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Changes in the Expression of the Tumor-Associated Antigen Recognized by Monoclonal Antibody 44-3A6 in A549 Cells due to Calcium

Key Words
Adenocarcinoma
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Calcium ion
CaM-Ca^{2+}-ATPase

Abstract

Although there is extensive data available on Ca^{2+} effects in normal tissues, comparatively little is known about its effects or regulation in tumor cells. The present studies were undertaken to investigate whether various extracellular calcium concentrations could modulate the expression of the tumor-associated antigen (TAA) recognized by monoclonal antibody (MAb) 44-3A6. It is highly expressed by the human lung adenocarcinoma cell line A549 and has been shown to be a 40-kD integral plasma membrane protein. Treatment of the A549 cell line with various concentrations of exogenous calcium showed a dose-dependent rise in the internal free calcium levels up to 2.4–2.9 m*M* (external calcium treatment). At higher concentrations, the internal calcium level showed a decline, indicating a higher calcium efflux. The calmodulin-dependent Ca^{2+}-ATPase enzyme involved in calcium homeostasis was assayed under these same conditions. The enzyme activity increased with increasing external calcium concentrations showing a 5-fold increase in cells treated with 4.05 m*M* calcium. These data suggest that as the internal calcium approaches toxic levels, the Ca^{2+}-regulated ATPase activity increases to reduce the calcium overload within the cell. Employing Western blot analysis and immunoperoxidase staining studies, this report shows that the antigen recognized by MAb 44-3A5 on A549 cells increased with an increase in calcium concentration. Evidence that this antigen is phosphorylated is presented using Western blot analysis of a radiolabeled antigen-enriched plasma membrane fraction. The previously reported subcellular localization, and now the phosphorylation and responsiveness to calcium by this TAA, gives it the properties predicted to be seen in a calcium 'pump-like' molecule. Thus, these studies support the hypothesis that this TAA may be important in intracellular calcium concentration control or that it is regulated via some calcium-mediated process.

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Introduction

Calcium has been implicated to play a critical role in the control of a wide range of cellular functions including excitability, motility and cell division [1–4]. It has long been known that changes in free cytosolic calcium can trigger cellular responses in nonneoplastic cells [5]. A definite but complex role of both extracellular and intracellular Ca^{2+} ions has been demonstrated in different cell types. The processes which are required for cell proliferation are highly regulated and depend on a variety of responses to the appropriate external signals such as hormones, growth factors and calcium ions. Considerable effort has been applied to understanding the roles of hormones and growth factors on neoplastic cells, where comparatively little is known about calcium ion effects.

Advances in tumor immunology have provided reagents which can be used to assess the biological properties of various tumors and tumor subsets. This approach has led to the elucidation of select antigens which are frequently expressed by morphologically similar neoplasms. One such tumor-associated antigen is detected by the monoclonal antibody (MAb) 44-3A6. This antigen has been extensively studied in human tumor tissue and has been shown to be a good marker for adenocarcinomas [6–11]. In the human pulmonary adenocarcinoma cell line A549, this antigen has been shown to be a 40-kD cell surface protein [12]. Studies using fluorescence-activated cell sorter analysis and immunogold-EM methods have demonstrated that this antigen is not cell cycle-specific, nonmodulated and expressed on the extracellular surface of the plasma membrane [13]. Like many of the tumor-associated antigens which have been studied because of their select expression by various morphological subtypes, little is known about the function of this antigen.

In view of our limited understanding of the effects of calcium on human pulmonary tumors, we questioned how the plasma membrane-bound calmodulin-dependent Ca^{2+}-ATPase (CaM-Ca^{2+}-ATPase) would respond in A549 cells upon exposure to various extracellular calcium concentrations. Changes in enzyme activity would serve as a physiological monitor for Ca_i^{2+} since it is important in the maintenance of calcium homenostasis. In addition, since little is known about the modulatory effects of calcium on tumor-associated antigen expression within these cells, we questioned whether the tumor-associated antigen recognized by MAb 44-3A6 could be modulated by calcium. The demonstration of a cell surface tumor-associated antigen which was modulated by extracellular calcium (which paralleled an increase in Ca^{2+}-ATPase activity) would be an important step toward understanding the possible function and/or regulation of that gene product.

Materials and Methods

Growth of A549 Cells

The human lung adenocarcinoma cell line was grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum, *L*-glutamine, and antibiotic-antimycotic solution as previously reported [9]. The RPMI 1640, besides other ingredients, contains 0.5 m*M* calcium. In experiments requiring extra calcium, the cells were grown in RPMI with added $CaCl_2$ solution corresponding to final calcium concentrations of 0.5, 1.3, 1.85, 2.4, 2.95, 3.55, and 4.05 m*M*. The cells were scraped from the flasks after 48 h and subjected to washing with cold physiological salt solution (PSS; all in m*M*: Na^+ = 190, K^+ = 5.0, Ca^{2+} = 0.5, Mg^{2+} = 1.2, glucose = 10, HEPES, pH 7.4 = 10) containing 0.1% bovine serum albumin. The cells from each group were suspended in the same volume of cold PSS. The PSS for treated groups was supplemented with additional calcium equivalent to their treatments. The cell suspensions were divided for four studies: (a) Ca_i^{2+} measurements, (b) Ca^{2+}-ATPase activity, (c) trypan blue exclusion, (d) Western blotting.

Simultaneously, cells were grown in chamber slides exposed to various concentrations of calcium. The cells were allowed to grow for 48 h, after which the medium was aspirated. The cells were fixed in 10% neutral buffered formalin for 1 h. The slides were washed in phosphate-buffered saline and immunostained using the avidin-biotin complex immunoperoxidase technique as routinely used in our laboratory [12, 13].

Ca_i^{2+} Measurements

The cells were loaded with Fura-2 by incubating them with 5 μ*M* Fura-2/AM at 37 °C for 45 min. The washings were carried out in the cold with PSS by centrifuging at 2,000 *g* for 10 min. The last wash was checked for background fluorescence. A known number of cells were added to the quartz cuvette containing normal PSS with 1 m*M* $LaCl_3$. The cells were equilibrated for 5–7 min at 37 °C with gentle stirring. Fluorescence was recorded at 510 nm emission and 340 nm excitation wavelength. Ca_i^{2+} was measured as previously reported using a Kilo-Dalton value of 224 n*M* [14].

CaM-Ca^{2+}-ATPase Assay

The cells were washed thoroughly with Ca^{2+}-free buffer containing 50 m*M* Tris-HCl, pH 7.5, at 0 °C and suspended in the same buffer. The enzyme was assayed according to the method described previously [15, 16]. Briefly, aliquots containing 50–100 μg cellular protein were preincubated in a medium containing 50 m*M* Tris buffer, pH 7.4, with and without 0.1 m*M* calmodulin antagonist, trifluoperazine (TFP) and 10 μM free Ca^{2+} buffered with EGTA solution (pH 7.5) as described previously [17]. ATP hydrolysis was initiated by the addition of 100 μl of buffer containing Tris-ATP and $MgCl_2$ to provide 0.5 m*M* ATP and 1 m*M* Mg^{2+} in the final volume of 500 μl. After incubation at 37 °C for 30 min (linear rate of ATP hydrolysis; data not shown), the reaction was stopped by the addition of 50 μl of cold 70% perchloric acid and P_i liberated was determined by the spectrophotometric method reported previously [18]. CaM-Ca^{2+}-ATPase activity was estimated as the difference of ATP hydrolysis in the presence and absence of TFP. One unit of enzyme activity is defined as the nanomoles P_i liberated per milligram of protein per 30 min at 37 °C.

Trypan Blue Exclusion Test

Equal volumes of each cell suspension and trypan blue stain (0.4%) were mixed together in a tube and the viable cells counted using a hemacytometer.

Western Blotting

Western blot analysis was performed as previously reported except that the antigen-antibody complexes were visualized by horseradish peroxidase-conjugated secondary antibody [12]. An equal number of cells (5×10^5) from each group was sonicated, centrifuged at 2,000 *g* for 10 min and the pellet suspended in 10% guanidine hydrochloride. After centrifugation at 12,000 *g*, the soluble fraction was precipitated by adding cold acetone. The pellet was dissolved in the loading buffer and the entire sample ran on a 10% SDS polyacrylamide gel as previously reported [12]. For equal protein loading experiments, the protein content was determined prior to solubilizing the samples. The volumes loaded onto these gels were adjusted so that 10 μg was loaded per lane.

MTT Assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was carried out as previously described [19]. In brief, cells (600 cells/well) were incubated at 37 °C in a 96-well plate with the required calcium concentrations for 48 h. MTT solution was added and the cells incubated for an additional 2 h. DMSO was added to each well and the plate was read at 540 nm on an ELISA plate reader.

Phosphorylation

The cells were thorougly washed with and suspended in PSS containing 0.5 m*M* Ca^{2+} and supplemented with 0.1% bovine serum albumin. To 5 ml of PSS in the flask, 200 μCi of ^{32}P-orthophosphate was added and the cells allowed to grow overnight at 37 °C. The cells were washed with cold PSS, harvested, and sonicated. The cell suspension was centrifuged at 2,000 *g* for 10 min to obtain a crude membrane pellet. The antigen, recognized by MAb 44-3A6, as well as several other proteins were extracted with 10% guanidine hydrochloride followed by acetone precipitation. The acetone pellet was treated with 10% Triton-X 100 and the soluble proteins were again acetone-precipitated to further enrich for antigen. This pellet was then subjected to Western blot analysis and autoradiography.

Results

The treatment of the A549 cells with various concentrations of calcium for 48 h resulted in a dose-dependent change in the intracellular free Ca^{2+}. The increase in free Ca^{2+}

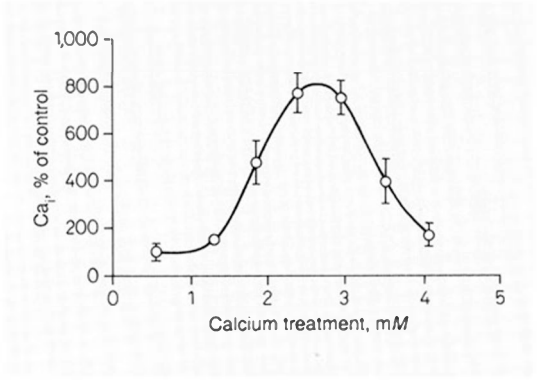


Fig. 1. Changes in free cytosolic calcium levels in A549 cells after exposure to external calcium concentrations. Control mean average value = 114 ± 7.6 n*M* with n = 5.

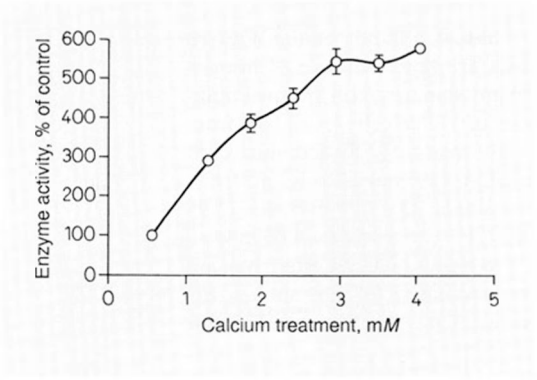


Fig. 2. Changes in Ca-ATPase activity in A549 cells in response to calcium treatment. CaM-Ca^{2+}-ATPase activity was measured as difference in ATP hydrolysis in the presence and absence of the CaM antagonist TFP. Control mean average values = 0.32 ± 0.02 μmol P_i liberated/mg protein/min (n = 5).

concentration was maximum when the exogenous calcium concentration was 2.4 m*M*. From thereon, the internal calcium showed a steady decrease (fig. 1). Exposure of A549 cells to different concentrations of exogenous Ca^{2+} caused a gradual increase in CaM-Ca^{2+}-ATPase activity (fig. 2). At 3 m*M* external Ca^{2+} concentration, there was a 5-fold increase in enzyme activity. At Ca^{2+} concentrations higher than 3 m*M*, the increase in enzyme activity was not significant. Under the assay conditions employed in these experiments, the activity of CaM-Ca^{2+}-ATPase (TFP-sensitive ATPase) amounted to about 20–30% of the total Ca^{2+}-ATPase activity.

Because it is well recognized that comparatively high extracellular calcium concentrations may be toxic to cells, it was necessary to assess the viability of A549 cells at various Ca^{2+} treatments. Using the MTT assay, it was observed that with the increase in exogenous calcium concentrations, the viability of the cells decreased (fig. 3). The data has been presented as a percentage of control. At 4.05 m*M* calcium concentration, almost 50% of the cells were nonviable. This data was confirmed using the trypan blue exclusion test (data not shown).

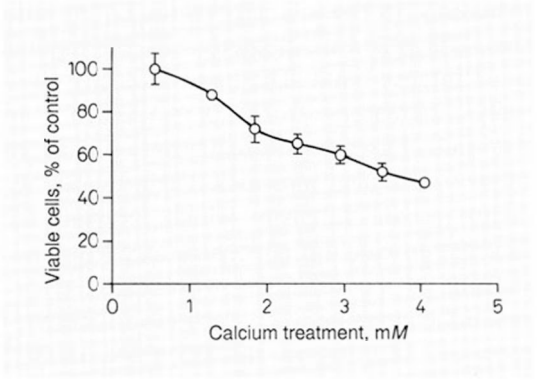


Fig. 3. MTT assay for viability of A549 cells after calcium treatment. The cells were grown in a 96-well plate at different calcium concentrations. The OD at 540 nm was the measure of the MTT reactivity, an index of cellular viability.

Western blot analysis (fig. 4a) using an equal number of viable cells from each treatment showed the expression of the antigen

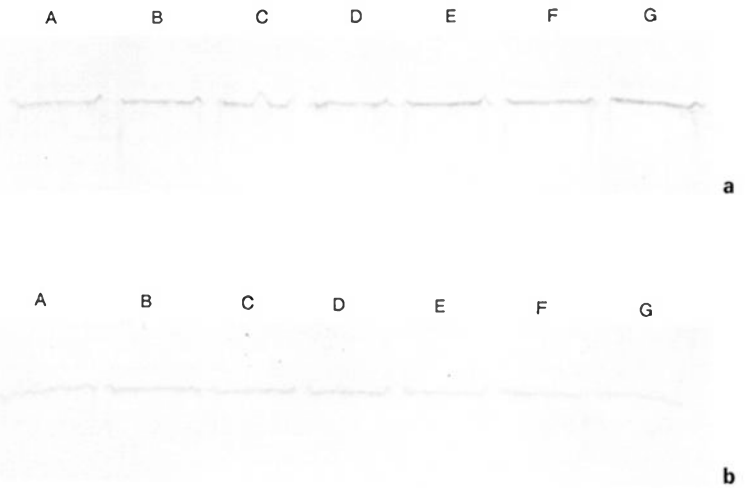


Fig. 4. a Bands on the Western blot are representative of antigen expression based on an equal number of viable cells (5×10^5) from each treatment loaded into each well. A = 0.5 m*M*, B = 1.3 m*M*, C = 1.85 m*M*, D = 2.4 m*M*, E = 2.95 m*M*, F = 3.5 m*M*, G = 4.05 m*M*.

b Bands on the Western blot are representative of the antigen expression based on an equal amount of protein loaded onto each well (10 µg). A = 0.5 m*M*, B = 1.3 m*M*, C = 1.85 m*M*, D = 2.4 m*M*, E = 2.95 m*M*, F = 3.5 m*M*, G = 4.05 m*M*.

recognized by MAb 44-3A6 at the expected 40-kD position. However, there was a visible difference in the intensity of the bands with the 4.05 m*M* Ca^{2+}-treated cells having the most intense band. The band intensity gradually increased from 0.5 (control) to 4.05 m*M*. Laser densitometry data (fig. 5a) of these bands also indicates a continuous increase in band intensity, which paralleles the increase in calcium concentration. Immunostaining of the slides (fig. 6) revealed a very gradual change between each group, with almost no staining in the 0.5 m*M* (control) treatment group. The cells grown in 4.05 m*M* Ca^{2+} showed the greatest intensity of immunostaining. In contrast, when an equal amount of protein was loaded onto each lane (fig. 4b), there appeared to be a steady decline in antigen density. Laser densitometry confirmed this observation (fig. 5b).

When the cells were grown in the presence of ^{32}P, and the antigen recognized by MAb 44-3A6 was enriched as outlined in 'Materials and Methods', a band on the Western blot was found to be both immunoreactive and radiolabeled. This finding suggests that the antigen is phosphorylated (fig. 7).

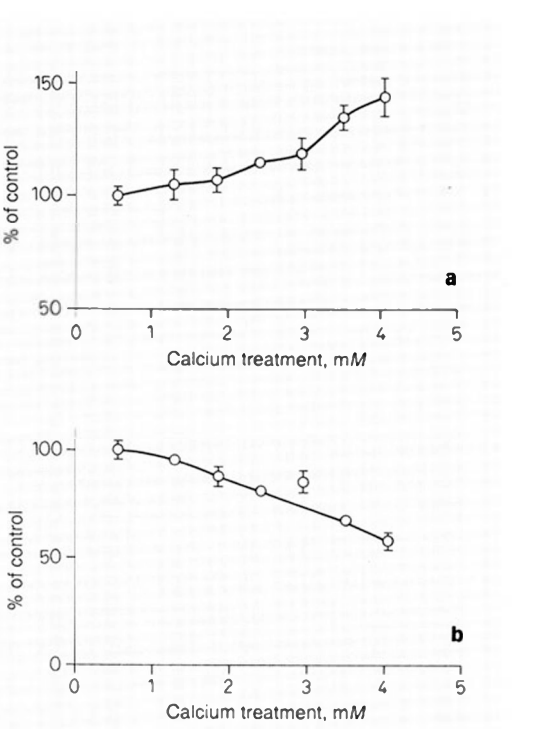
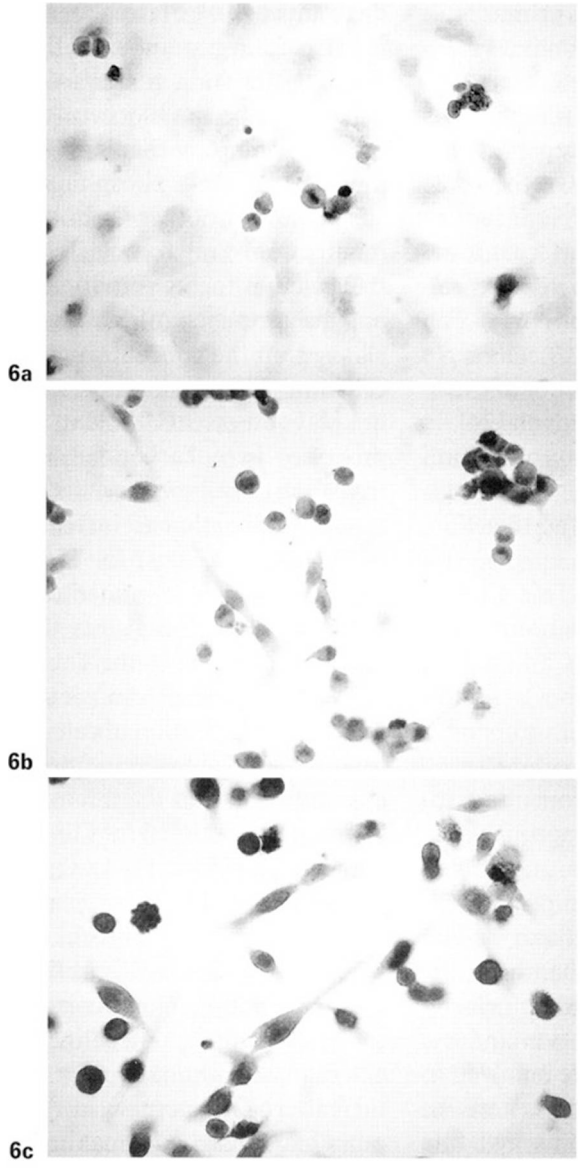


Fig. 5. a Laser densitometry of bands in figure 4a. Each point on the graph represents at least 3 scanning positions on the band. **b** Laser densitometry of bands in figure 4b. Each point on the graph represents at least 3 scanning positions on the band.



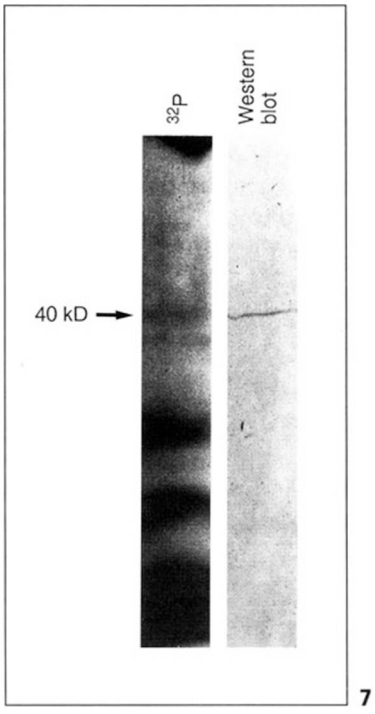


Fig. 6. Cells fixed and immunostained with MAb 44-3A6 after 48-hour incubation with different calcium concentrations. **a** Control. **b** 2.4 m*M*. **c** 4.05m*M*.

Fig. 7. Autoradiogram showing the phosphorylation of the antigen and the corresponding immunoreactivity band on the same Western blot.

Discussion

A dependency of normal cell proliferation on adequate extracellular Ca^{2+} levels was demonstrated in epidermal growth factor-induced rat liver epithelial (T51B) cells [20]. This was done by determining the role of Ca^{2+} influx during DNA synthesis. Low extracellular Ca^{2+} and the addition of La^{3+} prevented the rise in the labeling index of the nuclei stimulated by epidermal growth factor, indicating that movement of Ca^{2+} into the cell was required for DNA synthesis [20]. Induction of DNA synthesis has been shown to be pre-

ceded by an increase in calcium, primarily in the cytoplasm [21]. In human synovial cells, both DNA fragmentation and cell death were dependent on a sustained increase in the Ca^{2+} concentration [22]. It has been reported that some neoplastic cells do not have the same requirement for extracellular calcium ions as do normal cells [23]. However, at least some cancer cell lines were shown to require calcium ions for cell proliferation [24]. The highly malignant leukemia L1210 cells have been reported to require the presence of extracellular calcium for maximum growth [24].

Recently, employing a variety of stimuli (K+ depolatization, ionomycin, and acetylcholine), it has been demonstrated that Ca^{2+} influx into neuronal cells causes a significant increase in nuclear Ca^{2+} [25]. This finding supports the idea that gene regulation in cells may be mediated by changes in Ca^{2+} concentration at the DNA level. Whether a similar mechanism operates in tumor cells is open for further investigation.

The tachykinin family of neuropeptides induce a transient increase in intracellular free calcium concentration in human small cell lung carcinoma cells [26]. None of the peptides, however, showed a stimulatory effect on DNA synthesis. Evidence has been reported implying that two secondary cell-signalling pathways, Ca^{2+} mobilization and the activation of protein kinase C, are involved in the induction of spontaneous metastasis in mouse mammary adenocarcinoma cell line SP1 [27]. The Yoshida hepatoma cells, upon incubation with Ca^{2+} and a Ca^{2+} ionophore, showed cytoskeletal changes, which in late phases led to cell disorganization and necrosis [28].

For most of the cellular physiological responses, only micromolar concentrations of intracellular calcium are required. The maintenance of such a low internal calcium level is accomplished by an active Ca^{2+} transport mechanism [12]. Plasma membrane fractions isolated from a number of tissues have been shown to contain a Ca^{2+}-ATPase, which is believed to be the biochemical expression of the Ca^{2+} pump mechanism [29]. This plasma membrane-linked phosphoprotein is distinguishable from other Ca^{2+}-ATPase in that it requires only micromolar concentrations of free calcium for its activity and is dependent on the presence of calmodulin [15, 30]. Changes in the concentration of intracellular calcium, through the mediation of calmodulin, plays an essential role in many molecular processes in eukaryotic cells including the triggering of cell cycle events [31].

Our finding that an increase in the activity of CaM-Ca^{2+}-ATPase in A549 cells after the exposure of cells to graded concentrations of extracellular Ca^{2+} suggests that this enzyme plays a major role in the homeostasis of Ca^{2+} within these cells. Although the normal intracellular concentration of calcium is in the millimolar range, changes in free calcium (Ca_i^{2+}), estimated to be in the micromolar range, are believed to be critical for Ca^{2+}-mediated physiological responses [1–4, 32, 33]. The CaM-Ca^{2+}-ATPase, which is a plasma membrane-bound enzyme and a biochemical expression of Ca^{2+} pump activity, is utilized for pumping out excess Ca_i^{2+}. The increase in enzyme activity was approximately linear up to 3.0 m*M* external calcium, attaining a plateau level thereafter. The increase in free Ca_i^{2+}, on the other hand, reached a maximum level at 2.4–2.9 m*M*, beyond which it showed a decline. These results suggest that CaM-Ca^{2+}-ATPase activity in A549 cells is inducible and/or that increased activity results under these conditions. As anticipated from previous reports by others, we observed calcium toxicity at hyperphysiological Ca^{2+} concentrations (3.5 and 4.05 m*M*), which were documented both by cell counts and MTT assay results. That is, cell death was progressively greater as calcium

concentrations increased. Relatively high calcium concentrations may cause a calcium overload which eventually leads to cell disorganization and plasma membrane disruption. This has been well documented in other systems as well as coupling Ca^{2+} to a variety of cellular functions such as cell division, microtubular assembly-disassembly, exocytosis, cell adhesion and many other functions [1–4, 32, 33]. It is also important to note that in these high Ca^{2+} treatment groups, the surviving cells had lower internal free calcium than in cells exposed to lower concentrations of calcium. This finding suggests that at Ca^{2+} concentrations < 3 m*M* external Ca^{2+}, the CaM-Ca^{2+}-ATPase activity was induced, while at concentrations > 3 m*M*, other cellular mechanisms responsible for the maintenance of Ca^{2+} levels also participated in addition to Ca-pump activity. These cellular components may consist of mitochondria, endoplasmic reticulum and a number of calcium-binding proteins including calmodulin [1, 32]. Thus, despite a continued high rate of influx, the overall concentration of free Ca^{2+} is kept regulated.

It has been demonstrated that cells can employ Ca^{2+} as a messenger for regulating a sustained physiological response. In the case of insulin-like growth factor, when acting on competent-primed Balb 3T3 cells, exogenous calcium causes an immediate increase in the intracellular Ca^{2+} concentration [34]. This increase in calcium causes a specific 'calcium message' resulting in a sustained response of cellular functions which are cell type-dependent.

For this reason, we question whether the A549 cell surface tumor-associated antigen recognized by MAb 44-3A6 could be modulated as a consequence of intracellular calcium concentration. Using Ca^{2+}-ATPase as an indicator of the physiological effects of our calcium treatments, experiments were designed to assess the expression of the antigen recognized by MAb 44-3A6 in A549 cells. Western blot analysis demonstrated an increase in antigen expression (based on equal live cells), which paralleled the increase in CaM-Ca^{2+}-ATPase. Since high calcium caused significant toxicity, this increase could be due to immunoreactivity contributed by dead cells in the sample. If this were the case, then on Westerns loaded with equal amounts of protein (per sample), an increase (or at least a constant amount) of antigen density would be expected. A marked decrease was in fact observed, supporting the notion that the dead cells were not contributing to the detected antigen density and that the antigen density increased per unit viable cell. Even at near-toxic levels of external calcium, as indicated by a 50% cell death in culture, the antigen expression was significantly increased on Western blots loaded with equal cells per lane. This is further confirmed by immunoperoxidase staining, which shows an increase in both the frequency and intensity of staining as the calcium treatment concentration increases.

From the above observations, it appears that the expression of the antigen recognized by MAb 44-3A6 in the A549 cell line is modulated by the internal free calcium levels. The possibility that this effect is due to a direct result of the cation concentration is unlikely, since other cations (Cu^{2+}, Na^{+}, Zn^{2+}, K^{+}) at similar concentrations did not modulate antigen expression (unpublished data). Speculation that this cell surface tumor-associated antigen is directly linked to the regulation/modulation of intracellular calcium should be made with caution. Although it shares several properties of known calcium pump components, i.e., they have increased expression upon calcium esposure, are integral plasma membrane proteins, and are phosphorylated, these properties make this tumor-associated antigen only a reasonable candidate to be a

calcium 'pump-like' plasma membrane component. This is because the increase in the antigen expression may be simply a consequence of the autoregulatory mechanisms as discussed earlier, or it may be an epiphenomenon which is the result of the general effects of calcium stimulation.

Considerable work remains to be done regarding the biological function of this antigen. The study of the degree of phorsphorylation in response to calcium exposure, as well as other conditions, will be necessary to fully understand what role this protein plays in the cellular response to calcium. More detailed studies directed at linking the numerous Ca^{2+} signalling pathway components with the function of this protein and other calcium regulatory molecules is needed. The work reported herein is only the initial step in linking the increased expression of the tumor-associated antigen recognized by MAb 44-3A6 to intracellular free calcium levels. The understanding of how this protein functions may provide a new insight to what constitutes the adenocarcinoma phenotype and to possible approaches of how to modulate the growth of antigen-positive tumor cells.

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