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Alterations in Alveolar Type II Cell Metabolism Induced by Tetrandrine and Other Alkaloids

P. R. MILES,*† L. BOWMAN,* J. K. H. MA,‡ AND J. Y. C. MA*

*National Institute for Occupational Safety and Health, Division of Respiratory Disease Studies, Morgantown, West Virginia 26505; †Department of Physiology and ‡School of Pharmacy, West Virginia University, Morgantown, West Virginia 26506

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Tetrandrine (TT) and other bisbenzylisoquinoline alkaloids have been used in China as a treatment for fibrotic lung diseases. Because of their potential use as pulmonary therapeutic agents, we studied the effects of some of these compounds on energy metabolism in isolated rat alveolar type II cells, i.e., cells which play a critical role in maintaining normal lung function. Incubation of type II cells with most of the alkaloids produces a reduction in cellular ATP content. However, there is no effect of the alkaloids on cellular oxygen consumption. All of the alkaloids which produce reductions in cell ATP levels cause increases in internal calcium levels of type II cells. Incubation of the cells with the calcium ionophore, 4-bromo A-23187, leads to increased amounts of intracellular calcium and reductions in ATP levels, but has no effect on oxygen consumption. Exposure of isolated lung mitochondria to calcium produces a concentration-dependent reduction in ATP synthesis with no effect on mitochondrial oxygen consumption. Direct exposure of mitochondria to TT has no effect on ATP synthesis. These results are consistent with the notion that the alkaloids produce an increase in type II cell internal calcium levels which, in turn, leads to reduced rates of mitochondrial ATP synthesis.

The bisbenzylisoquinoline alkaloids are compounds which can be isolated from plants and which exhibit a broad range of pharmacological activities. Perhaps the best known alkaloid of this group is tetrandrine (TT), a compound isolated from the root of a traditional Chinese medicinal herb, *Stephania tetrandra*. It has been tested for its effects on the cardiovascular system, tumors, and the lungs. For example, tetrandrine has been shown to act as a calcium entry blocker in cardiac sarcolemmal vesicles (King *et al.*, 1988) and has been used in China as a treatment for angina and hypertension. Its possible effects as a tumor-inhibitory substance have also been investigated (Kupchan and Atland, 1973). In addition, there are several lines of evidence to suggest that it is a possible therapeutic agent for the treatment of silicosis. Yu *et al.* (1983) have shown that TT inhibits the development of experimental silicosis in rats when it is administered orally after intratracheal instillation of quartz. Other studies have shown that the alkaloid inhibits the synthesis of collagen by fibroblasts and the formation of silicotic nodules in rats exposed to silica (Huang *et al.*, 1981; Liu *et al.*, 1983). In fact, tetrandrine has been used clinically in China as an antifibrotic agent and has been shown to improve both the appearance of chest radiographs and the measurements of diffusing capacity in patients with silicosis (Li *et al.*, 1981).

Since tetrandrine may have a potential use as a therapeutic agent for fibrotic lung diseases, its effects on various pulmonary cells and tissues should be studied. Alveolar type II cells are located in the alveolar regions of the lungs and are important because they are responsible for the synthesis and secretion of pulmonary surfactant (Dobbs *et al.*, 1982; King, 1984), regeneration of the alveolar epithelium after lung injury (Kauffman *et al.*, 1974), and metabolism of foreign compounds (Bend *et al.*, 1985). Furthermore, in silicosis, for which TT has been suggested as a treatment, there are increased amounts of pulmonary surfactant produced (Dethloff *et al.*, 1986a,b) as a result of increased rates of surfactant synthesis by type II cells (Dethloff *et al.*, 1989; Miller *et al.*, 1988). Because type II cells play a critical role in maintaining normal lung function and because type II cells are affected in silicosis, for which TT has been used as a treatment, we studied the effects of tetrandrine on energy metabolism in these cells *in vitro*. Energy metabolism was chosen because it is a basic cellular function which is necessary for other cellular processes to occur. Since type II cells depend on mitochondrial metabolism for energy production (Fisher *et al.*, 1980), we examined the effects of tetrandrine on cellular and mitochondrial ATP production and oxygen consumption. Additional experiments were performed in an effort to determine if intracellular calcium levels may be involved in the TT-induced effects. A preliminary report of those results has appeared previously (Ma *et al.*, 1991).

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METHODS

Isolation of alveolar type II cells. Alveolar type II cells were obtained by digesting the lungs with elastase and purifying the cells by centrifugal elutriation as we reported previously (Jones *et al.*, 1982; Miles *et al.*, 1983). Male Sprague–Dawley rats (250–300 g; Hilltop Laboratories, Scottdale, PA) were anesthetized with sodium pentobarbital (150 mg/kg body wt). The heart and lungs were quickly removed, the lungs were perfused with 0.16 M NaCl to remove blood cells, and free alveolar macrophages were removed by tracheal lavage with ice-cold phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 9.35 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 5 mM glucose, pH 7.4). Then the lungs were filled with phosphate-buffered medium which contained elastase (40 U/ml, type I; ICN Biochemicals, Cleveland, OH) and deoxyribonuclease (DNase, 0.006%; U.S. Biochemical, Cleveland, OH) and were incubated for 30 min at 37°C to free lung cells. Following this enzymatic digestion, the lungs were minced with a McIlwain tissue chopper (slice thickness ≈ 0.5 mm; Mickle Engineering, Gomshall, Surrey, UK). The digestion was then arrested by placing the minced tissue in phosphate-buffered medium containing 25% fetal calf serum and 0.006% DNase (to help prevent cell clumping) and incubating it at 37°C for 10 min. The cell suspension was strained through nylon mesh of successively smaller pore sizes (150, 330, and 440 mesh). These cells were then loaded into an elutriation rotor (Model J-21 centrifuge equipped with a Model JE-6 rotor; Beckman Instrument Co., Fullerton, CA) at a flow rate of 10 ml/min and a rotor speed of 2000 rpm. Then 200 ml of phosphate-buffered medium were infused through the elutriator at this flow rate and rotor speed to remove cell fragments and small pneumocytes. Type II cells were then recovered at a flow rate of 19 ml/min and a rotor speed of 2000 rpm. The type II cells were washed once and resuspended in phosphate-buffered medium containing 1.8 mM $CaCl_2$ and 1.0 mM $MgCl_2$ for use in experiments. All studies were performed within 1 hr after the isolation procedure.

A Coulter Model Z_B electronic cell counter (Coulter Instrument, Hialeah, FL) was used to determine cell number. Phosphine 3R, a fluorescent dye, was routinely used to estimate the purity of the type II cell-enriched fraction as we reported previously (Miles *et al.*, 1983). In the experiments reported in this paper, we obtained 2.86 (±0.03) × 10^7 cells per rat in the type II cell fraction with a purity of 93(±1)% (means ± SE for 17 experiments). Contamination was due to alveolar macrophages. Cell viability was assessed by measuring membrane integrity as exclusion of trypan blue dye or release of lactate dehydrogenase (LDH). The percentage of cells that excluded trypan blue dye was determined as described previously (Philips, 1973). LDH release was also measured according to previously published methods (Wrobleski and Ladue, 1955). Membrane integrity was measured in untreated and in treated cells.

All measurements, which are described below, were made in untreated cells and in cells exposed to bisbenzylisoquinoline alkaloids, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; an uncoupler of oxidative phosphorylation), or to the calcium ionophore, 4-bromo A-23187. Some alkaloids (tetrandrine, fangchinoline, and berbamine) were kindly supplied by the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine, Beijing, China. Tubocurarine, another alkaloid, was obtained from Sigma Chemical Co. (St. Louis, MO). The bisbenzylisoquinoline alkoloids were dissolved in 0.1 M HCl which was then titrated to pH 6.5 with NaOH. Very small amounts of these stock solutions were added to the cell suspensions (<50 μl per milliliter). Addition of the HCl and NaOH alone had no effect on any of the measurements. CCCP (Sigma) and 4-bromo A-23187 (Molecular Probes, Inc., Eugene, OR) were dissolved in either DMSO or methanol, respectively, and then added to the cell suspensions. Neither DMSO nor methanol (maximal amount added was 20 μl/ml) had any effect on the measurements.

Measurement of cellular ATP levels and oxygen consumption. The ATP content of type II cells was determined by the firefly luciferase assay adapted from the method of Wulff and Doppen (1985). The cells (2 × 10^6 per milliliter) were incubated (37°C) for various times in phosphate-buffered medium containing Ca^{2+} and Mg^{2+} with and without alkaloids, CCCP, or 4-bromo A-23187. After incubation, the cells were spun down at 1000*g* for 10 min and the incubation medium removed. The cells were washed once in fresh incubation medium and then resuspended so there were 2 × 10^6 cells in 125 μl of 0.5 M tris(hydroxymethyl)aminomethane (Tris)–acetate (pH 7.4). Triton X-100 (125 μl; 1:200 in Tris–acetate) was added to disrupt cell membranes. The sample was then thoroughly mixed by vortexing for 10 sec and immediately analyzed for ATP content. The ATP concentration was determined by measuring the emission of light produced when 50 μl of the sample was mixed with 50 μl of firefly lantern extract (Sigma) in 0.4 ml of the Tris–acetate buffer. Light emission was measured with a Lumi-Aggregometer (Model 400, Chrono-Log, Havertown, PA). Cellular ATP content was calculated from a standard curve of ATP standard solutions (Sigma) and expressed as nmoles per 10^6 cells.

Oxygen consumption by type II cells was measured with a Gilson K-IC oxygraph fitted with a Clark electrode (Gilson Medical Instruments, Middletown, WI). Calibration of the oxygraph was done by measuring the oxygen levels in aliquots of incubation medium bubbled with gases of known oxygen concentration until saturation occurred. Type II cells (2 × 10^6 per milliliter) were incubated (37°C) for 1 hr in phosphate-buffered medium containing Ca^{2+} and Mg^{2+} with and without alkaloids, CCCP, or 4-bromo A-23187. The rate of oxygen consumption in 1.65 ml of this cell suspension was then measured for 10 min at a constant temperature of 37°C. Cellular oxygen consumption was expressed as nmoles per 10^6 cells per hour.

Estimation of internal calcium. The internal calcium concentration of alveolar type II cells was estimated according to a method described by Grynkiewicz *et al.* (1985). This method has been used by Wirtz and Dobbs (1990) in type II cells. We used the fluorescent dye, Indo-1-AM (Molecular Probes, Inc., Eugene, OR), for these experiments because it appeared to be uniformly distributed in type II cells, as was reported previously (Wirtz and Dobbs, 1990). The cells (5 × 10^6 per milliliter) were incubated (37°C) for 1 hr in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and 2 μM Indo-1-AM in order to load the cells with dye. The cells were then spun down, washed twice in the same medium without Indo-1-AM, resuspended in this medium, and incubated for another hour in order for hydrolysis of the dye to occur, i.e., conversion from the ester to the free acid form. Sodium pyruvate (10 mM) was included in all media used to incubate cells with Indo-1-AM and during hydrolysis to protect against ATP depletion caused by formaldehyde released during hydrolysis. Following the incubation period for hydrolysis, the cells were centrifuged, washed once, and resuspended at a concentration of 2 × 10^6 per milliliter in Hepes-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes); pH 7.4) containing Ca^{2+} and Mg^{2+} for use in these experiments.

In order to estimate internal calcium, the cell suspensions were placed in a Perkin Elmer LS 50 luminescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) equipped with a 37°C-thermostated cuvette holder with stirrer. Measurements of internal calcium in untreated cells were made after incubation of the samples for 5 min in the spectrometer. In other experiments, the bisbenzylisoquinoline alkaloids or 4-bromo A-23187 was added to the cell suspensions in the spectrometer and immediate effects on internal calcium were monitored. The fluorescence measurements were made by setting excitation at 350 nm and measuring emission at both 480 and 405 nm. We set the spectrometer in the ratio mode and measured the ratio of emission at 405 nm to that at 480 nm (R) as a function of time. The internal calcium was calculated according to

$$[Ca^{2+}]_i = 250 \frac{(R - R_{min})}{(R_{max} - R)} \frac{S_{f_2}}{S_{b_2}}$$ (Grynkiewicz *et al.*, 1985),

where R_{min} is the ratio when there is no Ca^{2+} bound to the dye, R_{max} is the ratio when the dye is saturated with Ca^{2+}, and S_{f_2}/S_{b_2} is the ratio of the

emission intensities at 480 nm for the free and bound forms of the dye. The value of 250 is the nanomolar dissociation constant for the dye at 37°C (Grynkiewicz *et al.*, 1985). To obtain R_{max}, the Ca^{2+} ionophore, 4-bromo A-23187 (Molecular Probes), which was dissolved in a small amount of methanol and then added to Hepes-buffered medium, was added until the dye was saturated with Ca^{2+}. R_{min} was obtained by lysing the cells with Triton X-100 (to 0.6%) and adding enough 3 mM EGTA to remove all Ca^{2+} from the dye.

There are at least three possible sources of error in these measurements. We checked for autofluorescence, i.e., baseline fluorescence at both emission wavelengths with no dye, and found none. We also looked for leakage of the dye from the cells into the medium. Leakage of the dye was not a problem when the cells were incubated for short periods of time, e.g., less than 10 min. However, when the cells are incubated for longer periods of time, there seems to be a gradual leakage of the dye from cells. Therefore, we report only immediate effects of alkaloids on internal calcium. It is also possible that the alkaloids and calcium entry blockers alter the binding of calcium with the dye. We studied this by using the free acid form of the dye and found no effects. Finally, the values we obtained for internal calcium levels in untreated type II cells are similar to those obtained by others (Wirtz and Dobbs, 1990; Sano *et al.*, 1987).

Isolation of lung mitochondria and measurement of ATP synthesis and oxygen consumption. Lung mitochondria were prepared as described by Spear and Lumeng (1978) in order to determine the effects of calcium and tetrandrine on mitochondrial function. Briefly, after the lungs were perfused and dissected free of the trachea, bronchi, and connective tissue, they were finely minced by chopping four times with a McIlwain tissue chopper (slice thickness = 0.5 mm). The mince was homogenized in cold (4°C) isolation medium (0.25 M sucrose, 2 mM EDTA, 5 mM Tris–HCl, and 1% fatty-acid poor BSA; pH 7.4) using a Potter–Elvehjehm homogenizer. Three strokes of the pestle at 1000 rpm was used. The concentration of tissue in the homogenate was 100 mg per milliliter. This crude homogenate was centrifuged at 2000*g* for 5 min and the pellet was discarded. The supernatant was centrifuged at 17,000*g* for 5 min to isolate the mitochondrial fraction. After the mitochondrial pellet was washed twice with cold isolation medium, the mitochondria were resuspended in an incubation medium containing 105 mM KCl, 2 mM KH_2PO_4, 30 mM Tris–HCl, 0.1 mM EDTA, and 1% fatty-acid poor BSA (pH 7.2) for use in all experiments. With this method, we obtained between 1.5 and 2.0 mg mitochondrial protein per gram of lung. To assess the condition of the isolated mitochondria, the respiratory control ratio (RCR), defined as the ratio of the rate of oxygen consumption in the presence of added ADP (0.3 mM) to the rate obtained following its (ADP) expenditure, was measured. The RCR was 2.7 ± 0.2 (mean ± SE for five experiments) which is comparable to that reported for others for rat lung mitochondria (Fisher *et al.*, 1973) and indicates that the membranes were intact and the rate of respiration acceptable.

The effects of small amounts of calcium on mitochondrial ATP synthesis and oxygen consumption were determined. In addition, the direct effect of tetrandrine on mitochondrial ATP synthesis was measured. Mitochondria were resuspended at a final concentration of 0.15 mg protein per milliliter in incubation medium containing succinate (5 mM) and ADP (10 μM) for all of these experiments. Varying amounts of $CaCl_2$ were added to the mitochondrial suspensions, and the final free calcium concentration of each suspension was measured by using the free acid form of the Indo dye as we described above. The calcium measurements were made at 30°C. Measurement of the exact calcium levels was necessary due to the presence of potential chelators such as EDTA, phosphate, ADP, succinate, and mitochondria. In these experiments, the final calcium levels were between 20 and 200 nM. Once the calcium concentrations were determined, ATP and oxygen consumption measurements were made in corresponding samples, i.e., other samples to which the same amount of $CaCl_2$ was added. To measure ATP production, the suspensions were incubated at 30°C for 5 min, centrifuged for 15 sec at 12,800*g* in the Eppendorf microfuge (Model 5412, Brinkmann Instrument, Westbury, NY), and ATP levels in the supernatants were determined with the firefly luciferase technique as described previously. No ATP was detectable in the pellet. (In a separate set of experiments, we determined that none of the effects of $CaCl_2$ are caused by chloride.) Five-minute incubations were used because the RCR tends to be reduced with longer incubation periods. In another type of experiment, mitochondria were incubated for 5 min with 20 μM TT and ATP synthesis was then measured as described above. Oxygen consumption by mitochondrial suspensions (0.15 mg protein/ml) was measured in the presence of succinate (5 mM) and ADP (0.3 mM) following incubation of the suspensions for 5 min with and without calcium. The measurements were made with the Gilson oxygraph as described previously.

Statistical analyses. All comparisons of statistical significance were made by using the unpaired Student *t* test. $p < 0.05$ was taken as the limit to indicate significance.

TABLE 1
Effects of Tetrandrine on Membrane Integrity of Alveolar Type II Cells[a]

TT concentration (μM)	Trypan blue exclusion (%)	LDH release (% untreated cells)[b]
0	94 (±1)	100
10	93 (±2)	95 (±5)
15	92 (±1)	91 (±4)
20	92 (±2)	102 (±6)
25	88 (±2)*	112 (±2)*
30	84 (±1)*	187 (±26)*

[a] Cells (2×10^6 per milliliter) were incubated at 37°C in phosphate-buffered medium containing Ca^{2+} and Mg^{2+} with and without various concentrations of tetrandrine for 2 hr. Then measurements were made. Values are means ± SE for five experiments.

[b] LDH in the medium from untreated cells was 141 (±27) U/10^6 cells.

* Values are significantly different from untreated animals ($p < .05$).

RESULTS

Membrane Integrity and Cell Viability

Experiments were first carried out to determine whether or not tetrandrine affects the membrane integrity and/or cell viability of type II cells. We studied the effects of incubating the cells for 2 hr with different concentrations of TT on the exclusion of trypan blue dye and the release of lactate dehydrogenase. The results are shown in Table 1. There seem to be no effects on membrane integrity with doses of TT up to 20 μM, i.e., doses which produce significant effects on type II cell metabolism as is shown later. Thus, the effects of the drug which are shown later in this paper are probably not due to gross membrane damage. In addition, TT (20 μM) has no effect on cellular oxygen consumption (Table 2), one of the most sensitive measures of cell viability. Therefore, these results show, to the best of our ability, that membrane integrity and/or cell viability are not impaired by concentrations of TT ≤ 20 μM.

TABLE 2

Effects of Bisbenzylisoquinoline Alkaloids and CCCP on Cellular ATP Content and Oxygen Consumption in Type II Cells[a]

Treatment (concentration)	Cellular ATP content (% untreated cells)[b]	Oxygen consumption (% untreated cells)[c]
Untreated cells	100	100
Tetrandrine (20 μM)	36 (±2)*	97 (±4)
CCCP (10 μM)	26 (±3)*	134 (±9)*
Fangchinoline (20 μM)	21 (±3)*	98 (±7)
Berbamine (20 μM)	54 (±8)*	99 (±6)
Tubocurarine (20 μM)	100 (±9)	107 (±7)

[a] Cells (2×10^6 per milliliter) were incubated for 1 hr at 37°C in phosphate-buffered medium containing Ca^{2+} and Mg^{2+} with and without bisbenzylisoquinoline alkaloids or CCCP. Then measurements were made. Values are means ± SE for six experiments.

[b] ATP content of untreated cells was 0.61 (±.06) nmol/10^6 cells.

[c] Oxygen consumption by untreated cells was 157 (±12) nmol/10^6 cells/hr.

* Values are significantly different from untreated animals ($p < .05$).

Cellular ATP Levels and Oxygen Consumption

Alveolar type II cells depend on mitochondrial (aerobic) metabolism for energy generation (Fisher *et al.*, 1980). Therefore, we studied the effects of tetrandrine on type II cell ATP levels. The time course for the effects of 20 μM TT on cellular ATP levels is shown in Fig. 1A. There is a reduction in ATP in as quickly as 5 min, and in 1 hr there is a 60% reduction. The concentration–response relationship for TT after 1 hr of incubation is shown in Fig. 1B. The decrease in cellular ATP content is concentration-dependent and the concentration which causes 50% inhibition is 16.6 (±1.4) μM. These results show that TT reduces cellular ATP levels in a time- and concentration-dependent manner.

Since cellular respiration is tightly coupled to ATP production, we measured oxygen consumption in untreated alveolar type II cells and in cells exposed to 20 μM tetrandrine for 1 hr (Table 2). Although exposure to TT results in a 64% reduction in cellular ATP content, cellular oxygen consumption is not affected by the alkaloid. One possible explanation for this result is that uncoupling of oxidative phosphorylation has occurred; i.e., electron transport proceeds without formation of ATP. We, therefore, tested the effects of a known uncoupler, CCCP (Table 2). In the presence of 10 μM CCCP, ATP content is reduced by 70–75% and cellular oxygen consumption is stimulated by 30–40%. The conventional definition of uncoupling is a reduction in ATP and an increase in oxygen consumption. Thus, CCCP acts as a traditional uncoupler but TT does not. The effect of TT on type II cell oxidative metabolism is a reduction in ATP levels with no change in oxygen consumption.

Several other bisbenzylisoquinoline alkaloids have been tested for antifibrotic potential in rats (Mo *et al.*, 1982). Therefore, we measured the effects of three other alkaloids on cellular ATP levels and on cellular oxygen consumption. The results are shown in Table 2. Fangchinoline and berbamine produce a reduction in cell ATP levels but have no effect on oxygen consumption. Tubocurarine has no effect on any of the measured parameters. It is interesting to note that the order of potency for effects of all bisbenzylisoquinoline alkaloids on cell ATP levels is similar to the reported order for antifibrotic potential (Mo *et al.*, 1982); i.e., TT and fangchinoline are more effective than berbamine, and tubocurarine has no effect. These results demonstrate that most of the alkaloids cause a reduction in cellular ATP levels but have no effect on oxygen consumption.

Alterations of Intracellular Calcium Levels

It is already known that tetrandrine affects cytosolic calcium levels in excitable tissues (King *et al.*, 1988). Therefore, one possible mechanism by which some bisbenzylisoquinoline alkaloids affect type II cell ATP levels may be by producing changes in intracellular calcium. Addition of tetrandrine to type II cells in suspension results in an immediate increase in the intracellular calcium concentration. A

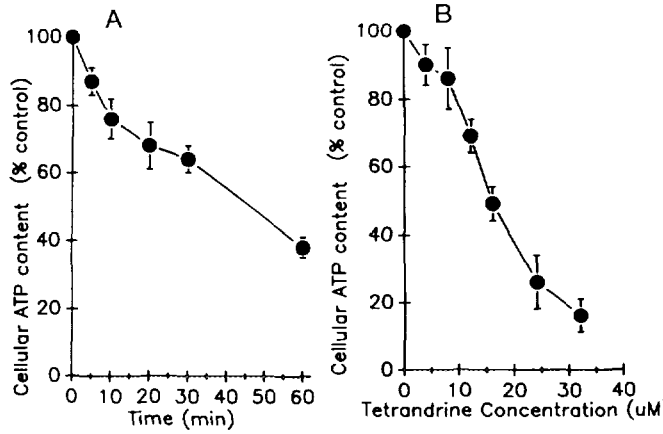

FIG. 1. (A) Time course for effect of tetrandrine (20 μM) on type II cell ATP content. Cells (2×10^6 per milliliter) were incubated at 37°C in phosphate-buffered medium containing Ca^{2+} and Mg^{2+} with and without TT (20 μM). After an appropriate incubation period, samples were taken and cellular ATP content was measured as described under Methods. The cell ATP contents are expressed as a percentage of those in untreated cells. The ATP content was 0.51 (±.07) nmol per 10^6 untreated cells and was constant during the 1-hr incubation period. The points are mean values for six experiments and the bars represent standard errors of the means. (B) Cellular ATP content as a function of the tetrandrine concentration. Cells (2×10^6 per milliliter) were incubated at 37°C with and without various concentrations of TT as described above for 1 hr. The ATP content was then measured as described under Methods and expressed as a percentage of that in untreated cells. The ATP content of untreated type II cells was 0.59 (±.12) nmol/10^6 cells. Points are mean values for five experiments and the bars represent the standard errors of the means.

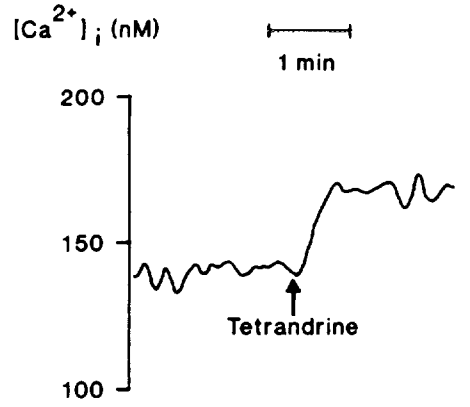

FIG. 2. Internal calcium concentrations before and after exposure of type II cells to tetrandrine (20 μM). The cells were loaded with Indo-1-AM as described under Methods. Following a 1-hr incubation period for hydrolysis of the dye to occur, cells (2×10^6 per milliliter) were resuspended in medium containing Ca^{2+} and Mg^{2+}. Internal calcium levels were calculated from continuously monitored fluorescence as described under Methods. Tetrandrine was added at the arrow. This tracing is representative of observations made in each of five different experiments.

tracing which is typical of our results is shown in Fig. 2. The internal calcium is increased by about 30–40 nM in the presence of TT. In these experiments, we measured only immediate effects of TT, i.e., within 1 min of addition of alkaloid. The effects of TT and other alkaloids on intracellular calcium are summarized in Table 3. The three alkaloids which cause a reduction in cell ATP levels, TT, fangchinoline, and berbamine, cause increases in intracellular calcium. Tubocurarine, on the other hand, has no effect on ATP or on internal calcium.

Since the preceding data suggest that the alkaloid-induced increases in intracellular calcium may be related to

TABLE 3
Effects of Bisbenzylisoquinoline Alkaloids on the Internal Calcium Levels of Alveolar Type II Cells[a]

Treatment	Internal calcium (% untreated cells)[b]
Control	100
Tetrandrine	122 (±1)*
Fangchinoline	135 (±2)*
Berbamine	122 (±6)*
Tubocurarine	100 (±1)

[a] The cells were first loaded with Indo-1-AM and then incubated while the ester was hydrolyzed as described under Methods. Then the cells (2×10^6 per milliliter) were incubated alone or in the presence of different bisbenzylisoquinoline alkaloids (20 μM). The internal Ca^{2+} levels were estimated immediately after addition of the alkaloids (i.e., within 1 min). Values are means ± SE for four experiments.

[b] Internal calcium in untreated cells was 141 (±9) nM.

* Values are significantly different from untreated animals ($p < .05$).

TABLE 4
Effects of the Calcium Ionophore, 4-Bromo A-23187, on Type II Cell ATP Content and Oxygen Consumption[a]

Ionophore concentration	Cellular ATP content (% control)[b]	Oxygen consumption (% control)[c]	Internal calcium (% control)[d]
0 (control)	100	100	100
0.5 μM	54 (±4)*	97 (±4)	150 (±7)*

[a] For measurements of cell ATP levels and oxygen consumption, cells (2×10^6 per milliliter) were incubated for 1 hr in phosphate-buffered medium containing Ca^{2+} and Mg^{2+} with and without 4-bromo A-23187. Then the measurements were made. The internal calcium level was measured immediately after the addition of ionophore. Values are means ± SE for five experiments.

[b] ATP content in untreated cells was 0.52 (±.05) nmol ATP/10^6 cells.

[c] Oxygen consumption by untreated cells was 121 (±6) nmol/10^6 cells/hr.

[d] Internal calcium in untreated cells was 144 (±10) nM.

* Values are significantly different from untreated animals ($p < .05$).

the reductions in cell ATP levels, we exposed the cells to the calcium ionophore, 4-bromo A-23187, in order to determine the effects of increased internal calcium. The results are summarized in Table 4. Addition of the ionophore to type II cells results in an increase in internal calcium. Exposure to the ionophore also results in reduced cellular ATP levels and has no effect on oxygen consumption, i.e., effects similar to those induced by the alkaloids. It must be pointed out that the reduction in cell ATP levels is less and the increase in internal calcium more in the presence of 0.5 μM ionophore than in the presence of alkaloids (20 μM). Although there is not a good quantitative comparison, these data, as well as those obtained with measurements of internal calcium, suggest that the alkaloid-induced reductions in type II cell ATP levels may be due, at least in part, to increased amounts of intracellular calcium.

Calcium Effects on Mitochondrial ATP Formation and Oxygen Consumption

Since a reduction in cell ATP levels may be related to increased intracellular calcium, it is possible that calcium affects mitochondria, the major site for cellular ATP synthesis. Therefore, we studied the effects of different concentrations of Ca^{2+} on lung mitochondrial ATP formation and oxygen consumption. In order to ascertain the exact concentrations in the mitochondrial suspensions, the Ca^{2+} levels were measured with Indo dye, because 0.1 mM EDTA, as well as other potential calcium chelators, are included in the incubation mixtures (Spear and Lumeng, 1978; Fisher *et al.*, 1973). In fact, very little ATP is formed in the absence of EDTA. The effects of different concentrations of Ca^{2+} (between 20 and 200 nM) on mitochondrial ATP formation

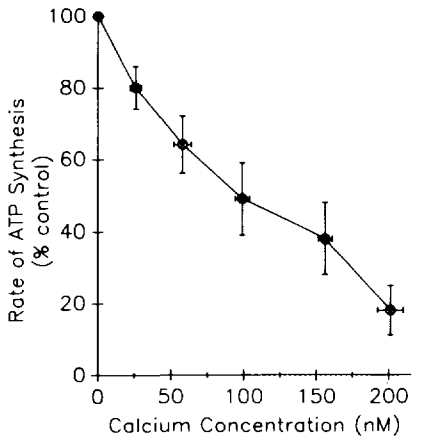

FIG. 3. Effects of calcium on the rate of ATP synthesis by isolated lung mitochondria. Mitochondria (0.15 mg protein/ml) were incubated for 5 min in medium containing 105 mM KCl, 2 mM KH_2PO_4, 30 mM Tris-HCl, 0.1 mM EDTA, 1% BSA, 5 mM succinate, 10 μM ADP, and different concentrations of $CaCl_2$ (pH 7.2). The exact calcium concentrations were determined in corresponding samples via measurements with Indo dye. The calcium levels varied slightly from experiment to experiment due to the presence of chelating agents in the mitochondrial suspensions. After the 5-min incubation, ATP production was measured as described under Methods. The results are expressed as a percentage of the rate of ATP synthesis in untreated (no Ca^{2+}) mitochondria. ATP synthesis in untreated mitochondria was 216 (±18) nmoles/min/mg protein. The points are mean values for five experiments and bars represent standard errors of the means.

are shown in Fig. 3. The results show that Ca^{2+} inhibits mitochondrial ATP synthesis in a concentration-dependent manner. Effects are seen with calcium concentrations as low as 25 nM. At the same time, there is no effect on mitochondrial oxygen consumption (in the presence of 5 mM succinate and 0.3 mM ADP); i.e., oxygen consumption in the presence of 200 μM added Ca^{2+} is 94(±5)% of that in the absence of Ca^{2+}. We also performed two experiments with mitochondria isolated from type II cells and obtained similar results (data not shown). Thus, the effects of Ca^{2+} on mitochondria are similar to the effects of bisbenzylisoquinoline alkaloids on type II cell ATP levels and oxygen consumption. In another set of experiments (Table 5), we determined that incubation of isolated lung mitochondria with TT has no effect on mitochondrial ATP formation. These results suggest that the TT-induced reduction in cellular ATP levels is not due to a direct effect of the alkaloid on mitochondria, but it may be due to the effects of increased internal calcium on mitochondria.

DISCUSSION

The results of this paper demonstrate that tetrandrine and other bisbenzylisoquinoline alkaloids, which have potential use as treatments for fibrotic lung diseases, are capable of altering the metabolism of isolated alveolar type II cells. The major effect seems to be a reduction in the level of cellular ATP. This effect may be initiated by an alkaloid-induced increase in the intracellular calcium concentration. The increase in internal calcium may, in turn, cause a reduction in mitochondrial ATP formation with no effect on mitochondrial oxygen consumption. Therefore, the overall effect is a reduced cellular ATP level which may impair the ability of type II cells to carry out some of their normal functions.

There are at least three lines of evidence to suggest that an alkaloid-induced increase in internal calcium is responsible, at least in part, for the reduction in cellular ATP levels. (1) All alkaloids which cause a reduction in cell ATP levels (with no change in oxygen consumption) produce an increase in intracellular calcium (Tables 2 and 3). (2) Similar effects on cell ATP levels and oxygen consumption are produced when internal calcium is elevated by exposing the cells to a calcium ionophore, 4-bromo A-23187 (Table 4). (3) When isolated mitochondria are incubated with increased amounts of calcium, the result is a reduction in mitochondrial ATP synthesis (Fig. 3) with no effect on oxygen consumption. Thus, type II cell ATP levels may be reduced when the alkaloid-induced elevation in internal calcium produces inhibition of mitochondrial ATP synthesis.

Several other investigators have studied the effects of calcium on mitochondrial function. Lindberg and Ernster (1954; Ernster and Lindberg, 1955) have shown that calcium ions cause uncoupling of oxidative phosphorylation in liver mitochondria. Chance and Williams (1956) have referred to calcium as being a "decontrolling" agent in mitochondria. Fisher *et al.* (1973) demonstrated that the calcium level in rat lung mitochondria is important in determining rates of substrate oxidation. They reported that addition of calcium depresses the oxidation of NAD-linked substrates and activates the mitochondrial glycerophosphate dehydrogenase. In our study, calcium causes an inhibition of ATP synthesis in lung mitochondria. Although the

TABLE 5
Effects of Tetrandrine on Mitochondrial ATP Synthesis[a]

Treatment	Rate of ATP synthesis (% untreated mitochondria)[b]
Control	100
Tetrandrine (20 μM)	107 (±6)

[a] Mitochondria (0.15 mg protein/ml) were incubated for 5 min in medium containing 105 mM KCl, 2 mM KH_2PO_4, 30 mM Tris-HCl, 0.1 mM EDTA, 1% BSA, 5 mM succinate, and 10 μM ADP (pH 7.2). Experiments were done in medium with and without TT. Then ATP levels were measured as described under Methods. Values are means ± SE for five experiments.

[b] ATP synthesis in untreated mitochondria was 205 (±21) nmoles/min/mg protein.

mechanism for this is not clear, it is apparent that varying the level of calcium around mitochondria has pronounced effects on mitochondrial function.

In these experiments, we encountered two technical problems which make it difficult to put the results together in a quantitative manner. First, due to leakage of the dye from the cells with increasing time of incubation (as mentioned under Methods), it is not possible to accurately follow intracellular calcium changes induced by the alkaloids and the calcium ionophore for more than a few minutes. However, the increases in intracellular calcium induced by these substances do seem to be sustained. This may account for the relatively substantial effects on ATP levels with what some may consider to be relatively modest increases in internal calcium; i.e., most intracellular calcium changes which are produced by various substances in other tissue and cells are more transient. Second, it is difficult to relate in a quantitative manner the effects of calcium on mitochondrial ATP synthesis (Fig. 3) to the effects of intracellular calcium changes on cellular ATP levels. The data in Fig. 3 show that if the calcium levels around mitochondria are increased from approximately 140 nM to 170 nM, i.e., a change similar to that induced by alkaloids, there is only a 20% reduction in mitochondrial ATP synthesis. Yet, cellular ATP levels are reduced much more. The problem is that mitochondria were incubated with calcium for only 5 min because the respiratory control ratio tends to be reduced with longer periods of incubation (as mentioned under Methods). Therefore, the calcium effects on mitochondrial ATP synthesis may be greater with exposure times similar to the 1-hr incubations used for cellular measurements.

The major effect of the bisbenzylisoquinoline alkaloids on type II cells is a dramatic reduction in the cellular ATP content. A reduced level of ATP may be due to a decrease in its rate of synthesis, an increase in its rate of degradation, or both of the above. In this manuscript, we have studied only ATP synthesis and have not measured ATP degradation and/or utilization. In addition to a reduction in the rate of ATP synthesis, there may be other factors involved in the lowered cellular ATP levels. We have shown in a previous report that glucose uptake and glycolysis may be impaired when type II cell ATP levels are reduced (LaCagnin *et al.*, 1990). This may further contribute to a decline in cellular ATP via a decline in the substrates available to mitochondria, e.g., pyruvate.

Finally, we also studied the effects of tetrandrine on the incorporation of choline into disaturated phosphatidylcholines (DSPC) in type II cells (data not presented). The results show that TT produces inhibition of DSPC synthesis in a time- and concentration-dependent manner. The mechanism(s) for this effect is not known. Two possible reasons for the inhibition are (1) a reduction in cellular levels of ATP, which serves as a cofactor for some enzymes involved in phospholipid synthesis, and (2) inhibition of choline-phosphotransferase by calcium, a potent inhibitor of this enzyme (Van Huesden *et al.*, 1981). In this regard, we have shown previously that incubation of type II cells in medium with calcium and no magnesium leads to reduced levels of DSPC synthesis (Miles *et al.*, 1983). These results do show that TT produces inhibition of surfactant DSPC synthesis, one of the most important functions of alveolar type II cells.

In summary, the results of this study demonstrate that the metabolism of alveolar type II cells is altered by tetrandrine and some other bisbenzylisoquinoline alkaloids. Although there may be other possible explanations, the results are consistent with the idea that the synthesis of ATP by mitochondria is inhibited by an alkaloid-induced increase in intracellular calcium. This may, in turn, lead to impairment of other type II cell functions, such as synthesis of pulmonary surfactant. In this regard, it may be interesting to study the pulmonary effects of *in vivo* exposure of animals to the alkaloids.

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