1993

Roles of Ion Channels in Nervous System Tumor Cell Growth

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ROLES OF ION CHANNELS IN NERVOUS SYSTEM TUMOR CELL GROWTH

by

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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

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 priciples 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

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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.

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4. **LEE, Y.S.**, Weber, M. and Wurster, R.D. 1992. Roles of Ca^{2+} and K^{+} in nervous system tumor cell growth. *Sociey 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).

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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	α-difluoromethylornithine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

EBSS	Earle's basal salt solution
EBV	Epstein-Barr virus
EC_{50}	half-maximal effective concentration
EGF	epidermal growth factor
EGTA	ethylene glycol-bis-(aminoethylether)N,N,N',N'-tetraacetic acid
E_m	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_3	inositol 1,4,5-triphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate

KRB	Krebs-Ringer buffer
MDR	multidrug resistance
MEM	Eagle's mininum essential medium
μl	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_2	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^{2+} entry
RNA	ribonucleic acid
RTK	receptor tyrosine kinase

SEM	standard error of the mean
SH	*src* 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 Unites 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 Ca^{2+} and 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 Ca^{2+} and 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 clarifing the mechanism of action of these drugs, their cytotoxicity was assayed by the trypan blue exclusion method.

Intracellular Ca^{2+} is known to modulate a variety of cellular functions including cell proliferation. The effects of growth-promoting agents on intracellular Ca^{2+} concentrations were studied to ascertain the role of intracellular Ca^{2+} in the proliferative signalling mechanisms in the tumor cell lines. Moreover, since Ca^{2+} and K^+ channel activities may regulate directly or indirectly intracellular Ca^{2+}, the effects of these ion channel modulators on the basal and agonist-induced elevations in intracellular Ca^{2+} concentrations were investigated in order to elucidate the mechanism of action of these drugs in the tumor cell growth. Intracellular 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 Ca^{2+} concentration in a dose-dependent manner and that these increases were due to release of Ca^{2+} from intracellular stores. Further, the results illustrate that Ca^{2+} and K^{+} channel modulators inhibited tumor cell growth in a dose-related fashion and reduced agonist-induced increased intracellular Ca^{2+} concentrations. These drugs did not induce significant alterations in basal intracellular Ca^{2+} concentration. The results suggest that an increase in intracellular 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 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 Ca^{2+} mobilization from intracellular stores without Ca^{2+} influx in tumor cell proliferation supports the notion of the existence of a diversity of cellular responses that are mediated by intracellular 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. Neverthless, 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 cowokers (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 (Rozengurt, 1989; Rozengurt, 1986; Rozengurt 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 (Rozengurt 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 (Reviwed 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 process 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 Na^+/H^+ antiport and increases in intracellular cyclic AMP levels, and the degradation of phosphoinositides to produce inositol triphosphates (IP_3) and diacylglycerol (DAG). These degradation products, in turn, affect the release of 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-γ1 (PLC-γ1) 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-γ1, 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).

Phophatidylinositol 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, PIP_2) through the activation of PLC.

Four distinct families of PLC are known, classified as α, β, γ and δ 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 Ca^{2+} stores sensitive to IP_3 via blockade of the store-specific 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 (Rozengurt and Sinnett-Smith, 1983)). Finally, microinjection of

either PLC (β or γ1) or antibodies against PLC-γ1 in NIH 3T3 cells induced bursts of cell division or blocked cell growth, respectively (Meldolesi and Magni, 1991).

IP_3 and Calcium

IP_3 induces the release of $Ca^{2+}{}_i$ from the Ca^{2+} storing pool, i.e., the endoplasmic reticulum (Berridge, 1987). Addition of PDGF to cells induces a rapid and transient rise in $Ca^{2+}{}_i$ (Moolenaar *et al.*, 1986; Villereal and Byron, 1992). This response does not require external Ca^{2+} and, thus, presumably is mediated by IP_3 which is also increased in PDGF-treated cells. Elevations in cytosolic Ca^{2+} 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^{2+} from extracellular medium abolished this effect, suggesting that EGF increases cellular Ca^{2+} by enhancing Ca^{2+} uptake. Thus, PDGF and EGF may modulate $Ca^{2+}{}_i$ levels via different mechanisms.

An optimal level of extracellular Ca^{2+} is required for the proliferation of cells *in vivo* (Rixon and Whitfield, 1976) and *in vitro* (Boynton *et al.*, 1984). The level of Ca^{2+} appears to vary depending on the origin of the cells. The extracellular Ca^{2+} is required at two brief and distinct points through the cell cycle (Boynton, 1988). The first Ca^{2+}-dependent state occurs immediately after stimulation of quiescent cells with serum and is referred to as G0-G1 transition, while the second Ca^{2+}-dependent state is late in the prereplicative period immediately before DNA synthesis, and is referred to as the G1-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 Ca^{2+}-binding protein which upon binding Ca^{2+} undergoes a conformational change that enables the protein to activate numerous enzymes (Carafoli, 1985). The second is cyclic AMP which, althrough not directly affected by 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 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 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 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 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 (Rozengurt, 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^+-H^+ Antiport and Cytoplasmic Alkalization

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

The addition of mitogens to quiescent cells results in stimulation of the Na^+-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 Na^+-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 Na^+-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 Na^+-H^+ antiport by external stimuli is mediated by PKC. First, biologically active phorbol esters like TPA stimulate Na^+-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 rasing intracellular pH (Nishizuka, 1984). Furthermore, trifluoperazine, an inhibitor of PKC *in vitro*, can block the activation of Na^+-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 transcriptonal 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 α 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 earlist 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 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). Neverthless, 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 α-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 constutively 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). 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 $[Ca^{2+}]_i$ ($\approx 10^{-7}$ M) is maintained by a complex interaction of several biochemical precesses. These include the regulation of plasma membrane Ca^{2+} channel opening, the binding of Ca^{2+} to membrane surfaces and Ca^{2+}-binding proteins, the extrusion of 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 Ca^{2+} from these intracellular compartments. In different cell types the $Ca^{2+}{}_i$ 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 Ca^{2+} concentration is 10^4 times higher outside the cell than in the cytosol, and since Ca^{2+} would move down an electrical gradient, the "opening" of the plasma membrane Ca^{2+} channels, even transiently, is a very effective mechanism for elevating intracellular Ca^{2+} concentration, as well as for depolarizing the cell. Depending on the

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

Excitable cells have voltage-sensitive 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, Ca^{2+} channels appear to be directly associated with membrane receptor proteins. The best example of a ligand-sensitive 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 Ca^{2+} as well as Na^+ and 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 Ca^{2+} from intracellular stores. Berridge and coworkers first described a new pool of phosphatidylinositol (i.e. PIP_2), which, when acted on by PLC, could release IP_3 and DAG (Berridge and Irvine, 1984). This work suggested that IP_3 could serve as a type of second messenger. Subsequent studies in numorous other cell types have confirmed that the release of IP_3 leads to the mobilization of $Ca^{2+}{}_i$ 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. $(1,3,4,5)IP_4$, is known to be produced when $(1,4,5)IP_3$ was phosphorylated by a $(1,4,5)IP_3$ 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 envelop 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 (DeLiscle *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 existance 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 ^{3}H-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 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 μM in various mammalian tissues, making it an important $Ca^{2+}{}_i$ buffer. Calmodulin has four binding sites for Ca^{2+} (two of high affinity and two of lower affinity) and two low-affinity metal-binding sites. The binding of each Ca^{2+} ion leads to stepwise conformational changes (the largest being with the binding of the third 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 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) 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 Ca^{2+} response (Villereal and Palfrey, 1989).

Role of Calcium in Normal Cell Proliferation

Increased free 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 Ca^{2+} in growth factors action comes from the numorous findings: PDGF caused a transient rapid increased 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 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 Ca^{2+} concentration in cell proliferation has been suggested from numorous studies which have used pharmacological agents to block growth factor-induced increased Ca^{2+}_i or used low amounts of 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 Ca^{2+}_i appears to result from the influx of 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, 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 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 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 Ca^{2+}_i. The critical time period for 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 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 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 (Serratose *et al.*, 1988). Specific CaM-binding proteins are found to be associated with DNA polymerase-α 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 Ca^{2+} requirement for the initiation of a later phase of prereplicative (G1) development (Balk *et al.*, 1973; Whitfield, 1990).

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

This limb of the 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 of Ca^{2+}_i through the interaction of IP_3 with Ca^{2+}-storage sites; (v) the quantity or structure of calmodulin resulting in alterations in its sensitivity to 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-γ (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 $Ca^{2+}{}_i$ due to altered responsiveness to IP_3 have not been reported. However, differences in the intracellular concentration of Ca^{2+} between normal and malignant cells have been observed. For example, an increased free $Ca^{2+}{}_i$ concentration was found in malignant compared with normal cells (Viegl *et al.*, 1982). Hickie and Kalant found a direct correlationship between the rate of growth of Morris hepatomas and their intracellular 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^+ (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^+ (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-β-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 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. Lous, 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_2O, 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_2O, 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-thereonine, 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_2O, 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. Lous, MO) was used. The components are the same as those of MEM without amino acids and vitamines. MEM or EBSS powders were dissolved in the sterilized water by gentle stirring. Supplements (i.e. 110 mg/L of sodium pyruvate (Sigma, St. Lous, MO) and 2.2 g/L of sodium bicarbonate (Sigma, St. Lous, 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^2 tissue culture flask. Cells were grown at 37°C in a humidified incubator under 5% CO_2/95% 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 μg/ml of streptomycin (Gibco BRL, Grand Island, NY) and 1 mM sodium pyruvate.

c) Culture Maintainance

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^2 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 $2x10^5$ 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. Ca^{2+} channel modulators such as verapamil, nifedipine, diltiazem and Bay K-8644 were used and purchased from ICN Chemicals (Cleveland, OH). As 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). Tetradotoxin, a Na^+ channel antagonist, and veratridine, a Na^+ ionophore, were also used and obtained from Sigma. Cobalt chloride, nickel chloride, ethylene glycol-bis (β-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 μl. Water-insoluble drugs were solubilized in ethanol, and the volume of these drug solutions was limited to 10 μl 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 μl 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 neddle 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^3 or 10^{-4} ml. The subsequent cell concentration per 1 ml (and the total number of cells) could be determined using the following calculations:

Cells per ml = the average count per square x 10^4

Total Cells = cells per ml x 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:

% of Control = [total cells (drug-treated)/total cells (control)] x 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-containg 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 throughly 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:

% Cell Death = [total dead cells (stained)/total cells (stained + unstained)] x 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 ± 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. 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 μg of Fura-2-AM (M.W. 1000) and vortexed for 15 minutes before making a 200 μM Fura-2-AM stock solution. This stock solution was aliquoted out to 50 μl 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 KH_2PO_4, 1.2mM $MgSO_4$, 5mM $NaHCO_3$, 1.3mM $CaCl_2$ (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. Ca^{2+}-free KRB was made by just omitting $CaCl_2$ 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 trysinization (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 $5x10^6$ 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 2μM. 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 (Ca^{2+}-containing or 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, $[Ca^{2+}]_i$

Resuspended cells were transferred to a quartz cuvette at a volume of 500 μl 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 Ca^{2+} concentrations. In order to see the effects of various drugs on basal, free intracellular 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 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 μl. All experiments were done at 37°C. Autofluorescence of the cells was almost negligable and corrected when needed. Fluorescence emission (510 nm) was monitored with excitation wavelenghs cycling between 340 and 380 nm. At the end of an experiment, fluorescence maximum and minimum values at each excitation wavelengh 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 Ca^{2+} concentrations using the formula and Fura-2 Ca^{2+} binding constant (224 nM) descibed 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 Ca^{2+} concentration that are essentially independent of the actual amount of dye in the cell. Ratioing considerably reduces problems associated with measuring 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 Ca^{2+} concentrations compared to those of basal levels. The quantatative data of the drug effects on the basal, free cytosolic Ca^{2+} concentrations were shown by % changes compared to control condition and determined using the following calculations:

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

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

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

where $\Delta[Ca^{2+}]_{i,d+a}$ and $\Delta[Ca^{2+}]_{i,a}$ represent agonist-induced increased intracellular 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 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 Ca^{2+} ions seems also to be involved in these processes (Rasmussen and Means, 1988). The intracellular 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 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 Ca^{2+} levels in many tumor cells (Veigl *et al.*, 1982). The high Ca^{2+} level seems to be due to either the excessive influx of extracellular Ca^{2+} or the ability of neoplastic mitochondria to retain high concentrations of Ca^{2+} (Metcalfe *et al.*, 1980). Thus, prolonged, abnormally high levels of intracellular Ca^{2+} may lead to increased activation of the 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 Ca^{2+}

concentration in medium (Swierenga *et al.*, 1978). The presence of 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 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 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 Ca^{2+}-dependent proliferation, while the growth of the L1210 leukemia cell line is inhibited by an increased extracellular Ca^{2+} concentration (Simpson and Arnold, 1986; Simpson and Taylor, 1988). Others have shown that removal of 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 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 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 Ca^{2+} in cellular proliferation stimulated many researchers to test the effect of drugs which reduce the transmembrane Ca^{2+} influx on cell growth. Using typical 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 messangeal 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). 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, Ca^{2+} channel blockers have shown to bind to other sites other than Ca^{2+} channels and produce non-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 Ca^{2+} channels as well as other effectors and are basically unknown.

In addition to the effect on tumor cell growth, 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 Ca^{2+} channel, possess a similar repeated transmembrane motif (Gottesman and Pastan, 1989). Although the Ca^{2+} regulatory effects of the 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 Ca^{2+} channel antagonists on both tumor cell growth and growth factor-induced increased intracellular 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 Ca^{2+}-related mechanisms responsible for the regulation of the tumor cell growth by these drugs. The data demonstrate that the increased intracellular Ca^{2+} concentration is an important signalling mechanism for growth factor-induced cell proliferation and that the most of the increased Ca^{2+} is from intracellular Ca^{2+} store release. Furthermore, 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 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 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.

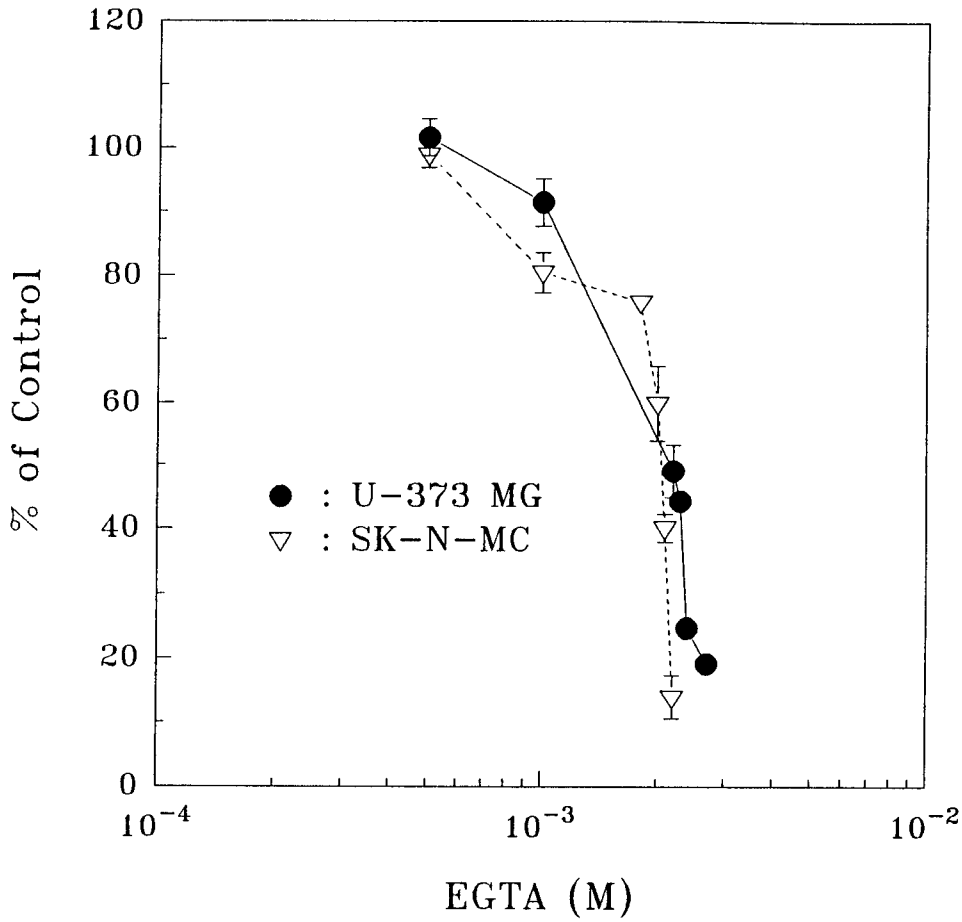

Fig. 4.1. Effect of EGTA on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of EGTA in the culture medium. The 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 Ca^{2+} may occur and that EGTA will reduce the transmembrane concentration gradient for Ca^{2+} ion by lowering available extracellular Ca^{2+}. Thus, the inhibition of Ca^{2+} influx ultimately leads to growth inhibition. However, since Ca^{2+} mobilization from internal stores without 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 Ca^{2+} response. If extracellular Ca^{2+} is lowered, then the cell will be gradually depleted of Ca^{2+}, i.e. intracellular stores will be reduced (Jin *et al.*, 1992; Murphy and Miller, 1988). Thus, transmembrane Ca^{2+} concentration gradient is important for maintaining Ca^{2+} stores involving Ca^{2+} mobilization in response to agonist stimulation. Therefore, the EGTA action on tumor cell growth may be due to the reduction of available Ca^{2+} of internal stores. Alternatively, long term exposure of EGTA may enter into the cells and thus, chelate intracellular Ca^{2+}, resulting in blunting growth factor-induced Ca^{2+} signalling mechanisms. However, these possibilities are only speculative, and the actual mechanism of EGTA is unknown.

The increased extracellular 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 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

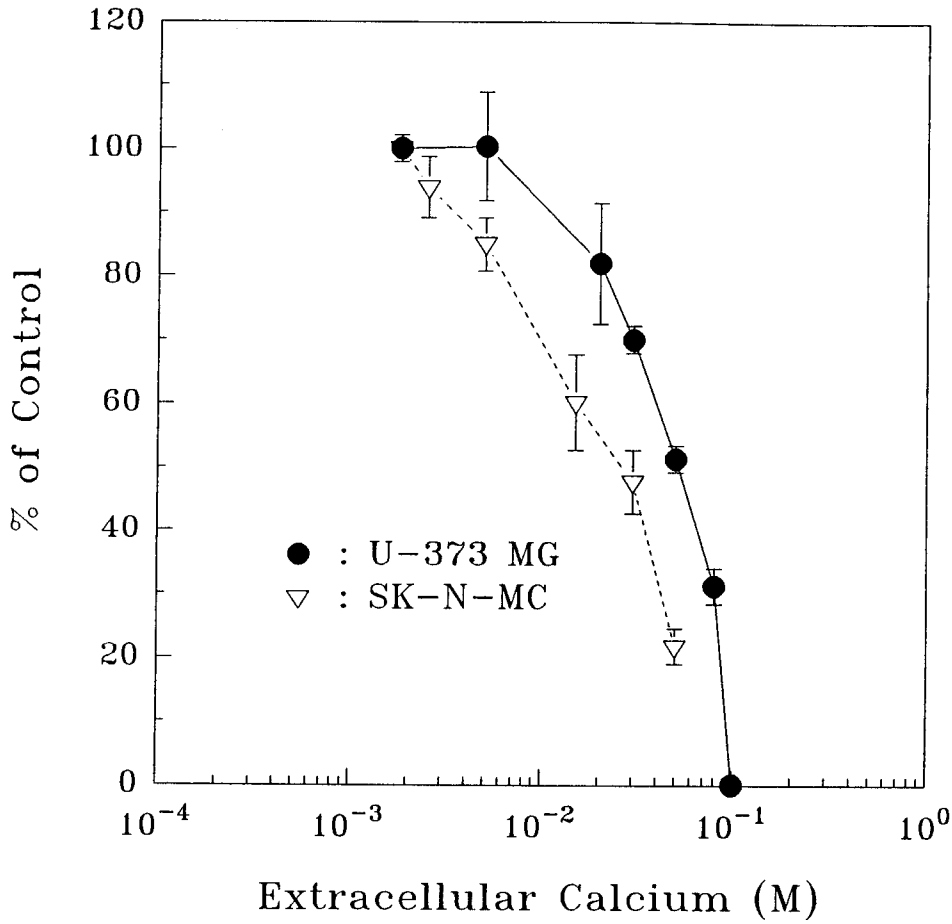

Fig. 4.2. Effect of high extracellular Ca^{2+} on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of Ca^{2+} in the culture medium. The cell number was counted 2 days after Ca^{2+} treatment. The results were expressed as a percent change of the number of the cells obtained in the culture medium containing normal 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 Ca^{2+}-stimulated catabolic enzymes, such as phospholipases, proteases and endonucleases. However, the effects of high extracellular Ca^{2+} concentration on the level of cytosolic Ca^{2+} has not been well described. If during cell proliferation Ca^{2+} influx occurs, then high extracellular Ca^{2+} concentration will increase the transmembrane concentration gradient for Ca^{2+} ions and, thus, possibly lead to a sustained increased intracellular Ca^{2+} concentration. However, the actual Ca^{2+} entry pathways are undefined, and how the intracellular Ca^{2+} regulatory mechanisms are disturbed is unknown.

Calcium Channel Antagonist-Induced Inhibition of Tumor Cell Growth

Structurally different Ca^{2+} channel antagonists were used in order to see the possible involvement of Ca^{2+} channels in tumor cell growth. The culture medium and 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 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 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 $\rangle$ nifedipine $\rangle$ diltiazem in both cell lines. However, the concentrations of half-maximum effect

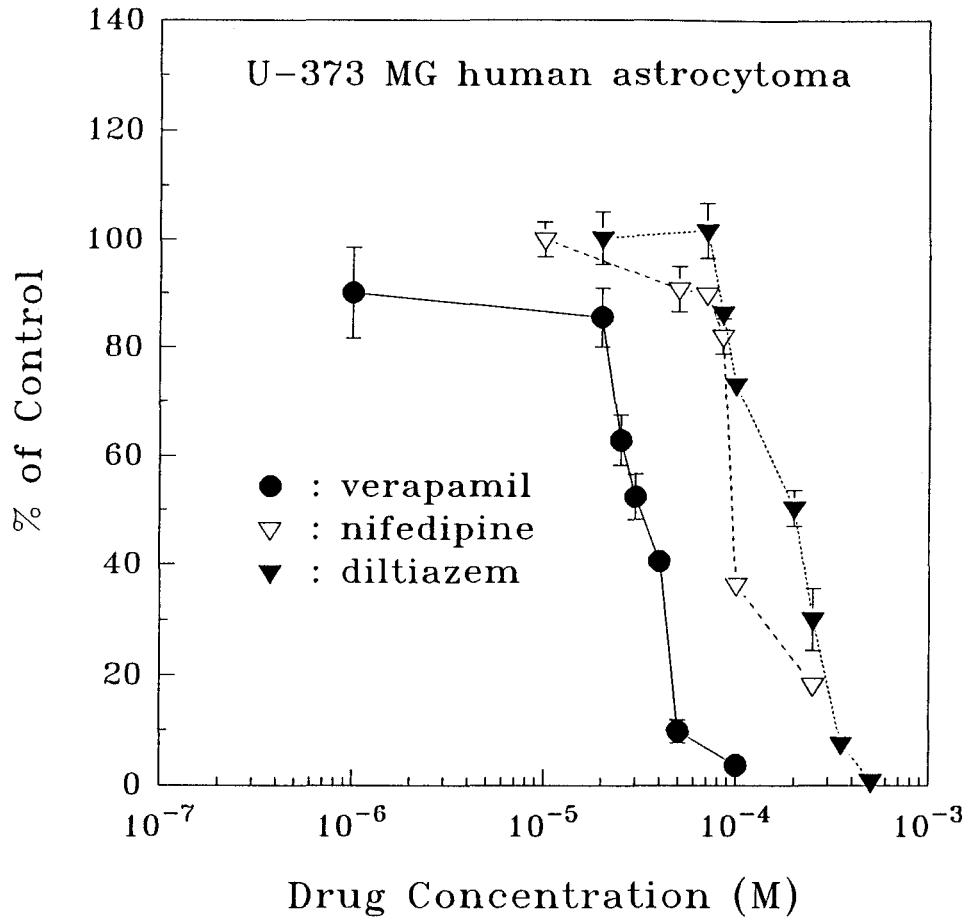

Fig. 4.3. Effect of Ca^{2+} channel antagonists on the growth of U-373 MG human astrocytoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of verapamil (closed circle), nifedipine (open inverted triangle) or diltiazem (closed 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.

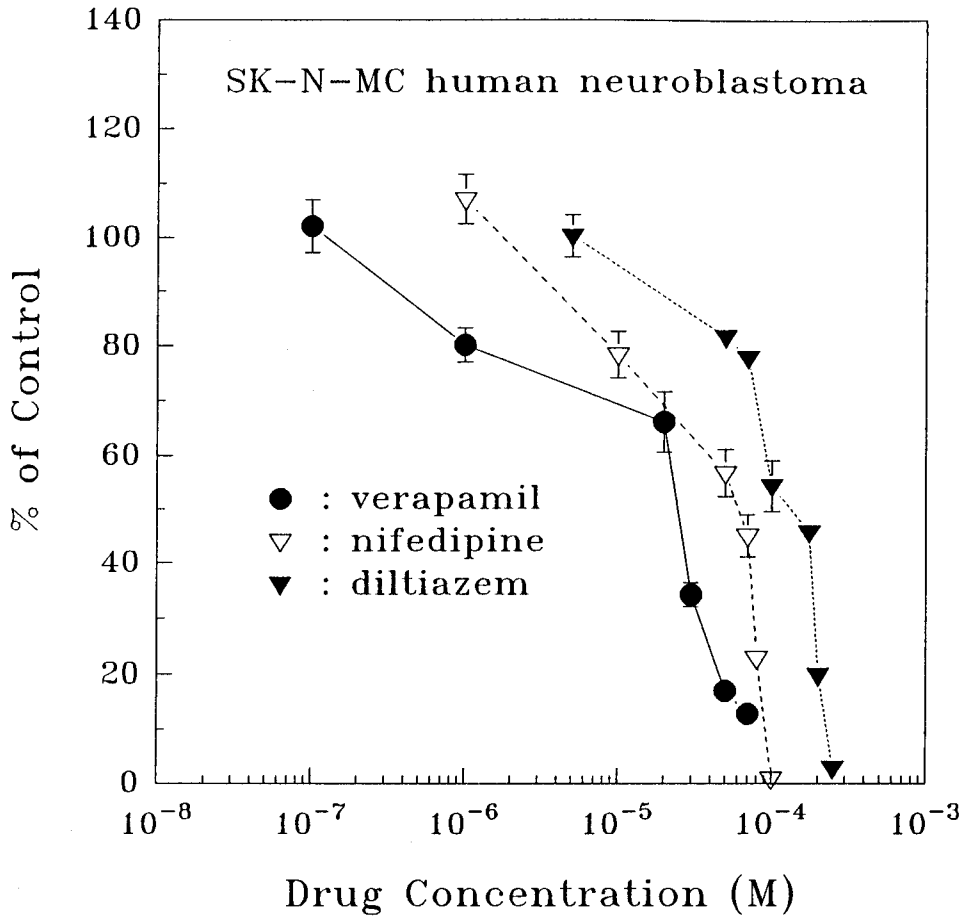

Fig. 4.4. Effect of Ca^{2+} channel antagonists on the growth of SK-N-MC human neuroblastoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of verapamil (closed circle), nifedipine (open inverted triangle) or diltiazem (closed 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 μM). Thus, this 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 Ca^{2+} channel antagonists, e.g. Co^{2+} and Ni^{2+}, also showed the inhibition of tumor cell growth in a dose-dependent manner illustrated in Figs. 4.5 and 4.6. Co^{2+} was more effective than Ni^{2+} in both cells. Since cytotoxicity assay (Figs. 4.9 and 4.10) showed that Ni^{2+} but not Co^{2+} was cytotoxic to both tumor cells, the growth-inhibitory action of Ni^{2+} may be due to also its cell-killing effect. In addition, Ni^{2+} has shown to be a potent carcinogen in experimental animals (Tveito *et al.*, 1989). This carcinogenic effect of Ni^{2+} may be related to its cytotoxicity since carcinogenecity induced by a certain chemical is often paradoxically caused by its cytotoxic effect (Ames and Gold, 1990). However, the actual mechanism of Ni^{2+} in carcinogenecity is not known (Tveito *et al.*, 1989). Interestingly, Ni^{2+} binds to CaM allosterically with 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 tumorigenecity.

The inhibitory effects of various organic and inorganic Ca^{2+} channel antagonists on the tumor cell growth may be related to their interactions with Ca^{2+} channels. If Ca^{2+} influx is important in tumor cell growth, the blockade of the Ca^{2+} entry pathway could give rise to the inhibition of cell growth. However, since the role of Ca^{2+} influx in tumor cell growth is not known and these drugs have multiple actions, the experiments dealing with intracellular 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 Ca^{2+} channel agonist, Bay K-8644 did not induce a significant alteration of the growth of both tumor cells up to 4μM. This result

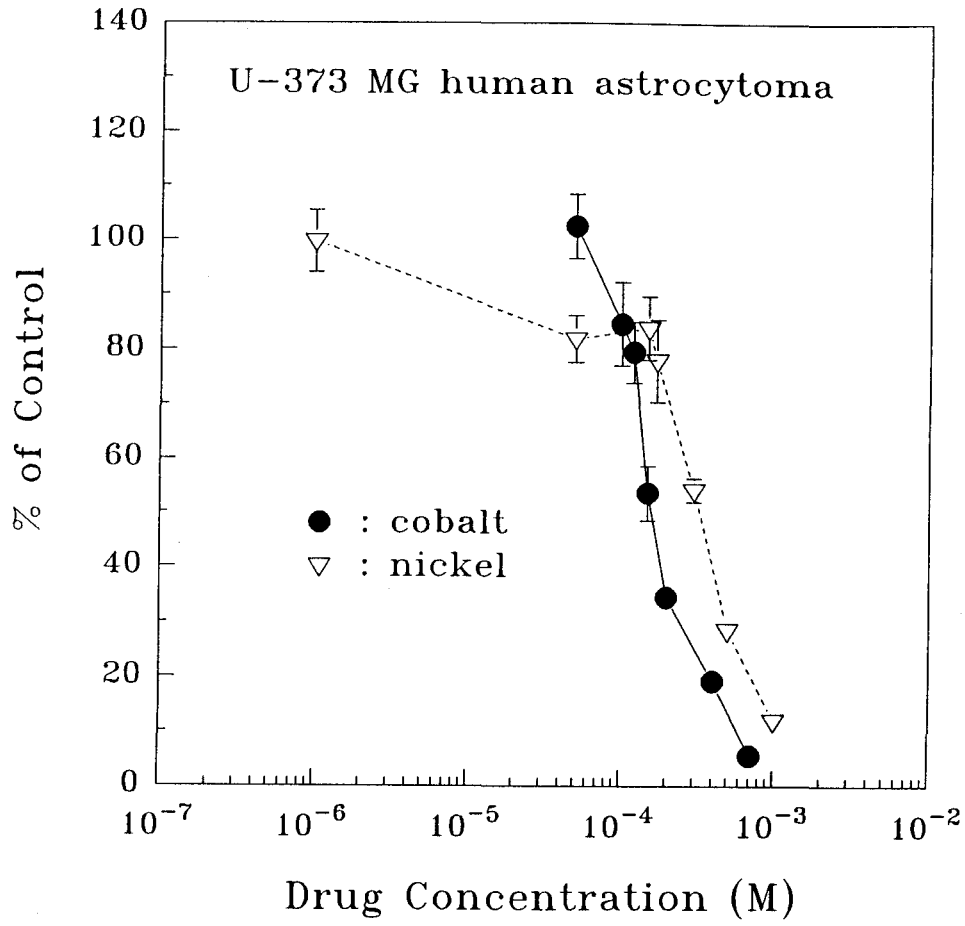

Fig. 4.5. Effect of inorganic Ca^{2+} channel antagonists on the growth of U-373 MG human astrocytoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of cobalt (closed circle) or nickel (open inverted triangle) in the culture 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.

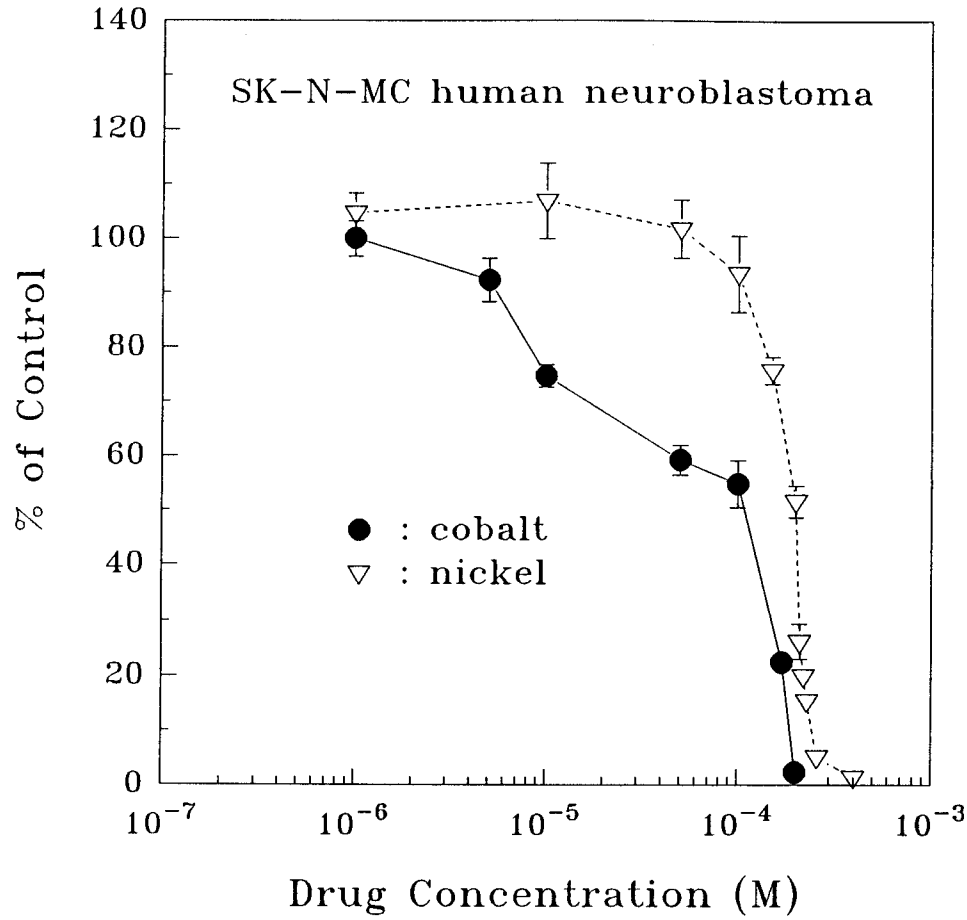

Fig. 4.6. Effect of inorganic Ca^{2+} channel antagonists on the growth of SK-N-MC human neuroblastoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of cobalt (closed circle) or nickel (open inverted triangle) in the culture 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.

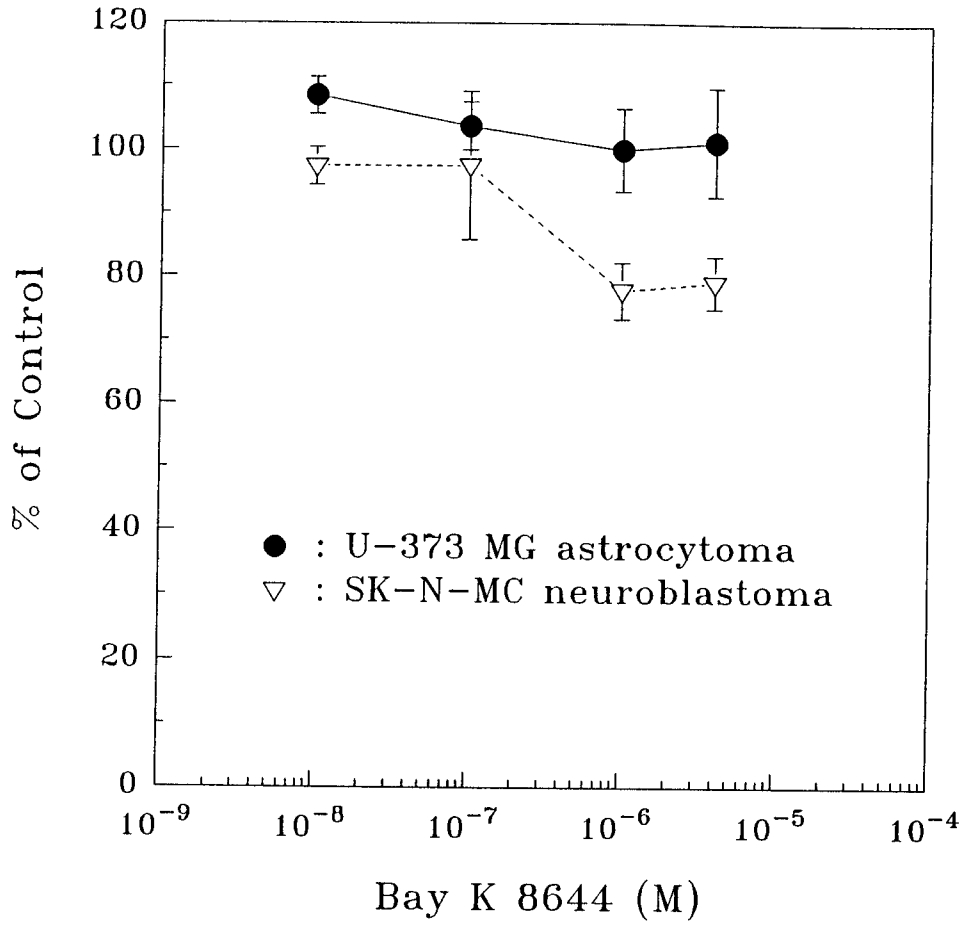

Fig. 4.7. Effect of Bay K-8644 on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of Bay K-8644 in the culture 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 Ca^{2+} channels are not involved in the tumor cell growth. However, this does not imply that Ca^{2+} signals are not important in the process of the cell proliferation. Internal Ca^{2+} release may be enough to trigger intracellular signalling mechanisms which ultimately lead to cell division. Thus, the intracellular Ca^{2+} measurements are required to determine the significance of Ca^{2+} signals as well as elucidating the targets for Ca^{2+} channel antagonists in terms of the regulation of the cell proliferation.

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

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

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

(Merritt *et al.*, 1990). This RMCE blocking action may result from either direct interaction with 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 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 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 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 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 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 Ca^{2+} stores, whereas a secondary, sustained elevation of intracellular Ca^{2+} (plateau) is maintained by Ca^{2+} entry from the extracellular space into the cytosol (Felder *et al.*, 1992; Lambert *et*

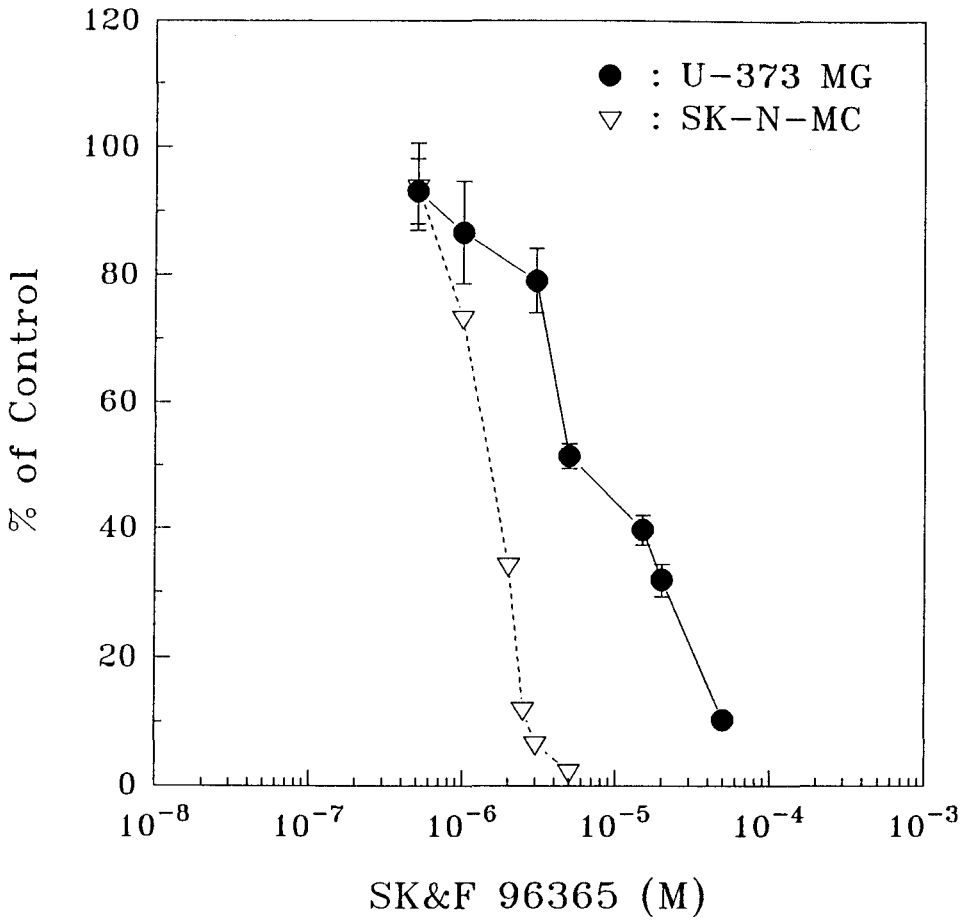

Fig. 4.8. Effect of SK&F 96365 on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of SK&F 96365, a receptor-operated 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.

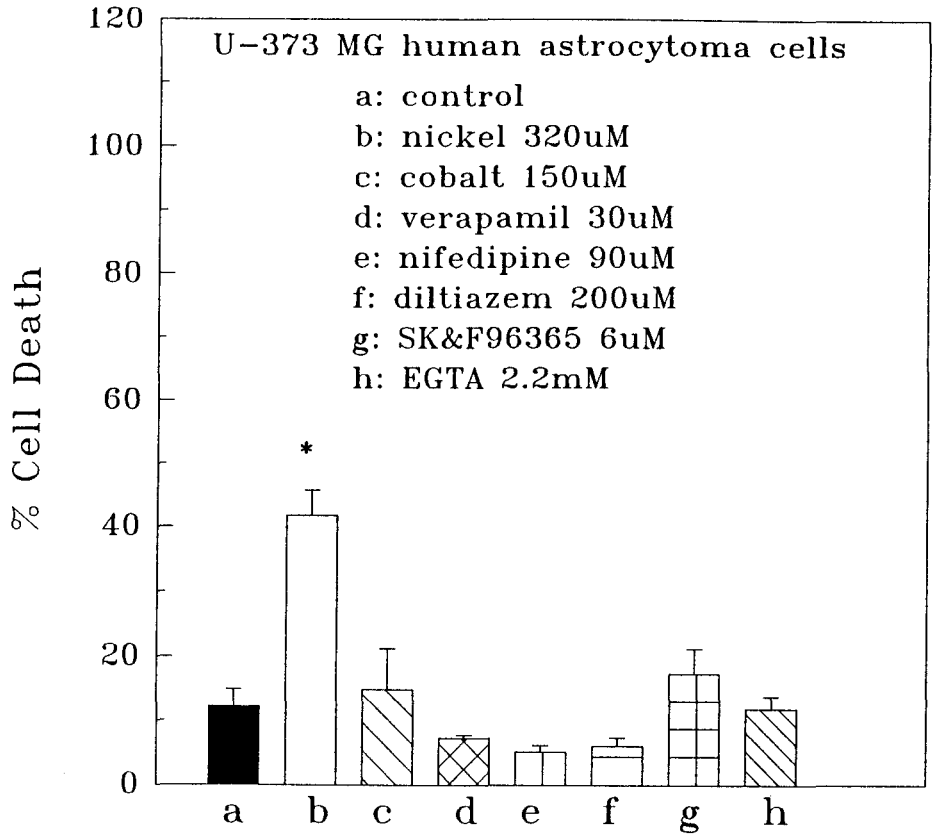

Fig. 4.9. Cytotoxic effect of EGTA or Ca^{2+} channel antagonists on U-373 MG human astrocytoma cell line. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of the drugs in the culture medium. Cell cytotoxicity was assayed by trypan blue extrusion method 2 days after 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).

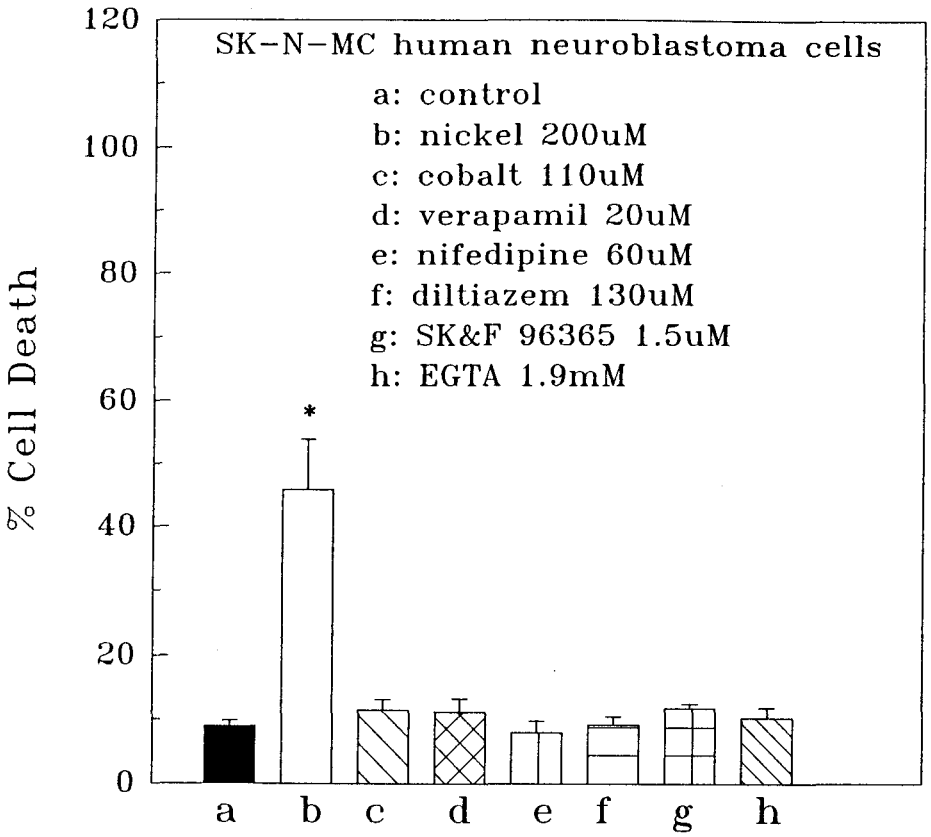

Fig. 4.10. Cytotoxic effect of EGTA or Ca^{2+} channel antagonists on SK-N-MC human neuroblastoma cell line. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of the drugs in the culture medium. Cell cytotoxicity was assayed by trypan blue extrusion method 2 days 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 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 Ca^{2+} concentration which was measured in Fura-2 loaded U-373 MG human astrocytoma cell line. In normal extracellular Ca^{2+} concentration (1.3mM), the response of these cells to Carb was transient without any significant, sustained increased intracellular 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 Ca^{2+} influx. Since this Ca^{2+} entry has been suggested to contribute to the refilling of empty 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 Ca^{2+} to pass from the extracellular space to the stores without entering the cytoplasm (Taylor, 1990). In order to ascertain no contribution of Ca^{2+} influx from the extracellular space to Carb-induced increased intracellular Ca^{2+} concentration, the experiment was done with the resuspending medium containing 0 Ca^{2+}. The results showed no differences of Carb-induced increased intracellular Ca^{2+} concentration between normal Ca^{2+}-containing and Ca^{2+}-free media (Fig. 4.11). Thus, the Carb-induced increased intracellular Ca^{2+} concentration is mainly due to internal store

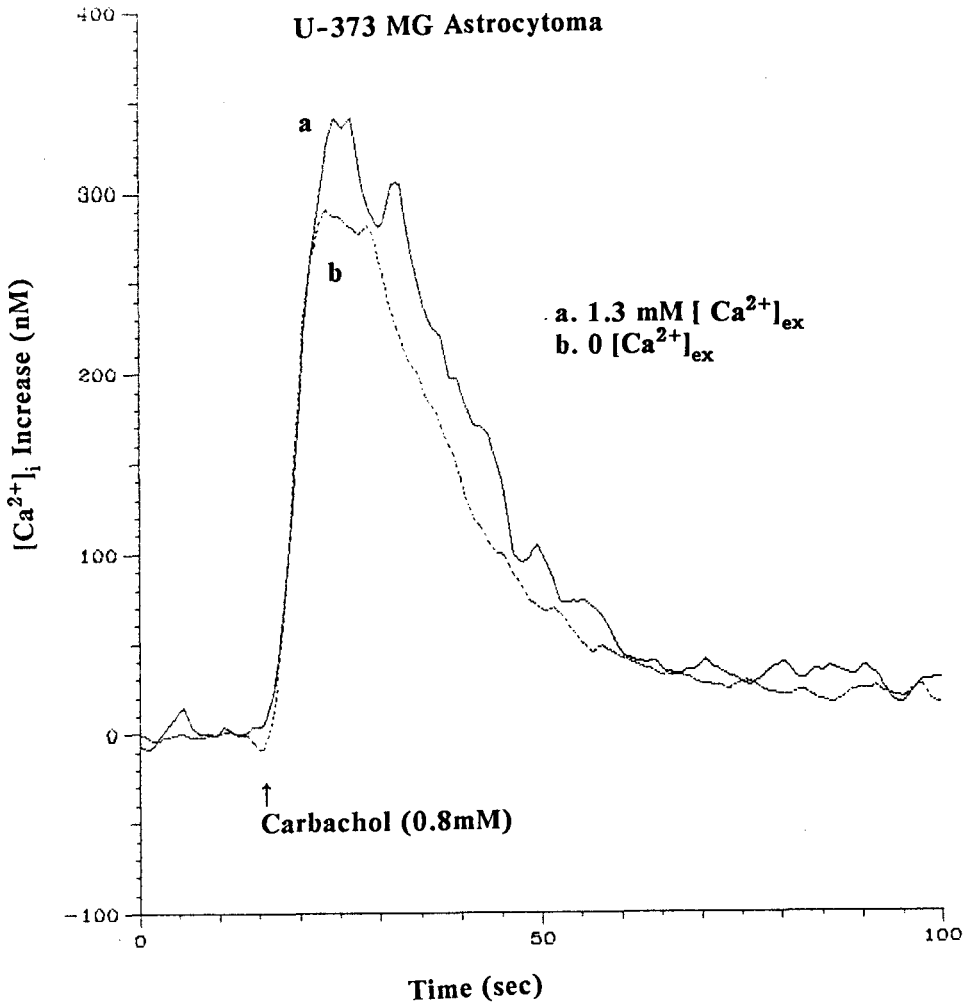

Fig. 4.11. Extracellular Ca^{2+} concentration independency of carbachol (0.8mM)-induced increased intracellular Ca^{2+} concentration in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with $2\mu M$ of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{2+} or 1.3mM 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 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 Ca^{2+} concentration. The results clearly illustrate a dose-dependent increase of intracellular 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 IP_3 which can open Ca^{2+} channels of the endoplasmic reticulum and release 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 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 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

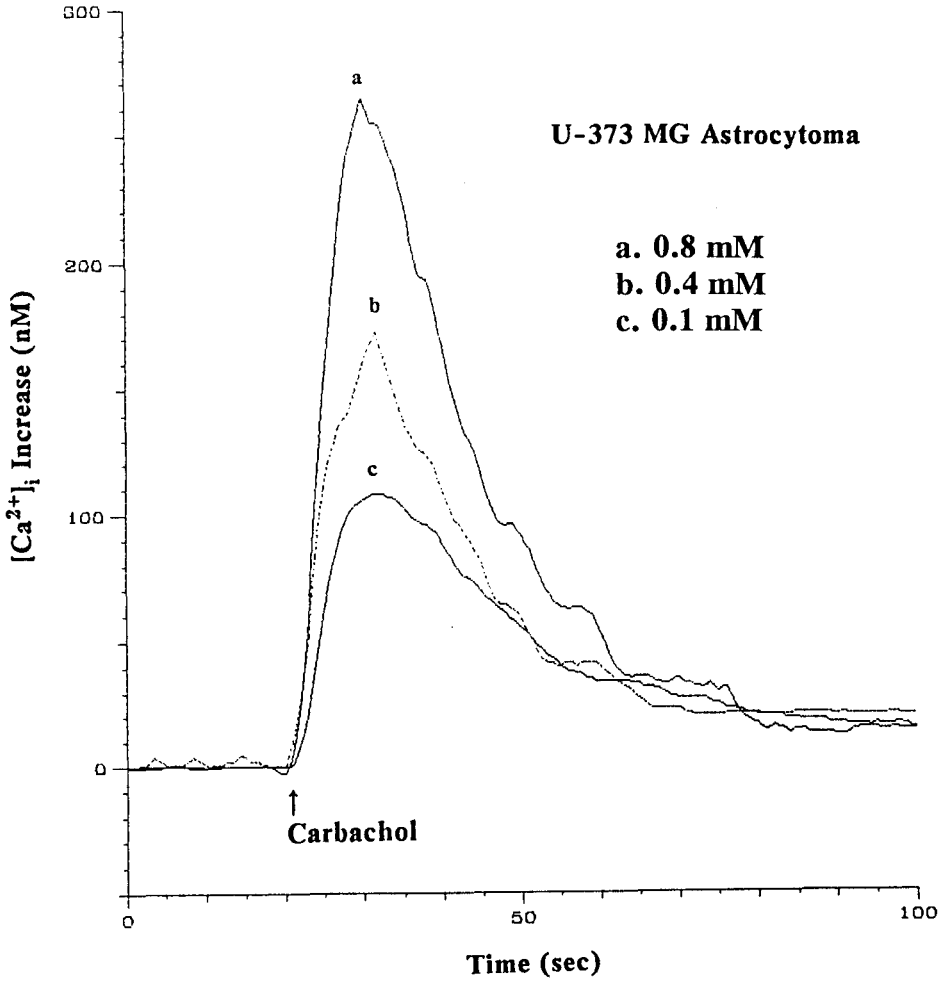

Fig. 4.12. Dose-dependent increase of intracellular Ca^{2+} concentration by carbachol in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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 various concentrations of carbachol.

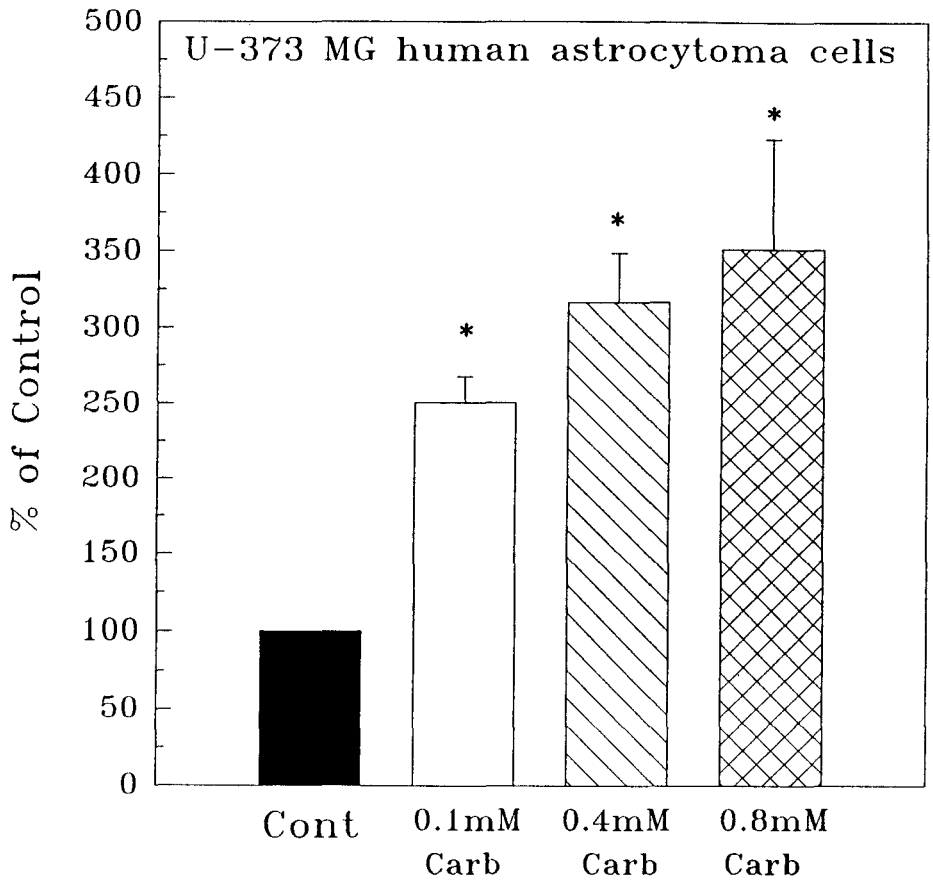

Fig. 4.13. Effect of carbachol on intracellular Ca^{2+} concentration in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{2+} and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of the intracellular 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).

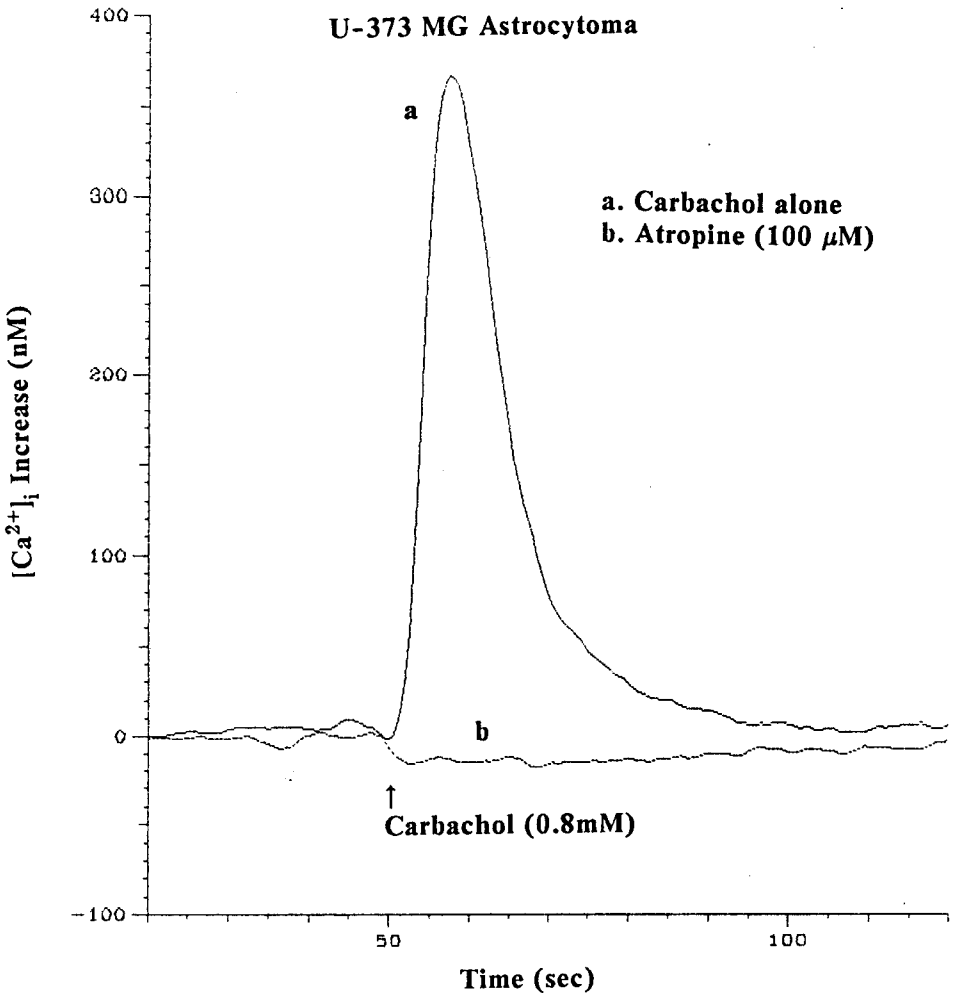

Fig. 4.14. Abolishment of carbachol (0.8mM)-induced increased intracellular Ca^{2+} concentration by the pretreatment of atropine (100μM) in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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 carbachol. Atropine was treated for 3 minutes before starting the experiment.

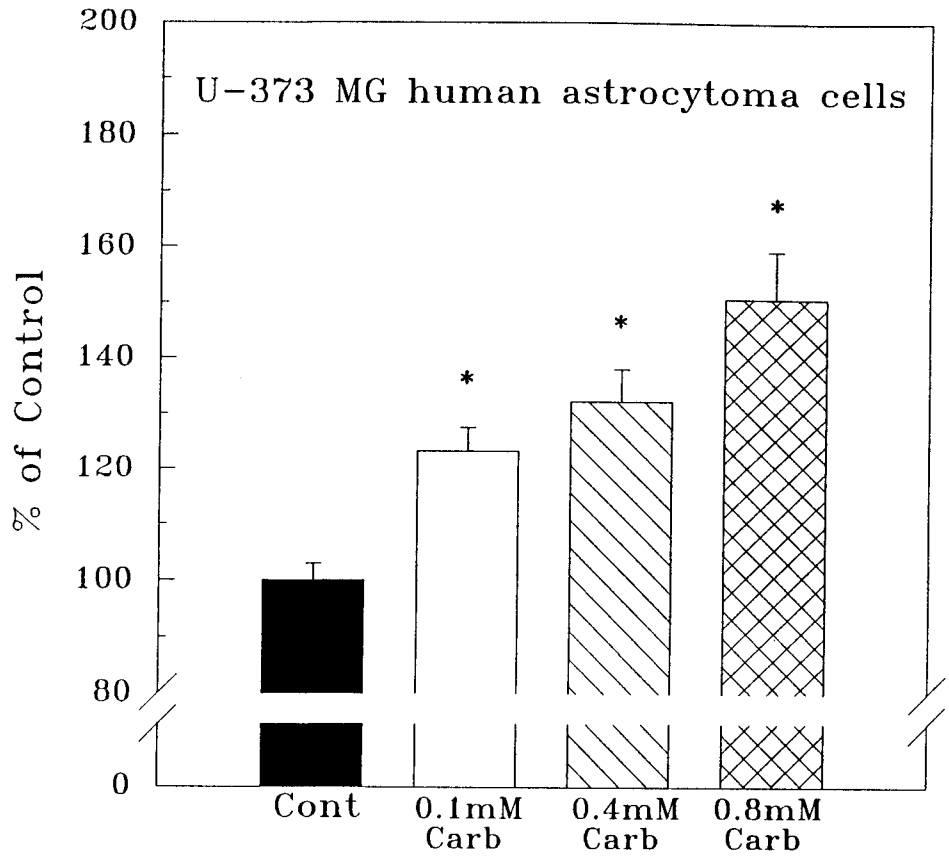

Fig. 4.15. Effect of carbachol on the growth of U-373 MG human astrocytoma cell lines. The cells were grown in MEM supplemented with 0.1% FBS in the absence or in the presence of various concentrations of carbachol in the culture medium. The cell number was counted 2 days after drug treatment. The results 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 Ca^{2+} concentration at a same concentration (Figs. 4.13 and 4.15). These results suggest that the increased intracellular 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 Ca^{2+} signals in the cell proliferation. Therefore, Carb was used as an agonist for the elevation of intracellular Ca^{2+} concentration in further experiments where the effects of 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 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 Ca^{2+} as illustrated in Fig. 4.17. These results suggest that serum component(s) can evoke intracellular Ca^{2+} increase through a receptor-mediated mechanism and that this increased intracellular Ca^{2+} is mainly due to internal Ca^{2+} release. These results further suggest that the increased intracellular 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 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 Ca^{2+}

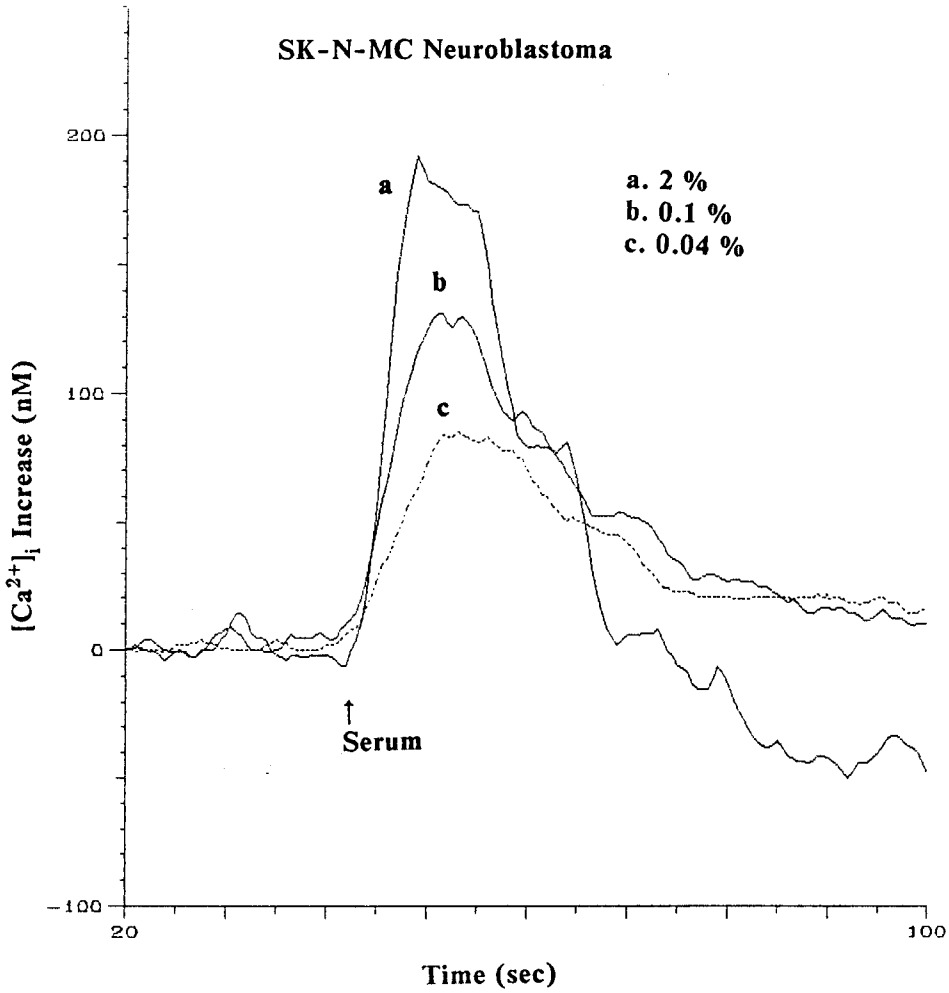

Fig. 4.16. Dose-dependent increase of intracellular Ca^{2+} concentration by serum in SK-N-MC human neuroblastoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{2+} 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 various concentrations of serum.

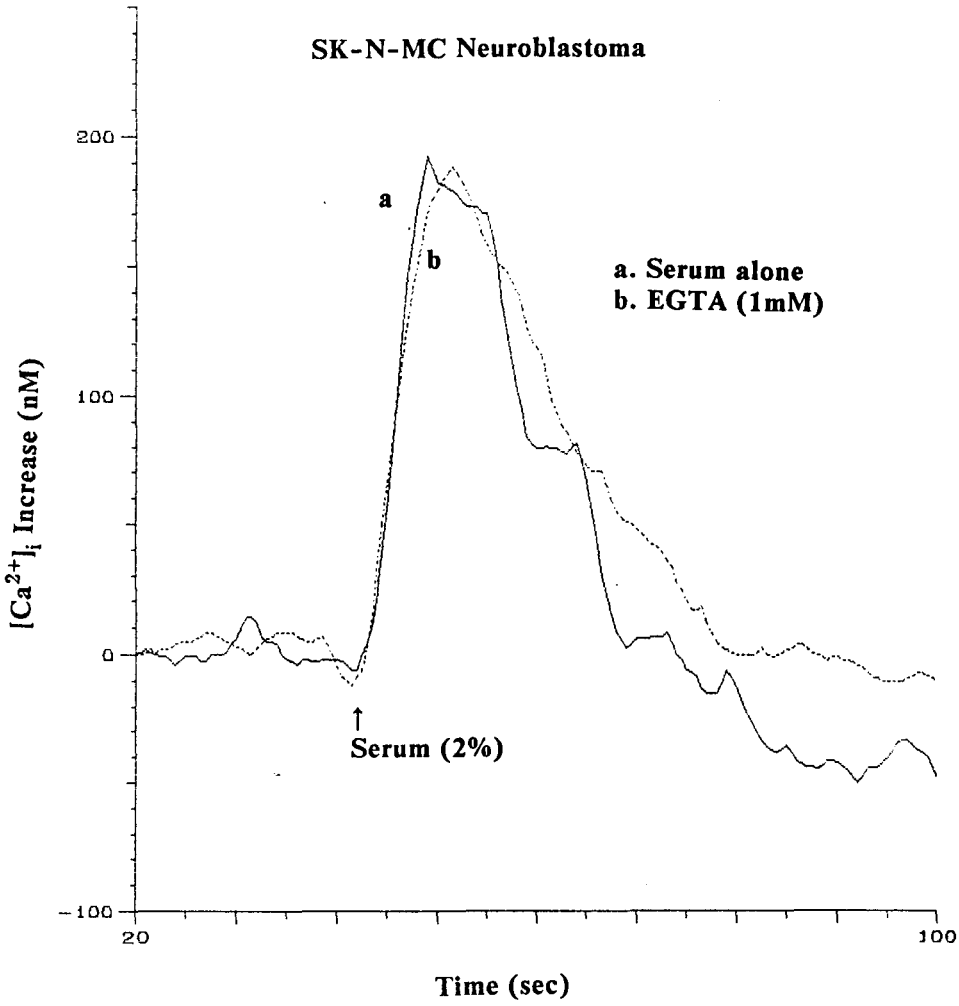

Fig. 4.17. Effect of EGTA (1mM) on serum (2%)-induced increased intracellular Ca^{2+} concentration in SK-N-MC human neuroblastoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 1.3mM Ca^{2+} and transferred to a quartz cuvette for fluorescence measurement. The data represent net increases of intracellular 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 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 Ca^{2+} for further experiments where the effect of various Ca^{2+} channel antagonists on an agonist-induced increased intracellular 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 Ca^{2+} from the internal stores seems to be an important signal transduction mechanism in the proliferation of tumor cells. Since the 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 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 Ca^{2+} channel antagonists on Carb-induced release of Ca^{2+} from the internal stores in Fura-2 loaded U-373 MG human astrocytoma cell line. Since the resuspending buffer solution containing 0 Ca^{2+} was used in these experiments, the Carb-induced increased intracellular Ca^{2+} concentration would result from the internal store release. The concentration of Ca^{2+} channel antagonists was chosen on the basis of their effects on the tumor cell growth. Fig. 4.19 shows the effects of 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 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 Ca^{2+} signals in its inhibitory effect on tumor cell growth.

The effects of Ca^{2+} channel antagonists on a serum-induced release of 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 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

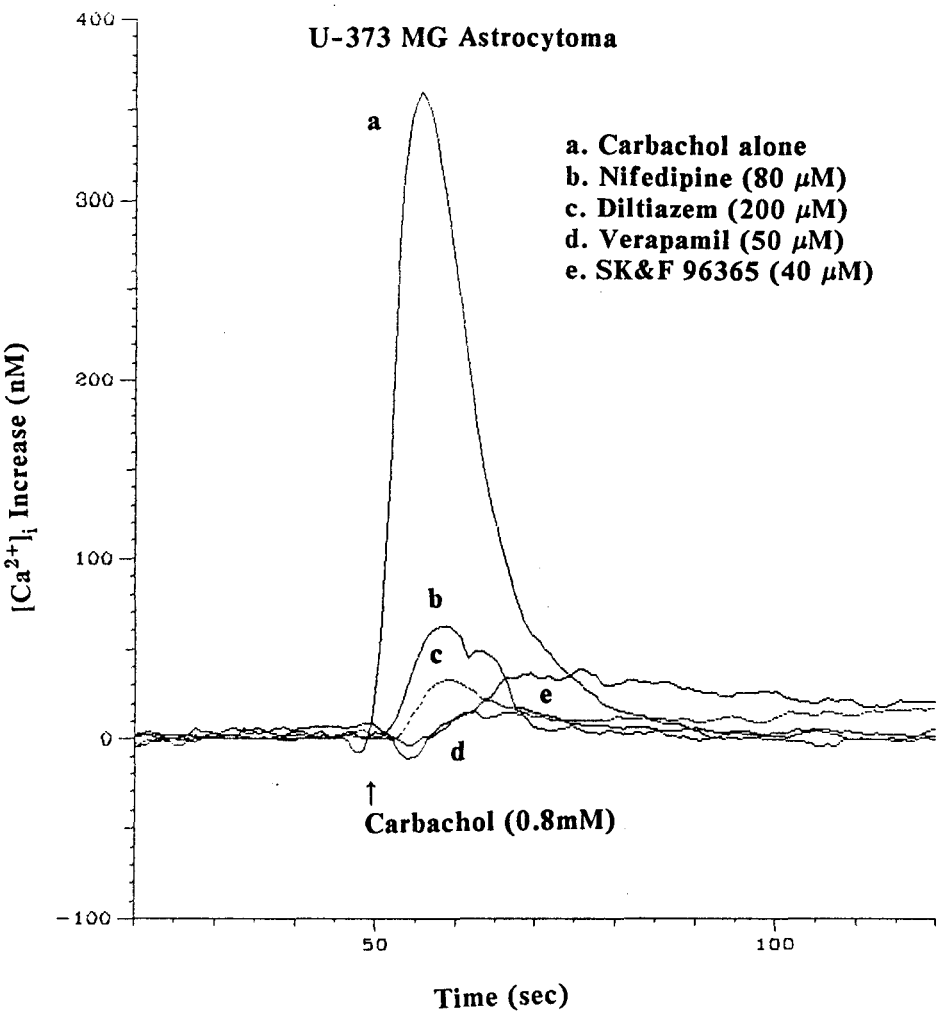

Fig. 4.18. Effect of Ca^{2+} channel antagonists on carbachol (0.8mM)-induced intracellular Ca^{2+} mobilization in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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 carbachol. Ca^{2+} channel antagonists were treated for 3 minutes before starting the experiment.

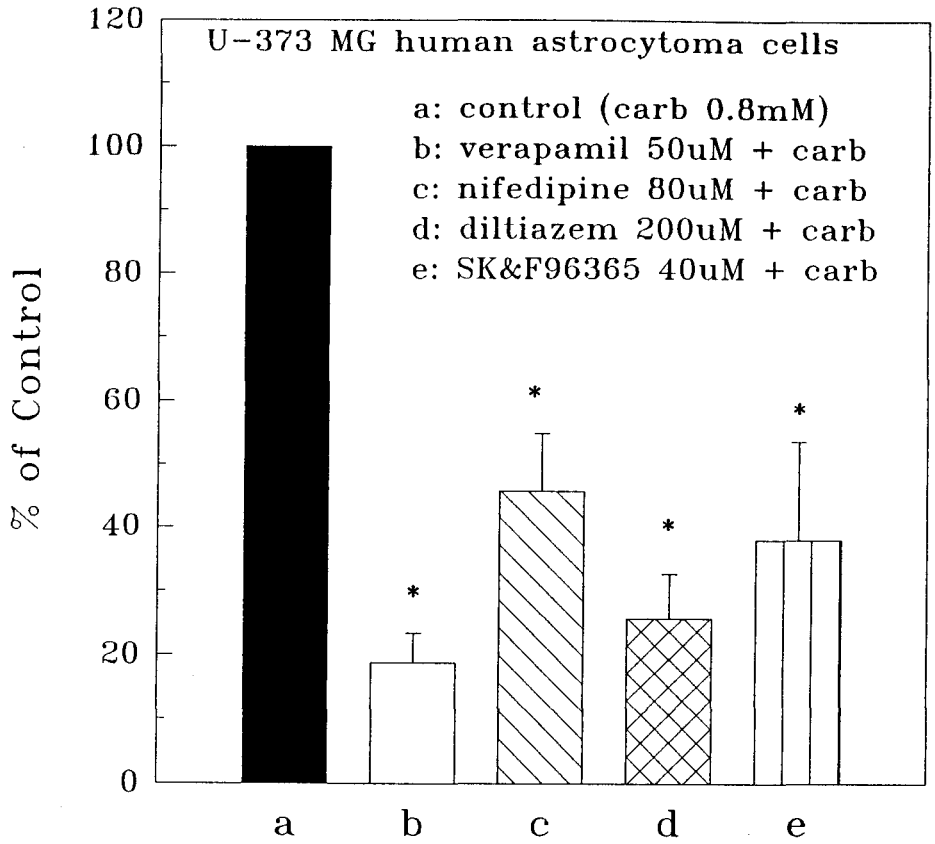

Fig. 4.19. Quantitative changes of carbachol (0.8mM)-induced intracellular Ca^{2+} mobilization by Ca^{2+} channel antagonists in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with $2\mu M$ of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended 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 the increased intracellular 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).

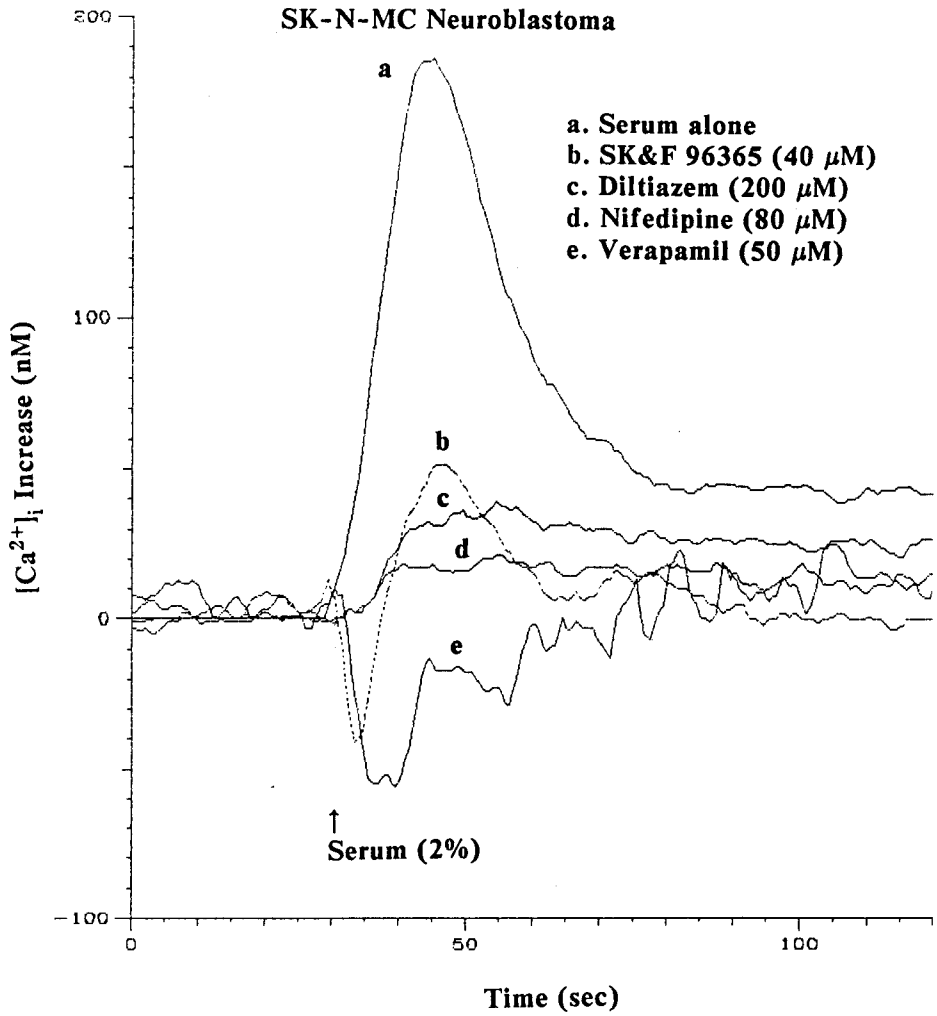

Fig. 4.20. Effect of Ca^{2+} channel antagonists on serum (2%)-induced intracellular Ca^{2+} mobilization in SK-N-MC human neuroblastoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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. Ca^{2+} channel antagonists were treated for 3 minutes before starting the experiment.

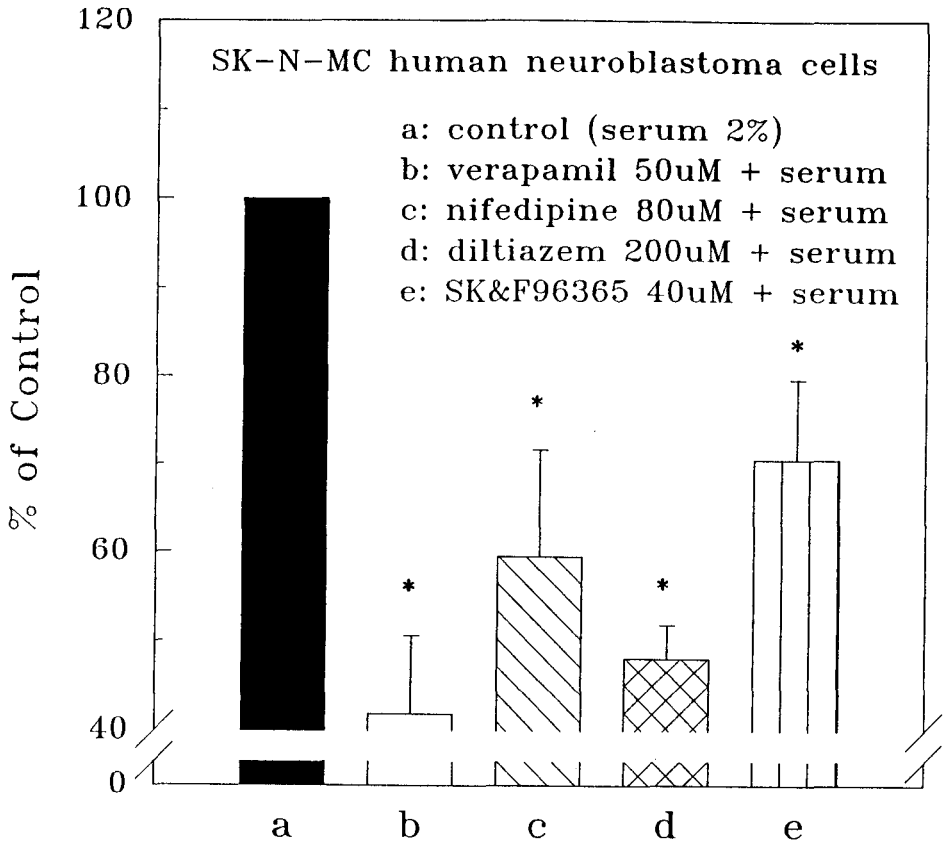

Fig. 4.21. Quantitative changes of serum (2%)-induced intracellular Ca^{2+} mobilization by Ca^{2+} channel antagonists in SK-N-MC human neuroblastoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with $2\mu M$ of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended 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 the increased intracellular 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 Ca^{2+} channel antagonists on the serum-induced intracellular 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 Ca^{2+} channel antagonists in tumor cell growth may differ in these tumor cell lines and that the signalling mechanism of increased intracellular 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 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 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 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 Ca^{2+} concentration in the isolated rat osteoblast (Zaidi *et al.*, 1990). In addition, Taylor and Simpson found that the treatment of amlodipine, a Ca^{2+} channel antagonist, caused a rapid concentration-dependent decrease of the intracellular Ca^{2+} concentration in the HT-39 human breast cancer cell line (Taylor and Simpson, 1992). Thus, these alterations of the basal, cytosolic Ca^{2+} concentration may result in an altered response to an agonist stimulation. Therefore, the inhibitory effects of Ca^{2+} channel antagonists on agonist-induced

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

In order to test this possibility, the effects of Ca^{2+} channel antagonists alone on the basal levels of free cytosolic 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 Ca^{2+} concentration in either cell lines. Therefore, these results further suggest that the inhibition of agonist-induced intracellular Ca^{2+} responses evoked by Ca^{2+} channel antagonists may be not due to their effects on the basal levels of free cytosolic Ca^{2+}.

Throughout these experiments involving intracellular Ca^{2+} measurements, the basal, free intracellular 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 Ca^{2+} levels. Thus, by monitoring basal intracellular Ca^{2+} levels, the existence of dead cells in the monitoring cell suspension can be indirectly checked. Variations of basal levels of intracellular Ca^{2+} concentration occured 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 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

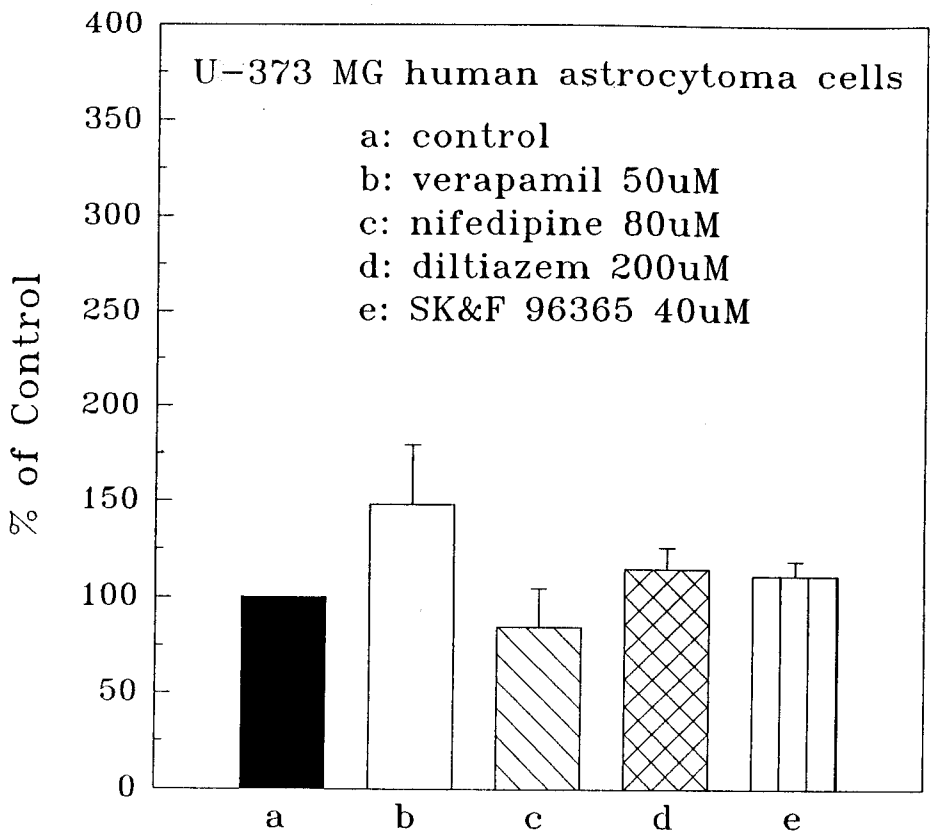

Fig. 4.22. No effect of Ca^{2+} channel antagonists on basal, free cytosolic Ca^{2+} concentration in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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.

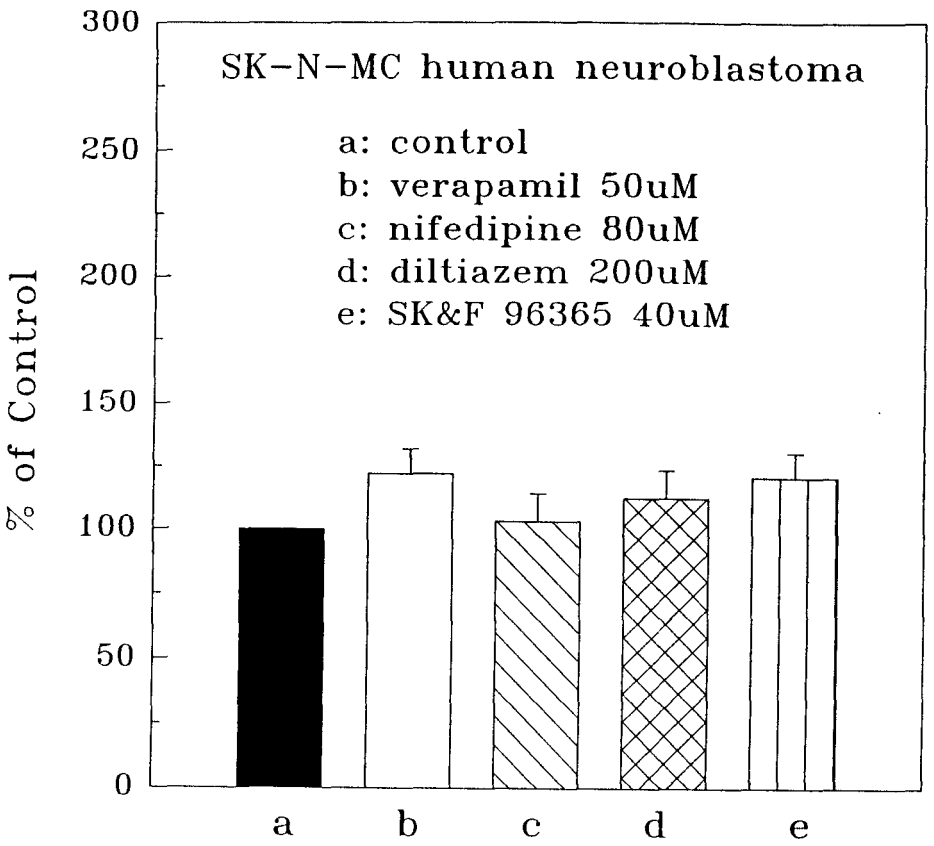

Fig. 4.23. No effect of Ca^{2+} channel antagonists on basal, free cytosolic Ca^{2+} concentration in SK-N-MC human neuroblastoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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.

Table 4.1. Basal, free intracellular Ca^{2+} concentrations of tumor cells

	Intracellular Ca^{2+} concentration [nM]	
Cell Line	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 Ca^{2+} concentration which results from the release of Ca^{2+} from the internal stores, is an essential signal transduction mechanism; 2) the blockade of this signalling mechanism by 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 Ca^{2+} concentration in cell proliferation has been suggested from many studies which have used pharamacological agents including Ca^{2+} channel blockers to inhibit growth factor-induced increased cytosolic Ca^{2+} concentration or used low amount of Ca^{2+} in the culture medium (Batra *et al.*, 1991; Block *et al.*, 1989; Ogata *et al.*, 1991; Olsen *et al.*, 1991; Shultz and Raij, 1990). Thus, these results suggest that during cell proliferation, the increased intracellular Ca^{2+} concentration occurs and that this Ca^{2+} increase is achieved through the influx of 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 Ca^{2+} from the internal stores without a significant 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 Ca^{2+} concentration due to the internal store release rather than 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 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 Ca^{2+} concentration (occuring 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 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 Ca^{2+} channel antagonists in the inhibition of tumor cell proliferation has been suggested to be due to their inhibitory effects on Ca^{2+} influx, the intracellular 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 Ca^{2+} channel antagonists in the regulation of cell proliferation has been challenged. For example, in the ^{45}Ca uptake studies, Schmidt and his associates suggested that the growth-inhibitory effects of verapamil appeared to be not due to Ca^{2+} influx or efflux in human medulloblastoma cells (Schmidt *et al.*, 1988). Thus, in order to elucidate the mechanism of action of Ca^{2+} channel antagonists in the present study, the effects of these drugs on both tumor cell growth and an agonist-induced increased intracellular Ca^{2+} concentration were investigated using human brain tumor cell lines.

The results of this study clearly show that Ca^{2+} channel antagonists inhibited both tumor cell growth (Figs. 4.3 - 4.6 and 4.8) and an agonist-induced release of 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 Ca^{2+} channel antagonists on tumor cell growth may be due to their blocking actions on the release of Ca^{2+} from the internal stores during cell proliferation.

A remaining important question is how Ca^{2+} channel antagonists can block an agonist-induced release of 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) IP_3-activated Ca^{2+} channels in the endoplasmic reticulum (ER); (v) indirect mechanism including the interaction with plama membrane K^+ channels.

The allosteric interaction of 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 Ca^{2+} channel antagonists (verapamil and diltiazem). Thus, the blockade of carbachol-binding to its receptors by 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 Ca^{2+} from internal stores shown in Figs. 4.18 and 4.19. However, the interaction of 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 Ca^{2+} channel antagonists may block IP_3-activated 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 IP_3-induced Ca^{2+} release was insensitive to a variety of Ca^{2+} channel blockers including verapamil and nifedipine (Shah and Pant, 1988). However, Ca^{2+} channel antagonists may affect indirectly IP_3-induced Ca^{2+} release through their inhibition of calmodulin (CaM) action. Of particular interest, CaM antagonists have shown to inhibit IP_3-stimulated Ca^{2+} mobilization, and thus, CaM has been suggested to be tightly associated with the intracellular membrane mechanism coupling IP_3 receptors to Ca^{2+} release channels (Hill *et al.*, 1988). Moreover, some 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 μM, can inhibit voltage-dependent 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 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 neutrophiles (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-γ1 (Alava *et al.*, 1992). Some Ca^{2+} channel blockers are known to inhibit CaM-dependent enzymes, particulary, cyclic AMP phosphodiesterase which degrades cylic 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 Ca^{2+} channel antagonist-induced inhibition of Ca^{2+} release from internal stores. Felodipine, a dihydropyridine 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 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, Ca^{2+} channel antagonist-induced blockade of intracellular Ca^{2+} mobilization may be possibly due to their modulatory effect on PKC. The whole cascades of these events which may be induced by Ca^{2+} channel antagonists are summarized diagramatically in Fig. 4.24.

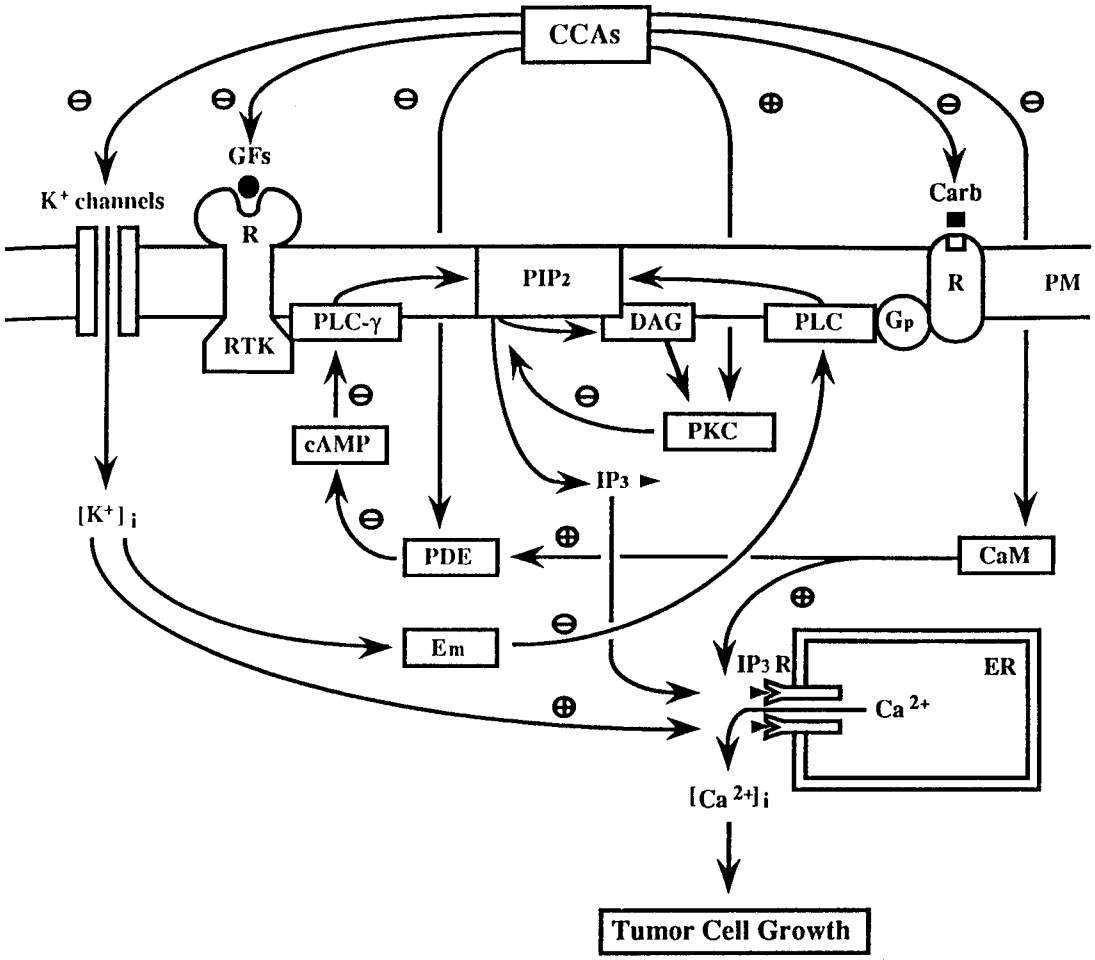

Fig. 4.24. Proposed inhibitory mechanisms of Ca^{2+} channel antagonists in tumor cell growth. This figure shows the signal transduction pathways of growth factors and carbachol-inducing tumor cell proliferation. In general, the stimulation of growth factor receptors can phosphorylate and then activate PLC-γ 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-γ and PLC degrade PIP_2 into DAG and IP_3. IP_3 binding to the receptors on the ER membrane can open IP_3-sensitive Ca^{2+} channels, and DAG can activate PKC. An increased intracellular 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 Ca^{2+} channel antagonists. Abbrevations used: CCAs, Ca^{2+} channel antagonists; GFs, growth factors; Carb, carbachol; R, receptor; RTK, receptor tyrosine kinase; PLC, phospholipase C; PLC-γ, phospholipase C-γ; G_p, GTP binding protein; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C; cAMP, cyclic adenosine 3',5'-monophosphate; PDE, cyclic AMP phosphodiesterase; CaM, calmodulin; E_m, membrane potential; $[K^+]_i$, intracellular K^+ concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3 R, IP_3 receptor 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 Ca^{2+}, H^+ and Na^+ (Geck and Bereiter-Hahn, 1991; Mendoza, 1988; Moolenaar *et al.*, 1981; Prasad *et al.*, 1987). Moreover, several pieces of evidence suggest that 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 K^+ channels. T cell mitogen, concanavalin A (ConA), increases the number of K^+ channels in murine T lymphocytes (DeCoursey *et al.*, 1987). In a murine noncytolytic T lymphocyte clone, L2, voltage-sensitive K^+ conductance is increased during interleukin 2 (IL2)-stimulated proliferation (Lee *et al.*, 1986). Ca^{2+}-activated 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 K^+ channel blockers have demonstrated a reasonable quantitative agreement between block of K^+ currents and inhibition of cell proliferation. Amigorena and coworkers have shown that K^+ channel blockers such as TEA, quinine and verapamil suppressed both K^+ current and DNA synthesis in murine B lymphocytes (Amigorena *et al.*, 1990). Similar

results have been found in neuroblastoma cells (Rouzaire-Dubois and Dubois, 1990; Rouzaire-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 correlationship 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 (Rouzaire-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 alterated E_m can affect other ion movements through the changes of transmembrane electrical gradients of these ions. Thus, interrelationship between the

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

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

B. Results

Inhibition of Tumor Cell Growth by High Extracellular Potassium

Dependence of tumor cell growth on the extracellular 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 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 K^+ in a dose-dependent manner.

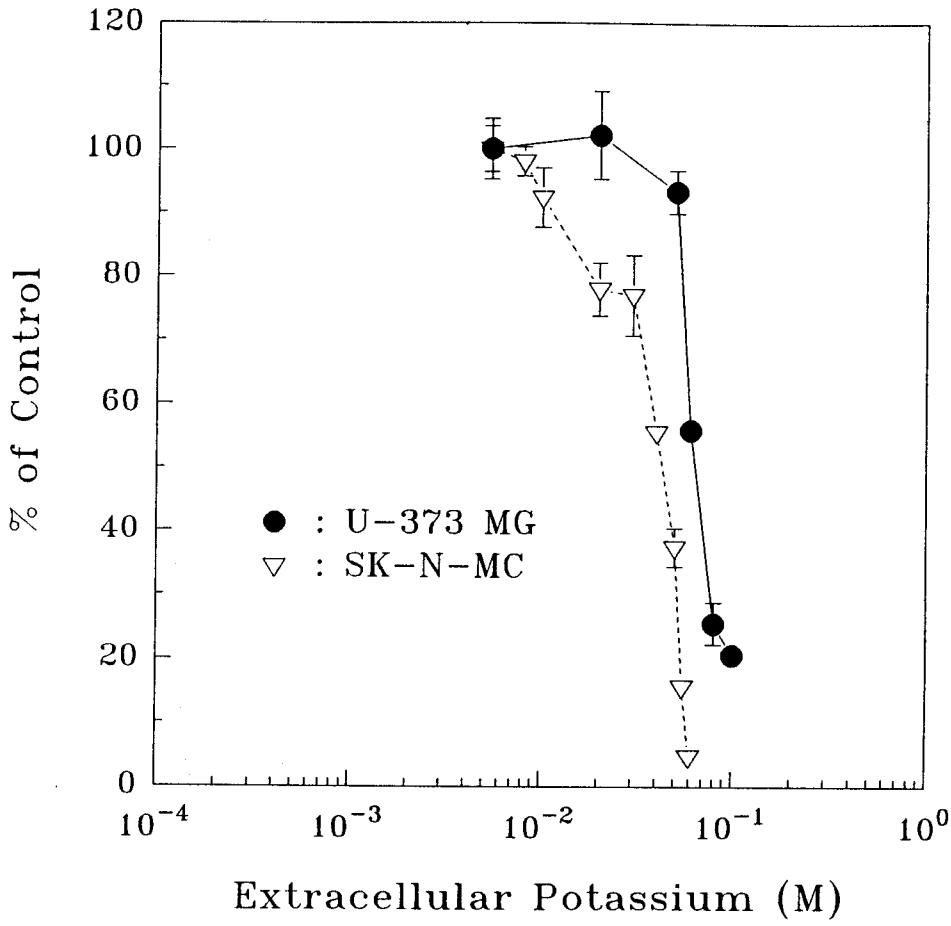

Fig. 5.1. Effect of high extracellular K^+ on the growth of U-373 MG human astrocytoma (closed circle) and SK-N-MC human neuroblastoma (open inverted triangle) cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of K^+ in the culture 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 Gowth

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

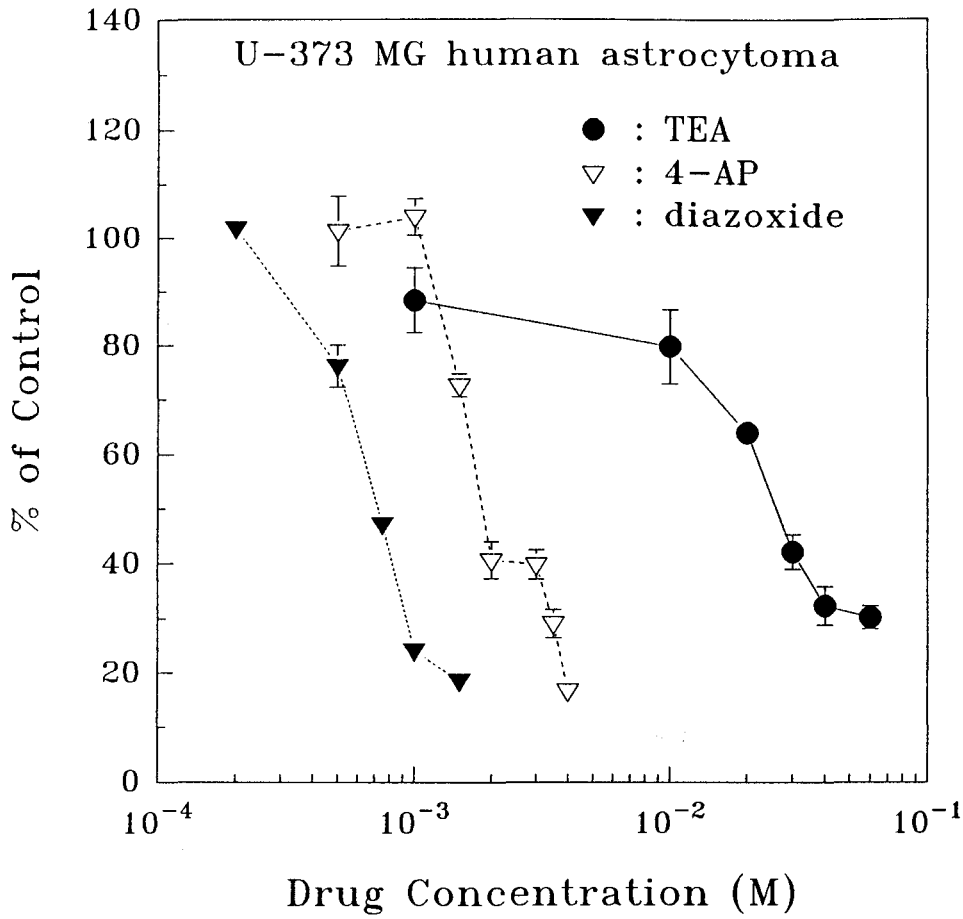

Fig. 5.2. Effect of K^+ channel modulators on the growth of U-373 MG human astrocytoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of TEA (closed circle), 4-AP (open inverted triangle) or diazoxide (closed inverted triangle) 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.

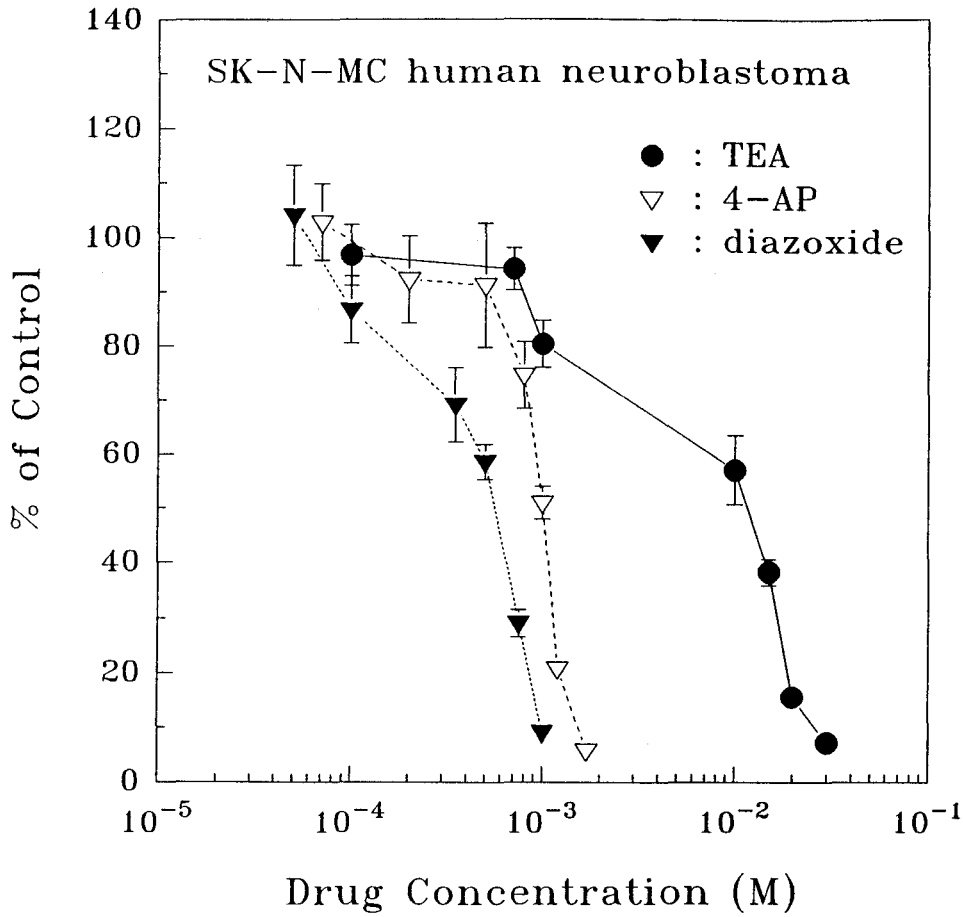

Fig. 5.3. Effect of K^+ channel modulators on the growth of SK-N-MC human neuroblastoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of TEA (closed circle), 4-AP (open inverted triangle) or diazoxide (closed inverted triangle) 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^-,

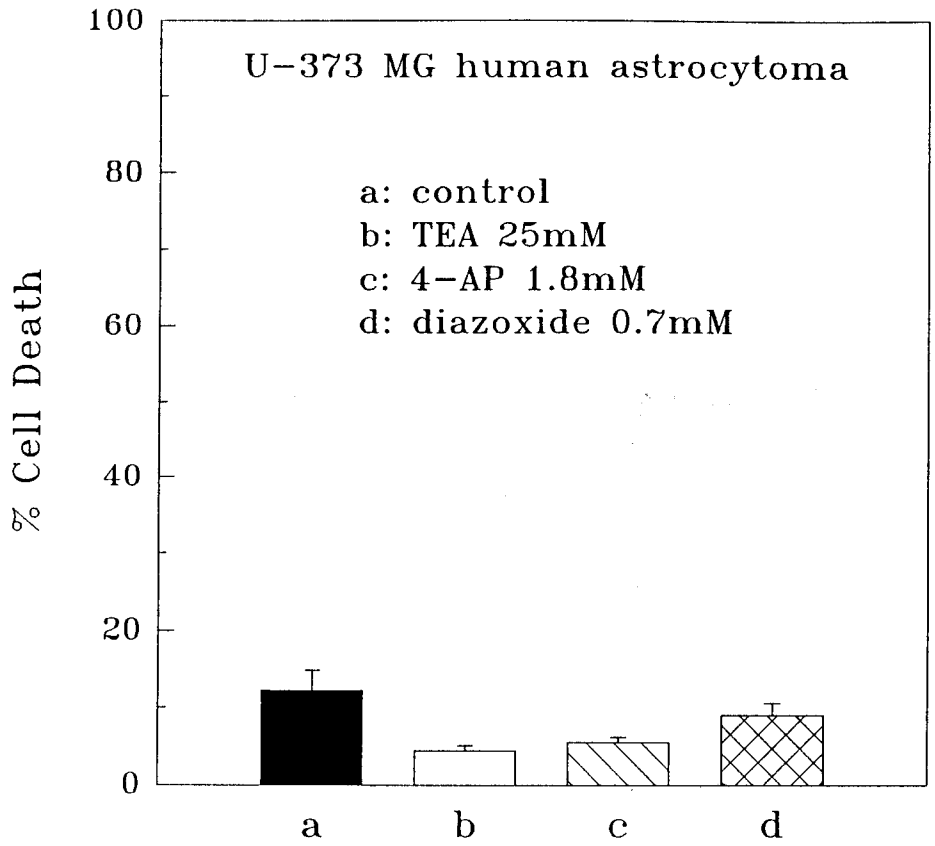

Fig. 5.4. No cytotoxic effect of K^+ channel modulators on U-373 MG human astrocytoma cell line. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of the drugs in the culture medium. Cell cytotoxicity was assayed by trypan blue extrusion method 2 days after drug 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.

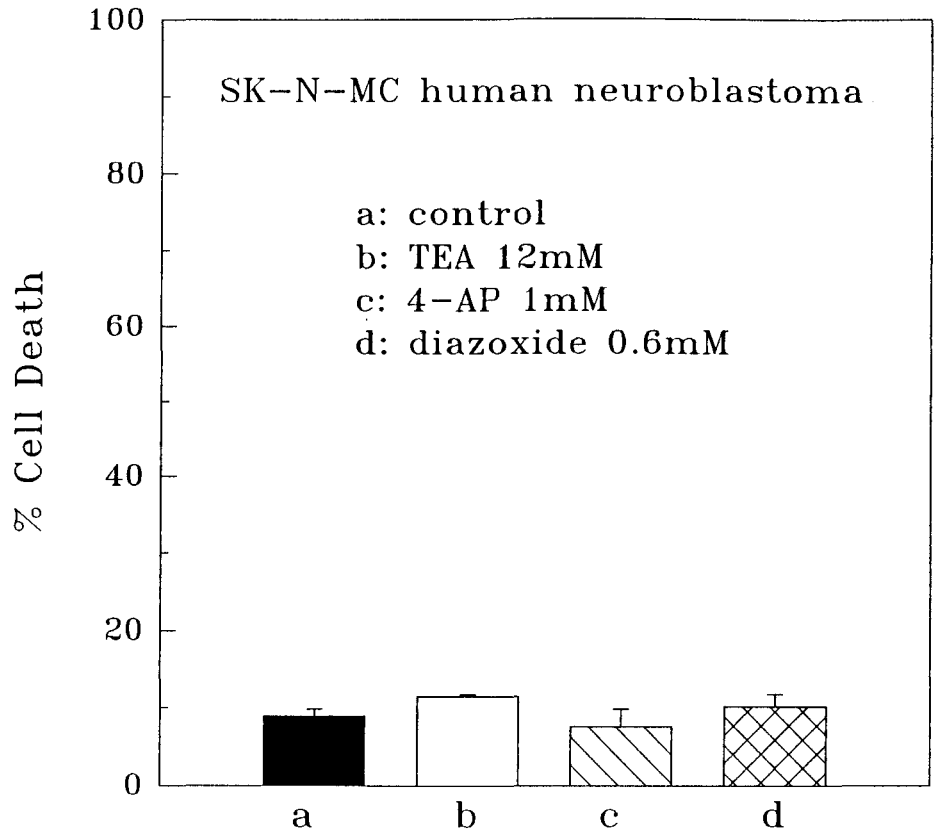

Fig. 5.5 No cytotoxic effect of K^+ channel modulators on SK-N-MC human neuroblastoma cell line. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of the drugs in the culture medium. Cell cytotoxicity was assayed by trypan blue extrusion method 2 days after drug 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 Na^+, whose concentration would remain more or less constant (Rouzaire-Dubois and Dubois, 1990). Thus, the blockade of K^+ channels can inhibit a mitogen-induced increased intracellular Na^+ concentration, resulting in the inhibition of cell proliferation. However, the role of increased intracellular Na^+ concentration has not been described in these tumor cells. Therefore, in order to test this possibility, the effects of veratridine, a Na^+ ionophore, and tetradotoxin (TTX), a voltage-sensitive 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 Na^+ concentration is not an important signal for cell proliferation in these tumor cells. These results further suggest that the growth-inhibitory effects of K^+ channel modulators shown in this study, are not due to their inhibition of mitogen-induced increased intracellular Na^+ concentration.

Second, Chandy *et al.* have suggested that in T lymphocytes Ca^{2+} entry is required for cell proliferation and that this Ca^{2+} entry may occur through the voltage-dependent K^+ channels (Chandy et al., 1984). Thus, the blockade of these K^+ channels may induce the inhibition of Ca^{2+} entry, resulting in the inhibition of cell proliferation. However, since in the tumor cells used in this study, 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 Ca^{2+} from the internal stores appeared to be an important signalling mechanism of growth factors in cell proliferation

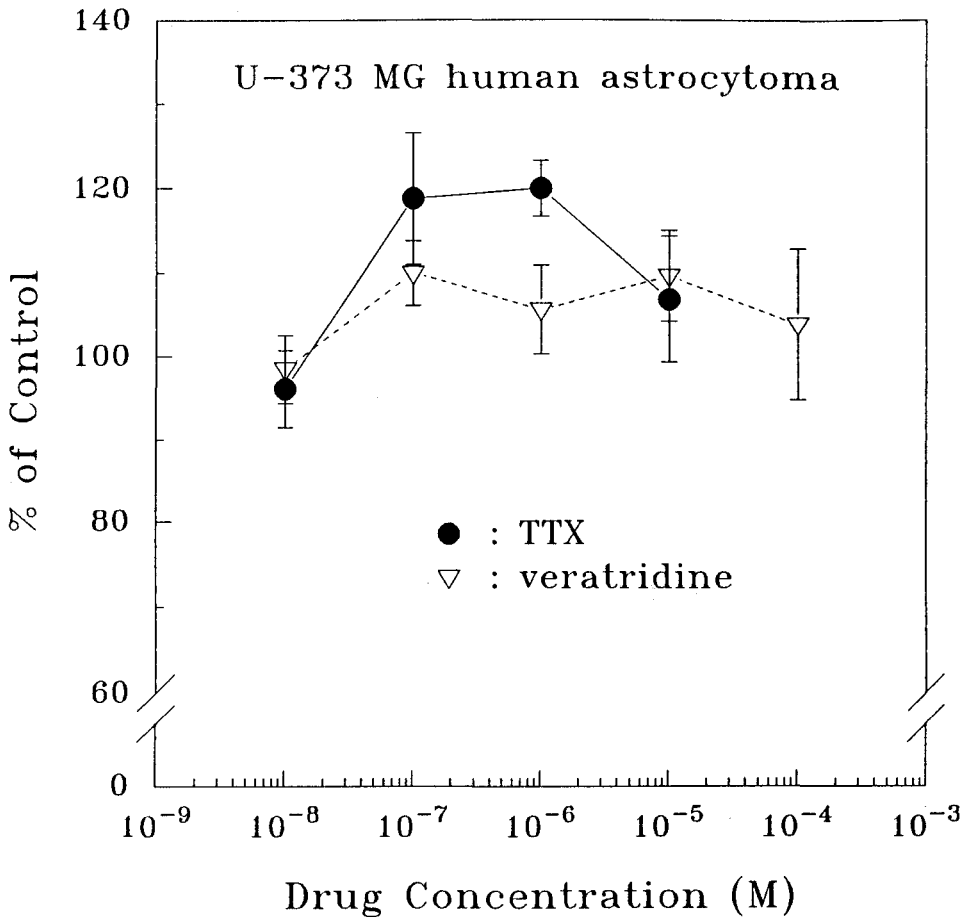

Fig. 5.6. Effect of veratridine and TTX on the growth of U-373 MG human astrocytoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of TTX (closed circle) and veratridine (open inverted triangle) in the culture medium. The 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.

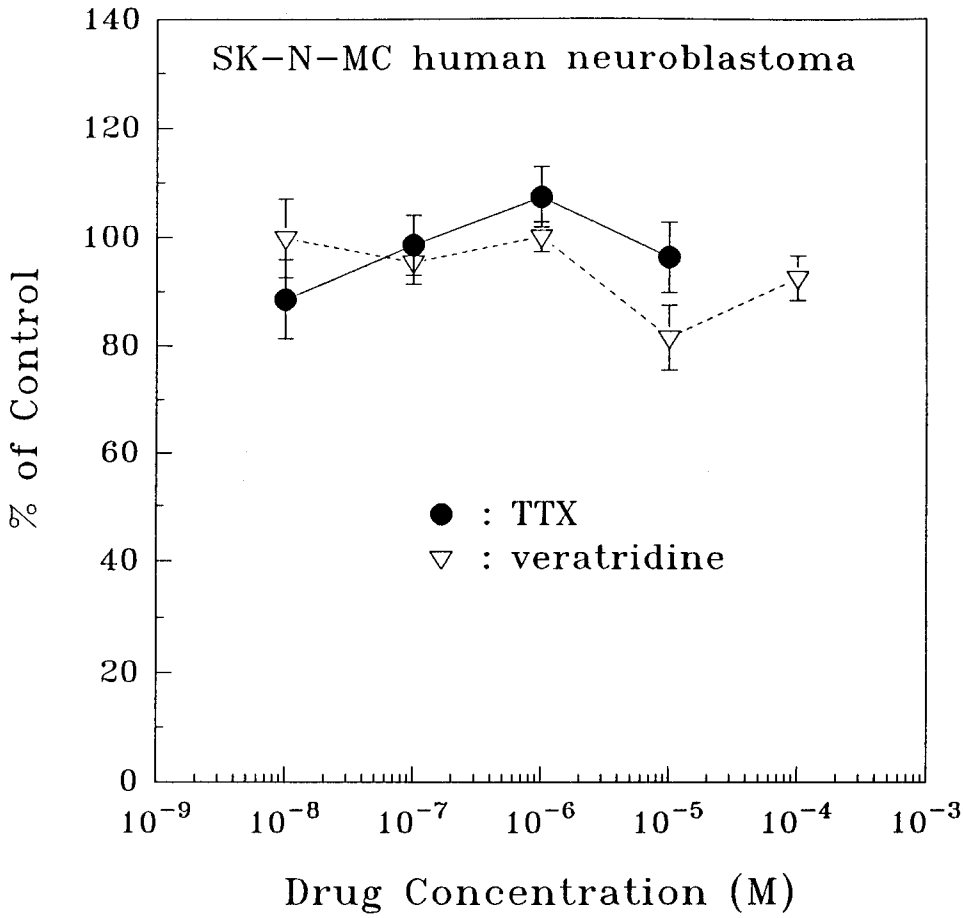

Fig. 5.7. Effect of veratridine and TTX on the growth of SK-N-MC human neuroblastoma cell lines. The cells were grown in MEM supplemented with 10% FBS in the absence or in the presence of various concentrations of TTX (closed circle) and veratridine (open inverted triangle) in the culture medium. The 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.

(decribed 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 Ca^{2+} was used, a agonist-induced increased intracellular Ca^{2+} concentration may result from the internal store release. The concentrations of 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 Ca^{2+} concentrations induced by Carb and the effects of pretreatment of 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 quantatitive comparison, percent changes of net increments of intracellular Ca^{2+} concentrations in the pretreatment of K^+ channel modulators compared to Carb or serum-induced 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 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 Ca^{2+} mobilization in these tumor cells.

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

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

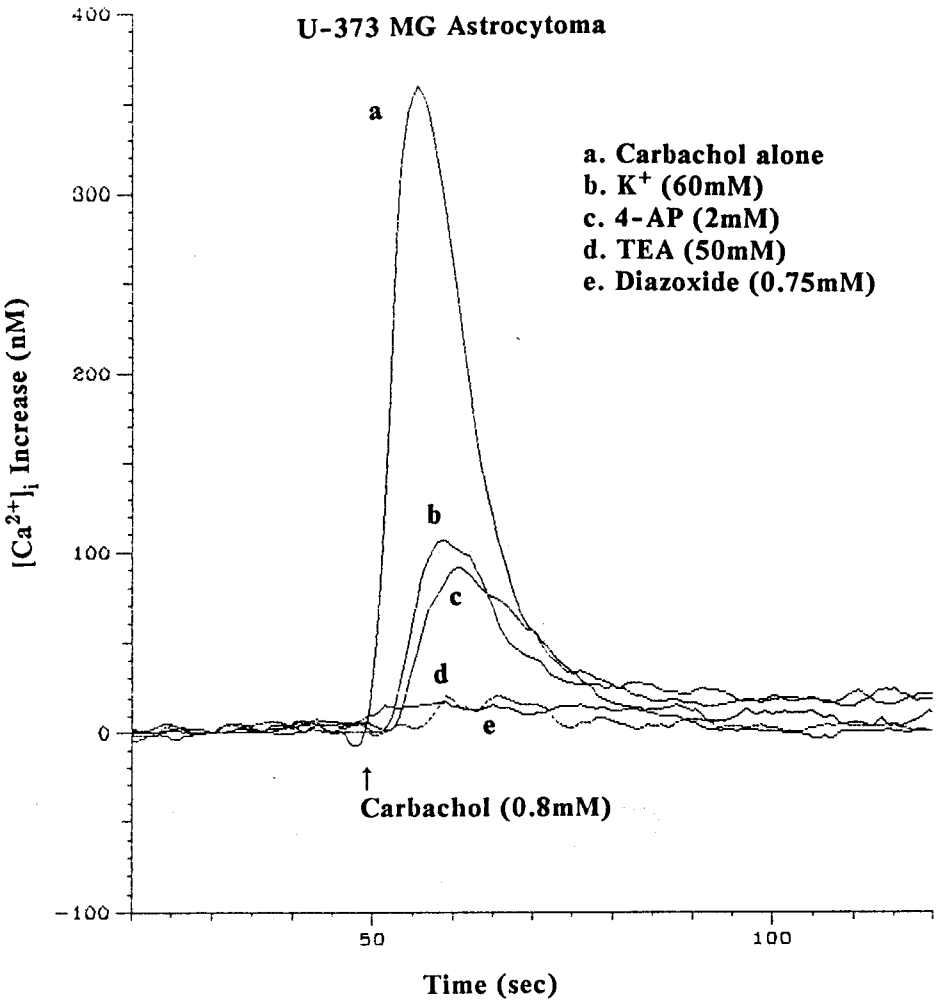

Fig. 5.8. Effect of high extracellular K^+ or K^+ channel modulators on carbachol (0.8mM)-induced intracellular Ca^{2+} mobilization in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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 carbachol. KCl or K^+ channel modulators were treated for 3 minutes before starting the experiment.

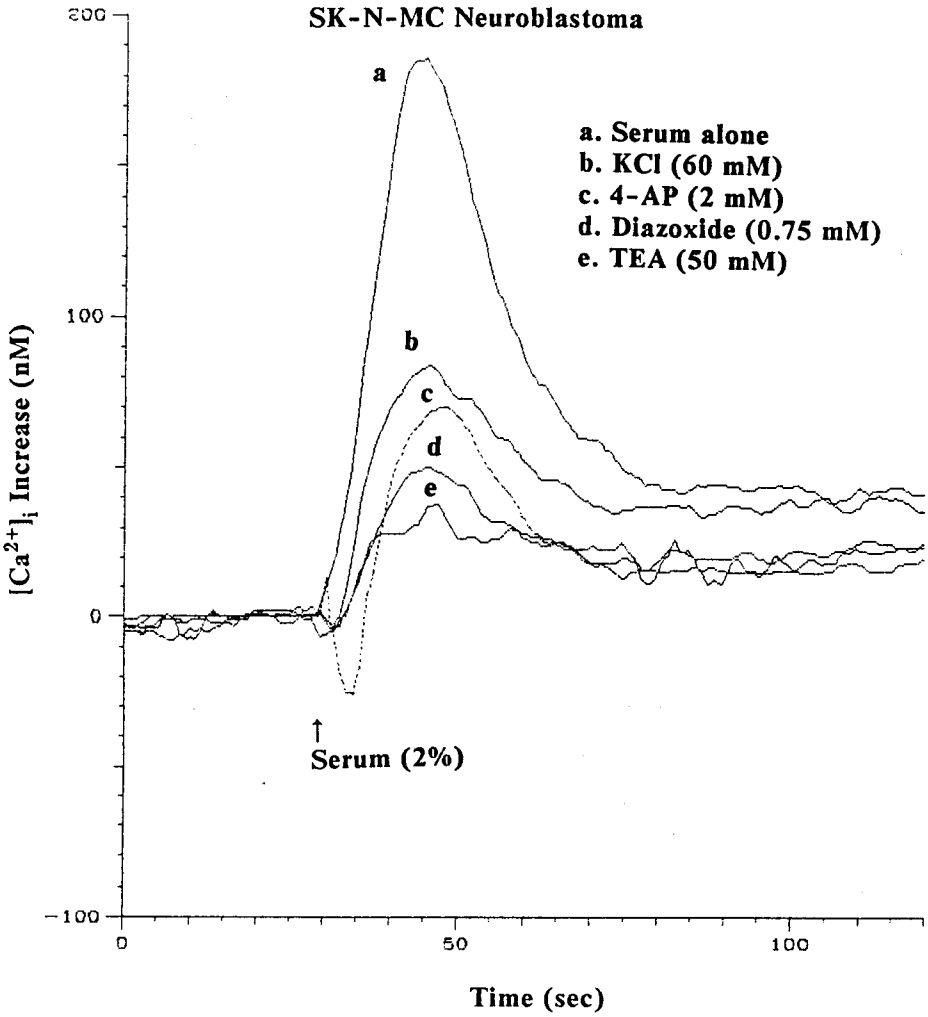

Fig. 5.9. Effect of high extracellular K^+ or K^+ channel modulators on serum (2%)-induced intracellular Ca^{2+} mobilization in SK-N-MC human neuroblastoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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.

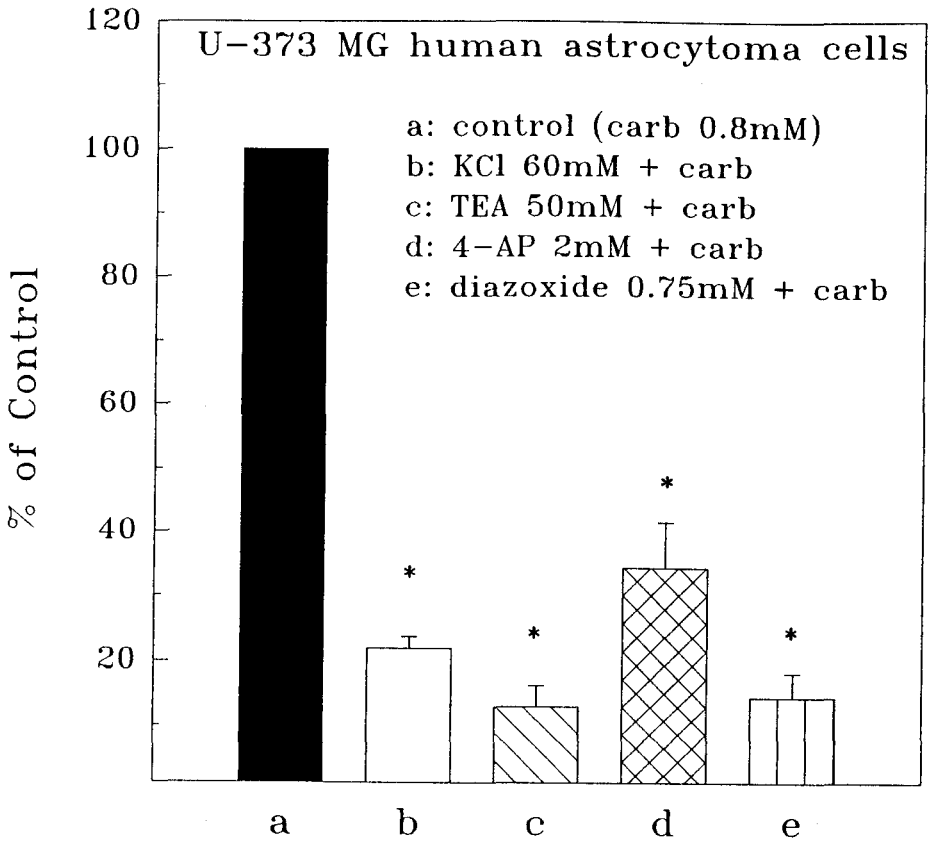

Fig. 5.10. Quantitative changes of carbachol (0.8mM)-induced intracellular Ca^{2+} mobilization by high extracellular K^+ or K^+ channel modulators in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{2+} and transferred to a quartz cuvette for fluorescence measurements. The results were expressed as a percent change of the increased intracellular 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)

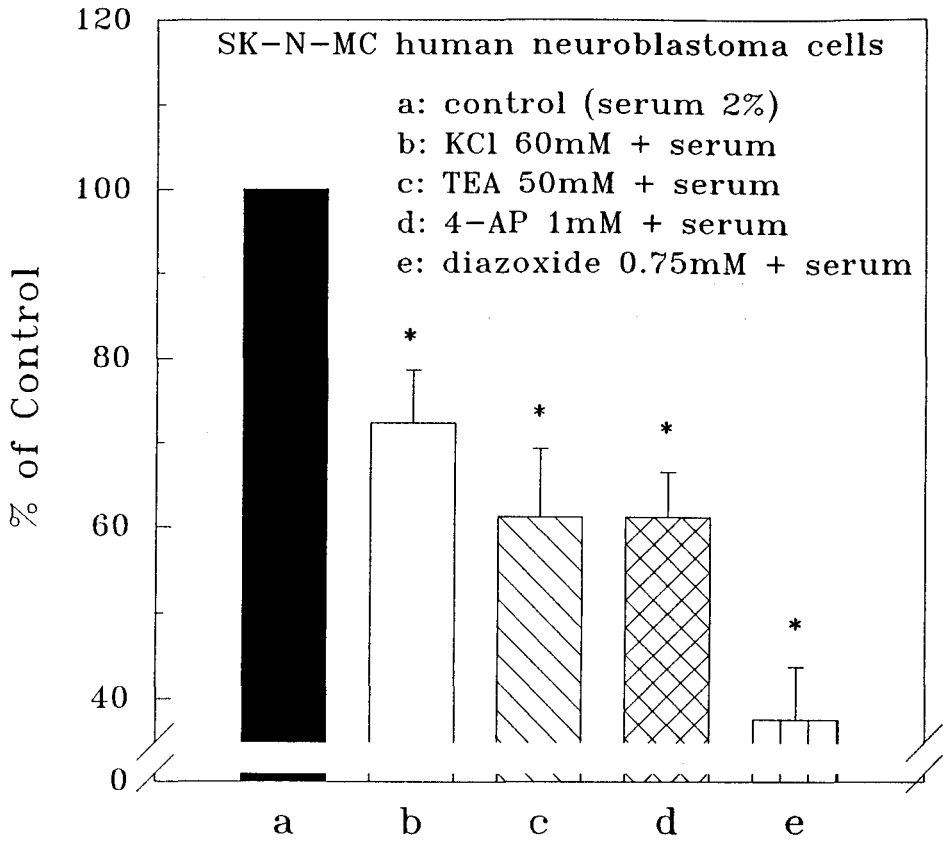

Fig. 5.11. Quantitative changes of serum (2%)-induced intracellular Ca^{2+} mobilization by high extracellular K^+ or K^+ channel modulators in SK-N-MC human neuroblastoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with $2\mu M$ of Fura-2-AME for 60 minutes at room temperature. The cells 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 the increased intracellular 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 Ca^{2+} concentrations were investigated. The results are shown in Figs. 5.12 and 5.13.

These results illustrate that K^+ channel modulators did not induce the significant alteration of the basal levels of intracellular Ca^{2+} concentrations. Thus, the blockade of agonist-induced intracellular Ca^{2+} increases by these drugs may be not due to the alteration of basal, free cytosolic Ca^{2+} concentration. These results further suggest that 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 K^+ and some 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, K^+ channel modulators evoked the inhibition of growth factor-induced increased intracellular Ca^{2+} concentrations as shown in Figs 5.8 - 5.11. These Ca^{2+} increases resulted from the internal store release. Thus, apparent correlationship between these two effects of 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 Ca^{2+} mobilization.

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

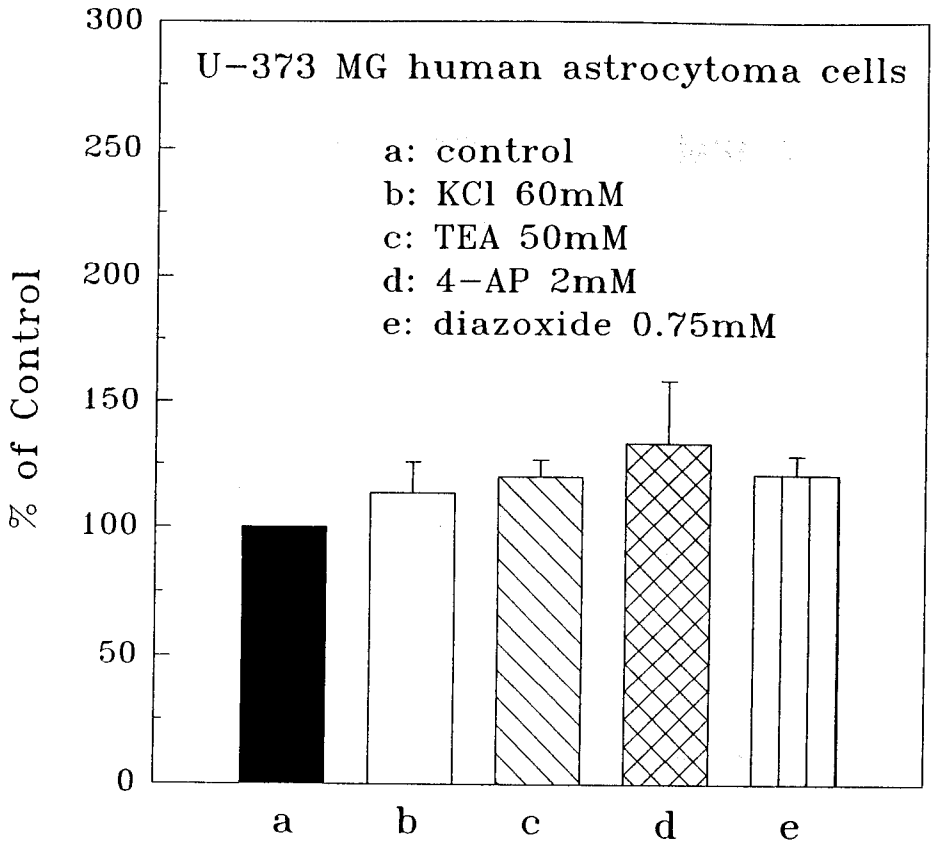

Fig. 5.12. No effect of high extracellular K^+ or K^+ channel modulators on basal, free cytosolic Ca^{2+} concentration in U-373 MG human astrocytoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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.

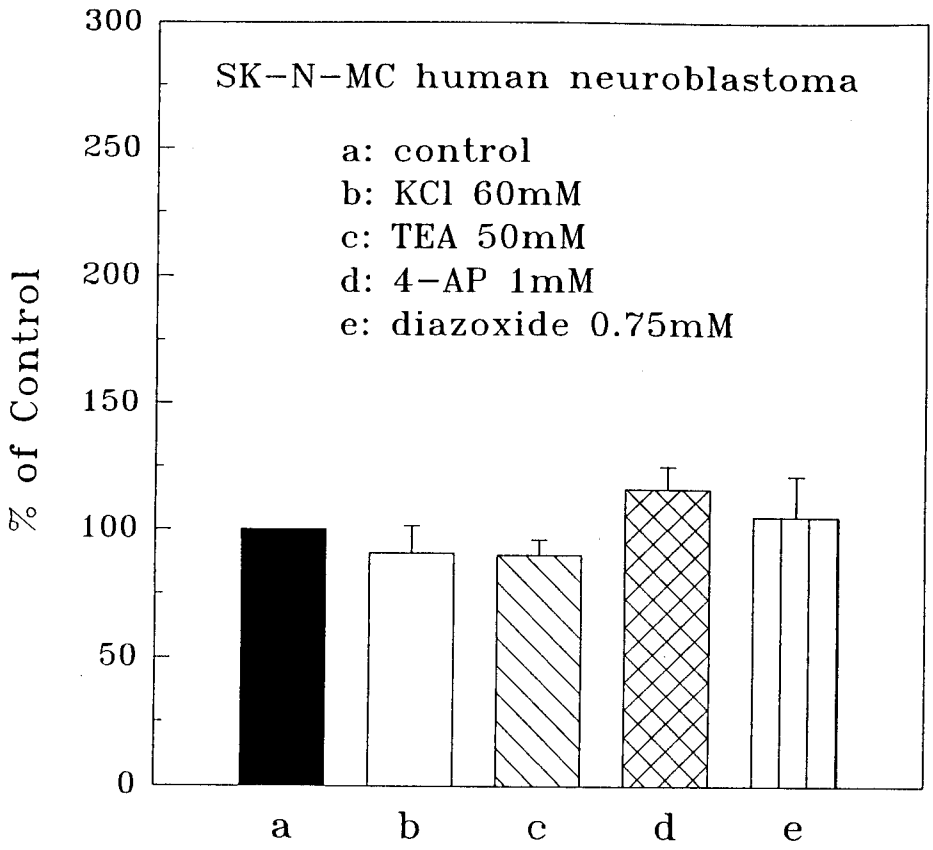

Fig. 5.13. No effect of high extracellular K+ or K+ channel modulators on basal, free cytosolic Ca2+ concentration in SK-N-MC human neuroblastoma cell line. Aliquots of $5x10^6$ cells/ml were incubated with 2μM of Fura-2-AME for 60 minutes at room temperature. The cells were washed, resuspended in the buffer solution containing 0 Ca^{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.

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 cytidylyltransferase (Cornell, 1991), protein kinase C (Epand 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_3 generation and, thus, the altered Ca^{2+} mobilization during agonist stimulation. Other possibilities may include changes in the accessibility of substrate (e.g. PIP_2), to the PLC, or uncoupling a regulatory subunit of G- protein from the PLC.

Interestingly, the same inhibitory effects of K^+ channel antagonists such as TEA and 4-AP, and K^+ channel activator such as diazoxide, on the tumor cell growth and agonist-induced Ca^{2+} mobilization were observed in the present study. If these agents simply acted by varying intracellular K^+ concentrations, diazoxide would be expected to have the opposite effect of K^+ channel antagonists, i.e. increased tumor cell growth and agonist-induced Ca^{2+} mobilization. Notable in this regard, Palade and his associates recently found that in canine microsomes, IP_3-induced Ca^{2+} release from the microsomes appeared to be dependent on the presence of K^+ in the medium (Palade *et al.*, 1989). Although a gradual reduction of K^+ concentration induced a parallel inhibition of IP_3-induced Ca^{2+} mobilization from the microsomes, the increase of K^+ permeability by the treatment of valinomycin, a K^+ ionophore, did not significantly affect on the IP_3-induced Ca^{2+} release. Thus, these results may suggest that K^+ channel blockers which can induce an increased intracellular K^+ concentration, would not have an effect on agonist-induced Ca^{2+} mobilization. However, diazoxide-induced reduction of cytoplasmic K^+

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 diaxoxide 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 diagramatically in Fig. 5.13.

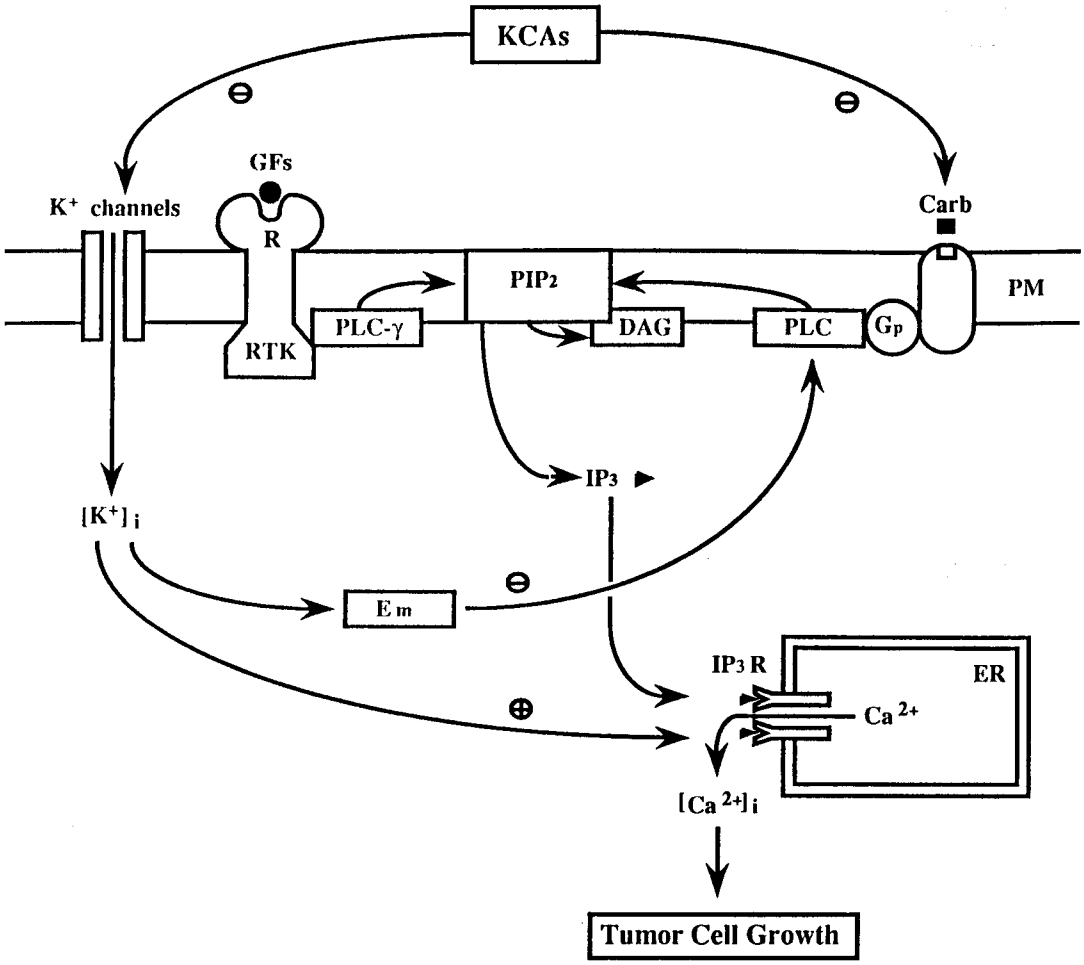

Fig. 5.14. Proposed inhibitory mechanisms of high extracellular K^+ or K^+ channel modulators in tumor cell growth. This figure shows the signal transduction pathways of growth factors and carbachol-inducing tumor cell proliferation. In general, the stimulation of growth factor receptors can phosphorylate and then activate PLC-γ 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-γ and PLC degrade PIP_2 into DAG and IP_3. IP_3 binding to the receptors on the ER membrane can open IP_3-sensitive Ca^{2+} channels, and DAG can activate PKC. An increased intracellular 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 high extracellular K^+ or K^+ channel modulators. Abbrevations used: KCAs, K^+ channel antagonists; GFs, growth factors; Carb, carbachol; R, receptor; RTK, receptor tyrosine kinase; PLC, phospholipase C; PLC-γ, phospholipase C-γ; G_p, GTP binding protein; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; DAG, diacylglycerol; E_m, membrane potential; $[K^+]_i$, intracellular K^+ concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3 R, IP_3 receptor Ca^{2+} channel; ER, endoplasmic reticulum; PM, plasma membrane.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Many studies suggest that the increased intracellular Ca^{2+} concentration is a critical event in the proliferative signalling mechanism of growth factors. Moreover, plasma membrane Ca^{2+} and K^+ channels are involved in the regulation of intracellular 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 disseration 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 Ca^{2+} and K^+ channels-regulating drugs on these tumor cell growth? 2) What are the characteristics and sources of increased intracellular 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 Ca^{2+} concentration changes induced by growth factors?

In order to explore the first question, the effects of various Ca^{2+} and 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 Ca^{2+} channel antagonists, verapamil, nifedipine and

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

2) Bay K-8644, a 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 Ca^{2+} channel antagonist, very effectively inhibited tumor cell growth.

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

5) Diazoxide, a 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 Ca^{2+} and K^+. Increased extracellular Ca^{2+} and K^+ concentrations inhibited tumor cell growth, and decreased extracellular Ca^{2+} concentrations by chelation with EGTA also induced the inhibition of tumor cell growth.

7) These ion channel modulators except 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 Ca^{2+} and K^+ channel-regulating drugs on these tumor cell proliferation.

The intracellular Ca^{2+} concentrations were measured in a variety of conditions in order to answer the second question. The levels of intracellular 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 Ca^{2+} concentration in U-373 MG human astrocytoma cells.

2) Carbachol induced a monophasic increase of intracellular Ca^{2+} concentration, and under the condition of 0 extracellular Ca^{2+} concentration, carbachol elicited a very similar response as that in a normal extracellular Ca^{2+} concentration.

3) Serum monophasically increased intracellular Ca^{2+} concentration in SK-N-MC human neuroblastoma cells, and this increase was insensitive to the alteration of extracellular Ca^{2+} concentration.

4) High extracellular K^+ or/and Bay K 8644 did not induce the significant alteration of intracellular Ca^{2+} concentration.

These results indicate that agents which act as growth factors, can induce the increased intracellular Ca^{2+} concentration and that this Ca^{2+} increase comes not from extracellular space but from intracellular stores. These results further suggest that no voltage-dependent Ca^{2+} channels are present and that intracellular 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 Ca^{2+} and K^+ channel modulators on agonist-induced intracellular 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 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 Ca^{2+} channel antagonists such as verapamil, nifedipine, diltiazem and SK&F 96365 inhibited significantly agonist-induced intracellular Ca^{2+} mobilization from intracellular stores in both model tumor cells.

2) The effective growth-inhibitory concentrations of K^+ channel modulators such as TEA, 4-AP and diazoxide induced significant reduction of increased intracellular 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 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 Ca^{2+} concentration mobilized from internal stores.

The mechanism of these ion channel modulators in the inhibition of agonist-induced 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 K^+ channel modulators may be explained by their modulation of membrane potential.

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

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

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

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