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# Non-ionophoretic elevation of intracellular $Ca^{2+}$ by Lonidamine

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**Abstract**—Lonidamine is an antispermatogenic and anticancer drug that is believed to act by inhibition of energy metabolism. In this study, the effects of Lonidamine on the concentration of intracellular free $Ca^{2+}$ of several tumor cell lines were assessed because of the important role that cytosolic $Ca^{2+}$ plays in cell viability and proliferation. The presence of 300 μM Lonidamine resulted in large elevations of cytosolic $Ca^{2+}$ (>100 nM) in AS-30D rat ascites hepatoma cells and in cultured EMT6 murine mammary adenocarcinoma cells but had little effect on cultured NCI-H345 human small cell lung cancer cells. The apparent $EC_{50}$ for Lonidamine was approximately 175 μM. The source of elevated cytosolic $Ca^{2+}$ was primarily intracellular stores, and the effects of Lonidamine on $Ca^{2+}$ efflux from these stores did not appear to be due to an ionophoretic action of this compound or to a decline in the level of cellular ATP. These results indicate that the $Ca^{2+}$ homeostasis of certain lines of tumor cells is specifically altered by Lonidamine at concentrations known to affect cell proliferation.

Lonidamine [1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carboxylic acid], an antispermatogenic and anticancer drug, is known to have a profound effect on cellular energy metabolism [1]. In both normal and neoplastic cells, oxygen consumption is impaired through inhibition of electron transport between FAD-linked dehydrogenases and the mitochondrial respiratory chain [2, 3]. Furthermore, Lonidamine stimulates aerobic lactate production in normal differentiated cells, but inhibits that of neoplastic cells [4]. This selective action on tumor glycolysis is related to its inhibitory effect on mitochondrially-bound hexokinase [4, 5], which is present at abnormally high levels on the outer membrane of tumor mitochondria [6]. Although previous studies indicate that the primary mechanism of action of Lonidamine is related to inhibition of ATP production, other mechanisms responsible for disturbing cellular homeostasis have not been excluded. One such mechanism is the alteration of intracellular $Ca^{2+}$ levels, which is known to inhibit cell proliferation and trigger irreversible cell injury in a number of systems [7]. The experiments described in this communication were undertaken to evaluate the potential effects of Lonidamine on intracellular $Ca^{2+}$.

## *Materials and Methods*

*Cell lines.* AS-30D rat ascites hepatoma cells [8] were harvested 7 days after intraperitoneal inoculation of ascites fluid (0.5 mL) from 100–125 g female Sprague–Dawley rats. Hepatoma cells were washed free of contaminating erythrocytes by multiple centrifugations at 4° (180 *g*, 5 min each) in a medium containing 150 mM NaCl, 5 mM KCl and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES*) (pH 7.4). Human small cell lung cancer cells (NCI-H345) were cultured in HITES medium (RPMI 1640 containing $3 \times 10^{-8}$ M $Na_2SeO_3$, 5 μg/mL insulin, $10^{-8}$ M β-estradiol and 10 μg/mL transferrin) supplemented with 2.5% heat-inactivated fetal bovine serum. EMT6 murine mammary adenocarcinoma cells were cultured in Waymouth's medium supplemented with 5% calf serum and 10% newborn calf serum plus antibiotics [9]. Twenty-four hours before experiments were performed, EMT6 cells were trypsinized and transferred into a spinner flask. Both cultured tumor cell lines were grown in a humidified atmosphere of 5% $CO_2$ and 95% air at 37° and harvested by centrifugation at 150 *g* for 5 min. Hepatoma cells and cultured cells were finally resuspended in RPMI 1640 that contained 20 mM HEPES/NaOH (pH 7.4). Cell viability was tested with the trypan blue exclusion method and was greater than 90%. ATP levels were assayed luminometrically by the luciferin/luciferase method [10].

*Liposomes.* Unilamellar liposomes [11] were prepared by sonicating 100 mg L-phosphatidylcholine (type X-E dried egg yolk; Sigma Chemical Co., St. Louis, MO) in 2 mL of medium (T = 25°) containing KCl (125 mM), HEPES (20 mM), $K_2HPO_4$ (2 mM), ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) (10 μM), at pH 7.4 in the presence or absence of the fluorescent $Ca^{2+}$ indicator fura 2 (100 μM) (Calbiochem, Inc., La Jolla, CA) until the suspension was translucent (approximately 60 min). The liposomes were applied to a Sephadex G-25 column (0.75 × 9 in) prewashed with and eluted with the same solution minus fura-2. Two milliliters of the turbid effluent was saved and 0.5 mL added to 1.5 mL of the RPMI medium maintained at 37°.

*Measurement of intracellular or intravesicular* $Ca^{2+}$ *concentration* ($[Ca^{2+}]_i$). Cell suspensions ($1.0 \times 10^8$ in 10 mL of RPMI medium) were incubated in the presence or absence of the cell permeant probe, fura 2/AM (5 μM; Calbiochem, Inc.), at 37° for 30 min in an orbital shaking water bath. Extracellular fura 2/AM was removed by centrifugation at 150 *g* for 5 min. Fura 2 -loaded and sham-loaded cells and liposomes were suspended in RPMI medium and then placed in a Perkin–Elmer LS-3 fluorescence spectrophotometer with a cuvette holder thermostatically maintained at 37°. Excitation and emission wavelengths were 340 and 510 nm, respectively. Cytosolic free $Ca^{2+}$ concentrations were calculated from the fluorescent signals as previously described [12].

## *Results and Discussion*

Recordings of the fluorescence generated by the $Ca^{2+}$-bound form of fura-2 loaded within three different cell lines and within phospholipid liposomes are shown in Fig. 1. The addition to these suspensions of Lonidamine at a concentration (300 μM) comparable to what is often used both *in vitro* and *in vivo* led to a rapid and sustained elevation of the fura-2 fluorescent signal in AS-30D hepatoma and EMT6 adenocarcinoma cells (Fig. 1, A and B), but had no effect on the fura-2 fluorescence of NCI-H345 small cell lung cancer cells (Fig. 1C) or that of the non-protein containing liposomes (Fig. 1D). No significant changes in fluorescence were observed during the addition of Lonidamine to sham-loaded cells or liposomes or by the addition of the dimethyl sulfoxide vehicle to fura-2 containing vesicular systems (not shown). The observation that the addition of the $Ca^{2+}$ ionophore ionomycin induced

* Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; and EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid.

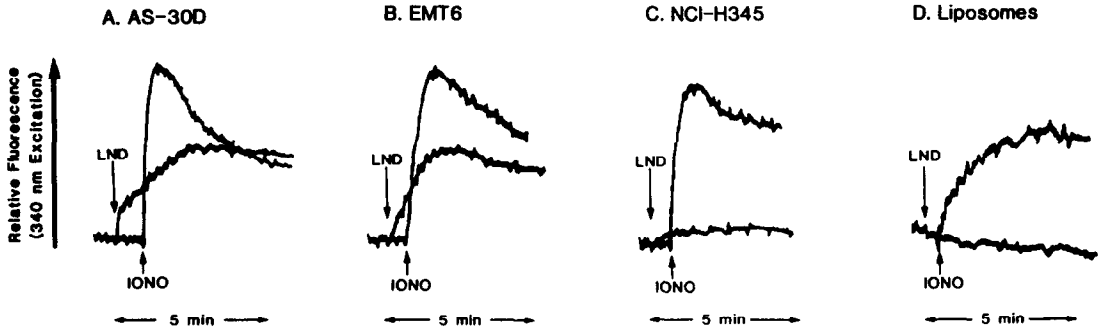


Fig. 1. Effect of 300 $\mu$M Lonidamine (LND) and 30 nM ionomycin (IONO) on the $[Ca^{2+}]_i$ of different cell lines and phospholipid liposomes in the presence of 0.4 mM extracellular $Ca^{2+}$. Each fura-2 fluorescent recording is representative of at least three experiments performed with different batches of cells or liposomes.

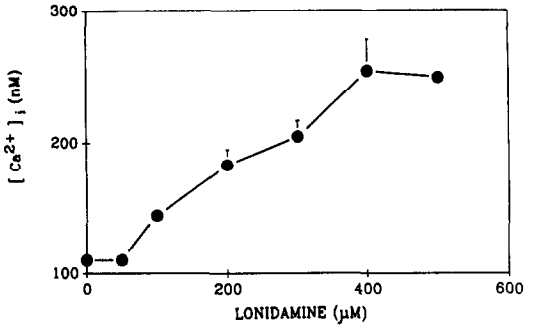


Fig. 2. Dependence of AS-30D hepatoma cell $[Ca^{2+}]_i$ on the concentration of Lonidamine added to the cell suspensions. Values are means ± SEM of five different experiments and were calculated from the fura-2 fluorescent signals corrected for the presence of extracellular fura-2.

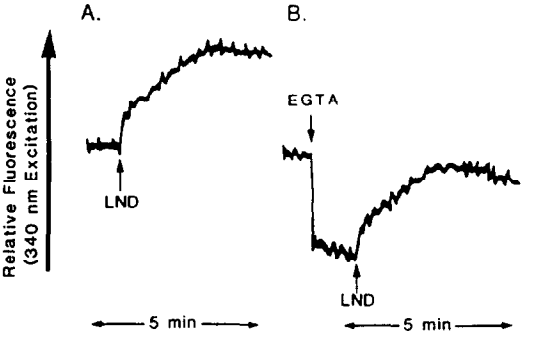


Fig. 3. Effect of chelating extracellular $Ca^{2+}$ on the increase in AS-30D hepatoma cell $[Ca^{2+}]_i$ induced by 300 $\mu$M Lonidamine. Fluorescence measurements of cytosolic $Ca^{2+}$ were made in the absence (A) and presence (B) of 12.5 mM EGTA. Addition of EGTA was accompanied by addition of sufficient NaOH to neutralize the drop in pH caused by $Ca^{2+}$ chelation. Each trace is representative of five different experiments.

a substantial, prolonged elevation in the fluorescent signal in all four systems (Fig. 1) indicates that each of the cell types and the liposomes were responsive to a rise in the intracellular or intravesicular $Ca^{2+}$ concentration. The finding that Lonidamine had no effect on the $Ca^{2+}$ levels present within one cell line or the liposomes strongly suggests that the positive response of some cell lines is due to specific effects on one or more $Ca^{2+}$ transport systems rather than to an ionophoretic activity of the drug or a non-specific effect on membrane ion permeabilities.

The concentration–response curve for the influence of Lonidamine on the fura-2-quantitated cytosolic free $Ca^{2+}$ concentration of AS-30D hepatoma cells is shown in Fig. 2. The effect of Lonidamine on these cells appears to saturate at between 300 and 400 $\mu$M with an apparent $EC_{50}$ of approximately 175 $\mu$M. The maximum change in $[Ca^{2+}]_i$ in these cells was approximately 150 nM. These results further support the specific nature of the effects of Lonidamine on intracellular $Ca^{2+}$.

In an initial attempt to focus on the site of action of Lonidamine on cytosolic $Ca^{2+}$, experiments were performed in the absence and presence of an excess of the $Ca^{2+}$ chelator EGTA (i.e. in the presence and absence of extracellular free $Ca^{2+}$) to determine whether Lonidamine primarily affects $Ca^{2+}$ flux across the plasma membrane or intracellular membranes. As seen in Fig. 3, addition of 300 $\mu$M Lonidamine in the absence of extracellular free $Ca^{2+}$ (Fig. 3B) resulted in a change of the intracellular $Ca^{2+}$ concentration that was comparable to the change observed in the presence of 0.4 mM extracellular free $Ca^{2+}$ (Fig. 3A). The average increase in cytosolic free $Ca^{2+}$ was 89 ± 8 and 66 ± 6 nM (SEM, N = 9) in the absence and presence of EGTA, respectively. Alternatively stated, the change in $Ca^{2+}$ induced by 300 $\mu$M Lonidamine in the absence of extracellular free $Ca^{2+}$ was on average 75 ± 6% of that observed in the presence of extracellular free $Ca^{2+}$. The primary site of action of Lonidamine on $Ca^{2+}$ flux is, therefore, at intracellular stores.

There are several possible mechanisms by which Lonidamine releases intracellular stores of $Ca^{2+}$. Based upon previous work demonstating the potential of Lonidamine to interfere with energy metabolism by inhibiting mitochondrially-bound hexokinase and the electron transport chain [2, 3, 5], it could promote net $Ca^{2+}$ efflux from the endoplasmic reticulum by lowering cellular ATP below the threshold necessary for maintaining $Ca^{2+}$-ATPase-dependent $Ca^{2+}$ influx. Measurement of ATP levels in suspensions of AS-30D cells immediately prior to and 5 min after the addition of 300 $\mu$M Lonidamine

indicated that no change in ATP occurred within this period in this system ($32.4 \pm 0.6$ vs $32.8 \pm 0.4$ $\mu$mol/$10^5$ cells, N = 4). It therefore appears likely that Lonidamine is either directly stimulating an efflux pathway or directly inhibiting the $Ca^{2+}$-transporting ATPase. Further studies are in progress to differentiate between these two modes of action.

Irrespective of the mechanism by which Lonidamine elevates cytosolic free $Ca^{2+}$ concentrations, this heretofore unknown effect of Lonidamine must be taken seriously into account when attempts are made to explain its antitumor and spermaticidal actions. The combined stress of inhibited energy metabolism and elevating intracellular $Ca^{2+}$ may thus make cells particularly susceptible to injury by this drug or by other pharmaceuticals commonly used together with Lonidamine.

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*Laboratory for Cell Metabolism and Pharmacokinetics
Regina Elena Institute for Cancer Research
Rome 00158, Italy
†Department of Pharmacology and
‡Department of Biochemistry and Molecular Biology
George Washington University Medical Center
Washington, DC 20037
U.S.A.

SUSANNA CASTIGLIONE*
KATHERINE A. KENNEDY†
ARISTIDE FLORIDI*
GARY FISKUM‡§

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§ Corresponding author. Tel. (202) 994-8819; FAX (202) 994-8974.