Bioscience Reports, Vol. 20, No. 3, 2000

Intracellular Free Calcium Concentration and Cisplatin Resistance in Human Lung Adenocarcinoma A_{549} Cells

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Received October 21, 1999; accepted May 11, 2000

Human lung adenocarcinoma A_{549} cells sensitive and A_{549}/DDP cells resistant to Cis-dichlorodiammine platinum[II] (cisplatin) exhibit different intracellular free calcium and calcium fluorescence images labeled with Fura-2/AM and Fluo-3/AM as judged by dual-excitation fluorescence assay, Miracal Imaging and Laser Scanning Confocal Microscopy (LSCM) of single cells. The concentration of intracellular free calcium of the resistant A_{549}/DDP cells is one third that of the sensitive A_{549} cells. The efflux of Rhodamine 123 in resistant A_{549}/DDP cells is faster than that in sensitive A_{549} cells. In addition, A_{549}/DDP cells have an increase of Phosphatidylinositol 4-kinase (PtdIns 4-kinase) activity in the plasma membrane. So it is tentatively suggested that the increase in PtIns 4-kinase activity resulting from lower intracellular Ca^{2+} concentration leads to an increase of its enzymatic products——PIP and PIP_2, which may stimulate the activity of P-glycoprotein.

KEY WORDS: Human lung adenocarcinoma A_{549} cells; cisplatin resistant; intracellular free Ca^{2+}.

ABBREVIATIONS: PtdIns 4-kinase, phosphatidylinositol 4-kinase; $[Ca^{2+}]$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_0$, extracellular Ca^{2+} concentration; PgP, P-glycoprotein; MDR, multidrug resistance.

INTRODUCTION

Chemotherapy can improve long term survival of cancer patients. However, it often results in the development of tumors which are resistant to the cytotoxic drugs most commonly used in chemotherapy. This phenomenon, known as multidrug resistance (MDR), is characterized by a decreased sensitivity of tumor cells not only to the particular drug employed for chemotherapy but also to broad spectrum of drugs with neither obvious structural similarity nor common targets [1]. MDR is a major obstacle in the treatment of cancer patients and the cause of MDR is associated with overexpression of an ATP-dependent plasma membrane transport protein known as P-glycoprotein (Pgp) [2, 3]. A central goal in the study of multidrug resistance of chemotherapy is to understand how tumor cells become drug resistant by lowering the intracellular concentration of antitumor agents [4].

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0144-8463/00/0600-0129$18.00/0

Axiotis *et al*. [5] reported that extracellular calcium ($[Ca^{2+}]_0$) could up-regulate Pgp expression of clonal parathyroid epithelial (PT-r) cells by Western and Northern blot analysis and immunohistochemistry. Furthermore, Mestdagh *et al*. [6] indicated that the intracellular Ca^{2+} concentration of MCF-7 cells sensitive to adriamycin was 2 times less than that observed in MCF-7/ADR resistant cells. But, Tsuruo *et al*. [7] did not find a significant difference of the intracellular free Ca^{2+} concentration between P388 leukemia cell lines sensitive and resistant to 5-fluorouraci. Possibly, this may be due to the different mechanisms of MDR for different tumor cells or for the same cell line treated with different drugs. In view of the multitude of factors involved in drug resistance of tumor cells, the molecular mechanism of MDR still remains poorly understood.

In the present paper, by using double excitation wavelength, fluorescence assay, Miracal Imaging System and Laser Scanning Confocal Microscopy (LSCM), the intracellular free calcium in, and calcium fluorescence images of A_{549} and A_{549}/DDP cells in the resting state at population and single cell levels were measured and compared. The efflux of Rhodamine 123 and actvity of PtdIns 4-kinase in the two cell lines were also studied. The results described here indicate a relationship between MDR and the change of intracellular free Ca^{2+} of human lung adenocarcinoma A_{549} cells and may provide a further basis for exploring the molecular mechanism of multidrug resistance in tumor cells.

MATERIALS AND METHODS

Reagents

Fluo-3/AM (acetoxymethyl ester) was purchased from Molecular Probes Co. (Eugene, OR, USA). Fura-2/AM was from Drug Institute of Chinese Medical Academy and dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 1 mmol/L and stored at –20°C. Pluronic F-127 and phosphatidylinositol were from Sigma (St. Louis, MO, USA). RPMI-1640 medium and fetal bovine serum were obtained from Gibco Co. (Chagrin Falls, OH, USA). [γ-^{32}P]ATP was from Beijing Yahun Biomedical Engineering Co. Rhodamine 123 was from Adrich Chemical Co. (Milwaukee, WI, USA). All other reagents were local products of analytical grade.

Cell Lines and Cell Culture

The parental cell line A_{549} (a human lung adenocarcinoma cell line) and the MDR cell line A_{549}/DDP, which is resistant to cisplatin (CDDP or DDP), were generously provided by Beijing Tumor Institute. Cells were grown as adherent monolayers on 75 cm flasks in RPMI-1640 (pH 7.35) medium supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 units/ml streptomycin at 37°C in a humidified incubator (Life Science Co) at 5% CO_2. A_{549}/DDP was cultured for 20 generations and did not alter its resistant phenotype. A_{549}/DDP cells were 8 times more resistant to cisplatin than A_{549} cells [8]. Cell cultures used for the experiments were in the logarithmic phase of growth.

A_{549} and A_{549}/DDP Loaded with Fura-2/AM

A_{549} and A_{549}/DDP cells were harvested from flasks with 0.25% trypsin and 0.02% EGTA, and washed twice with buffer A (5 mmol/L KCl, 2 mmol/L $CaCl_2$, 0.5 mmol/L KH_2PO_4, 137 mmol/L NaCl, 4 mmol/L $NaHCO_3$, 0.2 mmol/L Na_2HPO_4). The cells resuspended in the buffer A (10^5–10^6 cells/ml) were loaded with Fura-2/AM at a concentration of 4 μmol/L for 45 minutes [9]. Measurement of the fluorescence change of Fura-2 after Ca^{2+} binding was carried out in a F-4500 spectrofluorometer (Hitachi) at 37°C. The excitation wavelength was 340 nm and 380 nm, and emission wavelength was 500 nm. Calculation of intracellular free Ca^{2+} concentration was made according to the following equation [9]:

$$[Ca^2]_i = K_d \times \beta \times [(R - R_{min})/(R_{max} - R)]$$

Where K_d is the dissociation constant for Ca^{2+} bound to Fura-2 (224 nM), $R = F_{340}/F_{380}$; $\beta = F_{380;min}/F_{380;max}$; $R_{max} = F_{340;max}/F_{380;max}$; $R_{min} = F_{340,min}/F_{380,min}$. $F_{340,max}$ and $F_{380,max}$ are the fluorescence excited at 340 nm and 380 nm respectively at saturating Ca^{2+} concentration (when Fura-2 completely binds to Ca^{2+} after 0.3% Triton X-100 was added). $F_{340,min}$ and $F_{380,min}$ are the fluorescence excited at 340 nm and 380 nm respectively at zero Ca^{2+} concentration (when Ca^{2+} is completely chelated with 10 mmol/L EGTA) [10].

In addition, calcium images and the relative concentration of intracellular free Ca^{2+} were obtained using a Miracal Imaging System and Miracal Version 2.3 software (Life Sciences, Cambridge, UK). Loading of A_{549} and A_{549}/DDP cells with Fura-2/AM was performed as described above. The excitation wavelength was shifted from 380 to 340 nm after Ca^{2+} was bound to Fura-2. Therefore, the relative Ca^{2+} concentration was obtained based on the fluorescence ratio after dual-wavelength excitation.

Ca^{2+} Fluorescence Image Measured by Laser Scanning Confocal Microscopy

Cells (1×10^6 cells/ml) cultured in sterile Petri dishes (MatTek Co, USA) with quartz coverslips for 12 hours were loaded in RPMI-1640 medium containing 9 μmol/L Fluo-3/AM and 1 μl 25% Pluronic F-127 (dissolved in DMSO), and cultured at 37°C for 60 min in 5% CO_2 incubator. The labeled cells attached to coverslips were shaken at least 3 times during the incubation period and were washed three times with PBS to remove the unloaded Fluo-3/AM in the medium. After the loading with the fluorescence probes, the images of Ca^{2+} concentration in arbitrary single cells were monitored by Laser Confocal Scanning Microscopy (LSCM Leica, TCS-NT type, Germany) at 488 nm excitation wavelength.

Measurement of Rhodamine 123 Efflux by Flow Cytometry

The active efflux of Rhodamine 123 was measured using method of Dudeja *et al*. [11] with modification [11]. The logarithmic phase tumor cells were harvested from flasks with 0.25% trypsin and 1 mmol/L EDTA. The single cells suspended in RPMI-1640 (1×10^6 cells/ml) were incubated in 0.5 μg/ml Rhodamine 123 for 60

minutes at 37°C. After washing three times with RPMI-1640 medium, the stained tumor cells were analyzed immediately using a Coulter Epics XL Flow Cytometer (Coulter Co., USA), and after subseqent incubations, without dye, for up to 80 minutes.

Assay for PtdIns 4-kinase Activity of A_{549} and A_{549}/DDP Cell Membranes

PtdIns 4-kinase activity was assayed as described by Glenn *et al*. [12]. In brief, cells (4×10^9) were disrupted by repeatedly freeze-thawing in liquid nitrogen, the plasma membranes of the cells were prepared by two cycles of sucrose gradient (30%, w/v) ultracentrifugation at 18,000*g* for the first spin and 100,000*g* for the second spin (Beckman L8-80M, Ti80 rotor) for 60 minutes. 2 μl of solution containing 4.1 μCi [γ-^{32}P]ATP, 5 mmol/LATP, 50 mmol/L Tris-HCL, pH 7.5 was added to the reaction medium consisting of 15 μg of the membrane proteins and 45 μL of assay buffers (20 mmol/L Tris-HCL pH 7.5, 10 mmol/L DTT, 10 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.4% Triton X-100, 200 μg/mL PtdIns) to initiate the reaction, and incubated at 25°C for 30 minutes. The reaction was stopped with 80 μL of 1N hydrocloric acid. Lipids were extracted from the samples by twice washing with 600 μL of 2:1 (V/V) chloroform:methanol. The organic phase was collected and dried in vacuum at 120°C. ^{32}P was measured in a Pharmacia LKB-2202 automatic liquid scintillation counter.

RESULTS

The Free Ca^{2+} Concentration in A_{549}/DDP Cells Resistant to Cisplatin is Significantly Lower than That in A_{549} Sensitive Cells

The membrane-permeable Fura-2/AM is hydrolyzed by the endogenous acetoxymethyl esterase to form Fura-2 which is characterized by low fluorescence. Fura-2 specifically binds to intracellular free Ca^{2+} and the Fura-2-Ca^{2+} complex shows strong fluorescence [13]. The excitation wavelength of Fura-2 and the Fura-2-Ca^{2+} complex are 380 nm and 340 nm, respectively. We measured intracellular free Ca^{2+} by fluorescence spectroscopy using dual-exciting wavelengths [10]. The result shows that the intracellular free Ca^{2+} of A_{549} and A_{549}/DDP cells in the resting state were 294.86 ± 29.89 and 94.99 ± 5.74 nmol/L respectively (mean $\pm$ SEM of nine experiments). It indicated clearly that intracellular free Ca^{2+} concentration of A_{549} sensitive cells was about three times higher than that of A_{549}/DDP cells. The significant difference can be further confirmed by calcium image analysis and concentration measurement of the two cell lines using the Miracal Imaging System, which is designed specially for analyzing changes of intracellular free Ca^{2+}. Calcium images of ten cells of the two cell lines analyzed at three different areas within chosen cells were measured and statistically analyzed. The fluorescence intensity of each sample was recorded and the background fluorescence was subtracted. The fluorescence intensity for A_{549} and A_{549}/DDP were 265 ± 12.5 and 78.2 ± 4.1 (mean $\pm$ SEM of three experiments) respectively. The results also indicate that the relative intracellular Ca^{2+} concentration in A_{549} cells was three times more than in A_{549}/DDP- cells.

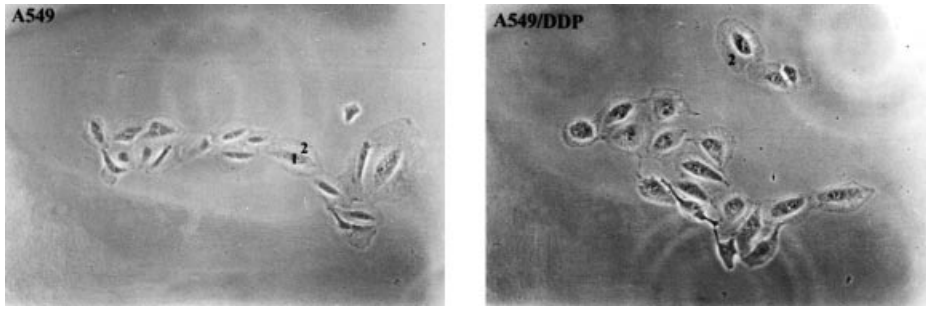

Fig. 1. Phase contrast images of the A_{549} (left) and A_{549}/DDP (right) cells. Cells cultured in RPMI-1640 were grown in the flask and viewed directly under the Nikon Phase Contrast Inverted Microscopy (Diaphot 300 type, Nikon Co., Japan). A_{549} cells (left) and A_{549}/DDP cells (right). The darker area surrounded by a halo (1) is the nucleus, and the less dark area around it (2) is the cytoplasm.

Intracellular Ca^{2+} Fluorescence Signal in A Single Cell Monitored by Laser Scanning Confocal Microscopy (LSCM)

Cells of the two cell lines were loaded with the fluorescent dye Fluo-3/AM as described above for Fura-2/AM [14]. Figure 1 shows phase contrast images of the A_{549} (left) and A_{549}/DDP (right) cells. There is no significant difference of morphology between the two cell lines. Fluorescence intensity and distribution of the Ca^{2+} fluorescence signal in a single cell were measured by LSCM at 488 nm excitation wavelength. Figure 2 shows confocal images and Ca^{2+} distribution in A_{549} single cells (left) and A_{549}/DDP single cells (right). The results clearly indicate that the Ca^{2+} fluorescence intensity and distribution in A_{549} (sensitive) and A_{549}/DDP cells

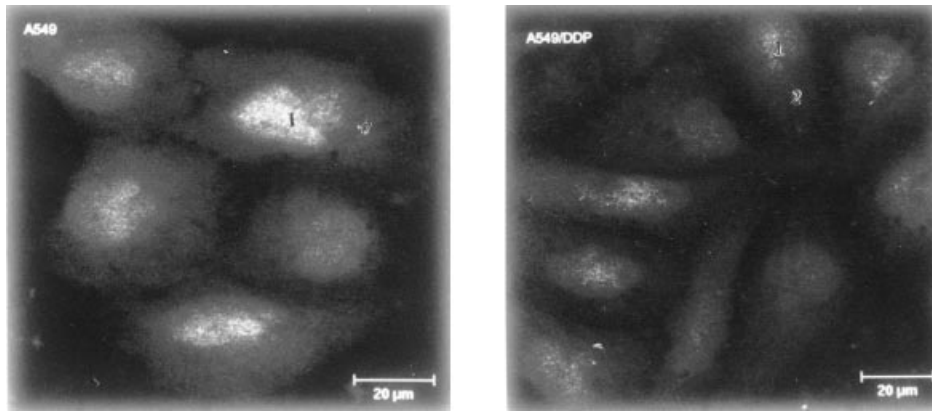

Fig. 2. Calcium fluorescence signal images of single A_{549} (left) and A_{549}/DDP (right) cells detected by LSCM. Cells were grown in the sterile Petri dish and then labelled as described in "Materials and Methods". Relative levels of intracellular free calcium are indicated by the fluorescence intensity of the Fluo-3-Ca^{2+} complex detected by LSCM (TCS-NT type, Leica Co., Germany) at 488 nm excitation wavelength. The bright area (1) corresponds to the nucleus, and the darker area surrounding it (2) is the cytoplasm.

(resistant to cisplatin) is different. It is also obvious that the distribution of Ca^{2+} within the two cells is heterogeneous. The differences observed in the Ca^{2+} fluroescence intensity and distribution (Fig. 2) can be further analyzed using image analysis software (Leica TCS NT/SP, Vers 1.6.582) of Leica Confocal Microscope Systems (Leica Co., Germany) (Fig. 3). The plot shows fluorescence intensity along an axis drawn through the cell; fluorescence intensity was automatically calculated by an internal calcium standard. The total fluorescence intensity of Ca^{2+} in an integral area for A_{549} and A_{549}/DDP were 138.6 ± 10.2 and 56.9 ± 6.8m, respectively (mean value $\pm$ SEM for 12 viable single cells). This confirms that a significant, three-fold difference of free Ca^{2+} concentration exists between sensitive A_{549} cells and resistant A_{549}/DDP cells. It is interesting to note that the Ca^{2+} fluorescence signal is strongest in the middle part (a in Fig. 3) (relative fluorescence intensity was 224) of the integral area curve measured for A_{549} cells compared with that of A_{549}/DDP cells (relative fluorescence intensity was 76.5). This could mean that the Ca^{2+} concentration in the nucleus was higher than that in the cytoplasm, but such distribution difference was not observed in the case of A_{549}/DDP cells. Anyway, all of the results obtained from three approaches above showed consistently that intracellular free Ca^{2+} concentration of A_{549} cells sensitive to cisplatin was significantly higher than that of A_{549}/DDP resistant to the drug.

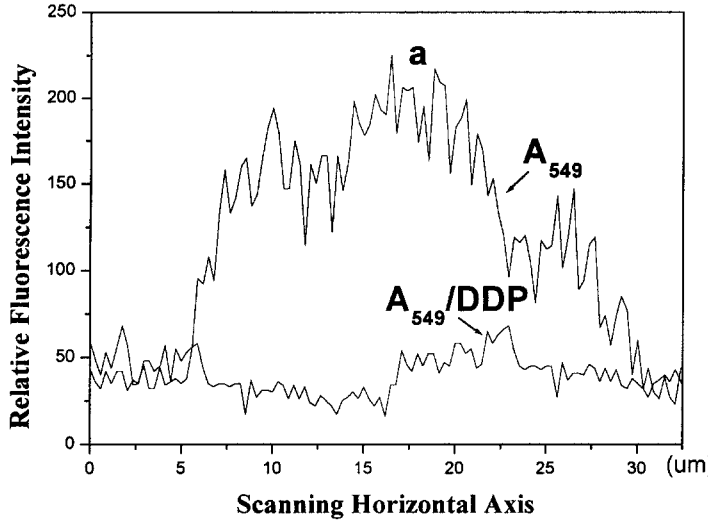

Fig. 3. Laser scanning picture of the Ca^{2+} fluorescence signal in A_{549} and A_{549}/DDP cells. Fluorescence signals of the two cells were scanned along an axis through the nuclei of a single cell. The data were collected with image analysis software (Leica TCS NT/SP, Vers 1.6.582) of Leica Confocal Microscope Systems. The calcium fluorescence images in Fig. 2 can be converted into fluorescence intensity and plotted automatically as indicated on the vertical axis in Fig. 3. Data represents the result from one of two single cell experiments which was repeated independently 6 times and the similar results were obtained.

Transmembrane Efflux Rate of Rhodamine 123 in A_{549}/DDP is Faster than That in A_{549} Cell

At present, is is mostly known that PgP170 (P-glycoprotein) contributes to the multidrug resistance of cancer cells [15], and one assay for the drug resistance is to monitor the efflux of a fluorescent dye like Rhodamine 123 [16]. So, this fluorescent probe was used to compare efflux characteristics from the resistant A_{549}/DDP and sensitive A_{549} cells using flow cytometry (Fig. 4). The results indicate that the efflux of Rodamine 123 from A_{549}/DDP cells is almost three times faster than that from A_{549} cells. This result is compatible with the notion that drug efflux is an important component of the drug-resistant phenotype of A_{549}/DDP cells.

Activity of PtdIns 4-kinase in A_{549} and A_{549}/DDP Cells

A suitable transmembrane Ca^{2+} gradient across the plasma membrane is important for the proper conformation and activities of membrane proteins including membrane bound enzymes [17, 18]. Low intracellular free Ca^{2+} in resistant of A_{549}/DDP cells would result in a change of the transmembrane Ca^{2+} gradient which may affect activities of membrane enzymes. PtdIns 4-kinase, an integral membrane protein, plays an important role in signal transduction and its enzymatic products (PIP, PIP_2) are involved in modulating the function of channels and other transmembrane proteins. We measured and compared the activity of PtdIns 4-kinase in A_{549} and A_{549}/DDP cells. PtdIns 4-kinase activity for sensitive A_{549} and resistant A_{549}/

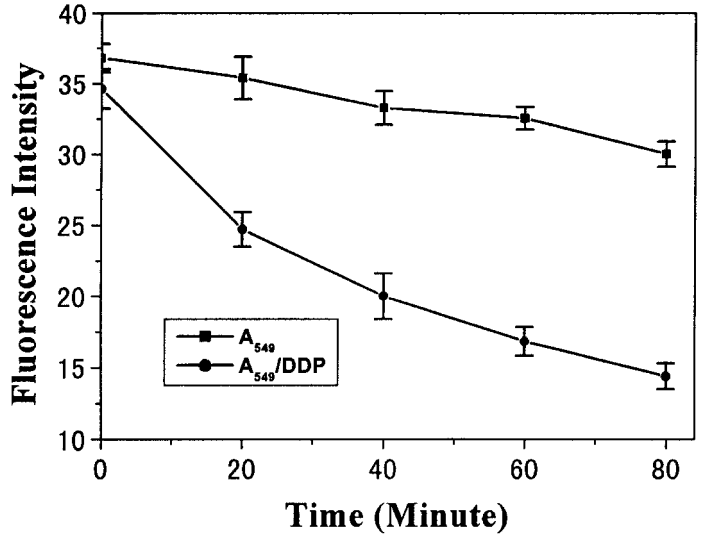

Fig. 4. Change of fluorescence intensity of Rhodamine-123 during effluxing of the dye from A_{549} and A_{549}/DDP cells. The stained cells were analyzed by flow cytometry with confocal optics for Rhodamine 123 fluorescence. Excitation was 48 nm by an argon ion laser, data were obtained from 10,000 viable cells. Values were means ± SEM of three independent determinations.

DDP cells were 0.35 ± 0.003 and 0.46 ± 0.005 pmol/mg · min (mean ± SEM of three experiments, $p < 0.05$) respectively, the activity of PtdIns 4-kinase of the latter was increased about 31% compared with that of the former.

DISCUSSION

Calcium, as a second messenger, takes part in many cellular functions such as secretion, movement, metabolism, differentiation and proliferation in cellular life cycles [19]. Ca^{2+} homeostasis is critical for regulation of cell function. Therefore, studies on changes of intracellular Ca^{2+} are of significant importance in understanding functional changes of cells in physiological and pathological conditions. Multidrug resistance is characterized by a decreased sensitivity of tumor cells to a broad spectrum of drugs. Measurements of intracellular Ca^{2+} concentration in drug sensitive and resistant cells from different laboratories are inconsistent Mestdagh *et al*. [6] indicate that the Ca^{2+} concentration of MCF-7 cells sensitive to adriamycin was 2 times less than that observed in MCF-7/ADR resistant cells. Nygren *et al*. [20] also report that the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was elevated in the cisplatin-resistant U1285-250 lung cancer cells by 51% compared with parental cells. Tsuruo *et al*. [7] found no significant difference of the intracellular free Ca^{2+} concentration between sensitive and resistant cell lines. The precise mechanisms setting intracellular Ca^{2+} and MDR of tumor cells have not yet been fully elucidated. It is possible that there are different mechanisms of MDR for different cell lines or for the same cell line treated with different drugs. A_{549} cells are human airway epithelial cells. Ca^{2+} plays a central role in epithelial cell function, including regulation of ion transport, mucus secretion, and ciliary beat frequency [21]. A_{549}/DDP cells were derived from wild type A_{549} cells using a stepwise selection protocol of increasing cisplatin concentration which has some special properties with respect to its biological activity [22, 23]. The intracellular free Ca^{2+} concentration determined here were obtained using three different approaches. In all cases, significantly lower intracellular free Ca^{2+} concentrations were found in the resistant A_{549}/DDP cells than in the sensitive A_{549} cells. Values of intracellular free Ca^{2+} of human lung adenocarcinoma cells resistant to cisplatin have not previously been reported. Our results show that there is a difference both in the average intracellular free Ca^{2+} and in Ca^{2+} distribution (in time and space) between the sensitive A_{549} and resistant A_{549}/DDP cell lines.

PtdIns 4-kinase is an integral membrane protein, that catalyzes the conversion of PtdIns to PIP and PIP2 which play an important role in phosphatidylinositol signal transduction pathway [24, 25]. Reinhard *et al*. [26] reported that the activity of purified PtdIns 4-kinase from the human erythrocyte membrane *in vitro* was affected by Ca^{2+} and was inhibited with increasing of Ca^{2+} concentration. We found that a higher PtdIns 4-kinase actvity was observed in the resistant A_{549}/DDP cells with a lower intracellular free Ca^{2+} concentration.

What is the relationship betwee the changes of intracellular free Ca^{2+} concentration, activity of PtdIns 4-kinase and multidrug resistance of A_{549} cells? It is well known that efflux of Rhodamine 123, a fluorescence dye, can be used to detect cells resistant or sensitive to drugs [16]. Our results clearly show that efflux of Rhodamine

123 is significantly faster from resistant A_{549}/DDP cells which have lower intracellular free Ca^{2+} concentration (see Fig. 4). We have detected the presence of Pgp on the plasma membrane of A_{549} cells, using Pgp monoclonal antibody McAb F4 (from Sigma Co., Clone No. F4) assay (data not shown). The difference of efflux of Rhodamine 123 between the two cells might correlate with the Pgp activity of the cells. Axiotis *et al*. [5] indicated that the P-glycoprotein expression is regulated by extracellular calcium [5]. Witkowski *et al*. [27] also demonstrated that T lymphocyte cells that express high levels of P-glycoprotein (Pgp^+ cells) may have a specific defect in intracellular calcium signal generation, and do indeed show smaller changes in intracellular calcium ion concentration than Pgp^- cells when activated by activators. However, the molecular basis for the abnormality in intracellular calcium signal still needs to be further elucidated for various cancer cells resistant to different drugs [28]. In the present paper, we also show that the activity of PtdIns 4-kinase of A_{549}/DDP cells with lower intracellular Ca^{2+} concentration was higher than that of sensitive A_{549} cells. Misra *et al*. [29] revealed that PI_3-Kinase activity and lipid product (PIP_2) were implicated in regulation of ATP-dependent transport by P-glycoprotein and multidrug resistance associated protein. So, it is possible that enzymatic products like PIP or PIP_2 produced by PtdIns 4-kinase with higher activity modulated by lower intracellular Ca^{2+} concentration may stimulate the P-glycoprotein which pumps the drugs out of the tumor cells.

In conclusion, intracellular free Ca^{2+} in the A_{549}/DDP cell line resistant to cisplatin monitored by three different approaches was threefold lower than that of the sensitive A^{2+} cells. The decreased intracellular free Ca^{2+} may regulate the activity of PtdIns 4-kinase to increase its products, PIP and PIP_2, which could in turn stimulate the activity of P-glycoprotein to pump cisplatin out of the cells. Related studies are still in progress.

ACKNOWLEDGEMENT

This work was generously supported by project 39730130 from the National Natural Science Foundation of China and key project KJ 951-B1-609 from Chinese Academy of Sciences. The authors are grateful to Prof. Liu Xuyi for kindly providing the cell lines, and the helpful discussions with Prof. Yang Fuyu as well as help by Drs. Gao Jin and Yue Jiachang in the studies.

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