheung, J.Y. 1986. Vasopressin increases cytosolic free calcium concentration in glomerulosa mesangial cells. *Am. J. Physiol.* 20:F94-F102.

Bosland, M.C. 1988. The etiopathogenesis of prostatic cancer with special reference to environmental factors. *Adv. Cancer Res.* 51:1-106.

Boveri, T. 1914. *Zur Trage der Entstehung maligner Tumoren*, edited by Jena, Fischer.

- Bowles, Jr., A.P., Pantazis, C.G. and Wansley, W. 1990. Use of verapamil to enhance the antiproliferative activity of BCNU in human glioma cells: an *in vitro* and *in vivo* study. *J. Neurosurg.* 73:248-253.
- Boynton, A.L. 1988. Calcium and epithelial cell proliferation. *Mineral Electrolyte Metab.* 14:86-94.
- Boynton, A.L., Kleine, L.P. and Whitfield, J.F. 1984. Relation between colony formation in calcium-deficient medium, colony formation in soft agar and tumor formation and T51B rat liver cells. *Cancer Lett.* 21:293-302.
- Brock, T.A., Rittenhouse, S.E., Powers, C.W., Ekstein, L.S., Gimbrone, M.A. and Alexander, R.W. 1985. Phorbol ester and 1-oleoyl-2-acetyl glycerol inhibit angiotensin activation of phospholipase C in cultured vascular smooth muscle. *J. Biol. Chem.* 260:14158-14162.
- Brodie, C. and Sampson, S.R. 1991. Verapamil regulation of Na-K pump levels in rat skeletal myotubes: role of spontaneous activity and Na channels. *J. Neurosci. Res.* 28:229-235.
- Bruserud, O. 1992. Effect of dipyridamole, theophyllamine and verapamil on spontaneous *in vitro* proliferation of myelogenous leukaemia cells. *Acta. Oncol.* 31:53-58.
- Cahalan, M.D. and Lewis, R.S. 1990. Functional roles of ion channels in lymphocytes. *Semin. Immunol.* 2:107-117.
- Canellakis, Z.N., Marsh, L.L. and Bondy, P.K. 1989. Polyamines and their derivatives as modulators in growth and differentiation. *Yale J. Biol. Med.* 62:481-491.
- Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. 1991. Oncogenes and signal transduction. *Cell.* 64:281-302.
- Carafoli, E. 1985. Membrane transport regulates the signalling function of calcium. In *Cell Membrane and Cancer*, edited by Galeotti *et al.*, p 147-151, Elsevier, Amsterdam.
- Carafoli, E. 1987. Intracellular calcium homeostasis. *Ann. Rev. Biochem.* 56:395-433.
- Carpenter, G. 1992. Receptor tyrosine kinase substrates: *src* homology domains and signal transduction. *FASEB J.* 6:3283-3289.
- Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd, A.E.III and Means, A.R. 1982. Calmodulin and the cell cycle: Involvement in regulation of cell-cycle progression. *Cell.* 28:41-50.

- Chan, T.C.K. 1989. Calcium-independent growth of human ovarian carcinoma cells. *J. Cell. Physiol.* 141:461-466.
- Chan, J. and Greenberg, D.A. 1991. SK&F 96365, a receptor-mediated calcium entry inhibitor, inhibit calcium responses to endothelin-1 in NG 108-15 cells. *Biochem. Biophys. Res. Commun.* 177: 1141-1146.
- Chang, B.K. 1991. Inhibitory effects of a calcium antagonist on ornithine decarboxylase induction in pancreatic cancer cell lines. *Pancrease.* 6:631-636.
- Chiarugi, V., Ruggiero, M., and Porciatti, F. 1987. Oncogenes and transmembrane cell signalling. *Cancer Invest.* 5:215-229.
- Chiu, S.Y. and Wilson, G.F. 1989. The role of potassium channels in Schwann cell proliferation in Wallerian degeneration of explant rabbit sciatic nerves. *J. Physiol.* 448:199-222.
- Clarkson, B. and Baserga, R. 1974. *Control of Proliferation in Animal Cells.* New York, Cold Spring Harbor Laboratory.
- Cohen, S.M. and Ellwein, L.B. 1990. Cell proliferation in carcinogenesis. *Science.* 249:1007-1011.
- Cohen, S. and Fava, R.A. 1985. Internalization of functional epidermal growth factor:receptor/kinase complexes in A-431 cells. *J. Biol. Chem.* 260:12351-12358.
- Cohen, S.M., Purtilo, D.T., and Ellwein, L.B. 1991. Pivotal role of increased cell proliferation in human carcinogenesis. *Modern Pathology.* 4:371-382.
- Connor, C.G., Moore, P.B. and Brandy, R.C. 1983. The role of calmodulin in cell transformation. *Biochem. Biophys. Res. Commun.* 112:647-654.
- Constantious, A.I., Squinto, S.P. and Jungmann, R.A. 1985. The phosphoform of the regulatory subunit RII of cyclic AMP-dependent protein kinase possesses intrinsic topoisomerase activity. *Cell.* 42:429-437.
- Cornell, R.B. 1991. Regulation of CTP:phosphocholine cytidylyltransferase by lipids. 1. Negative surface charge dependence for activation. *Biochemistry.* 30:5873-5880.
- Corral, M., Tichonicky, L., Guguen-Guillouzo, C., Corcos, D., Raymondjean, M., Paris, B., Kruh, J., and Defer, N. 1985. Expression of *c-fos* oncogene during hepatocarcinogenesis, liver regeneration and in synchronized HTC cells. *Exp. Cell Res.* 160:427-434.

- Criss, W. and Kakiuchi, S. 1982. Calcium: calmodulin and cancer. *Federation Proc.* 41:2289-2291.
- Cross, M. and Dexter, T.M. 1991. Growth factors in development, transformation, and tumorigenesis. *Cell.* 64:271-280.
- Cukierman, S. 1992. Characterization of K<sup>+</sup> currents in rat malignant lymphocytes (Nb2 cells). *J. Membrane Biol.* 126:147-157.
- DeCoursey, T. E., Chandy, K.G., Gupta, S. and Cahalan, M.D. 1987. Mitogen induction of ion channels in murine T lymphocytes. *J. Gen. Physiol.* 89:405-420.
- DeCoursey, T.E., Chandy, K.G., Gupta, S. and Cahalan, M.D. 1985. Voltage-dependent ion channels in T-lymphocytes. *J. Neuroimmunol.* 10:71-95.
- DeCoursey, T.E., Chandy, K.G., Gupta, S., and Cahalan, M.D. 1984. Voltage-gated K<sup>+</sup> channels in human T lymphocytes: a role in mitogenesis? *Nature.* 307:465-468.
- DeLisle, S., Pittet, D., Potter, B.V.L., Lew, P.D. and Welsh, M.J. 1992. InsP<sub>3</sub> and Ins(1,23,4,5)P<sub>4</sub> act in synergy to stimulate influx of extracellular Ca<sup>2+</sup> in *Xenopus oocytes*. *Am. J. Physiol.* 262:C1456-C1463.
- Delvaux, M., Bastie, M.J., Chentoufi, J. and Vaysse, R.N. 1990. Na<sup>+</sup>/H<sup>+</sup> exchange in AR 4-2J cells: stimulation by growth factors and pancreatic secretagogues. *Digestion.* 46(suppl):156-161.
- Dere, W.H., Hirayu, H. and Rapoport, B. 1986. Thyrotropin and cyclic AMP regulation of *ras* proto-oncogene expression in cultured thyroid cells. *FEBS Lett.* 196:305-308.
- Deutsch, C. K<sup>+</sup> channels and mitogenesis. 1990. In *Potassium channels: Basic Function and Therapeutic Aspects*. Alan R. Liss, Inc. pp. 251-271.
- Di Virgilio, F., Lew, P.D., Anderson, T. and Pozzan, T. 1987. Plasma membrane potential modulates chemotactic peptide-stimulated cytosolic free Ca<sup>2+</sup> changes in human neutrophils. *J. Biol. Chem.* 262:4574-4579.
- Diliberto, P.A., Hubbert, T. and Herman, B. 1991. Early PDGF-induced alterations in cytosolic free calcium are required for mitogenesis. *Res. Comm. Chem. Pathol. Pharmacol.* 72:3-12.
- Drummond, A.H. and Macintyre, D.E. 1985. Protein kinase C as a bidirectional regulator of cell function. *Trends Pharmacol. Sci.* 6:233-234.
- Dumont, J.E., Jauniaux, J.C. and Roger, P.P. 1989. The cyclic AMP-mediated

stimulation of cell proliferation. *Trends Biol. Sci.* 14:73-77.

Dunsford, H.A., Sell, S. and Chisari, F.V. 1990. Hepatocarcinogenesis due to chronic liver cell injury in hepatitis B virus transgenic mice. *Cancer Res.* 50:3400-3407.

Epand, R.M. and Lester, D.S. 1990. The role of membrane biophysical properties in the regulation of protein kinase C activity. *Trend Pharmacol Sci.* 11:317-320.

Felder, C.C., Poulter, M.O. and Wess, J. 1992. Muscarinic receptor-operated  $\text{Ca}^{2+}$  influx in transfected fibroblast cells is independent of inositol phosphates and release of intracellular  $\text{Ca}^{2+}$ . *Proc. Natl. Acad. Sci. USA.* 89:509-513.

Ferris, C.D. and Snyder, S.H. 1992. Inositol 1,4,5-triphosphate-activated calcium channels. *Ann. Rev. Physiol.* 54:469-488.

Feth, F., Rascher, W. and Michel, M.C. 1991. G-protein coupling and signalling of  $\text{Y}_1$ -like neuropeptide Y receptors in SK-N-MC cells. *Naunyn-Schmiedeberg's Arch Pharmacol.* 344:1-7.

Filep, J.G. and Foldes-Filep, E. 1990. Inhibition by calcium channel blockers of the binding of platelet-activating factor to human neutrophil granulocytes. *Eur. J. Pharmacol.* 190:67-73.

Fischer, H., Illek, B., Negulescu, P.A., Clauss, W. and Machen, T.E. 1992. Carbachol-activated calcium entry into HT-29 cells is regulated by both membrane potential and cell volume. *Proc. Natl. Acad. Sci.* 89:1438-1442.

Fisher, S.K., Heacock, A.M. and Agranoff, B.W. 1992. Inositol lipids and signal transduction in the nervous system: An update. *J Neurochem.* 58:18-38.

Flockhart, D.A. and Corbin, J. 1982. Regulatory mechanisms in the control of protein kinases. *CRC Crit. Rev. Biochem.* 12:133-186.

Frace, A.M. and Gargus, J.J. 1989. Activation of single-channel currents in mouse fibroblasts by platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA.* 86:2511-2515.

Gallin, E.K. 1991. Ion channels in leukocytes. *Physiol Rev.* 71:775-811.

Garcia-Sancho, J., Alvarez, J.A., Montero, M and Villalobos, C 1992.  $\text{Ca}^{2+}$  influx following receptor activation. *Trends Pharmacol. Sci.* 13:12-13.

Gardner, P. 1990. Patch clamp studies of lymphocyte activation. *Annu. Rev. Immunol.* 8:231-252.

- Geck, P. and Bereiter-Hahn, J. 1991. The role of electrolytes in early stages of cell proliferation. *Cell. Biol. Rev.* 25:85-104.
- Gelfand, E.W., Cheung, R.K., Mills, G.B. and Grinstein, S. 1987. Role of membrane potential in the response of human lymphocytes to phytohemagglutinin. *J. Immunol.* 138:527-531.
- Gerry, R.H., Rauch, B., Colvin, R.A., Adler, P.N. and Messineo, F.C. 1987. Verapamil interaction with the muscarinic receptor: stereo-selectivity at two sites. *Biochem. Pharmacol.* 36:2951-2956.
- Ginty, D.D. and Seidel, E.R. 1989. Polyamine-dependent growth and calmodulin-regulated induction of ornithine decarboxylase. *Am. J. Physiol.* 256:G342-G348.
- Goustin, A.S., Leof, E.B., Shipley, G.D., and Moses, H.L. 1986. Growth factors and cancer. *Cancer Res.* 46:1015-1029.
- Green, S. 1992. Nuclear receptors and chemical carcinogenesis. *Trends Pharmacol. Sci.* 13:251-255.
- Greenfield, R.E., Ellwein, L.B. and Cohen, S.M. 1984. A general probabilistic model of carcinogenesis: analysis of experimental urinary bladder cancer. *Carcinogenesis.* 5:437-445.
- Grinstein, S., Rotin, D., and Mason, M.J. 1989. Na<sup>+</sup>/H<sup>+</sup> exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta.* 988:73-97.
- Gross, J.L., Morrison, R.S., Eidsvoog, K., Herblin, W.F., Kornblith, P.L. and Dexter, D.L. 1990. Basic fibroblast growth factor: a potential autocrine regulator of human glioma cell growth. *J. Neurosci. Res.* 27:689-696.
- Gruver, C.L., George, S.E. and Means, A.R. 1992. Cardiomyocyte growth regulation by Ca<sup>2+</sup>-calmodulin. *Trends Cardiovasc. Med.* 2:226-231.
- Grynkiewicz, G., Poene, M. and Tsien, R.Y. 1985. A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450.
- Hait, W.N. 1987. Increased biological activity of calmodulin (CaM) in leukemic lymphocytes. *Proc. Am. Assoc. Cancer Res.* 28:99.
- Hait, W.N. and DeRosa, W.T. 1988. Calmodulin as a target for new chemotherapeutic strategies. *Cancer Invest.* 6:499-511.

- Hammond, R.A., Foster, K.A., Berchthold, M.W., Gassmann, M., Holmes, A.M., Hubscher, U. and Brown, N.C. 1988. Calcium-dependent calmodulin-binding proteins associated with mammalian DNA polymerase- $\alpha$ . *Biochim. Biophys. Acta.* 951:315-321.
- He, X., Wu, X. and Baum, B.J. 1988. Protein kinase C differentially inhibits muscarinic receptor operated  $\text{Ca}^{2+}$  release and entry in human salivary cells. *Biochem. Biophys. Res. Commun.* 152:1062-1069.
- Heldin, C.H., Betsholtz, C., Claesson-Welsh, L. and Westermark, B. 1987. Subversion of growth regulatory pathways in malignant transformation. *Biochim. Biophys. Acta.* 907:219-244.
- Helson, L. 1984. Calcium channel blockers enhancement of anticancer drug cytotoxicity-a review. *Cancer Drug Delivery.* 1:353-361.
- Henderson, B.E. 1989. Establishment of an association between a virus and a human cancer. *J. Natl. Cancer Inst.* 81:320-321.
- Henderson, B.E., Ross, R.K., and Pike, M.C. 1991. Toward the primary prevention of cancer. *Science.* 254:1131-1138.
- Henderson, B.E., Ross, R.K., and Pike, M.C. 1982. Endogenous hormones as a major factor in human cancer. *Cancer Res.* 42:3232-3239.
- Henry-Toulme, N., Sarthou, P. and Bolard, J. 1990. Early membrane potential and cytoplasmic calcium changes during mitogenic stimulation of WEHI 231 cell line by polyene antibiotics, lipopolysaccharide and anti-immunoglobulin. *Biochim. Biophys. Acta.* 1051:285-292.
- Henzi, V. and MacDermott, A.B. 1992. Characteristics and function of  $\text{Ca}^{2+}$ - and inositol 1,4,5-triphosphate-releasable stores of  $\text{Ca}^{2+}$  in neurons. *Neurosci.* 46:251-273.
- Herlyn, M. and Malkowicz, S.B. 1991. Regulatory pathways in tumor growth and invasion. *Lab. Invest.* 65:262-271.
- Herlyn, M., Kath, R., Williams, N., Valyi-Nagy, I. and Rodeck, U. 1990. Growth-regulatory factors for normal, premalignant, and malignant human cells *in vitro*. *Adv. Cancer Res.* 54:213-234.
- Herschman, H.R. 1991. Primary response genes induced by growth factors and tumor promoters. *Ann. Rev. Biochem.* 60:281-319.
- Hesketh, T.R., Morris, J.D.H., Moore, J.P., and Metcalfe, J.C. 1988.  $\text{Ca}^{2+}$  and pH responses to sequential additions of mitogens in single 3T3 fibroblasts: correlations with

DNA synthesis. *J. Biol. Chem.* 263:11879-11886.

Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.V. and Metcalfe, J.C. 1983. Free cytosolic calcium concentration and the mitogenic stimulation of lymphocytes. *J. Biol. Chem.* 258:4876-4882.

Hickie, R.A. and Kalant, H. 1967. Calcium and magnesium content of rat liver and Morris hepatoma 5123 + C. *Cancer Res.* 27:1053-1057.

Hiely, C.R., Cowley, D.J., Pelton, J.T. and Hargreaves, A.C. 1992. BQ-123, cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu), is a non-competitive antagonist of the actions of endothelin-1 in SK-N-MC human neuroblastoma cells. *Biochem. Biophys. Res. Commun.* 184:504-510.

Hill, R.N., Erdreich, L.S., Paynter, O.E., Roberts, P.A., Rosenthal, S.L. and Wilkinson, C.F. 1989. Thyroid follicular cell carcinogenesis. *Fundam. Appl. Toxicol.* 12:629-697.

Hill, T.D., Campos-Gonzalez, R., Kindmark, H. and Boynton, A.L. 1988. Inhibition of inositol triphosphate-stimulated calcium mobilization by calmodulin antagonists in rat liver epithelial cells. *J. Biol. Chem.* 263:16479-16484.

Hiramatsu, Y., Baum, B. and Ambudkar, I.S. 1992. Elevation of cytosolic  $[Ca^{2+}]$  due to intracellular  $Ca^{2+}$  release retards carbachol stimulation of divalent cation entry in rat parotid gland acinar cells. *J. Membrane Biol.* 129:277-286.

Hollstein, M. and Yamasaki, H. 1986. Tumor promoter-mediated modulation of cell differentiation and communication: the phorbol ester-oncogene connection. In *Tumor Cell Differentiation*, edited by Aarbakke *et al.*, p 317-339, Clifton, NJ: Humana Press.

Holt, J.T., Gopal, T.V., Moulton, A.D. and Nienhaus, A.W. 1986. Inducible production of *c-fos* antisense RNA inhibits 3T3 cell proliferation. *Proc. Natl. Acad. Sci. USA.* 83:4794-4798.

Homa, S.T., Khan, S.N., Conroy, D.M., Speak, A.E., and Smith, A.D. 1990. Verapamil inhibits phosphatidic acid formation and modifies phosphoinositide metabolism in stimulated platelets. *Eur. J. Pharmacol.* 182:457-464.

Housey, G.M., Johnson, M.D., Hsiao, W.L., O'Brian, C.A. and Murphy, J.P. 1988. Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. *Cell.* 52:343-354.

Hunter, T. 1991. Cooperation between oncogenes. *Cell.* 64:249-270.



Hupe, D.J., Boltz, R., Cohen, C.J., Felix, J., Ham, E., Miller, D., Soderman, D., and van Skiver, D. 1991. The inhibition of receptor-mediated and voltage-dependent calcium entry by the antiproliferative L-651, 582. *J. Biol. Chem.* 266:10136-10142.

Hynes, R.O. 1976. Cell surface proteins and malignant transformation. *Biochim. Biophys. Acta.* 158:73-107.

IARC Monograph. 1975. Some naturally occurring substances. *Eval. Carcinog. Risk Chem. Man.* 10:153.

Irvine, R. and Moore, R. 1986. Microinjection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external  $\text{Ca}^{2+}$ . *Biochem. J.* 240:917-920.

Isobe, T., Ishioka, N. and Okuyama, T. 1981. Isolation and characterization of Des (alanyl) calmodulin in porcine brain. *Biochem. Biophys. Res. Commun.* 102:279-286.

Jacob, R., Merritt, J.E., Hallam, T.J. and Rink, T.J. 1988. Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. *Nature.* 335:40-45.

Janne, J., Alhonen, L. and Leinonen, P. 1991. Polyamines: from molecular biology to clinical applications. *Ann. Med.* 23:241-259.

Jin, W., Lee, N.M., Loh, H.H. and Thayer, S.A. 1992. Dual excitatory and inhibitory effects of opioids on intracellular calcium in neuroblastoma x glioma hybrid NG108-15 cells. *Mol. Pharmacol.* 42:1083-1089.

Jones, A., Boyton, A.L. and MacManus, J.P. 1982.  $\text{Ca}^{2+}$ -calmodulin mediates the DNA-synthetic response of calcium deprived liver cells to the tumor promoter TPA. *Exp. Cell Res.* 138:87-93.

Jones, T.R., Bigner, S.H., Schold, S.C. Jr., Eng, L.F. and Bigner, D.D. 1981. Anaplastic human gliomas grown in athymic mice. Morphology and glial fibrillary acidic protein expression. *Am. J. Pathol.* 105:316-327.

Julius, D., Livelli, T.J. and Axel, R. 1989. Ectopic expression of the serotonin 1c receptor and the triggering of malignant transformation. *Science.* 224:1057-1062.

Kaczmarek, L., Hyland, J.K., Watt, R., Rozenberg, M. and Baserga, R. 1985. Microinjected *c-myc* as competence factor. *Science.* 228:1313-1315.

Karin, M. 1992. Signal transduction from cell surface to nucleus in development and disease. *FASEB J.* 6:2581-2590.

- Katayma, N., Nishikawa, M., Komada, F., Minami, N. and Shirakawa, S. 1990. A role for calmodulin in the growth of human hematopoietic progenitor cells. *Blood*. 75:1446-1454.
- Katayama, S., Kito, S., Miyoshi, R. and Matsubayashi, H. 1987. Effects of calcium antagonists on muscarinic receptor subtypes in the rat brain. *Brain Res*. 422:168-171.
- Kates, R.E. 1987. Pharmacokinetics of calcium antagonists. In *The Role of Calcium in Biological Systems*. Vol. IV., pp. 37-64, edited by Anghileri, L.J., CRC Press, Inc.
- Kelly, K., Cochran, B.H., Stiles, C.D. and Leder, P. 1983. Cell-specific regulation of the *c-myc* gene by lymphocyte mitogens and platelet-derived growth factor. *Cell*. 35:603-610.
- Kiefer, H., Blume, A.J. and Kaback, H.R. 1980. Membrane potential changes during mitogenic stimulation of mouse spleen lymphocytes. *Proc Natl. Acad. Sci. USA*. 77:2200-2204.
- Kikkawa, U., Kaibuchi, K., Castagna, M., Yamanishi, J., Sano, K., Tanaka, Y., Miyake, R., Takai, Y., and Nishizuka, Y. 1984. Protein phosphorylation and mechanism of action of tumor-promoting phorbol esters. *Adv. Cyclic Nucleotide Res*. 17:437-442.
- Kim, H.K., Kim, J.W., Zilberstein, A. Margolis, B., Kim, J.G., Schlessinger, J., and Rhee, S.G. 1991. PDGF stimulation of inositol phospholipid hydrolysis requires PLC- $\gamma$ 1 phosphorylation on tyrosine residues 783 and 1254. *Cell*. 65:435-441.
- Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. and Pawson, T. 1991. SH2 and SH3 domains: elements that control interaction of cytoplasmic signalling proteins. *Science*. 252:668-674.
- Kostyuk, P.G. 1989. Diversity of calcium ion channels in cellular membranes. *Neurosci*. 28:253-261.
- Kostyuk, P.G., Krishtal, O.A. and Doroshenko, P.A. 1975. Outward currents in isolated snail neurons. III. Effects of verapamil. *Comp. Biochem. Physiol*. 51C:269-274.
- Kunert-Radek, J., Stepien, H., Lyson, K., and Pawlikowski, M. 1990. Effects of calcium channel modulators on the proliferation of mouse spleen lymphocytes *in vitro*. *Agent Action*. 29:254-258.
- Kunert-Radek, J., Stepien, H., Radek, A. Lyson, K. and Pawlikowski, M. 1989. Inhibitory effect of calcium channel blockers on proliferation of human glioma cells *in vitro*. *Acta. Neurol. Scand*. 79:166-169.

- Kusano, K. and Gainer, H. 1991. Bombesin-like peptides induce  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  conductance increases in mouse fibroblasts. *Am. J. Physiol.* 260:C701-C707.
- Kuno, M., Goronzy, J., Weyand, C.M., and Gardner, P. 1986. Single-channel and whole-cell recordings of mitogen-regulated inward currents in human cloned helper T lymphocytes. *Nature.* 323:269-273.
- Labarca, R., Janowsky, A., Patel, J. and Paul, S.M. 1984. Phorbol esters inhibit agonist-induced [ $^3\text{H}$ ]inositol-1-phosphate accumulation in rat hippocampal slices. *Biochem. Biophys. Res. Commun.* 123:703-709.
- L'Allemain, G., Franchi, A., Cragoe, E. JR., and Pouyssegur, J. 1984. Blockade of the  $\text{Na}^+/\text{H}^+$  antiport abolishes growth factor-induced DNA synthesis in fibroblasts. *J. Biol. Chem.* 259:4313-4319.
- Lambert, D.G., Whitham, E.M., Baird, J.G. and Nahorski, S.R. 1990. Different mechanisms of  $\text{Ca}^{2+}$  entry induced by depolarization and muscarinic receptor stimulation in SH-SY5Y human neuroblastoma cells. *Mol. Brain Res.* 8:263-266.
- Lang, R.A. and Burgess, A.W. 1990. Autocrine growth factors and tumourigenic transformation. *Immunol. Today.* 11:244-249.
- Lee C.M., Kum, W., Cockram, C.S., Teoh, R. and Young, J.D. 1989. Functional substance P receptors on a human astrocytoma cell line (U-373 MG). *Brain Res.* 488:328-331.
- Lee, S.C., Price, M., and Deutsch, C. 1986. K-channel blockers and T-lymphocyte proliferation. *Biophys. J.* 49:167a.
- Lee, S.C., Sabath, D.E., Deutsch, C., and Prystowsky, M.B. 1986. Increased voltage-gated potassium conductance during Interleukin 2-stimulated proliferation of a mouse helper T lymphocyte clone. *J. Cell. Biol.* 102:1200-1208.
- Leeb-Lundberg, L.M.F., Cotecchia, S., Lomasney, J.W., DeBernardis, J.F., Lefkowitz, R.J. and Caron, M.G. 1985. Phorbol esters promote  $\alpha_1$ -adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc. Natl. Acad. Sci. USA.* 82:5651-5655.
- Lester, D.S., Doll, L., Brumfeld, V. and Miller, I.R. 1990. Lipid dependence of surface conformations of protein kinase C. *Biochim. Biophys. Acta* 1039:33-41.
- Lichtman, A.H., Segel, G.B. and Lichtman, M.A. 1983. The role of calcium in lymphocyte proliferation. *Blood.* 61:413-422.

- Lin, W.-W., Kiang, J.G., and Chuang, D.-M. 1992. Pharmacological characterization of endothelin-stimulated phosphoinositide breakdown and cytosolic free  $\text{Ca}^{2+}$  rise in rat  $\text{C}_6$  glioma cells. *J. Neurosci.* 12:1077-1085.
- Lonchampt, M.O., Pinelis, S., Goulin, J., Chabrier, P.E., and Braquet, P. 1991. Proliferation and  $\text{Na}^+/\text{H}^+$  exchange activation by endothelin in vascular smooth muscle cells. *Am. J. Hypertens.* 4:776-779.
- MacManus, G.P., Whitfield, J.F. and Stewart, D.J. 1984. The presence in human tumors of a Mr 11,700 calcium-binding protein similar to rodent oncomodulin. *Cancer Lett.* 21:309-315.
- Maeda, N., Kawasaki, T., Nakade, S., Yokoda, N., Taguchi, T, Kasai, M. and Mikoshiba, K. 1991. Structural and functional characterization of inositol 1,4,5-triphosphate receptor channel from mouse cerebellum. *J. Biol. Chem.* 266:1109-1116.
- Magni, M., Meldolesi, J., and Pandiella, A. 1991. Ionic events induced by epidermal growth factor. *J. Biol. Chem.* 266:6329-6335.
- Mahaut-Smith, M.P. and Mason, M.J. 1991.  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in rat thymic lymphocytes: Activation by Concanavalin A. *J. Physiol.* 439:513-528.
- Maino, W.C., Green, H.M. and Crumpton, M.J. 1974. The role of calcium ions in initiating transformation of lymphocytes. *Nature.* 251:324-327.
- Manalan, A. and Klee, C.B. 1984. Calmodulin. *Adv. Cyclic Nucleotide and Protein Phosphorylation Res.* 18:227-278.
- Matthew, J.B. 1985. Electrostatic effects in proteins. *Ann. Rev. Biophys. Biophys. Chem.* 14:387-417.
- McDonald, J.W. and Johnston, M.V. 1990. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Brain Res. Rev.* 15:41-70.
- McMahon, S.B. and Monroe, J.G. 1992. Role of primary response genes in generating cellular responses to growth factors. *FESB J.* 6:2707-2715.
- Meldolesi, J. and Magni, M. 1991. Lipid metabolites and growth factor action. *Trends Pharmacol. Sci.* 12:362-364.
- Mendoza, S.A. 1988. The role of ion transport in the regulation of cell proliferation. *Pediatr. Nephrol.* 2:118-123.

- Merritt, J.E., Armstrong, W.P., Benham, C.D., Hallam, T.J., Jacob, R., Jaxa-Chamiec, A., Leigh, B.K., McCarthy, S.A., Moores, K.E. and Rink, T.J. 1990. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.* 271:515-522.
- Metcalfe, J.C., Moore, J.P., Smith, G.A., and Hesketh, T.R. 1986. Calcium and cell proliferation. *Br. Med. Bull.* 42:405-412.
- Michel, M.C., Feth, F. and Rascher, W. 1992. NPY-stimulated  $Ca^{2+}$  mobilization in SK-N-MC cells is enhanced after isoproterenol treatment. *Am. J. Physiol.* 262:E383-E388.
- Michell, R.H. 1992. Inositol lipids in cellular signalling mechanisms. *Trends Biol. Sci.* 17:274-276.
- Moolenaar, W.H., Aerts, R.J., Tertoolen, L.G.J. and de Laat, S.W. 1986. The epidermal growth factor-induced calcium signal in A431 cells. *J Biol. Chem.* 261:279-284.
- Moolenaar, W.H., Mummery, C.L., van der Saag, P.T. and de Laat, S.W. 1981. Rapid ionic events and the initiation of growth in serum-stimulated neuroblastoma cells. *Cell.* 23:789-798.
- Moolenaar, W.H., Tertoolen, L.G.J. and de Laat, S.W. 1984. Growth factors immediately raise cytoplasmic free  $Ca^{2+}$  in human fibroblasts. *J. Biol. Chem.* 259:8066-8069.
- Moolgavkar, S.H., Knudson, A.G. Jr. 1981. Mutation and cancer: a model for human carcinogenesis. *J. Natl. Cancer Inst.* 66:1037-1052.
- Moolgarvkar, S.H., Luebeck, G. and deGunst, M. 1990. Two mutation model for carcinogenesis: relative roles of somatic mutations and cell proliferation in determining risk. In *Scientific Issues in Quantitative Cancer Risk Assessment*, edited by Moolgavkar, S.H., p. 136-152. New York, Birkhaeuser Boston, Inc.
- Morris, A.P., Gallacher, D.V., Irvine, R.F. and Peterson, O.H. 1987. Synergism of inositol triphosphate and tetrakisphosphate in activating  $Ca^{2+}$ -dependent  $K^+$  channels. *Nature.* 33):653-655.
- Mozhayeva, G.N., Naumov, A.P., and Kuryshev, Y.A. 1991. Variety of  $Ca^{2+}$ -permeable channels in human carcinoma A431 cells. *J. Membrane Biol.* 124:113-126.
- Muller, R., Bravo, R., Burckhardt, J. and Curran, T. 1984. Induction of *c-fos* gene and protei by growth factors precedes activation of *c-myc*. *Nature.* 312:716-720.

- Muller, R., Curran, T., Muller, D. and Guilbert, L. 1985. *Nature*. 314:546-548.
- Murphy, P. and Hart, D.A. 1992. Modulation of plasminogen activator and plasminogen activator inhibitor expression in the human U373 glioblastoma/astrocytoma cell line by inflammatory mediators. *Exp. Cell Res.* 198:93-100.
- Murphy, S.N. and Miller, R.J. 1988. A glutamate receptor regulates  $Ca^{2+}$  mobilization in hippocampal neurons. *Proc. Natl. Acad. Sci. USA*. 85:8737-8741.
- Nagamine, Y. and Reich, E. 1985. Gene expression and cAMP. *Proc. Natl. Acad. Sci. USA*. 82:4606-4610.
- Nandi, J., King, R.L., Kaplan, D.S. and Levine, R.A. 1990. Mechanisms of gastric proton pump inhibition by calcium channel antagonists. *J. Pharmacol. Exp. Ther.* 252:1102-1107.
- Nicotera, P., Bellomo, G., and Orrenius, S. 1992. Calcium-mediated mechanisms in chemically induced cell death. *Annu. Rev. Pharmacol. Toxicol.* 32:449-470.
- Nigg, E.A. 1990. Mechanisms of signal transduction to the cell nucleus. *Adv. Cancer Res.* 55:271-310.
- Nishimoto, I., Ghkuni, Y., Ogata, E. and Kojima, I. 1987. Insulin-like growth factor II increases cytoplasmic free calcium in competent Balb/c 3T3 cells treated with epidermal growth factor. *Biochem. Biophys. Res. Commun.* 142:275-286.
- Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*. 308:693-698.
- Nishizuka, Y. 1986. Studies and prospectives of protein kinase C. *Science*. 233:305-312.
- Nordling, C.O. 1953. A new theory on the cancer-inducing mechanism. *Br. J. Cancer*. 7:68-72.
- O'Brien, T.G. and Diamond, L. 1977. Ornithine decarboxylase induction and DNA synthesis in hamster embryo cell cultures treated with tumor-promoting phorbol diesters. *Cancer Res.* 37:3895-3900.
- Ogata, E., Nishimoto, I., Matsunaga, H., Murayama, Y., Okamoto, T and Kojima, I. 1991. Calcium as an intracellular signal for cell proliferation response. *Contrib. Nephrol.* 91:2-6.
- Ohira, K., Vayuvegula, B., Murakami, M., Gollapudi, S., Frohman, E., van der Noort, S., and Gupta, S. 1991. Tetraethylammonium, a  $K^+$  channel blocker, inhibits interferon-

$\gamma$ -induced major histocompatibility class II antigen (Ia) expression and DNA synthesis in rat astrocytes. *J. Neuroimmunol.* 31:43-49.

Olashaw, N.E. and Pledger, W.J. 1988. Cellular mechanisms regulating proliferation. *Adv. Second Mess. Phospho. Res.* 22:139-173.

Olsen, R., Seewald, M., and Powis, G. 1989. Contribution of external and internal  $\text{Ca}^{2+}$  to changes in intracellular free  $\text{Ca}^{2+}$  produced by mitogens in Swiss 3T3 fibroblasts: the role of dihydropyridine sensitive  $\text{Ca}^{2+}$  channels. *Biochem. Biophys. Res. Commun.* 162:448-455.

Orrenius, S., McConkey, D.J., Bellomo, G. and Nicotera, P. 1989. Role of  $\text{Ca}^{2+}$  in toxic cell killing. *Trends Pharmacol. Sci.* 10:281-285.

Palade, P., Dettbarn, C., Volpe, P., Alderson, B. and Otero, A.S. 1989. Direct inhibition of inositol-1,4,5-triphosphate-induced  $\text{Ca}^{2+}$  release from brain microsomes by  $\text{K}^+$  channel blockers. *Mol. Pharmacol.* 36:664-672.

Pancrazio, J.J., Viglione, M.P., Kleiman, R.J., and Kim, Y.I. 1991. Verapamil-induced blockade of voltage-activated  $\text{K}^+$  current in small-cell lung cancer cells. *J. Pharmacol. Exper. Ther.* 257:184-191.

Parsons, P.G., Musk, P., Gross, P.D. and Leah, J. 1983. Effects of calcium depletion on human cells in vitro and the anomalous behavior of the human melanoma cell line MM170. *Cancer Res.* 43:2081-2087.

Paul, D. and Ristow, H.J. 1979. Cell cycle control by  $\text{Ca}^{2+}$  ions in mouse 3T3 cells and in transformed 3T3 cells. *J. Cell. Physiol.* 98:31-39.

Peppelenbosch, M.P., Tertoolen, L.G.J., den Hertog, J., and de Laat, S.W. 1992. Epidermal growth factor activates calcium channels by phospholipase  $\text{A}_2/5$ -lipoxygenase-mediated Leukotriene  $\text{C}_4$  production. *Cell.* 69:295-303.

Peres, A., Racca, C., Zippel, R and Sturani, E. 1990. Cytosolic calcium and membrane conductance in response to platelet-derived growth factor and bradykinin stimulation in single human fibroblasts. *Eur. J. Cell Biol.* 53:290-295.

Pincus, D.W., DiCicco-Bloom, E., and Black, I.B. 1991. Role of voltage-sensitive calcium channels in mitogenic stimulation of neuroblasts. *Brain Res.* 553:211-214.

Pittet, D., Di Virgilio, F., Pozzan, T., Monod, A. and Lew, D.P. 1990. Correlation between plasma membrane potential and second messenger generation in the promyelocytic cell line HL-60. *J. Biol. Chem.* 265:14256-14263.

Pledger, W.J., Stiles, C.D., Antoniades, H.N. and Scher, C.D. 1977. Induction of DNA synthesis in BALB/c-3T3 cells by serum components: Reevaluation of the commitment process. *Proc. Natl. Acad. Sci. USA.* 74:4481-4485.

Ponten, G. 1971. *Spontaneous and Virus-Induced Transformation in Cell Culture.* Berlin, Springer Verlag.

Ponten, J. and Macintyre, E.H. 1968. Long term culture of normal and neoplastic human glia. *Acta Path. Microbiol. Scand.* 74:465-486.

Porter, C.W. and Bergeron, R.J. 1988. Enzyme regulation as an approach to interference with polyamine biosynthesis-an alternative to enzyme inhibition. *Adv. Enz. Regul.* 27:57-79.

Potter, L.T., Ferrendelli, C.A., Hanchett, H.E., Hollifield, M.A. and Lorenzi, M.V. 1989. Tetrahydroaminoacridine and other allosteric antagonists of hippocampal M1 muscarine receptors. *Mol. Pharmacol.* 35:652-660.

Pouyssegur, J. and Seuwen, K. 1992. Transmembrane receptors and intracellular pathways that control cell proliferation. *Annu. Rev. Physiol.* 54:195-210.

Prasad, K.V.S., Greer, W.L., Severini, A.S. and Kaplan, J.G. 1987. Increase in intracellular Na<sup>+</sup>: Transmembrane signal for rejoining of DNA strand breaks in proliferating lymphocytes. *Cancer Res.* 47:5397-5400.

Preston-Martin, S., Pike, M.C., Ross, R.K., Jones, P.A., and Henderson, B.E. 1990. Increased cell division as a cause of human cancer. *Cancer Res.* 50:7415-7421.

Price, M., Lee, S.C. and Deutsch, C. 1989. Charybdotoxin inhibits proliferation and interleukin 2 production in human peripheral blood lymphocytes *Proc. Natl. Acad. Sci. USA.* 86:10171-10175.

Puro, D.G. 1991. A calcium-activated, calcium-permeable ion channel in human retinal glial cells: modulation by basic fibroblast growth factor. *Brain Res.* 548:329-333.

Puro, D.G. and Mano, T. 1991. Modulation of calcium channels in human retinal glial cells by basic fibroblast growth factor: a possible role in retinal pathophysiology. *J. Neurosci.* 11:1873-1880.

Puro, D.G., Roberge, F. and Chan, C.C. 1989. Retinal glial cell proliferation and ion channels: a possible link. *Invest. Ophthalmol.* 30:521-529.

Putney, Jr., J.W. 1990. Receptor-regulated calcium entry. *Pharmacol. Ther.* 48:427-434.



- Radinsky, R. 1991. Growth factors and their receptors in metastasis. *Sem. Cancer Biol.* 2:169-177.
- Ramsdell, J.S. 1991. Voltage-dependent calcium channels regulates GH<sub>4</sub> pituitary cell proliferation at two stages of the cell cycle. *J. Cell. Physiol.* 146:197-206.
- Rane, S.G. 1991. A Ca<sup>2+</sup>-activated K<sup>+</sup> current in ras-transformed fibroblasts is absent from nontransformed cells. *Am. J. Physiol.* 260:C104-C112.
- Raos, N and Kasprzak, K.S. 1989. Allosteric binding of nickel(II) to calmodulin. *Fund Appl Toxicol.* 13:816-822.
- Rasmussen, H. and Rasmussen, J.E. 1990. Calcium as intracellular messenger: From simplicity to complexity. *Curr. Top. Cell. Reg.* 31:1-109.
- Reddy, P.V., Reed, W.C., Deacon, D.H. and Quesenberry, P.J. 1992. Growth factor-dependent proliferative stimulation of hematopoietic cells is associated with the modulation of cytoplasmic and nuclear 68-Kd calmodulin-binding protein. *Blood.* 79:1946-1955.
- Rhee, S.G., Suh, P.G., Ryu, S.H. and Lee, S.Y. 1989. Studies of inositol phospholipid-specific phospholipase C. *Science.* 244:546-550.
- Rink, T.J. 1990. Receptor-mediated calcium entry. *FEBS Lett.* 268:381-385.
- Rixon, R.H. and Whitfield, J.F. 1976. The control of liver regeneration by parathyroid hormone and calcium. *J. Cell. Physiol.* 87:147-156.
- Roe, M.W., Hepler, J.T., Harden, T.K. and Herman, B. 1989. Platelet-derived growth factor and angiotensin II cause increases in cytosolic free calcium by different mechanisms in vascular smooth muscle cells. *J. Cell. Physiol.* 139:100-108.
- Rouzair-Dubois, B and Dubois, J.M. 1990. Tamoxifen blocks both proliferation and voltage-dependent K<sup>+</sup> channels of neuroblastoma cells. *Cell. Signalling.* 2:387-393.
- Rouzair-Dubois, B and Dubois, J.M. 1991. A quantitative analysis of the role of K<sup>+</sup> channels in mitogenesis of neuroblastoma cells. *Cell. Signalling.* 3:333-339.
- Rozengurt, E. 1989. Signal transduction pathways in mitogenesis. *Br. Med. Bull.* 45:515-528.
- Rozengurt, E. 1986. Early signals in the mitogenic response. *Science.* 234:161-166.
- Rozengurt, E. and Mendoza, S.A. 1985. Synergistic signals in mitogenesis: role of ion

fluxes, cyclic nucleotides and protein kinase C in Swiss 3T3 cells. *J. Cell Sci. Suppl* 3:229-242.

Rozengurt, E. and Ober, S.S. 1990. The role of early signaling events in the mitogenic response. *News Physiol. Sci.* 5:21-24.

Rozengurt, E. and Sinnett-Smith, J. 1988. Early signals underlying the induction of the *c-fos* and *c-myc* genes in quiescent fibroblasts: studies with bombesin and other growth factors. *Prog. Neucleic Acid Res. Mol. Biol.* 35:261-295.

Russo, J., Gusterson, B.A., Rogers, A.E., Russo, I.H., Wellings, S.R. and van Zwieten, M. 1990. Biology of disease: comparative study of human and rat mammary tumorigenesis. *Lab. Invest.* 62:244-278.

Sage, S.O., Mahaut-Smith, M.P. and Rink, T.J. 1992. Calcium entry in nonexcitable cells: lessons from human platelets. *News Physiol. Sci.* 7:108-113.

Schanne, F.A.X., Kane, A.B. and Farber, J.L. 1979. Calcium dependence of toxic cell death: A final common pathway. *Science.* 206:700-702.

Schmidt, W.F., Huber, K.R., Ettinger, R.S., and Neuberger, R.W. 1988. Antiproliferative effect of verapamil alone on brain tumor cells *in vitro*. *Cancer Res.* 48:3617-3621.

Schoepp, D.D. and Johnson, B.G. 1988. Selective inhibition of excitatory amino acid-stimulated phosphoinositide hydrolysis in the rat hippocampus by activation of protein kinase C. *Biochem. Pharmacol.* 37:4299-4305.

Schuller, H.M., Orloff, M. and Reznik, G.K. 1991. Antiproliferative effects of the  $Ca^{2+}$ /calmodulin antagonist B859-35 and the  $Ca^{2+}$ /channel blocker verapamil on human lung cancer cell lines. *Carcinogenesis.* 12:2301-2303.

Serratose, J., Pujol, M.J., Bachs, O. and Carafoli, E. 1988. Rearrangement of nuclear calmodulin during proliferative liver cell activation. *Biochem. Biophys. Res. Commun.* 150:1162-1169.

Sethi, T. and Rozengurt, E. 1991. Galanin stimulates  $Ca^{2+}$  mobilization, inositol phosphate accumulation, and clonal growth in small cell lung cancer cells. *Cancer Res.* 51:1674-1679.

Shah, J. and Pant, H.C. 1988. Potassium channel blockers inhibit inositol triphosphate-induced calcium release in the microsomal fractions isolated from the rat brain. *Biochem. J.* 250:617-620.

Sheng, M., McFadden, G. and Greenberg, M.E. 1990. Membrane depolarization and

calcium induce *c-fos* transcription via phosphorylation of transcription factor CREB. *Neuron*. 4:571-582.

Shirakawa, F., Yamashita, U., Oda, S., Chiba, S., Eto, S. and Suzuki, H. 1986. Calcium dependency in the growth of adult T-cell leukemia cells *in vitro*. *Cancer Res*. 46:658-661.

Shultz, P.J. and Raj, L. 1990. Inhibition of human mesangial cell proliferation by calcium channel blockers. *Hypertension*. 15(Suppl I):I-76-I-80.

Shuttleworth, T.J. and Thompson, J.L. 1992. Modulation of inositol (1,4,5)triphosphate-sensitive calcium store content during continuous receptor activation and its effects on calcium entry. *Cell Calcium*. 13:541-551.

Simpson, R.U. and Arnold, A.J. 1986. Calcium antagonizes 1,25-dihydroxy-vitamin D<sub>3</sub> inhibition of breast cancer cell proliferation. *Endocrinology*. 119:2284-2289.

Simpson, R.U. and Taylor, J.M. 1988. Regulation of cancer cell proliferation by calcium antagonists. *Exp. Oncol. (Life Sci. Adv.)*. 7:81-89.

Smith, J.B. 1983. Vanadium ions stimulate DNA synthesis in Swiss mouse 3T3 and 3T6 cells. *Proc. Natl. Acad. Sci. USA*. 80:6162-6166.

Soergel, D.G., Yasumoto, T., Daly, J.W., and Gusovsky, F. 1992. Maitotoxin effects are blocked by SK&F 96365, an inhibitor of receptor-mediated calcium entry. *Mol. Pharmacol*. 41:487-493.

Spengler, B.A., Biedler, J.L., Helson, L. and Freedman, L.S. 1973. Morphology and growth, tumorigenicity and cytogenetics of human neuroblastoma cells established *in vitro*. *In Vitro*. 8:410.

Sternweis, P.C. and Smrcka, A. V. 1992. Regulation of phospholipase C by G proteins. *Trends Biol. Sci*. 17:502-506.

Stiles, C.D. 1985. The biological role of oncogene-insights from platelet-derived growth factor: Rhoads Memorial Award lecture. *Cancer Res*. 45:5215-5218.

Stys, P.K., Waxman, S.G., and Ransom, B.R. 1991. Na<sup>+</sup>-Ca<sup>2+</sup> exchanger mediates Ca<sup>2+</sup> influx during anoxia in mammalian central nervous system white matter. *Ann. Neurol*. 30:375-380.

Subramanyam, C., Honn, S.C., Reed, W.C. and Reddy, G.P.V. 1990. Nuclear localization of 68 kDa calmodulin-binding protein is associated with the onset of DNA replication. *J. Cell. Physiol*. 144:423-428.

- Sutherland, C. and Walsh, M.P. 1989. Activation of protein kinase C by the dihydropyridine calcium channel blocker, felodipine. *Biochem. Pharmacol.* 38:1263-1270.
- Swarup, G., Cohen, C. and Garbers, D.L. 1982. Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate. *Biochem. Biophys. Res. Commun.* 130:1193-1200.
- Swierenga, S.H., Whitfield, J.F. and Morris, H.P. 1978. The reduced extracellular calcium requirement for proliferation by neoplastic hepatocytes. *In Vitro.* 14:527-535.
- Szollosi, J. Feuerstein, B.G., Vereb, G., Pershadsingh, H.A., and Marton, L. 1991. Calcium channels in PDGF-stimulated A172 cells open after intracellular calcium release and are not voltage-dependent. *Cell Calcium.* 12:477-491.
- Tabor, C.W. and Tabor, H. 1984. Polyamines. *Annu. Rev. Biochem.* 53:749-790.
- Takada, K., Amino, N., Tada, H., and Miyai, K. 1990. Relationship between proliferation and cell cycle-dependent  $Ca^{2+}$  influx induced by a combination of thyrotropin and insulin-like growth factor-1 in rat thyroid cells. *J. Clin. Invest.* 86:1548-1555.
- Takuwa, N., Iwamoto, A., Kumada, M., Yamashita, K., and Takuwa, Y. 1991. Role of  $Ca^{2+}$  influx in bombesin-induced mitogenesis in Swiss 3T3 fibroblasts. *J. Biol. Chem.* 266:1403-1409.
- Tatham, P.E.R., O'Flynn, K. and Linch, D.C. 1986. The relationship between mitogen-induced membrane potential change and intracellular free calcium in human T-lymphocytes *Biochim. Biophys. Acta.* 856:202-211.
- Taylor, C.W. 1990. Receptor-regulated  $Ca^{2+}$  entry: secret pathway or secret messenger? *Trends Pharmacol Sci.* 11:269-271.
- Taylor, C.W. and Marshall, C.B. 1992. Calcium and inositol 1,4,5-triphosphate receptors: a complex relationship. *Trends Biol. Sci.* 17:403-407.
- Taylor, J.M. and Simpson, R.U. 1992. Inhibition of cancer cell growth by calcium channel antagonists in athymic mouse. *Cancer Res.* 52:2413-2418.
- Thastrup, O. 1990. Thapsigargin, a tumor promoter, discharges intracellular  $Ca^{2+}$  stores by specific inhibition of the endoplasmic reticulum  $Ca^{2+}$ -ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466-2470.
- Tsien, R.Y., Pozzan, T. and Rink, T.J. 1982. T-cell mitogens cause early changes in

- cytoplasmic free  $\text{Ca}^{2+}$  and membrane potential in lymphocytes. *Nature*. 295:68-71.
- Tucker, R.W., Chang, D.T. and Maede-Coburn, K. 1989. Effects of platelet-derived growth factor and fibroblast growth factor on free intracellular calcium and mitogenesis. *J. Cell. Biochem.* 39:139-151.
- Tveito, G., Hansteen, I.L., Dalen, H. and Haugen, A. 1989. Immortalization of normal human kidney epithelial cells by nickel(II). *Cancer Res.* 49:1829-1835.
- Valdivia, H.H., Valdivia, C., Ma, J. and Coronado, R. 1990. Direct binding of verapamil to the ryanodine receptor channel of sarcoplasmic reticulum. *Biophys. J.* 58:471-481.
- Vallance, S.J., Downes, C.P., Cragoe, E.J. and Whetton, A.D. 1990. Granulocyte-macrophage colony-stimulating factor can stimulate macrophage proliferation via persistent activation of  $\text{Na}^+/\text{H}^+$  antiport. *Biochem. J.* 265:359-364.
- Valone, F.H. 1987. Inhibition of platelet-activating factor binding to human platelets by calcium channel blockers. *Thromb. Res.* 45:427-435.
- Varmus, H. 1988. Retroviruses. *Science*. 240:1427-1443.
- Veigl, M.L., Sedwick, W.D. and Vanaman, T.C. 1982. Calmodulin and  $\text{Ca}^{2+}$  in normal and transformed cells. *Federation Proc.* 41:2283-2288.
- Vicentini, L.M. and Villereal, M.L. 1986. Inositol phosphates turnover, cytosolic  $\text{Ca}^{2+}$  and pH: putative signals for the control of cell growth. *Life Sci.* 38:2269-2276.
- Villereal, M.L. and Byron, K.L. 1992. Calcium signals in growth factor signal transduction. *Rev. Physiol. Biochem. Pharmacol.* 119:67-121.
- Waelbroeck, M.P., Robberecht, P., de Neef, P. and Christophe, J. 1984. Effects of verapamil on the binding properties of rat heart muscarinic receptors: evidence for an allosteric site. *Biochem. Biophys. Res. Commun.* 121:340-345.
- Walsh, M.P., Sutherland, C. and Scott-Woo, G.C. 1988. Effects of felodipine (a dihydropyridine calcium channel blocker) and analogues on calmodulin-dependent enzymes. *Biochem. Pharmacol.* 37:1569-1580.
- Wang S.A., Castle, N.A., and Wang, G.K. 1992. Identification of RBK1 potassium channels in C6 astrocytoma cells. *Glia.* 5:146-153.
- Wegman, E.A., Young, J.A., and Cook, D.I. 1991. A 23-pS  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel in MCF-7 human breast carcinoma cells: an apparent correlation of channel incidence

with the rate of cell proliferation. *Pflugers Arch.* 417:562-570.

Whitfield, J.F. 1990. *Calcium, Cell Cycles and Cancer*. Boca Raton, CRC Press, Inc.

Whitfield, J.F., Durkin, J.P., Franks, D.J., Kleine, L.P., Raptis, L., Rixon, R.H., Sikorska, M. and Walker, P.R. 1987. Calcium, cyclic AMP and protein kinase C-partners in mitogenesis. *Cancer Meta. Rev.* 5:205-250.

Whitney, R.B. and Sutherland, R.M. 1972. Requirement for calcium ions in lymphocyte transformation stimulated by phytohemagglutinin. *J Cell. Physiol.* 80:329-338.

Wigler, M. and Weinstein, I.B. 1976. Tumor promoter induces plasminogen activator. *Nature.* 259:232-233.

Wolf, M., Cuatrecasas, P. and Sahyoun, N. 1985. Interaction of protein kinase C with membranes is regulated by  $Ca^{2+}$ , phorbol esters and ATP. *J. Biol. Chem.* 260:15718-15722.

Yarden, Y. 1988. Growth factor receptor tyrosine kinases. *Annu. Rev. Biochem.* 57:443-478.

Zagari, M., Stephens, M., Earp, H.S. and Herman, B. 1989. Relationship of cytosolic ion fluxes and protein kinase C activation to platelet-derived growth factor induced competence and growth in BALB/c-3T3 cells. *J. Cell. Physiol.* 139:167-174.

Zaidi, M., MacIntyre, I. and Datta, H. 1990. Intracellular calcium in the control of osteoclast function. II. Paradoxical elevation of cytosolic calcium by verapamil. *Biochem. Biophys. Res Commun.* 167:80

Zernig, G. 1990. Widening potential for  $Ca^{2+}$  antagonists: non-L-type  $Ca^{2+}$  channel interaction. *Trends Pharmacol. Sci.* 11:38-44.

Ziel, H.K. and Finkle, W.D. 1975. Increased risk of endometrial carcinoma among users of conjugated estrogens. *N. Engl. J. Med.* 293:1167-1170.

Zimmer, M. and Hofmann, F. 1987. Differentiation of the drug-binding sites of calmodulin. *Eur. J. Biochem.* 164:411-420.

Zschauer, A., van Breemen, C., Buhler, F.R., and Nelson, M.T. 1988. Calcium channels in thrombin-activated human platelet membrane. *Nature.* 334:703-705.

zur Hausen, H. 1989. Papillomaviruses as carcinoma viruses. In *Advances in Viral Oncology*, edited by Klein, G., Vol 8, p.1, New York, Raven Press.

## APPROVAL SHEET

The dissertation submitted by Yong Soo Lee has been read and approved by the following committee:

Dr. Robert D. Wurster, Director  
Professor, Physiology, Loyola

Dr. Mohammed M. Sayeed  
Professor, Physiology, Loyola

Dr. Thomas C. Origitano  
Assistant Professor, Neurosurgery and Physiology, Loyola

Dr. Russell Pieper  
Assistant Professor, Pharmacology, Loyola

Dr. Ronald R. Fiscus  
Associate Professor, Physiology and Biophysics  
University of Kentucky

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

*March 29, 1993*

-----  
Date

*Robert D. Wurster*

-----  
Director's Signature