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ATP promotes cell survival via regulation of cytosolic [Ca^{2+}] and Bcl-2/Bax ratio in lung cancer cells

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Song S, Jacobson KN, McDermott KM, Reddy SP, Cress AE, Tang H, Dudek SM, Black SM, Garcia JG, Makino A, Yuan JX. ATP promotes cell survival via regulation of cytosolic [Ca^{2+}] and Bcl-2/Bax ratio in lung cancer cells. Am J Physiol Cell Physiol 310: C99–C114, 2016. First published October 21, 2015; doi:10.1152/ajpcell.00092.2015.—Adenosine triphosphate (ATP) is a ubiquitous extracellular messenger elevated in the tumor microenvironment. ATP regulates cell functions by acting on purinergic receptors (P2X and P2Y) and activating a series of intracellular signaling pathways. We examined ATP-induced Ca^{2+} signaling and its effects on antiapoptotic (Bcl-2) and proapoptotic (Bax) proteins in normal human airway epithelial cells and lung cancer cells. Lung cancer cells exhibited two phases (transient and plateau phases) of increase in cytosolic [Ca^{2+}] ([Ca^{2+}]_{cyt}) caused by ATP, while only the transient phase was observed in normal cells. Removal of extracellular Ca^{2+} eliminated the plateau phase increase of [Ca^{2+}]_{cyt} in lung cancer cells, indicating that the plateau phase of [Ca^{2+}]_{cyt} increase is due to Ca^{2+} influx. The distribution of P2X (P2X1-7) and P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) receptors was different between lung cancer cells and normal cells. Proapoptotic P2X7 was nearly undetectable in lung cancer cells, which may explain why lung cancer cells showed decreased cytotoxicity when treated with high concentration of ATP. The Bcl-2/Bax ratio was increased in lung cancer cells following treatment with ATP; however, the antiapoptotic protein Bcl-2 demonstrated more sensitivity to ATP than proapoptotic protein Bax. Decreasing extracellular Ca^{2+} or chelating intracellular Ca^{2+} with BAPTA-AM significantly inhibited ATP-induced increase in Bcl-2/Bax ratio, indicating that a rise in [Ca^{2+}]_{cyt} through Ca^{2+} influx is the critical mediator for ATP-mediated increase in Bcl-2/Bax ratio. Therefore, despite high ATP levels in the tumor microenvironment, which would induce cell apoptosis in normal cells, the decreased P2X7 and elevated Bcl-2/Bax ratio in lung cancer cells may enable tumor cells to survive. Increasing the Bcl-2/Bax ratio by exposure to high extracellular ATP may, therefore, be an important selective pressure promoting transformation and cancer progression.

Energy source, also functions as an extracellular messenger (2). ATP receptors are purinergic receptors (P2 receptors) and include the ligand-gated ion channel family of P2 receptors (P2X1-7) as well as the G protein-coupled receptor (GPCR) family of P2 receptors (P2Y1-2, P2Y4, P2Y6, and P2Y11-14) (3, 31). Activation of P2X receptors, which are nonselective cation channels formed by three homomeric or heteromeric P2X subunits, directly results in Na^{+} and Ca^{2+} influx through the cell plasma membrane, leading to membrane depolarization, which in turn activates voltage-gated Na^{+} and Ca^{2+} channels and causes the firing of action potentials. Activation of P2Y receptors, which are GPCRs, increases cytosolic free Ca^{2+} concentration ([Ca^{2+}]_{cyt}) by inducing Ca^{2+} release from intracellular stores (e.g., sarcoplasmic or endoplasmic reticulum) and Ca^{2+} influx through store-operated (SOC) and/or receptor-operated (ROC) Ca^{2+} channels. P2X and P2Y signaling is not only responsible for inducing action potentials in excitable cells (e.g., neurons), but also has been implicated in cell proliferation, differentiation, and apoptosis in nonexcitable cells (e.g., epithelial cells) (5). In a lung cancer cell line, A549, extracellular ATP, UTP, and UDP have been shown to stimulate proliferation that is dependent on the P2Y2 and P2Y6 receptors (44).

Extracellular or intercellular ATP concentration is reported to be very low (1–5 μM) in normal healthy tissues; however, it is significantly increased (to >100 μM) in the tumor microenvironment (26). The effect of increased extracellular ATP on cancer cell function is, however, dependent on the expression of different P2 receptors and changes in cytosolic [Ca^{2+}]_{cyt} due to activation of the different P2 receptors. Recent observations imply that differential expression of P2X and P2Y receptors may determine the kinetically distinct patterns of ATP-mediated increases in [Ca^{2+}]_{cyt}; the different patterns of Ca^{2+} signaling may lead to differential effects on cancer cells (e.g., proliferation vs. apoptosis) (34). This underscores the necessity of further characterization of P2 receptors in tumor cells, which could lead to novel therapeutic strategies that target purinergic signaling in the treatment of cancer.

Intracellular Ca^{2+} plays a critical role in the regulation of processes relevant to tumorigenesis, including cell proliferation, migration, and apoptosis (1, 22, 34). The role of Ca^{2+} channels and Ca^{2+} pumps in tumorigenesis is achieved through...
altered global or local changes in $[\text{Ca}^{2+}]_{\text{cyt}}$. The remodeling of $\text{Ca}^{2+}$ signals in cancer cells is due potentially to aberrant expression of different $\text{Ca}^{2+}$ channels, $\text{Ca}^{2+}$ pumps, and $\text{Ca}^{2+}$ transporters (22, 30). For example, it has been shown that P2Y$_2$ and P2Y$_4$ receptor expression in A549 cells promotes an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and induces $\text{Ca}^{2+}$-dependent release of ATP and UTP as part of a positive feedback loop (4).

The Bcl-2 protein family consists of both antiapoptotic and proapoptotic members, which are traditionally considered to reside in or translocate to mitochondria and function as apoptotic regulators by modulating mitochondrial membrane permeability. The ratio between anti- and proapoptotic Bcl-2 proteins determines whether cells survive or die (13). The Bcl-2 family of proteins has also been detected in other subcellular compartments including the endoplasmic reticulum, nuclear membrane, plasma membrane and within the subcellular compartments (15, 28, 41), while changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ also exert effects on the expression of the anti- or proapoptotic Bcl-2 proteins. The Bcl-2 family of proteins also functions to relay $\text{Ca}^{2+}$ signals to regulate cell apoptosis and proliferation. We hypothesize that there is a feedback loop in which $\text{Ca}^{2+}$ signaling regulates the expression of Bcl-2 family members, resulting in an increase of the Bcl-2/Bax ratio.

In this report, we show that extracellular ATP induces only a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in normal lung epithelial cells; however, ATP induces a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ followed by a plateau phase (or a slowly declining phase) increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ in lung cancer cells (H23 and A549) that is dependent on extracellular $\text{Ca}^{2+}$. Furthermore, extracellular treatment with ATP increases the ratio of the antiapoptotic protein, Bcl-2, to the proapoptotic protein, Bax (Bcl-2/Bax ratio) in lung cancer cells, but not in normal lung epithelial cells. We also demonstrate that chelation of intracellular $\text{Ca}^{2+}$ (with BAPTA-AM) or removal of extracellular $\text{Ca}^{2+}$ inhibits the ATP-induced increase of Bcl-2/Bax ratio in lung cancer cells. These data imply that extracellular ATP, by selectively inducing a sustained or long-lasting increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in lung cancer cells (but not in normal lung epithelial cells), promotes cell survival and enhances lung tumor growth through increase of the Bcl-2/Bax ratio. Inhibiting ATP-induced $\text{Ca}^{2+}$ influx in lung cancer cells may lead to novel therapeutic approaches for lung cancer.

**MATERIALS AND METHODS**

**Chemicals and drugs.** Adenosine 5-triphosphate disodium salt hydrate (ATP), uridine 5-triphosphate trisodium salt hydrate (UTP), $\alpha$-methylene adenosine 5-triphosphate lithium salt ($\alpha$-mATP), $\beta$-y-methyleneadenosine 5-triphosphate disodium salt ($\beta$-y-mATP), and TNP-ATP hydrate were prepared as concentrated stock solutions in distilled water. U-73122 hydrate, BAPTA-AM, and cyclopiazonic acid (CPA) were prepared as concentrated stock solutions in dimethyl sulfoxide (DMSO). All stock solutions (in water, DMSO) were aliquoted and kept frozen at $-20^\circ\text{C}$ until use. On the day of experiments, aliquots of the stock solutions were diluted in HEPES-buffered bath solution to the final concentrations for each drug. The pH values of all solutions were measured after addition of the drugs and adjusted to 7.4. All drugs were from Sigma Chemical, unless otherwise indicated.

**Cell culture.** Human non-small cell lung cancer cell lines A549 and H23 and human airway epithelial cell line BEAS-2B (American Type Culture Collection, Manassas, VA) were cultured in DMEM medium (Corning Cellgro, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY), 100 IU/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were routinely cultured in 20% $\text{O}_2$ and 5% $\text{CO}_2$ at 37°C.

**Measurement of $[\text{Ca}^{2+}]_{\text{cyt}}$.** Human non-small cell lung cancer cell lines A549 and H23 and human airway epithelial cell line were grown at 50%–60% confluence on 25-mm diameter circular glass coverslips. Cells were loaded for 60 min in room temperature (23°C) with 4 $\mu$M fura-2 acetoxyethyl ester (fura-2/AM; Invitrogen/Molecular Probes, Eugene, OR) in HEPES-buffered solution. Coverslips with cells were placed into the recording chamber of an inverted fluorescent microscope (Eclipse Ti-E; Nikon, Tokyo, Japan) equipped with an objective lens (S Plan Fluor 20/0.45 ELWD; Nikon), an EM-CC camera (Evolve; Photometrics, Tucson, AZ) and the NIS Elements 3.2 software (Nikon), and superfused with HEPES-buffered solution for 30 min to wash out residual extracellular fura-2/AM and allow sufficient time for intracellular esterase to convert fura-2/AM to fura-2. With excitation at 340 and 380 nm, the emitted fluorescence was acquired at 520 nm by a fluorescent objective lens and an EM-CCD camera using NIS Elements 3.2 software. Intracellular $\text{Ca}^{2+}$ concentration was expressed as 340/380 fluorescence ratio within an area of interest in a cell recorded every 2 s. The HEPES-buffered solution contained (in mM) 137 $\text{NaCl}$, 5.9 $\text{KCl}$, 1.8 $\text{CaCl}_2$, 1.2 $\text{MgCl}_2$, 14 glucose, and 10 HEPES (pH was adjusted to 7.4 with 10 N NaOH). The $\text{Ca}^{2+}$-free solution was prepared by replacing 1.8 mM CaCl$_2$ with equimolar MgCl$_2$ and adding 1 mM EGTA to chelate residual Ca$^{2+}$. Measurement of $[\text{Ca}^{2+}]_{\text{cyt}}$ was carried out at room temperature (23°C). For each experimental replicate we quantitated the fluorescence ratios for at least 20 cells.

**Western blotting.** Total protein was isolated from human non-small cell lung cancer cell lines A549 and H23 and human airway epithelial cell line by the TRIzol method. The extracted RNA was quantified by spectrophotometry at 260 nm. The synthesis and polymerase chain reaction were carried out using a Platinum PCR SuperMix (Invitrogen, Grand Island, NY) with 3-step cycling. The relative expression levels of P2X and P2Y receptors were normalized against the amount of GAPDH mRNA in the same RNA extract. Quantitative real-time RT-PCR was performed on a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad) using an iTaq Universal SYBR Green Supermix (Bio-Rad) following the instructions of the manufacturer. All samples were run in triplicate, and GAPDH was used as an internal control. The expression level of P2YR mRNA or P2XR mRNA was normalized to GAPDH and is expressed as a ratio relative to GAPDH.

**Cell viability assay.** Viability of human non-small cell lung cancer cell line A549 and H23 and normal human airway epithelial cell line after treatments with DMEM with 10% FBS, 100 $\mu$M ATP in DMEM with 10% FBS, and 1 mM ATP DMEM with 10% FBS was determined by the Trypan blue dye exclusion assay. Control and treated cells were incubated with Trypan blue solution for 3–5 min and then counted on Countess (Invitrogen, Grand Island, NY). Cell numbers per milliliter cell suspension were determined in each preparation, and the percentage of cells that excluded Trypan blue stain is indicated as a measure of cell viability, which is expressed in percentage (%).
ATP INCREASES Bcl-2/Bax RATIO BY INDUCING SUSTAINED Ca\textsuperscript{2+} INFLUX
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Statistical analysis. Pooled data are shown as means ± SE. The statistical significance between two groups was determined by Student’s t-test. The statistical significance among groups was determined by Scheffé’s test after one-way analysis of variance. Significant difference is expressed in the figures or figure legends as P < 0.05.

RESULTS

Extracellular ATP induces a prolonged [Ca\textsuperscript{2+}]\textsubscript{cyt} response in lung cancer cells but not in normal lung epithelial cells. Extracellular ATP increases [Ca\textsuperscript{2+}]\textsubscript{cyt} through activation of different P2 receptors. To determine whether extracellular application of ATP induces different patterns of intracellular Ca\textsuperscript{2+} signaling in normal and lung cancer cells, we treated cells with 100 μM of ATP and measured [Ca\textsuperscript{2+}]\textsubscript{cyt} using the Ca\textsuperscript{2+} indicator fura-2. We compared a normal lung airway epithelial cell line (BEAS-2B) to two lung cancer cell lines (H23 and A549) that reflect the heterogeneity of human lung cancer (see Table 1 for details). Two different patterns ATP-induced increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} were observed in normal and lung cancer cells: a transient increase or slowly declining increase (Fig. 1, A–C). ATP induced a transient increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} in normal cells with a mean duration of 120 s (Fig. 1, A–C). In lung cancer cells, ATP induced the same rapid increase in [Ca\textsuperscript{2+}]\textsubscript{cyt}; however, the [Ca\textsuperscript{2+}]\textsubscript{cyt} rise that followed the transient increase was sustained for a longer period of time (a plateau increase) in H23 (mean duration of 660 s) and A549 cells (mean duration of 700 s). While 95% and 99% of H23 and A549 lung cancer cells responded to ATP, only 30% of normal cells responded (Fig. 1D). Of the responding cells, we observed three patterns: 1) Pattern 1, a transient increase followed by a plateau increase; 2) Pattern 2, a transient increase followed by a slowly declining increase; and 3) Pattern 3, only a transient increase (Fig. 1B). Pattern 3 represents the highest proportion of normal responding cells (78%) (Fig. 1E). In contrast, Pattern 1 represents the highest proportion of responding lung cancer cells (H23, 70%; A549, 55%) (Fig. 1E).

The [Ca\textsuperscript{2+}]\textsubscript{cyt} increase during the plateau phase was higher in A549 cells (mean of 0.06 [ratio 340/380]) compared with H23 cells (mean of 0.18 [ratio 340/380]). The results demonstrate that lung cancer cells respond to extracellular ATP with a prolonged increase in [Ca\textsuperscript{2+}]\textsubscript{cyt}. Since the ATP-induced increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} was mainly caused by activation of different P2 receptors, the ATP-induced plateau phase of increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} in lung cancer cells (but not in normal cells) suggests that ATP receptors (P2X or P2Y receptors) may be expressed differently in lung cancer cells versus normal cells.

In the next set of experiments, we performed RT-PCR and real-time RT-PCR to examine and compare the relative expression levels of P2X receptors (P2X2, P2X3, P2X4, P2X5, P2X6, P2X7) and P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) in normal and lung cancer cells. We observed a statistically significant (P < 0.05) increase in expression of several of the P2X receptors and the P2Y receptors in lung cancer cells compared with normal cells (P2X5: H23 = 2.05-fold, A549 = 3.02-fold; P2X4: H23 = 4.05-fold, A549 = 4.08-fold; P2X5: H23 = 11.00-fold, A549 = 3.45-fold. P2Y1: H23 = 4.55-fold, A549 = 2.37-fold; P2Y2: H23 = 3.44-fold, A549 = 14.53-fold; P2Y4: H23 = 7.12-fold, A549 = 4.18-fold; P2Y6: H23 = 12.64-fold, A549 = 24.84-fold) (Fig. 1, F–J). Our findings suggest that the prolonged increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} induced by ATP in lung cancer cells may be due to upregulated expression of P2 receptors.

Lung cancer cells are more sensitive to extracellular ATP than normal cells. To determine whether the pattern of ATP-induced increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} is concentration-dependent, we treated normal (BEAS-2B) and lung cancer cells (H23 and A549) with different concentrations of ATP. In lung cancer cells, ATP induced an increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} starting at 1 μM; however, ATP induced an increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} that was detectable at a higher concentration of 10 μM in normal cells (Fig. 2A). Both H23 and A549 cells that respond to ATP have a plateau phase of increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} when treated with higher concentrations of ATP (100 μM). While H23 cells also respond to lower concentrations of ATP (1 μM and 10 μM) with a plateau phase of increase in [Ca\textsuperscript{2+}]\textsubscript{cyt}, A549 cells respond to the lower concentrations of ATP with Ca\textsuperscript{2+} oscillations. To determine the total amount of increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} over time, we calculated the area under the curve (AUC). Lung cancer cells exhibited a dose-dependent increase in the AUC starting at 10 μM compared with normal cells (P < 0.05) (Fig. 2B). We observed that the transient phase of increase (induced by ATP starting at 1 μM) and plateau phase of increase (induced by ATP starting at 10 μM) in [Ca\textsuperscript{2+}]\textsubscript{cyt} were significantly higher in H23 and A549 cells than in normal cells (Fig. 2, C and D). Furthermore, we observed that in all cell types the amplitude of the transient increase (Fig. 2C) and the amplitude of the plateau increase (Fig. 2D) maximized at the ATP concentration of 10−6 to 10−5 M. Further increasing ATP concentration from 10−5 M to 10−3 M did not further increase the amplitude of both transient and plateau increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} (Fig. 2, C and D). These observations suggest that lung cancer cells are more sensitive to extracellular ATP and that the downstream intracellular Ca\textsuperscript{2+} signaling may be of higher intensity with a more prolonged duration in lung cancer cells than normal cells.

Ca\textsuperscript{2+} influx is required for ATP-induced plateau phase of increase of [Ca\textsuperscript{2+}]\textsubscript{cyt} in lung cancer cells. It is known that ATP can increase [Ca\textsuperscript{2+}]\textsubscript{cyt} by inducing Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores and/or Ca\textsuperscript{2+} influx from extracellular source via the GPCR P2Y receptors. To determine whether the observed ATP-induced plateau phase of increase of [Ca\textsuperscript{2+}]\textsubscript{cyt} is dependent on extracellular Ca\textsuperscript{2+}, we treated cells with ATP in the absence of extracellular Ca\textsuperscript{2+}. In the absence of extracellular Ca\textsuperscript{2+}, only 5% of the normal cells (BEAS-2B) responded.

<table>
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<th>src\textsuperscript{+} Expression</th>
<th>ras\textsuperscript{+} Expression</th>
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<td>Normal</td>
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<td>Carcinoma</td>
<td>–</td>
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*Gene. †Protein. References in parentheses.
Fig. 1. ATP-induced increase in cytosolic free Ca$^{2+}$/H$^{+}$ concentration ([Ca$^{2+}$/H$^{+}$]$_{cyt}$) and P2X and P2Y purinergic receptor expression in normal airway epithelial cell line and lung cancer cell lines. A: representative images showing different patterns of [Ca$^{2+}$/H$^{+}$]$_{cyt}$ changes in normal (top) and lung cancer cells (middle and bottom) before, during, and after application of 100 μM ATP. B: representative traces showing different patterns of [Ca$^{2+}$/H$^{+}$]$_{cyt}$ changes in normal (top) and lung cancer cells (middle and bottom) before, during, and after application of 100 μM ATP. C: representative traces from B were overlaid to show the differences of [Ca$^{2+}$/H$^{+}$]$_{cyt}$ changes in normal and lung cancer cells in each pattern. D: summarized data showing the proportion of activated cells in each of normal and lung cancer cells (n = 4–14). E: summarized data showing the proportion of each pattern of changes of [Ca$^{2+}$/H$^{+}$]$_{cyt}$ induced by ATP in normal and lung cancer cells (n = 4–14 separated experiments). F: RT-PCR data showing the expression level of P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7 purinergic receptors in normal and lung cancer cells (n = 5). G: RT-PCR data showing the expression level of P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 purinergic receptors in normal and lung cancer cells (n = 4). H: summarized data (means ± SE) from real-time RT-PCR showing the expression level of P2X7 purinergic receptors in normal (Nor) and lung cancer cells (n = 3, *P < 0.05 vs. Normal; #P < 0.05 vs. Normal and A549, Normal and H23). I: summarized data (means ± SE) from real-time PCR showing the expression level of P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 purinergic receptors in normal and lung cancer cells (n = 3, *P < 0.05 vs. Normal; *P < 0.05 vs. Normal and A549, Normal and H23).
to ATP with a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 3, A–C). In H23 cells, ATP induced a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in 95% of the cells; however, the plateau phase increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ was no longer observed (Fig. 3, A–C). In A549 cells, ATP induced either transient increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ or $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations in 97% of the cells and, similar to H23 cells, the plateau phase of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ was no longer observed (Fig. 3, A–C). Extracellular ATP induced transient increases in

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Fig. 2. Dose-response curves of area under the curve, transient, and plateau amplitude of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by ATP in normal and lung cancer cells. A: representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ before, during, and after application of 1 nM, 100 nM, 1 μM, 10 μM, 100 μM, and 1 mM ATP in normal (top) and lung cancer cells (middle and bottom). B: summarized data (means ± SE) showing the dose-response curve of area under the curve of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by ATP in normal and lung cancer cells ($n = 3$, *P < 0.05 vs. Normal). C: summarized data (means ± SE) showing dose-response curve of transient amplitude of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by ATP in normal and lung cancer cells ($n = 3$, *P < 0.05 vs. Normal). D: summarized data (means ± SE) showing dose-response curve of plateau amplitude of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by ATP in normal and lung cancer cells ($n = 3$, *P < 0.05 vs. Normal).

Fig. 3. ATP-induced changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the absence of extracellular $\text{Ca}^{2+}$ in normal and lung cancer cells. A: representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ in normal and lung cancer cells before, during, and after application of 100 μM ATP when depriving extracellular $\text{Ca}^{2+}$ in individual cells. B: representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ in normal and lung cancer cells before, during, and after application of 100 μM ATP when depriving extracellular $\text{Ca}^{2+}$ in average. C: summarized data showing the proportion of activated cells in 1.8 mM and 0 mM extracellular $\text{Ca}^{2+}$ (OCa$^{2+}$) in normal and lung cancer cells, respectively ($n = 3–14$).
[Ca^{2+}]_{\text{cyt}} in both H23 and A549 cells superfused either with Ca^{2+}-containing (99% of H23 and A549 cells) solution or with Ca^{2+}-free solution (95% of H23 and A549 cells) (Fig. 3C). These data suggest that normal cells require extracellular Ca^{2+} for ATP to induce a transient increase in [Ca^{2+}]_{\text{cyt}}. In contrast, cancer cells do not require extracellular Ca^{2+} for ATP to induce a transient increase in [Ca^{2+}]_{\text{cyt}}. Our results also suggest that Ca^{2+} influx from extracellular source is responsible for the ATP-induced plateau phase of increase in [Ca^{2+}]_{\text{cyt}} observed in lung cancer cells.

Next, we wanted to investigate the potential mechanism that mediates Ca^{2+} influx in lung cancer cells (H23 and A549). Upon activation of P2Y receptors, ATP may mobilize Ca^{2+} from intracellular Ca^{2+} stores by inositol trisphosphate (IP_{3}) and activation of the IP_{3} receptor (a Ca^{2+} release channel) on the sarcoplasmic (SR) or endoplasmic (ER) reticulum membrane. To determine whether depletion or partial depletion of intracellular Ca^{2+} stores (SR) by ATP could induce store-operated Ca^{2+} entry (SOCE), we first treated cells with ATP in Ca^{2+}-free solution and observed a transient increase in [Ca^{2+}]_{\text{cyt}} (due apparently to Ca^{2+} leak from the intracellular stores) in both normal and lung cancer cells. We then superfused the cells with 1.8 mM Ca^{2+}-containing solution and observed that restoration of extracellular Ca^{2+} induced a second increase in [Ca^{2+}]_{\text{cyt}} which was due apparently to Ca^{2+} influx through store-operated Ca^{2+} channels (Fig. 4A). In both normal and lung cancer cells, the second increases in [Ca^{2+}]_{\text{cyt}} induced by ATP-mediated SOCE showed no statistically significant differences [0.08 ± 0.0013 (340/380 ratio) for normal cells; 0.08 ± 0.003 for H23 cells; and 0.11 ± 0.01 for A549 cells] (Fig. 4B) (19, 44). Next, we treated cells with cyclopiazonic acid (CPA), a reversible inhibitor of the SR/ER Ca^{2+}-pump (SERCA), passively depletes Ca^{2+} from the SR and induces SOCE. We observed that the second increase in [Ca^{2+}]_{\text{cyt}} due to extracellular Ca^{2+} restoration-mediated SOCE was significantly lower in H23 cells (0.10 ± 0.004) than in normal cells (0.45 ± 0.022, P < 0.05); however, we did not observe a significant difference in A549 cells (0.40 ± 0.010) compared with normal cells (Fig. 4, C and G). When ATP was then introduced to the cells in the presence of extracellular Ca^{2+}, we observed that the second increase in [Ca^{2+}]_{\text{cyt}} was significantly higher than that in the presence of extracellular Ca^{2+} without ATP in both normal and lung cancer cells (0.51 ± 0.010 for normal cells; 0.13 ± 0.003 for H23 cells; 0.47 ± 0.008 for A549 cells; P < 0.05 vs. the value in the absence of ATP) (Fig. 4, D, F, and G).

To determine whether P2X receptors are involved, we pretreated cells with TNP-ATP (P2X receptor inhibitor). TNP-ATP treatment significantly attenuated the amplitude of the second increase in [Ca^{2+}]_{\text{cyt}} induced by extracellular Ca^{2+} restoration with ATP in both normal and lung cancer cells (0.46 ± 0.012 for normal cells; 0.01 ± 0.003 for H23 cells; 0.44 ± 0.0047 for A549 cells; P < 0.05 vs. the value in the absence of TNP-ATP) (Fig. 4, E, F, and G). Taken together, these data suggest that ATP-induced Ca^{2+} influx is at least partially mediated through store-operated Ca^{2+} channels (SOCC) and P2X receptors.

P2X and P2Y receptors are both required for ATP-induced increases in [Ca^{2+}]_{\text{cyt}} in lung cancer cells. Activation of P2X and P2Y receptors is known to mediate changes in intracellular Ca^{2+} through different mechanisms. For example, activation of P2X receptors, nonsel ective cation channels, directly mediate Ca^{2+} influx through the receptors, while activation of P2Y receptors, GPCRs, mediates Ca^{2+} influx through receptor-operated and store-operated Ca^{2+} channels and/or Ca^{2+} mobilization from the SR/ER as a result of G protein-mediated increases in diacylglycerol (DAG) and IP_{3}. To determine whether P2X receptors are responsible for the ATP-induced plateau phase of [Ca^{2+}]_{\text{cyt}} increase in lung cancer cells (H23 and A549), we treated cells with the P2X receptor inhibitors PPADS and TNP-ATP (14, 16). We observed that both PPADS and TNP-ATP decreased the ATP-induced transient phase of [Ca^{2+}]_{\text{cyt}} increase in normal cells and the ATP-induced transient and plateau phases of [Ca^{2+}]_{\text{cyt}} increases in lung cancer cells (Fig. 5A). To determine whether P2X receptor activation can directly increase [Ca^{2+}]_{\text{cyt}}, by Ca^{2+} influx through the receptors, we treated cells with P2X receptor activators β,γ-meATP and αβ,meATP (11, 23). We observed that αβ-meATP induced a small increase in [Ca^{2+}]_{\text{cyt}} in both normal and lung cancer cells, while β,γ-meATP only induced a small increase in [Ca^{2+}]_{\text{cyt}} in A549 cells but not in normal cells and H23 cells (Fig. 5B).

It is known that P2Y receptors are GPCRs that can activate phospholipase C (PLC) to increase [Ca^{2+}]_{\text{cyt}} via increasing IP_{3} and DAG. U-73122, a PLC inhibitor, attenuated ATP-induced transient phase of [Ca^{2+}]_{\text{cyt}} increase in both normal and lung cancer cells. Only a small increase in [Ca^{2+}]_{\text{cyt}} was induced by ATP in H23 and A549 cells, but not in normal cells, after U-73122 treatment (Fig. 5C) (42).

We next examined whether the P2Y receptor activator UTP can increase [Ca^{2+}]_{\text{cyt}} (35). We observed that only transient or oscillatory increases of [Ca^{2+}]_{\text{cyt}} (without the plateau phase) were observed in normal and lung cancer cells in response to UTP (Fig. 5C). These observations suggest that both P2X and P2Y receptors are involved in the ATP-induced increase in [Ca^{2+}]_{\text{cyt}} in normal and lung cancer cells, of which P2X receptors are involved in Ca^{2+} influx from extracellular source and P2Y receptors are involved in Ca^{2+} release from intracellular Ca^{2+} stores. The specific subtypes of P2X receptors or P2Y receptors involved cannot be discriminated due to the nonspecificity of the inhibitors and activators. Future studies are needed to identify the specific subunits of P2X and P2Y receptors involved in ATP-mediated increases in [Ca^{2+}]_{\text{cyt}}.

Lung cancer cells have a decreased cytotoxic response to extracellular ATP compared with normal cells. RT-PCR data have previously shown that the expression level of P2X_{7} mRNA was very low in lung cancer cells (H23 and A549) compared with normal cells (BEAS-2B) (20, 21, 39). The P2X_{7} receptor is known to be involved in non-Bcl-2/Bax-mediated cell apoptosis (29, 44). The loss of P2X_{7} in lung cancer cells is surprising, given that numerous studies have reported that ATP can exert a cytotoxic effect on several tumor cell types (6, 12, 33, 43). Therefore, we investigated whether extracellular ATP has a differential effect on cell growth processes (including cell apoptosis and cell proliferation) in normal cells versus lung cancer cells.

There was no effect of 100 μM ATP on cell viability in both normal and lung cancer cells in comparison to their control during ATP stimulation at 24, 48, and 72 h, respectively (Fig. 6A). However, a decrease in cell viability was observed when normal cells were treated with 1 mM ATP for 48 h (75.33% ± 9.23 vs. control of 89.25% ± 3.74, P < 0.05) and 72 h...
Decreased cell viability was also observed in H23 lung cancer cells at 72 h (65.00% ± 6.61 vs. control of 81.00% ± 3.88, \(P < 0.05\)) with 1 mM ATP, while no change of cell viability was observed in A549 lung cancer cells (Fig. 6A).

We next examined the ability of ATP to affect cell proliferation. Cell proliferation was inhibited in normal cells treated with 100 \(\mu\)M ATP for at 72 h (4.25 \(\times 10^6\) ± 0.45 vs. control of \(7.22 \times 10^6\) ± 2.00, \(P < 0.05\)) and with 1 mM ATP for 48 h (3.06 \(\times 10^6\) ± 0.81 vs. control of \(5.69 \times 10^6\) ± 0.89, \(P < 0.05\)) and 72 h (3.06 \(\times 10^6\) ± 1.03 vs. control of \(7.22 \times 10^6\) ± 2.00, \(P < 0.05\)) (Fig. 6B). There was also a small decrease in cell proliferation in lung cancer cells at 72 h when cells were treated with 100 \(\mu\)M ATP (1.03 \(\times 10^6\) ± 0.59 vs. control of 1.74 \(\times 10^6\) ± 0.50 for H23 cells; 4.08 \(\times 10^6\) ± 0.88 vs. control of 5.82 \(\times 10^6\) ± 0.97 for A549 cells, \(P < 0.05\)). However, no
change in cell proliferation was observed in lung cancer cells when treated with 1 mM ATP (Fig. 6B).

These data indicate that relatively low concentrations of ATP (micromole) negatively modulate cell proliferation but not cell viability in lung cancer cells. Higher concentrations of ATP (in the millimolar range), however, do not affect cell proliferation in lung cancer cells, but induce decreased cell viability in H23 cells. In normal cells, in which P2X7 receptors are expressed at a higher level, we observed consistent effects of ATP on cell viability and cell proliferation. Future studies are needed to determine whether the absence of P2X7 uncouples cell viability and proliferation in lung cancer cells.

**ATP increases the Bcl-2/Bax ratio in lung cancer cells.** Since the lung cancer cells (H23 and A549) express low levels of the P2X7 receptor, we considered whether ATP could modulate cell apoptosis by other means. Antiapoptotic Bcl-2 is well known for its classic role in blocking cytochrome c release from mitochondria and thereby inhibiting apoptosis; its expression is upregulated in many tumor cells (9, 10). Bax, a member of the Bcl-2 family of proteins, has a proapoptotic role that opposes Bcl-2; as such, high Bcl-2/Bax ratio represents an antiapoptotic cell state. Therefore we examined the expression levels of both Bcl-2 and Bax proteins to determine whether extracellular ATP affects Bcl-2/Bax ratio in normal and lung cancer cells.

The cells were first treated with vehicle (water) for 0, 2, 4, 8, 16, and 24 h; Bcl-2 and Bax protein expression levels, as well as Bcl-2/Bax ratio, showed no change in both normal and lung cancer cells (Fig. 7, A, D, and E). We observed that Bcl-2 protein expression level was higher in basal conditions (0 h) in lung cancer cells than in normal cells, while the Bax protein expression level showed no difference between normal and lung cancer cells (Fig. 7, A and D). We observed that Bcl-2 protein expression level was significantly increased following treatment with ATP (100 μM and 1 mM) for 0, 2, 4, 8, 16, 24 h. In normal cells, both Bcl-2 and Bax protein expression levels, as well as Bcl-2/Bax ratio, were not changed following treatment with ATP (100 μM and 1 mM) (Fig. 7, B, C, F, G, H, and I). In lung cancer cells (H23 and A549), Bcl-2 protein expression was upregulated following treatment with ATP (100 μM and 1 mM) from 2 h to 24 h, while Bax protein level was negligibly changed or slightly (but not significantly) increased (Fig. 7, B, D, F, and H). The Bcl-2/Bax ratio was significantly increased following treatment of lung cancer cells (H23 and A549) with ATP (100 μM and 1 mM) (Fig. 7, G and I). We observed that Bcl-2, rather than Bax, was more sensitive to ATP in lung cancer cells H23 and A549. In normal cells, because of the low expression level of Bcl-2 protein, the Bcl-2/Bax ratio was much lower and less affected by ATP than in lung cancer cells. However, consistent with the decreased viability observed in
Intracellular Ca$^{2+}$ induced only transient or oscillatory increases in [Ca$^{2+}$]$_{cyt}$ in lung cancer cells. We observed that intracellular Ca$^{2+}$ is required for regulation of Bcl-2 and Bax protein levels, as well as Bcl-2/Bax ratio, measured expression levels of Bcl-2 and Bax proteins. In both normal and lung cancer cells, Bcl-2 and Bax protein expression showed no change during ATP treatment. These data suggest that Ca$^{2+}$ influx is required for ATP-induced increases of the Bcl-2/Bax ratio in lung cancer cells, following treatment with ATP. These data also suggest that the Ca$^{2+}$ influx is responsible for the enhancement of Bcl-2/Bax ratio in both normal and lung cancer cells.

Intracellular Ca$^{2+}$ is required for extracellular ATP to increase Bcl-2/Bax ratio in lung cancer cells. To understand the potential mechanism by which ATP increases Bcl-2/Bax ratio, we focused on ATP-induced [Ca$^{2+}$]$_{cyt}$ increase, which is one of the major downstream messengers of P2 receptor (P2X and P2Y) activation. Cells were treated with BAPTA-AM, a membrane-permeable Ca$^{2+}$ chelator, for 30 min before treatment with ATP (100 μM and 1 mM) to determine whether intracellular Ca$^{2+}$ is required for regulation of Bcl-2 and Bax protein expression. BAPTA-AM pretreatment blocked the ATP-induced increase of [Ca$^{2+}$]$_{cyt}$ in both normal and lung cancer cells (H23 and A549) (Fig. 8, A and B). We also treated cells with ATP (100 μM and 1 mM) for 0, 2, 4, 8, 16, 24 h, respectively, after BAPTA-AM treatment to determine whether intracellular Ca$^{2+}$ is required for ATP-induced increase in Bcl-2/Bax ratio. In both normal and lung cancer cells, chelation of intracellular Ca$^{2+}$ with BAPTA-AM precluded the ATP-induced changes in Bcl-2 and Bax protein expression and Bcl-2/Bax ratio (Fig. 8, C–H). These data suggest that an increase of intracellular Ca$^{2+}$ is required for extracellular ATP to increase Bcl-2/Bax ratio in lung cancer cells.

Ca$^{2+}$ influx is required for ATP-induced increase of Bcl-2/Bax ratio in lung cancer cells. ATP induces two phases of increase in [Ca$^{2+}$]$_{cyt}$ in lung cancer cells (H23 and A549), the transient phase (due to Ca$^{2+}$ release) and the plateau phase (due to Ca$^{2+}$ influx), which we investigated. To determine the role of Ca$^{2+}$ influx, we decreased the extracellular Ca$^{2+}$ concentration in the media to 80 nM to inhibit Ca$^{2+}$ influx while avoiding cell death. Under these conditions, we observed that ATP (100 μM and 1 mM) induced only transient or oscillatory increases in [Ca$^{2+}$]$_{cyt}$ without a plateau phase in both normal and lung cancer cells (Fig. 9, A and B). We also treated cells with ATP (100 μM and 1 mM) for 0, 2, 4, 8, 16, 24 h in 80 nM Ca$^{2+}$ media and measured expression levels of Bcl-2 and Bax proteins. In both normal and lung cancer cells, ATP-induced increase of the Bcl-2/Bax ratio is not mediated by Ca$^{2+}$ influx through P2X receptors. However, U-73122 has been shown to inhibit phospholipase A2 (PLA2) and 5-lipoxygenase as well. To our knowledge, these enzymes are not involved in Bcl-2/Bax protein regulation. Further stud-
ies are needed to focus on investigating the channels that mediate ATP-induced Ca\textsuperscript{2+} influx required for the plateau phase in lung cancer cells.

**DISCUSSION**

In addition to functioning as a molecular energy source, ATP is also an important extracellular messenger and agonist. In this study, we have shown that ATP induces different Ca\textsuperscript{2+} signaling patterns in normal lung airway epithelial cells versus lung cancer cells. Purinergic receptors P2Xs and P2Ys are ATP receptors that are widely expressed on multiple cell types, including tumor cells. Our study showed that P2Xs and P2Ys exhibit different expression distribution in normal and lung cancer cells. Our data show that P2X3, P2X4 and P2X5 recep-

Fig. 7. Effect of ATP on Bcl-2 and Bax expression and Bcl-2/Bax ratio in normal and lung cancer cells. A: Western blot analysis of Bcl-2 and Bax expression in normal (top) and lung cancer cells (middle and bottom) treated with vehicle (water) for 0, 2, 4, 8, 16, and 24 h. β-Actin was used as a control. B: Western blot analysis of Bcl-2 and Bax expression in normal (top) and lung cancer cells (middle and bottom) treated with 100 µM ATP for 0, 2, 4, 8, 16, and 24 h. β-Actin was used as a control. C: Western blot analysis of Bcl-2 and Bax expression in normal (top) and lung cancer cells (middle and bottom) treated with 1 mM ATP for 0, 2, 4, 8, 16, and 24 h. β-Actin is used as a control. D: summarized data (means ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with vehicle for 0, 2, 4, 8, 16, and 24 h. E: summarized data (means ± SE) showing the changes of Bcl-2/Bax ratio level in normal and lung cancer cells treated with vehicle for 0, 2, 4, 8, 16, and 24 h (n = 3). F: summarized data (means ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 100 µM ATP for 0, 2, 4, 8, 16, and 24 h. G: summarized data (means ± SE) showing the changes of Bcl-2/Bax ratio level in normal and lung cancer cells treated with 100 µM ATP for 0, 2, 4, 8, 16, and 24 h (n = 8, *P < 0.05 vs. Normal). H: summarized data (means ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16, and 24 h. I: summarized data (means ± SE) showing the changes of Bcl-2/Bax ratio level in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16, and 24 h (n = 7, *P < 0.05 vs. Normal).
tors and P2Y1, P2Y2, P2Y4 and P2Y6 receptors are expressed more highly in lung cancer cells than in normal cells. Indeed, P2Xs and P2Ys are known to respond to ATP to regulate tumorigenic behaviors including cell proliferation and apoptosis.

The Ca$^{2+}$ signal is also known to be involved in many cellular processes that promote tumorigenesis (1, 22). Ca$^{2+}$ signaling components that determine Ca$^{2+}$ signaling homeostasis change their expression or function in the occurrence and development of disease (30). The remodeling of Ca$^{2+}$ signaling that occurs in cancer cells is achieved through differential expression of specific Ca$^{2+}$ pumps and channels (30). Although alternative Ca$^{2+}$ signaling is not necessary for cancer initiation, the consequence of onset may contribute to tumor progression. Our study showed that ATP induces markedly Ca$^{2+}$ influx in lung cancer cells but not in normal cells. The most probable candidate channels for Ca$^{2+}$ influx are P2Xs, because P2Xs per se are Ca$^{2+}$-permeable cation channels (31).

Fig. 8. Effect of ATP on Bcl-2 and Bax expression and Bcl-2/Bax ratio in normal and lung cancer cells after application of BAPTA-AM. A: representative traces showing changes in \([Ca^{2+}]_{cyt}\) before, during, and after application of 100 \(\mu\)M ATP after treatment with 25 \(\mu\)M BAPTA-AM in normal and lung cancer cells. B: representative traces showing changes in \([Ca^{2+}]_{cyt}\) before, during, and after application of 1 mM ATP after treatment with 25 \(\mu\)M BAPTA-AM in normal and lung cancer cells. C: Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 100 \(\mu\)M ATP for 0, 2, 4, 8, 16, and 24 h after application of 25 \(\mu\)M BAPTA-AM. Bcl-2 and Bax were used as a control. D: Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16, and 24 h after application of 25 \(\mu\)M BAPTA-AM. Bcl-2 and Bax were used as a control. E: summarized data (means ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 100 \(\mu\)M ATP for 0, 2, 4, 8, 16, and 24 h after application of 25 \(\mu\)M BAPTA-AM. F: summarized data (means ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer cells treated with 100 \(\mu\)M ATP for 0, 2, 4, 8, 16, and 24 h after application of 25 \(\mu\)M BAPTA-AM. G: summarized data (means ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16, and 24 h after application of 25 \(\mu\)M BAPTA-AM. H: summarized data (means ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer cells treated with 1 mM ATP for 0, 2, 4, 8, 16, and 24 h after application of 25 \(\mu\)M BAPTA-AM.
As discussed earlier, H23 and A549 lung cancer cells had significantly higher levels of P2X3, P2X4, and P2X5 expression compared with normal cells. Studies have shown that P2X receptors desensitize ATP fast and mediate short-time inward current (36), which may underlie the limited Ca\(^{2+}\) influx induced by the activation of P2X receptors shown in P2X inhibitor and agonist data.

Store-operated Ca\(^{2+}\) entry (SOCE) through GPCR-activated store-operated Ca\(^{2+}\) channels (SOC) become the next most plausible overfunctioning Ca\(^{2+}\) influx pathway in consideration of overexpressed P2Y receptors (GPCRs) in lung cancer cells. P2Ys function as G protein-coupled receptors and can cause Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores to increase cytosolic [Ca\(^{2+}\)] upon ATP activation (3, 31, 44).

Fig. 9. Effect of ATP on Bcl-2 and Bax expression and Bcl-2/Bax ratio in normal and lung cancer cells in low extracellular Ca\(^{2+}\) culture conditions. A: representative traces showing changes in [Ca\(^{2+}\)]\(_{cyt}\) in normal and lung cancer cells before, during, and after application of 100 µM ATP in media containing 80 nM extracellular Ca\(^{2+}\). B: representative traces showing changes in [Ca\(^{2+}\)]\(_{cyt}\) in normal and lung cancer cells before, during, and after application of 1 mM ATP in media containing 80 nM extracellular Ca\(^{2+}\). C: Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 100 µM ATP in media containing 80 nM extracellular Ca\(^{2+}\), for 0, 2, 4, 8, 16, and 24 h. β-Actin was used as a control. D: Western blot analysis of Bcl-2 and Bax expression in normal and lung cancer cells treated with 1 mM ATP in media containing 80 nM extracellular Ca\(^{2+}\), for 0, 2, 4, 8, 16, and 24 h. E: summarized data (means ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 100 µM ATP in media containing 80 nM extracellular Ca\(^{2+}\), for 0, 2, 4, 8, 16, and 24 h. F: summarized data (means ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer cells treated with 100 µM ATP in media containing 80 nM extracellular Ca\(^{2+}\), for 0, 2, 4, 8, 16, and 24 h. G: summarized data (means ± SE) showing the changes of Bcl-2 and Bax expression level in normal and lung cancer cells treated with 1 mM ATP in media containing 80 nM extracellular Ca\(^{2+}\), for 0, 2, 4, 8, 16, and 24 h. H: summarized data (means ± SE) showing the changes of Bcl-2/Bax ratio in normal and lung cancer cells treated with 1 mM ATP in media containing 80 nM extracellular Ca\(^{2+}\), for 0, 2, 4, 8, 16, and 24 h. (n = 3).
ATP (by active depletion of Ca\(^{2+}\) from the intracellular stores) and CPA (by passive depletion of Ca\(^{2+}\) from the intracellular stores) both induced SOCE, but ATP/CPA-induced SOCE in cancer cells was not enhanced, but rather reduced, in H23 lung cancer cells. This result was surprising because aggressive cancer cells would be expected to show stronger Ca\(^{2+}\) signaling intensity in consideration of sustained proliferation and migration. Although it has been demonstrated in proliferative pulmonary artery smooth muscle cells that enhanced SOCE promotes cell proliferation and...
growth, its effect on tumor growth is less clear (38, 46). Several studies have shown that, in cancer, decreased SERCA2 expression leads to low ER Ca$^{2+}$ capacity, which then lowers the risk of mitochondrial Ca$^{2+}$ overload that triggers cell apoptosis (27, 40). In addition, overexpressed antiapoptotic protein Bcl-2 was shown to reduce ER Ca$^{2+}$ content in part by interaction with IP3 receptors (25). However, other studies have shown that overexpressed Bcl-2 protein conserved SR/ER Ca$^{2+}$ content by upregulating SERCA2 (7). These conflicting results suggest that the relationship between Bcl-2 protein and ER Ca$^{2+}$ content depends on cell context. It is of great interest that our results show that both H23 and A549 lung cancer cells had significantly higher levels of Bcl-2 protein expression compared with normal epithelial lung cells. Thus, the low SOCE in cancer cells may result from reduced ER Ca$^{2+}$ capacity, which is due to overexpressed Bcl-2 protein. Therefore, it is possible that low SOCE is important to survival if it normally couples to reduced ER Ca$^{2+}$ capacity to regulate mitochondrial Ca$^{2+}$ loading. Both SOCE and P2X-mediated Ca$^{2+}$ influx participate in the Ca$^{2+}$ influx induced by ATP; however, they are not critically significant to the different kinetic Ca$^{2+}$ signal in lung cancer cells compared with normal cells.

Cell viability results showing that lung cancer cells exhibit resistance to the cytotoxicity of high concentration of ATP in comparison to normal cells are consistent with published data that showed low (nearly undetectable) expression levels of P2X$\cdot$R in lung cancer cells (20, 21, 39). P2X$\cdot$ is considered to act as a proapoptotic factor through increasing cytomembrane permeability activated by a high concentration of ATP (9, 29). However, significantly decreased cell viability was observed in H23 lung cancer cells at late (72 h) culture but not in A549 lung cancer cells. These two lung cancer cell lines chosen in this study reflect the heterogeneity of the human lung cancer progression pathway, involving, for example, the expression of P53, Myc, and Ras. This allows us to speculate that, despite being lung carcinoma, each has its own particular response to environmental stimuli. We also showed in both normal and lung cancer cells that ATP affected cell proliferation. It is interesting to note that a lower concentration of ATP reduced cell proliferation in both H23 and A549 lung cancer cells, while reduced cell proliferation was not observed with a higher concentration of ATP. These findings are inconsistent with cell viability results that indicated more cell death in a high concentration of ATP while the cells remained healthy in low concentration. These results suggest that ATP-induced cell apoptosis and cell proliferation are through separate pathways, and each sensitizes to a specific concentration range of ATP.

It is worth mentioning here that A549 lung cancer cells have been reported to secrete extracellular matrix components that have ATP binding sites. In our study, A549 lung cancer cells start to respond to ATP at 1 μM, which is the same as H23 lung cancer cells. A strong increase in intracellular Ca$^{2+}$ increase was observed in A549 lung cancer cells after ATP stimulation, and P2X and P2Y receptor inhibitors weakened ATP-induced intracellular Ca$^{2+}$ increase in A549 lung cancer cells. These observations suggest that although extracellular matrix components secreted by A549 lung cancer cells might bind ATP, the binding ability is not sufficient to eliminate ATP action on P2 receptors. Therefore, the lack of effect of ATP on cell viability of A549 lung cancer cells is not due to competing binding of ATP to extracellular matrix components.

There are data suggesting that very high intracellular Ca$^{2+}$ levels can promote cell death through necrosis, whereas sustained and lower intracellular Ca$^{2+}$ increases induced by milder insults promote cell death through apoptosis (8, 24). In this study, our data showed that ATP induces slow and long term phase of [Ca$^{2+}$]$\text{_{cyt}}$ increase in lung cancer cells, but that ATP at both high and relatively lower levels rarely affected cell viability, which indicates cell apoptosis. This leads us to propose that ATP may enhance the ability of antiapoptosis of lung cancer cells in some way to desensitize the cytoxicity of Ca$^{2+}$ overload. Thus, we focused on the Bcl-2/Bax ratio, the change of which determines cell fate. Our data showed that ATP promotes antiapoptosis by increasing Bcl-2/Bax ratio in lung cancer cells. It is of great interest that cancer cells respond to extracellular ATP by increasing survival capacities via multiple anti-death pathways that include low P2X$\cdot$ expression, high Bcl-2 expression, and an increased Bcl-2/ Bax ratio.

To provide some insight on the mechanism of ATP-modulated Bcl-2/Bax ratio in lung cancer cells, we abolished ATP-induced [Ca$^{2+}$]$\text{_{cyt}}$ increase by using BAPTA-AM and eliminated Ca$^{2+}$ influx through treating cells in low extracellular Ca$^{2+}$ media, and both ways led to unchanged Bcl-2/Bax ratio in lung cancer cells. Additionally, U-73122 was applied to abolish Ca$^{2+}$ mobilizing from intracellular Ca$^{2+}$ stores rather than extracellular Ca$^{2+}$ influx. However, calcium image data showed that U-73122 also reduced Ca$^{2+}$ influx significantly. Cells treated with U-73122 showed no change in the ratio of Bcl-2/Bax after ATP treatment. PLC inhibition leads to attenuation or blockade of two other Ca$^{2+}$ influx pathways, store-operated Ca$^{2+}$ entry through SOC and receptor-operated Ca$^{2+}$ entry through ROC, even when P2Y receptors are activated by ATP. The U-73122 data indicate that, although the P2X family is one of the mechanisms that mediated ATP-induced Ca$^{2+}$ influx, the P2X contribution to ATP-induced total Ca$^{2+}$ influx is limited or, by itself, is insufficient to affect Bcl-2 expression and the Bcl-2/Bax ratio. These results suggest that a sustained increase in cytosolic Ca$^{2+}$ due to Ca$^{2+}$ influx from the extracellular environment is the critical mediator between extracellular ATP and an enhanced Bcl-2/Bax ratio in lung cancer cells. Thus, determining Ca$^{2+}$ channels and transporters leading to sustained [Ca$^{2+}$]$\text{_{cyt}}$ increase in lung cancer cells would provide an important therapeutic target eliminating ATP-mediated increase in the Bcl-2/Bax ratio and, therefore, attenuating the ability of antiapoptosis of lung cancer cells. In this study, we did not determine possible pathways that lead to enhanced Ca$^{2+}$ influx in lung cancer cells compared with normal cells. Ca$^{2+}$ influx through P2X channels or SOCE showed no difference between cancer cells and normal cells. Other channels like VDCC and ROCC or Ca$^{2+}$ extrusion and sequestration mechanisms such as the Ca$^{2+}$-Mg$^{2+}$-ATPase in the plasma membrane or SERCA on the membrane of SR or ER might be the reasons, which would be of great interest in a future study.
This study has highlighted the role of ATP in the extra-cellular microenvironment with regard to tumor cell growth in vitro. Enhancing the antiapoptotic ability of ATP may be an important selective pressure promoting oncogenesis transformation and cancer progression. We have shown for the first time that ATP increases the Bcl-2/Bax ratio through a sustained increase in [Ca\(^{2+}\)]\(_{cyt}\) in lung cancer cells. Additionally, we have demonstrated that Ca\(^{2+}\) from the extra-cellular environment mediates this ATP-induced increase in Bcl-2/Bax ratio. This study has several implications for lung cancer biology. It suggests that tumor cells may adapt to high ATP in the tumor microenvironment through low P2X7 receptor expression to escape apoptosis and also turn ATP into a stimulus to inhibit apoptosis. This conversion of harm to benefit is important for the aggressive behavior of tumors. Despite the exogenous application of ATP having antineoplastic action on tumors, as implicated by several studies, because of its cytotoxicity on low-tolerance normal cells, this side effect weakens its therapeutic value. Thus, understanding this ATP-induced antiapoptotic pathway observed in lung cancer cells would be of therapeutic value to facilitate the development of cancer-specific targeted therapies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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