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Mediation of Intracellular Ca^{2+} in the Phospholipase A_2-induced Cell Proliferation in Human Neuroblastoma Cells

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The role of phospholipase A_2 (PLA_2) in tumor cell growth was investigated using SK-N-MC human neuroblastoma cells. 4-Bromophenacyl bromide (BPB) and mepacrine (Mep), known PLA_2 inhibitors, suppressed growth of the tumor cells in a dose-dependent manner without a significant cytotoxicity. Melittin (Mel), a PLA_2 activator, enhanced the cell growth in a concentration-dependent fashion. The growth-enhancing effects of Mel were significantly reversed by the co-treatment with PLA_2 inhibitors. In addition, Mel induced intracellular Ca^{2+} release from internal stores like as did serum, a known intracellular Ca^{2+} agonist in the tumor cells. Intracellular Ca^{2+} release induced by these agonists was significantly blocked by PLA_2 inhibitors at growth-inhibitory concentrations. Arachidonic acid (AA), a product of the PLA_2-catalyzed reaction, induced cell growth enhancement and intracellular Ca^{2+} release. These effects of AA were significantly blocked by BAPTA/AM, an intracellular Ca^{2+} chelator. Taken together, these results suggest that the modulation of PLA_2 activity may be one of the regulatory mechanisms of cell growth in human neuroblastoma cells. Intracellular Ca^{2+} may act as a key mediator in the PLA_2-induced growth regulation.

Key Words: Phospholipase A_2, Human neuroblastoma cells, Cell proliferation, Intracellular Ca^{2+}

INTRODUCTION

Phospholipase A_2 (PLA_2) is a lipolytic enzyme which hydrolyzes the acyl group from the *sn-2* position of glycerophospholipids, generating free fatty acids and lysophospholipids (van den Bosch et al, 1990). The products of the PLA_2-catalyzed reaction are known to act as second messengers themselves, or be further metabolized to eicosanoids, platelet-activating factor and lysophosphatidic acid (Farooqui et al, 1997). These metabolites are recognized as bioactive lipids which can potentially alter many ongoing cellular processes (Farooqui et al, 1997). Recently, PLA_2 and its enzymatic product, arachidonic acid (AA) have been reported to be involved in regulating cellular proliferation in a variety of cell types (Butcher et al, 1993; Anderson et al, 1997). Moreover, PLA_2 is activated by many growth factors (Nakazato, 1991; Chepenik et al, 1994). However, the role of PLA_2 in the regulation of cell growth in human neuronal tumor cells has not been clearly determined yet.

An increasing body of evidence suggests that intracellular Ca^{2+} has an important role in the process of cellular proliferation (Villereal & Byron, 1992). Previously, we have shown that intracellular Ca^{2+} signalling mechanisms are involved in the modulation of cell growth in human brain tumor cells (Lee et al, 1994; Lee et al, 1995). Recent reports show that AA has an influence on the intracellular Ca^{2+} signalling mechanisms in a variety cell types (Alonso-Torre & Garcia-Sancho, 1997; Striggow et al, 1997).

Thus, in this study we investigated the possible role of PLA_2 in the growth of human neuronal tumor cells and examined the involvement of intracellular Ca^{2+} signalling mechanisms in the PLA_2 action, using SK-N-MC human neuroblastoma cells as a model cellular system.

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METHODS

Materials

SK-N-MC human neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), trypsin solution, trypan blue, sodium pyruvate, ethylene glycol-bis-(aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), melittin (Mel), arachidonic acid (AA), 4-bromophenacyl bromide (BPB), mepacrine (Mep), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). 1-(2,5-Carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'-amino-methylphenoxy)-ethan e-N,N,N'N'-tetraacetoxylmethyl ester (Fura-2/AM) and bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) were from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). The stock solution of drugs was sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

Cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in a MEM supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

Cell growth assay (MTT staining)

Cell growth was assessed as described by Mosmann (1983). Cells from 4-5-day-old cultures were incubated in 1 ml of media in 24-well plates at an initial density of 5×10^4 cells/dish. Drugs to be tested were added to cultures 1 day after seeding to ensure uniform attachment of cells at the onset of the experiments. The cells were grown for an additional 2 days. Drugs and culture medium were replaced every day. In control experiments cells were grown in the same media containing drug-free vehicle. After a period of incubation, 100 μl of MTT (5 mg MTT/ml in H_2O) were added and cells incubated for a further 4 hr. One hundred microliters of acid-isopropanol (0.04 N HCl in isopropanol) were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell growth was obtained by scanning with an ELISA reader (Molecular Devices, Menlo Park, CA) with a 570 nm filter.

Cell cytotoxicity assay

Cell cytotoxicity was assessed by the trypan blue exclusion method (Bowles et al, 1990). The experimental procedure was the same as the cell growth assay except that total and viable cells which were stained with 0.2% trypan blue after trypsinization, were counted using a hemocytometer. The results are presented as percent of the number of dead cells.

Intracellular Ca^{2+} measurement

Aliquots of the tumor cells, cultured for 3~5 days, were washed in EBSS. Then, 2 μM of Fura-2/AM was added, and the cells were incubated for 60 min at room temperature (22~23°C). Unloaded Fura-2 was removed by centrifugation at $150 \times g$ for 3 min. Cells were resuspended at a density of 2×10^6/ml in Ca^{2+}-free Krebs-Ringer buffer (KRB) containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES and 6 mM glucose (pH 7.4), transferred to a quartz cuvette and stirred continuously. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm at 37°C using a Hitachi F4500 fluorescence spectrophotometer. Intracellular Ca^{2+} concentration was calculated from the fluorescence ratio excited at 340 and 380 nm, following the method described by Grynkiewicz et al (1985).

Data analysis

All experiments were done four times. All data were displayed as % of control condition. Data were expressed as mean$\pm$standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered statistically significant.

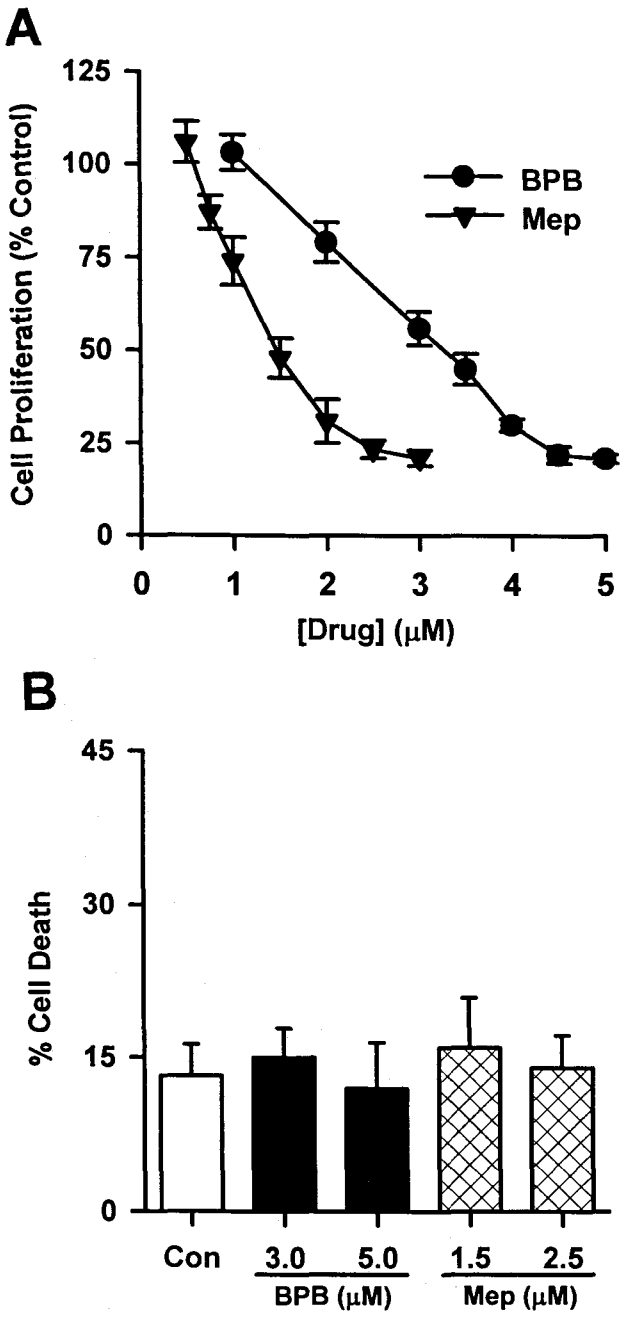

Fig. 1. Effects of BPB and Mep, PLA_2 inhibitors, on cell growth (A) and cytotoxicity (B) in SK-N-MC human neuroblastoma cells. Cell growth and cytotoxicity assays were performed by MTT staining and trypan blue exclusion methods, respectively. Results are expressed as percent change of control condition in which the cells were grown in medium containing drug-free vehicle (A) or percent of the number of dead cells (B). Data points (A) or columns (B) represent the mean values of four replications with bars indicating SEM.

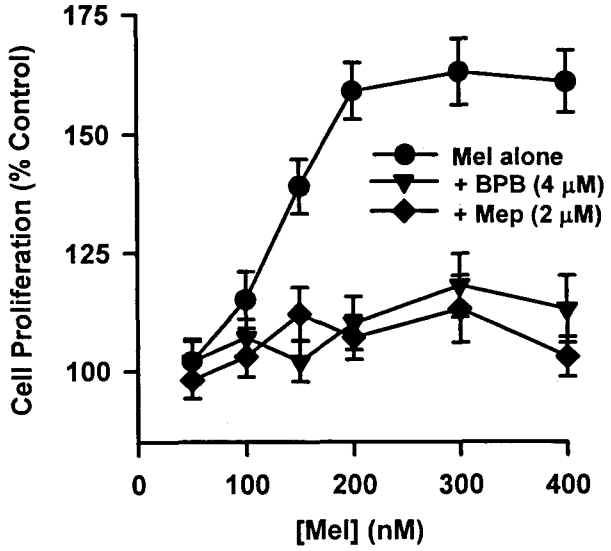

Fig. 2. Growth-enhancing activity of Mel, a PLA_2 activator, and its reversal by BPB or Mep, PLA_2 inhibitors, in SK-N-MC human neuroblastoma cells. Cell growth assay was done by MTT staining method. Results are expressed as percent change of control condition in which the cells were grown in medium containing drug-free vehicle. Data points represent the mean values of four replications with bars indicating SEM.

RESULTS

Effects of PLA_2 inhibitors on the growth of neuroblastoma cells

To determine the role of PLA_2 in the growth of human neuronal tumor cells, we investigated the effect of PLA_2 inhibitors on the growth of SK-N-MC human neuroblastoma cells. In these experiments BPB (Vargaftig et al, 1980) and Mep (Estevez & Phillis, 1997) were used as PLA_2 inhibitors. These PLA_2 inhibitors suppressed cell growth in a dose-dependent manner in the tumor cells as shown in Fig. 1A. The concentrations of half-maximum effects (EC_{50}) of BPB and Mep were 3 and 1.5 μM, respectively. Since the inhibition of cell growth may result from either the inhibition of cell proliferation or direct cytotoxicity, the cytotoxic effects of these drugs were examined using trypan blue exclusion method (Bowles et al, 1990). These PLA_2 inhibitors did not induce a significant cytotoxicity compared to control condition in the tumor cells as depicted in Fig. 1B. These results suggest that the inhibition of tumor cell growth induced by these PLA_2 inhibitors

may be mediated not by a direct cytotoxicity, but by the interference with tumor cell proliferation.

Effects of a PLA_2 activator on the growth of neuroblastoma cells

The effects of Mel, a PLA_2 activator (Shaposhnikova et al, 1997), on tumor cell growth were also studied, and the results are depicted in Fig. 2. The results show that Mel induced a dose-dependent enhancement of tumor cell growth. Maximum enhancement of cell growth induced by Mel was about 160% compared to control condition. The EC_{50} value of the growth-enhancing activity of Mel was 150 nM. The growth-enhancing action of Mel was significantly reversed by the co-treatment with the PLA_2 inhibitors, either 4 μM of BPB or 2 μM of Mep.

Effects of PLA_2 inhibitors on agonist-induced intracellular Ca^{2+} release

To examine the relationship between the observed growth-regulating actions of these PLA_2 modulators and the intracellular Ca^{2+} regulating mechanisms, we measured the change of intracellular Ca^{2+} concentration using Fura-2 fluorescence technique. Previously, we reported that in the SK-N-MC human neuroblastoma cell line serum induced a transient increase in intracellular Ca^{2+} concentrations without a sustained increase (plateau phase) in normal Ca^{2+} medium (Lee et al, 1993a). Furthermore, these intracellular Ca^{2+} increases were not altered in the Ca^{2+}-free medium, indicating that Ca^{2+} influx from extracellular compartment is not involved in the serum-induced increased intracellular Ca^{2+} concentration (Lee et al, 1993b). Throughout the present study intracellular Ca^{2+} concentrations were monitored using the Ca^{2+}-free medium and thus, any increases in intracellular Ca^{2+} concentrations represent internal Ca^{2+} release from the intracellular Ca^{2+} stores.

Fig. 3A show the effects of BPB and Mep on serum-induced intracellular Ca^{2+} release in SK-N-MC human neuroblastoma cells. The results illustrate that the growth-inhibitory concentrations of these drugs significantly blocked the serum-induced intracellular Ca^{2+} release. The growth-stimulatory concentration (0.2 μM) of Mel increased intracellular Ca^{2+} concentration as a similar pattern as serum shown in Fig. 3A(d). In addition, BPB and Mep significantly

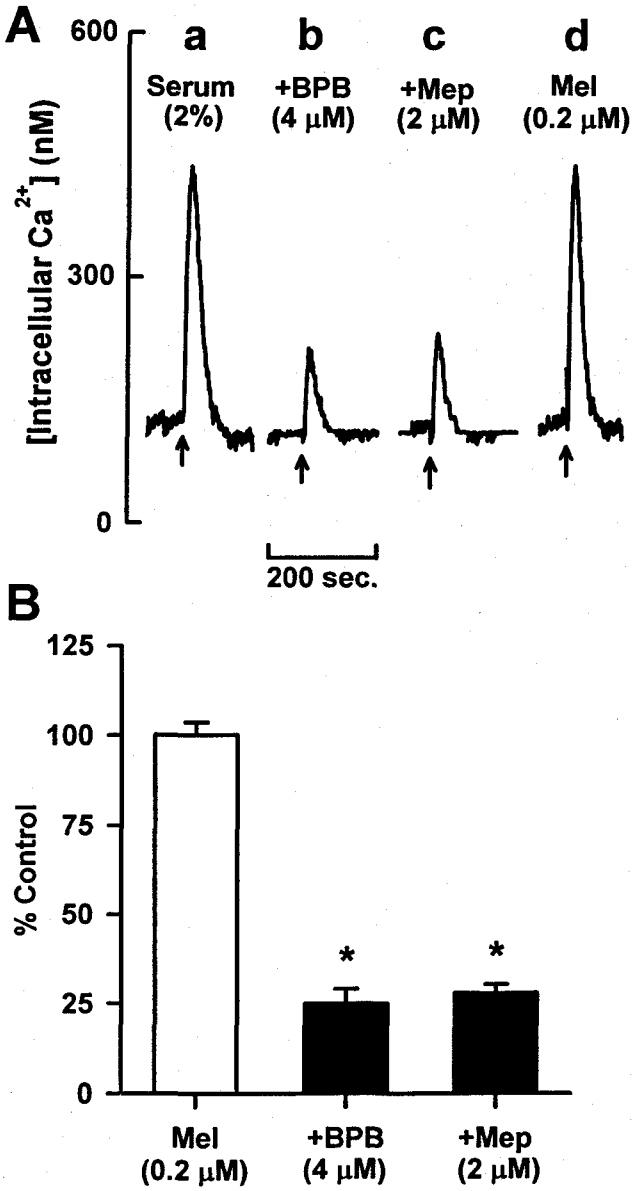

Fig. 3. Effects of PLA_2 inhibitors on serum or Mel-induced intracellular Ca^{2+} release in SK-N-MC human neuroblastoma cells. Aliquots of 2×10^6 cells/ml were incubated with 2 μM of Fura-2/AM for 60 min at room temperature (22~23°C). The cells were washed, resuspended in Ca^{2+}-free buffer solution, and transferred to a quartz cuvet for fluorescence measurements. The data (A) represent intracellular Ca^{2+} changes with time. Arrows show the time points for addition of 2% of serum (a, b and c) or 0.2 μM of Mel (d). In these experiments 4 μM of BPB (b) or 2 μM of Mep (c) were applied 3 min before fluorescence measurements. Quantitative changes (B) were expressed as percent changes of the increased intracellular Ca^{2+} concentration induced by the drug compared to Mel alone. Each column represents the mean value of four replications with bars indicating SEM (*$p<0.05$ compared to Mel alone).

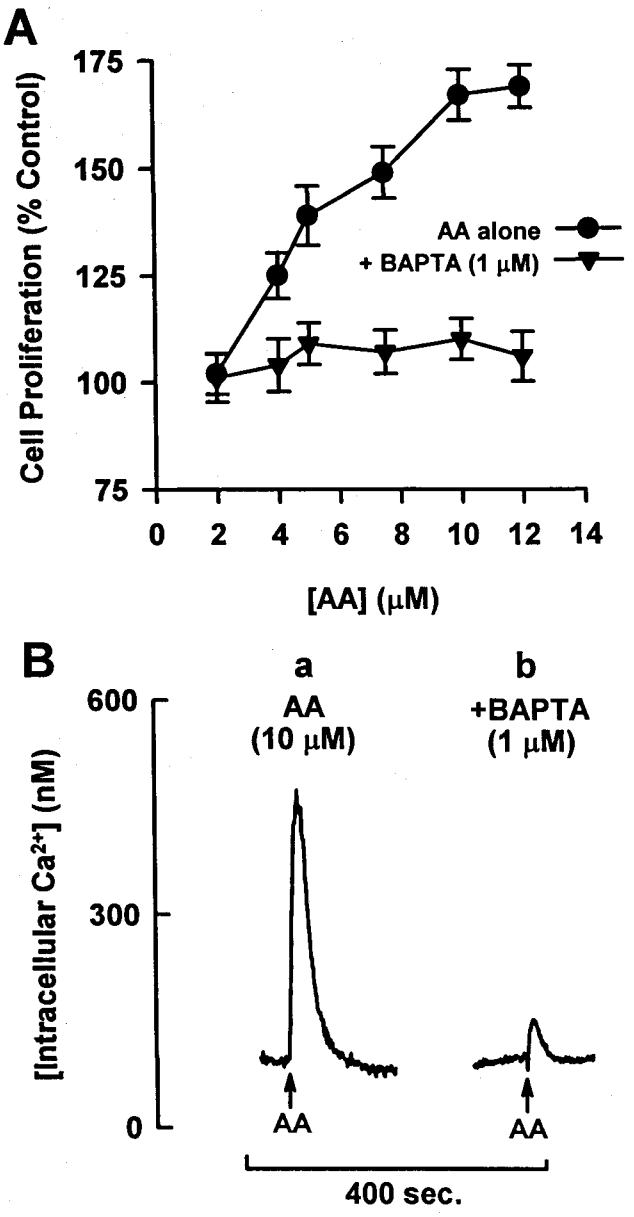

Fig. 4. Inhibitory effects of BAPTA/AM, an intracellular Ca^{2+} chelator, on AA-induced enhancement of cell growth (A) and increased intracellular Ca^{2+} concentration (B) in SK-N-MC human neuroblastoma cells. Cell growth and intracellular Ca^{2+} concentration were assessed by MTT staining and Fura-2/AM fluorescence techniques, respectively. Experimental procedures of fluorescence measurements were the same as Fig. 3. The results (A) are expressed as percent change of control condition in which the cells were grown in medium containing drug-free vehicle. Data points represent the mean values of four replications with bars indicating SEM. The data (B) represent intracellular Ca^{2+} changes with time. Arrows show the time points for addition of 10 μM of AA. One micromole of BAPTA/AM was applied 3 min before fluorescence measurements (b).

blocked the Mel-induced intracellular Ca^{2+} release shown in Fig. 3B.

Involvement of intracellular Ca^{2+} signals in the stimulation of cell growth by AA

To clarify the involvement of PLA_2 in the cell growth regulation, the effects of AA, a product of the PLA_2-catalyzed reaction, on the tumor cell growth were examined. Exogenous administration of AA stimulated the tumor cell growth in a dose-dependent fashion shown in Fig. 4A. The role of intracellular Ca^{2+} in the mechanism by which AA stimulates the tumor cell growth was also tested. The growth-stimulatory effects of AA were significantly reversed by the co-treatment with 1 μM of BAPTA/AM, an intracellular Ca^{2+} chelator (Jiang et al, 1994), shown in Fig. 4A. In addition, AA induced intracellular Ca^{2+} release at the growth-stimulatory concentration (10 μM) and pretreatment with 1 μM of BAPTA/AM significantly inhibited the AA-induced intracellular Ca^{2+} release as depicted in Fig. 4B.

DISCUSSION

The results of the present study clearly demonstrate that PLA_2 regulates the growth of the human neuroblastoma cells. The following observations give evidence on the growth-regulatory action of PLA_2. Inhibition of PLA_2 by BPB or Mep suppressed the growth of the tumor cells in a dose-dependent manner without cytotoxicity (Fig. 1). The stimulation of PLA_2 activity by Mel enhanced the growth of the tumor cells in a concentration-dependent manner (Fig. 2). The Mel-induced growth enhancement was significantly reversed by the co-treatment with PLA_2 inhibitors (Fig. 2). In addition, AA stimulated the tumor cell growth in a dose-related fashion (Fig. 4A).

The exact mechanism by which PLA_2 influences cellular proliferation is not known. However, the results of this study suggest that intracellular Ca^{2+} may mediate the regulatory effects of PLA_2 on the tumor cell growth (Figs. 3 and 4). The PLA_2 activation by Mel induced intracellular Ca^{2+} release (Fig. 3A). The Mel-induced intracellular Ca^{2+} release was significantly blocked by pre-treatment with PLA_2 inhibitors (Fig. 3B). Interestingly, the intracellular Ca^{2+} release by serum, a known intracellular Ca^{2+} releasing agent in the tumor cells, was also

significantly inhibited by pre-treatment with PLA_2 inhibitors (Fig. 3A). The growth-stimulatory effects of AA were significantly reversed by the co-treatment with BAPTA/AM, an intracellular Ca^{2+} chelator (Fig. 4A). AA induced intracellular Ca^{2+} release, which was significantly blocked by the pre-treatment with BAPTA/AM (Fig. 4B). Moreover, the results of our previous studies also showed that in the human neuroblastoma cells increased intracellular Ca^{2+} concentration stimulates cellular proliferation (Lee et al, 1994; Lee et al, 1995).

Other studies also imply that intracellular Ca^{2+} plays an important role in cellular proliferation in many cell types (Metcalfe et al, 1986; Whitfield et al, 1987; Geck & Bereiter-Hahn, 1991; Ogata et al, 1991). The intracellular Ca^{2+} is increased at anaphase initiation of the cell cycle (Boynton, 1988). Various growth factors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF) and insulin-like growth factor II (IGF-II), have been shown to increase intracellular Ca^{2+} levels in a variety of cell types, including human fibroblast cells (Moolenaar et al, 1984), vascular smooth muscle cells (Roe et al, 1989), osteoblastic cells (Loza et al, 1995) and BALB/c-3T3 cells (Nishimoto et al, 1987). Total cellular Ca^{2+} levels are increased in exponentially growing transformed cells (Veigl et al, 1982). The critical role of intracellular Ca^{2+} in cell proliferation is also derived from the results of the experiments in which the researchers have used inhibitors of intracellular Ca^{2+} increases. In addition to inhibition of intracellular Ca^{2+} increases, these agents have also been shown to block cellular proliferation (Jensen et al, 1995; Kataoka et al, 1997). The downstream events of increased intracellular Ca^{2+} levels as a signal transduction mechanism of cellular proliferation appear to be the activation of calmodulin (CaM), a Ca^{2+}-binding protein (Rasmussen & Means, 1989; Katayama et al, 1990), and cyclin-dependent protein kinases (Whitfield et al, 1995).

In conclusion, the modulation of PLA_2 activity is a regulatory mechanism of cell growth in human neuroblastoma cells. Intracellular Ca^{2+} may act as a key mediator in these actions. The results of this study further suggest that PLA_2 may be a good target enzyme for the study on the chemotherapeutic intervention of human neuroblastomas.

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