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The role of intracellular pH in cell growth arrest induced by ATP

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Humez, Sandrine, Michaël Monet, Fabien van Coppenolle, Philippe Delcourt, and Natalia Prevarskaya. The role of intracellular pH in cell growth arrest induced by ATP. Am J Physiol Cell Physiol 287: C1733–C1746, 2004. First published September 8, 2004; doi:10.1152/ajpcell.00578.2003.—In this study, we investigated ionic mechanisms involved in growth arrest induced by extracellular ATP in androgen-independent prostate cancer cells. Extracellular ATP reversibly induced a rapid and sustained intracellular pH (pHi) decrease from 7.41 to 7.11. Inhibition of Ca2+ influx, lowering extracellular Ca2+, and buffering cytoplasmic Ca2+ inhibited ATP-induced acidification, thereby demonstrating that acidification is a consequence of Ca2+ entry. We show that ATP induced reuptake of Ca2+ by the mitochondria and a transient depolarization of the inner mitochondrial membrane. ATP-induced acidification was reduced after the suppression of the mitochondrial proton gradient by rotenone and carbonyl cyanide p-trifluoromethoxyphenylhydrazone, after inhibition of Ca2+ uptake into the mitochondria by ruthenium red, and after inhibition of the F0F1-ATPase with oligomycin. ATP-induced acidification was not induced by either stimulation of the Cl-/HCO3- exchanger or inhibition of the Na+/H+ exchanger. In addition, intracellular acidification, induced by an ammonium prepulse method, reduced the amount of releasable Ca2+ from the endoplasmic reticulum, assessed by measuring change in cytosolic Ca2+ induced by thapsigargin or ATP in a Ca2+-free medium. This latter finding reveals cross talk between pH1 and Ca2+ homeostasis in which the Ca2+-induced intracellular acidification can in turn regulate the amount of Ca2+ that can be released from the endoplasmic reticulum. Furthermore, pH decrease was capable of reducing cell growth. Taken together, our results suggest that ATP-induced acidification in DU-145 cells results from specific effect of mitochondrial function and is one of the major mechanisms leading to growth arrest induced by ATP.

pH plays a central role in the regulation of many aspects of cell physiology, and protons may function as a second messenger in a manner similar to that of Ca2+ (3). Relatively small changes in pH1 could have a profound effect on a variety of cellular functions. For example, pH1 plays a role in the control of DNA synthesis, cellular proliferation, protein synthesis rate, cell fertilization, cell volume regulation, muscle contractility, and neurotransmitter reuptake, and apoptosis. pH is also one of the factors thought to control the rate of cell proliferation and transformation (22, 37). It is now established that the pH1 of transformed cells is often more alkaline than that of normal cells (22, 32). In addition, because intracellular alkalinization has been shown to be involved in cell proliferation, a correlation between pH1 and cell cycle has been suggested (32). Thus a causative link between cellular pH homeostasis and tumor development has been suggested repeatedly, and elevated pH1 has been demonstrated to parallel both cell transformation and cell proliferation. Furthermore, Reschkin et al. (31) demonstrated that alkalinization is an early event in malignant transformation. In addition, the acid extrusion mechanism Na+/H+ exchanger (NHE) isozyme 1 (NHE1) has been shown to play a key role in cell survival and proliferation (26), and NHE1 transcription is known to be strongly enhanced during cell proliferation (44). More recently, it was clearly shown that increased pH1 promotes the timing of the entry and transition of second growth phase and mitosis (G2/M) (30). It is therefore possible to suggest that lowering pH1 may also reduce cell proliferation and/or induce apoptosis. To date, results concerning pH1 regulation and its involvement in prostate cancer cell physiology are lacking. Thus the main goal of this study was to evaluate the role of pH1 in the regulation of androgen-independent prostate cancer cell physiology. More particularly, this work focuses on the effect of external ATP on DU-145 cells because 1) ATP regulates the growth of these cells and 2) ATP

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has been shown to modulate pH$_i$ in epithelial cells (19, 25, 28, 42) by mechanisms that are poorly understood.

It is now well established that cross talk exists between pH$_i$ and intracellular Ca$^{2+}$. In effect, pH$_i$ has been described as being able to affect intracellular Ca$^{2+}$ homeostasis and contribute to the length, magnitude, and frequency of the Ca$^{2+}$ signal through the modulation of voltage-dependent or -independent plasma membrane Ca$^{2+}$ channels and/or through regulation of the mobilization of Ca$^{2+}$ from internal stores (3, 7, 8, 16). On the other hand, Ca$^{2+}$ has been described as inducing pH$_i$ variation, particularly in neurons (1, 4, 36, 40, 47). In androgen-independent prostate cancer cells, external nucleotides induce a rapid intracellular Ca$^{2+}$ increase (a few seconds after ATP application) and a long-term decrease in releasable Ca$^{2+}$ from intracellular stores (after ATP has been applied for 2 days) (43). Thus the aim of the present study was to evaluate possible cross talk between the change in intracellular Ca$^{2+}$ and pH$_i$ and vice versa, as well as the possible implication of this cross talk in the growth regulation induced by ATP in androgen-independent prostate cancer cells.

The results of the present study show that ATP induces intracellular acidification of DU-145. This acidification is clearly linked to Ca$^{2+}$ entry because of the ATP exposure of these cells. In addition, we show that acidification is linked to mitochondrial function and particularly to the F$_0$F$_1$-ATPase. We also demonstrate that ATP-induced acidification was not induced by stimulation of the Cl$^{-}$/HCO$_3^-$ exchanger or by inhibition of NHE. Our finding leads us to suggest the existence of cross talk between pH$_i$ and Ca$^{2+}$ homeostasis in which the Ca$^{2+}$-induced intracellular acidification can in turn reduce the amount of releasable Ca$^{2+}$ from the endoplasmic reticulum.

We also suggest that ATP-induced acidification in DU-145 cells induced by the short-term Ca$^{2+}$ response to ATP is one of the mechanisms leading to the long-term effect of ATP on Ca$^{2+}$ homeostasis previously described in our model (43). Furthermore, we show that a decrease in pH$_i$, induced by 48-h dimethyl amiloride (DMA) treatment, was able to reduce cell growth. In conclusion, the present results suggest that acidification is one of the major mechanisms leading to growth arrest induced by ATP. Our results also highlight the physiological role of pH$_i$ in the growth of prostate cancer cells and the cross talk between pH$_i$ and Ca$^{2+}$ response in these cells.

**METHODS**

**Cell culture.** The androgen-independent human prostate cancer cell line DU-145, obtained from the American Type Culture Collection (Manassas, VA), was maintained in culture in RPMI 1640 medium (Gibco/Life Technologies) supplemented with 10% fetal calf serum (Seromed; Poly-Labo, Strasbourg, France) and 5 mM l-glutamine (Sigma, L’Isle d’Abeau, France). Cells were grown at 37°C in a humidified atmosphere containing 5% CO$_2$. Before fluorescence measurements, the cells were trypsinized and transferred to glass coverslips. Cells were used 1–4 days after trypsinization. The medium was replaced every 24 h.

**Ca$^{2+}$ measurements using fura-2 AM.** The culture medium was replaced by HBSS containing (in mM) 142 NaCl, 5.6 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 0.34 Na$_2$HPO$_4$, 0.44 KH$_2$PO$_4$, 4.2 NaHCO$_3$, 10 HEPES, and 5.6 glucose. The osmolality and pH of this solution were adjusted to 310 mosM and 7.4, respectively. When a Ca$^{2+}$-free medium was required, CaCl$_2$ was omitted and replaced by equimolar MgCl$_2$. Dye loading was achieved by transferring the cells into a standard HBSS solution containing 3 µM fura-2 acetoxymethyl ester (fura-2 AM; Calbiochem, Meudon, France) for 40 min at room temperature, then rinsing them three times with dye-free solution. Intracellular Ca$^{2+}$ was measured using an imaging system (Princeton, Evry, France). The glass coverslip was mounted in a chamber on an Olympus microscope equipped for fluorescence. Fura-2 fluorescence was excited at 340 and 380 nm, and emitted fluorescence was measured at 510 nm (long-pass filter). The cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$_i]) was derived from the ratio of fluorescence intensities for each of the excitation wavelengths (F$_{340}$/F$_{380}$) and the Grynkiewicz equation. Ca$^{2+}$ measurements using fura-2 AM were performed at 33°C. The cells were continuously perfused with HBSS solution, and chemicals were added via a whole chamber perfusion system. The flow rate of the whole chamber perfusion system was set at 1 ml/min, and the chamber volume was 500 µl.

**Direct quantification of endoplasmic reticulum Ca$^{2+}$ concentration.** To obtain images of [Ca$^{2+}$_i] within the endoplasmic reticulum ([Ca$^{2+}$_ER]), DU-145 cells were loaded with 2 µM Mag fura-2 (the AM derivative of Mag fura-2) for 45 min at 37°C. After incubation with the dye, the cells were rinsed briefly in a high-K$^+$ solution (in mM) composed of 125 KCl, 25 NaCl, 10 HEPES, and 0.1 MgCl$_2$, pH 7.2, and then exposed for 2 min to an intracellular buffer at 33°C and 5 µM/mL digitonin. Diginon-permeabilized cells were continuously superfused with a digitonin-free intracellular buffer supplemented with 0.2 mM MgATP and free [Ca$^{2+}^+]$ clamped to 170 nM using a Ca$^{2+}$-ethylene glycol-bis(2-aminoethylether)-tetraacetic acid (EGTA) buffer. The Mag fura-2 fluorescence ratio was calibrated using exposure to 10 µM ionomycin and 15 mM Ca$^{2+}$ or 10 mM EGTA, assuming a dissociation constant for Ca$^{2+}$-Mag fura-2 at room temperature of 53 µM. Ratio imaging measurements of Mag fura-2 fluorescence were obtained using an imaging system (Princeton).

**pH$_i$ measurements with BCECF.** Dye loading was achieved by transferring the cells into a standard HBSS solution containing 1 µM 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM for 20 min at room temperature, then rinsing the cells three times with dye-free solution. pH$_i$ was measured using an imaging system (Princeton). The glass coverslip was mounted in a chamber on an Olympus microscope equipped for fluorescence. BCECF fluorescence was excited at 490 and 440 nm, and emitted fluorescence was measured at 530 nm (long-pass filter). The F$_{490}$/F$_{440}$ emission ratio was converted to a linear pH scale using in situ calibration data obtained according to the nigericin technique. The cells were continuously perfused with HBSS, and chemicals were added via a whole chamber perfusion system. The flow rate of the whole chamber perfusion system was set at 1 ml/min, and the chamber volume was 500 µl. pH$_i$ measurements with BCECF were performed at 33°C. In some experiments, extra-cellular Na$^+$ ions were replaced with N-methyl-d-glucamine, or Cl$^-$ ions were replaced by methane sulfonate.

**Mitochondrial [Ca$^{2+}$_i] measurements.** Fluorescence analysis was performed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Le Pecq, France) connected to a Zeiss Axiovert 200 M with a ×63 oil-immersion objective lens (numerical aperture 1.4). The image acquisition characteristics (e.g., pinhole aperture, laser intensity, scan speed) were the same throughout the experiments to ensure the comparability of the results. The confocal microscope software AIM 3.2 (Carl Zeiss) was used for data acquisition and analysis.

Changes in mitochondrial [Ca$^{2+}^+]$ were monitored with the membrane-permeable dihydro rhod-2 AM. Dihydro rhod-2 AM was formed by reacting 10 µl of 1 mg/ml NaBH$_4$ with 40 µl of 1 mM rhod-2 AM stock solution. Chemical reduction of rhod-2 AM with sodium borohydride before loading enhanced the mitochondrial localization of the indicator. Dye loading was achieved by transferring the cells into a standard HBSS solution containing 5 µM dihydro rhod-2 AM for 30 min at 37°C, followed by rinsing with dye-free solution for at least 3 h before the onset imaging. The dye was excited by a 543-nm laser line, and emission from the dye was collected through a long-pass filter of 560 nm. Stimulation-induced increases in dihydro rhod-2 fluorescence...
were plotted as F/Frest, where F is the measured fluorescence and Frest is the resting (i.e., prestimulation) fluorescence.

Mitochondrial potential measurements. Changes in mitochondrial potential were monitored with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Fluorescence analysis was performed using a Zeiss LSM 510 confocal microscope. JC-1 was first dissolved in DMSO (5 mg/ml). Dye loading was achieved by transferring the cells into a standard HBSS solution containing 5 \( \mu \)g/ml of JC-1 for 20 min at 37°C. After the loading period, the cells were rinsed three times with dye-free solution. At high mitochondrial membrane potentials, JC-1 accumulates sufficiently in the mitochondria to form aggregates (J aggregates) that fluoresce red. At lower mitochondrial potentials, less dye enters the mitochondria, resulting in monomers that fluoresce green. Cells were excited by a 488-nm laser line, and fluorescence emission was recorded at 530 and 590 nm. Mitochondrial potential was expressed as the red-green fluorescence ratio.

Measurements of in vitro cell growth. Cells were seeded at an initial density of 900 cells/well in 96-well plates (Poly Labo, Strasbourg, France). After 48 h, cells were cultured in treatment medium (day 0). From day 0, the treatment medium was changed daily for each condition. Cells were harvested on day 2, and the cell number was determined using a colorimetric method. The CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega, Madison, WI) was used to determine the number of viable cells. This commercial assay is composed of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) and phenazine methosulfate (PMS), an electron coupling reagent. MTS is bioreduced by cells into a formazan that is soluble in the cell culture medium. The absorbance of formazan at 490 nm is measured directly and fluorescence emission was recorded at 530 and 590 nm. Mitochondrial potential was expressed as the red-green fluorescence ratio.

Data analysis. Statistical data refer to fluorimetric measurements of Ca\(^2+\) and pH in single cells from each coverslip. Data were normalized for presentation of some results, and values are relative to basal pH, or [Ca\(^{2+}\)], designated as 1. Data analysis was performed using Origin 5.0 software (Microcal Software); graphs are expressed as means ± SE. Each experiment was repeated several times. The Tukey-Kramer test was used for statistical comparison among means and differences, and \( P < 0.05 \) was considered significant.

Chemicals. Rotenone, nigericin, FCCP, 2-amino-phenyl borate (2-APB), oligomycin, thapsigargin (TG), DIDS, DMA, ATP, EGTA-AM, ruthenium red, ionomycin, fura-2 AM, and BCECF-AM were purchased from Sigma. JC-1 and rhod-2 AM were obtained from Interchim.

RESULTS

Effect of ATP on pH \(_i\) and the involvement of PLC. Under control conditions, pH\(_i\) measured in DU-145 cells was 7.41 ± 0.18 (n = 208). A typical effect of extracellular ATP (100 \( \mu \)M) on pH\(_i\) in DU-145 cells is illustrated in Fig. 1A. In the presence of external Ca\(^{2+}\) (2 mM) exposure of DU-145 cells to ATP (100 \( \mu \)M) produced a rapid decrease of 0.31 ± 0.09 pH units (n = 163). This effect was reversible: removal of ATP from the bath solution was followed by pH\(_i\) recovery toward basal levels (Fig. 1B).

The activation of P2X receptor in DU-145 cells was reported previously (18). Figure 1C shows that intracellular acidification induced by ATP is not linked to the activation of P2X receptors, because inhibition of these P2X receptors by 30 \( \mu \)M pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) did not affect the ATP response. It also was previously shown

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**Fig. 1.** ATP induces acidification linked to activation of PLC by the P\(_2\) purinergic receptor. A: time course of change in intracellular pH (pHi) after the application of 100 \( \mu \)M ATP to the extracellular medium. B: time course of change in pHi after a transient application of 100 \( \mu \)M ATP to the extracellular medium. C: time course of changes in pHi after application of 100 \( \mu \)M ATP to the extracellular medium (n = 30) or in the presence of 30 \( \mu \)M pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS; n = 56). D: time course of change in pH after application of 100 \( \mu \)M ATP to the extracellular medium in the presence of 20 \( \mu \)M U-73122 (a PLC inhibitor; n = 38) or in the presence of 20 \( \mu \)M U-73343 (inactive analog of U-73122, n = 36). These results are representative of the mean.
that DU-145 express P2Y receptors coupled to PLC (10). To determine PLC involvement in ATP-induced acidification, we inhibited the PLC pathway by preincubating cells for 45 min with U-73122 (20 μM), a PLC inhibitor, before applying ATP. U-73122 abolished the pH↓ decrease (n = 36; Fig. 1D), whereas the same concentration of the inactive analog, U-73343, did not alter the ATP-induced intracellular acidification (n = 38; Fig. 1D).

Role of cytoplasmic Ca2+ increase in ATP-induced acidification. In DU-145 cells, external ATP is known to produce a large [Ca2+]i increase (18, 43). We therefore investigated the role of Ca2+ in the ATP-induced acidification. Figure 2A clearly shows that 100 μM 2-APB, which blocks the ATP-induced Ca2+ entry in DU-145 (43), inhibits the effect of ATP on pH↓. In addition, incubation of DU-145 cells with the permeable Ca2+ chelator EGTA-AM (50 μM) also greatly reduced ATP-induced acidification (Fig. 2B). In effect, ATP induced a decrease of 0.33 ± 0.03 pH units (n = 24) in control conditions, whereas it induced decreases of 0.04 ± 0.01 (n = 26) and 0.05 ± 0.01 (n = 30) pH units when cells were treated with 100 μM 2-APB and 50 μM EGTA-AM, respectively (Fig. 2C). When applied to a Ca2+-free medium, ATP induced only slight acidification (0.09 ± 0.02 pH units; n = 25) that greatly increased (to 0.29 ± 0.056 pH units; n = 25) when Ca2+ was readmitted to the bathing medium (Fig. 2D).

To further confirm the role of Ca2+ in ATP-induced acidification, we then tested the hypothesis that TG (1 μM), a sarcoplasmic Ca2+-ATPase inhibitor that induces intracellular Ca2+ release from endoplasmic reticulum stores followed by the well-described capacitive Ca2+ entry, could mimic the acidification induced by ATP. We also investigated whether Ca2+-induced intracellular acidification could be produced by the [Ca2+]i increase induced by Ca2+ release and/or by Ca2+ influx. As shown in Fig. 3A, TG (1 μM) application to cells bathed in a Ca2+-free medium were unable to induce intracellular acidification, whereas Ca2+ readmission to the bathing medium induced sustainable intracellular acidification. Furthermore, ionomycin (1 μM), which is widely used to increase intracellular Ca2+, was also unable to induce intracellular acidification in a Ca2+-free medium, whereas Ca2+ readmission to the bathing medium induced sustainable intracellular acidification (Fig. 3B). The magnitude of the intracellular acidification induced by TG and ionomycin was of the same order as that induced by ATP (Fig. 3C). Moreover, the addition of ATP after TG pretreatment failed to induce an additional and significant pH↓ acidification (Fig. 3D). Taken together, these results clearly show that Ca2+ influx is involved in ATP-induced acidification.

Involvement of mitochondrial F0F1-ATPase in ATP-induced pH↓ drop. Mitochondria are known to reuptake Ca2+ ions released into the cytoplasm during agonist stimulation, a process dependent on the membrane potential across the inner membrane (27, 34). In effect, when [Ca2+]i reaches the level at which the rate of Ca2+ influx into the mitochondria exceeds the rate of Ca2+ extrusion from the mitochondria, the mitochondria start to accumulate Ca2+, which depolarizes the inner mitochondrial membrane. To compensate for the mitochondrial membrane potential drop, regulatory mechanisms that extrude extra protons from the mitochondrial matrix are activated. For example, protons may be extruded by the mitochondrial F0F1-ATPase at the expense of cytoplasmic ATP (6). Recognizing that F0F1-ATPase can operate bidirectionally (2, 24), we investigated whether this ATPase could be responsible for the change in cytoplasmic pH induced by ATP.

To assess the role of mitochondria in ATP-induced acidification, we looked into whether ATP stimulation was able to induce Ca2+ influx into mitochondria and depolarization of the inner mitochondrial potential. To determine directly whether mitochondria take up Ca2+ after ATP stimulation, we imaged mitochondria using confocal microscopy and the mitochondrial Ca2+-sensitive dye dihydro-rhod2. Dihydro-rhod2 imaging of DU-145 cells showed patchy staining in the cytosol, a
pattern compatible with preferential staining of mitochondria using dihydro-rhod2 (Fig. 4A). The application of 100 μM ATP resulted in a large increase in fluorescence (F/F_{rest} = 1.46 ± 0.22; n = 15), suggesting that mitochondria take up Ca^{2+} after ATP stimulation (Fig. 4B). Changes in mitochondrial potential were investigated using confocal microscopy and the mitochondrial-specific, voltage-sensitive dye JC-1. Unstimulated cells displayed patchy red fluorescence, which indicates a polarized state (Fig. 4Ca). As shown in Fig. 4Cb, the addition of ATP induced dissipation of the mitochondrial potential. FCCP (5 μM) cause the mitochondrial potential to collapse dramatically and served as a positive control (Fig. 4Cc). The variation in the mitochondrial potential was plotted as the red-green fluorescence ratio. When cells were perfused with 100 μM ATP, the depolarization of the mitochondria was observed and identified as a decrease in the JC-1 fluorescence ratio (Fig. 4D). ATP-evoked depolarization of mitochondria was transient (n = 41), indicating a regulation of the mitochondrial potential.

We therefore reduced the proton gradient across the inner mitochondrial membrane with 10 μM rotenone, a mitochondrial toxin that is a potent and competitive inhibitor of the complex I respiratory chain. Treating DU-145 cells with 10 μM rotenone for up to 30 min decreased the ATP-induced acidification (Fig. 5A). As shown in Fig. 5B, in the presence of rotenone, the ATP-induced acidification was 0.07 ± 0.07 pH units (n = 55), corresponding to 41% of the pH_{i} variation induced by ATP under control conditions (0.17 ± 0.04 pH units; n = 47). As shown in Fig. 5, C and D, rotenone was unable to modify the amount of [Ca^{2+}], increase induced by ATP in our experimental conditions. Indeed, the [Ca^{2+}]_{i} increase induced by ATP was 786 ± 268 nM (n = 45) in control conditions, whereas it was 756 ± 190 nM (n = 53) when cells were treated for 30 min with 10 μM rotenone. This result suggests that rotenone does not act on pH_{i} by altering the Ca^{2+} response to ATP.

We then used a protonophore (H^{+} ionophore) to collapse the mitochondrial membrane potential. FCCP uncouples mitochondrial respiration and ATP production by dissipating the proton gradient across the inner mitochondrial membrane, and it is also known to prevent mitochondrial Ca^{2+} uptake (13, 14, 32). Cells were treated for 30 min with 5 μM FCCP, and then ATP (100 μM) was applied. This treatment reduced the ATP-induced acidification by 48% (Fig. 6A). Indeed, the magnitude of the ATP-induced acidification in control conditions was 0.29 ± 0.06 pH units (n = 64), whereas it was 0.15 ± 0.06 pH units (n = 62) when cells were treated with FCCP (Fig. 6D). FCCP-treated cells exhibit a smaller [Ca^{2+}]_{i} increase when challenged by 100 μM ATP compared with cells treated with ATP alone (Fig. 6G). The variation of [Ca^{2+}]_{i} induced by ATP was 786 ± 268 nM (n = 49) under control conditions, compared with 627 ± 103 nM (n = 30) when cells were treated with 5 μM FCCP.

Oligomycin is a selective inhibitor of membrane-bound mitochondrial F0F1-ATPase. In the presence of oligomycin, both synthesis and hydrolysis of ATP by the mitochondria, as well as proton movement, are prevented (24). The incubation of DU-145 cells with 2 μM oligomycin reduced the magnitude of ATP-induced acidification (Fig. 6B). In the presence of oligomycin, ATP-induced acidification was 0.11 ± 0.05 pH
units (n = 61), corresponding to 39% of the control conditions (0.28 ± 0.05 pH units; n = 60). Oligomycin (2 μM) was unable to significantly modify the amplitude of the ATP-induced increase in [Ca^{2+}]. In the presence of oligomycin, the amplitude of the [Ca^{2+}] increase induced by ATP (100 μM) was 730 ± 200 nM (n = 40), whereas it was 786 ± 268 nM (n = 49) when cells were kept under control conditions. Furthermore, specific inhibition of the vacuolar type H^+ -ATPase with bafilomycin had no effect on cytoplasmic acidification induced by ATP (data not shown).

Inhibition of the mitochondrial Ca^{2+} uniporter with ruthenium red also reduced ATP-induced acidification. Treating DU-145 cells with 30 μM ruthenium red for 30 min decreased ATP-induced acidification by 67% (Fig. 6C). In the presence of ruthenium red, ATP-induced acidification was 0.10 ± 0.01 pH units (n = 45), whereas it was 0.32 ± 0.02 pH units (n = 44) for untreated cells (Fig. 6F). As shown in Fig. 6G, ruthenium red was unable to modify the amount of [Ca^{2+}] increase induced by ATP in our experimental conditions.

Taken together, these data indicate that a specific effect on the mitochondrial function accounts for ATP-induced acidification in DU-145 cells. We therefore conclude that FoF1-ATPase may participate in pH_i acidification induced by ATP.

Cl^-/HCO_3^- exchanger and NHE involvement in ATP-induced acidification. We also tested the possibility of the involvement of the Cl^-/HCO_3^- exchanger in the extracellular ATP effect, because its binding to a P2 purinergic receptor leads, in some models, to an enhancement of the Cl^-/HCO_3^- exchanger (49). The presence of the Cl^-/HCO_3^- exchanger was studied by measuring the change in pH_i induced by a Cl^-/HCO_3^- exchanger inhibitor (Fig. 7A). To investigate the involvement of exchange in the ATP effect, we measured the change in pH_i induced by ATP in Cl^-/HCO_3^- free medium and in the presence of

Fig. 4. ATP increases mitochondrial [Ca^{2+}] and decreases the mitochondrial potential. A: confocal microscopic image showing a DU-145 cell loaded with dihydro-rhod2. The rhod-2 fluorescence is localized in mitochondria. B: time course of ATP-induced increase in mitochondrial [Ca^{2+}]. C: confocal microscopic images showing a DU-145 cells loaded with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1) before ATP stimulation (a), during ATP (100 μM) stimulation (b), and during carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP; 5 μM) application (c). D: time course of ATP induced a transient loss of mitochondrial potential and subsequent effect of FCCP. Stimulation of DU-145 cells led to a decrease in fluorescence of JC-1 ratio, as an index of mitochondrial depolarization. Bars, 20 μM.
DIDS. Figure 7B illustrates that the effect of ATP on pH$_i$ was not inhibited in the Cl$^-$-free medium, with all cells showing a pH$_i$ decrease of $\sim0.25 \pm 0.10$ pH units ($n = 14$). As shown in Fig. 7C, the effect of ATP on pH$_i$ was not inhibited by DIDS. Indeed, ATP-induced acidification was identical to the control conditions when cells were treated with 100 μM DIDS (100 ± 17%; $n = 23$). These latter results suggest that ATP-induced acidification is not mediated by activation of the Cl$^-$/HCO$_3^-$ exchanger. In contrast, the activity of the Cl$^-$/HCO$_3^-$ exchanger was reduced when ATP was applied to DU-145 cells, because the pH$_i$ variation induced by the Cl$^-$-free medium was smaller when ATP was present in the bathing solution (Fig. 7, D–F). Indeed, pH$_i$ increase induced by the Cl$^-$-free medium was 0.58 ± 0.17 pH units ($n = 72$) in control condition, while it was 0.41 ± 0.12 pH units ($n = 51$) for 100 μM ATP-treated cells.

We then tested whether NHE could be involved in the ATP-induced pH$_i$ decrease, because it has been described that NHE can be involved in ATP-induced intracellular acidification (42). Figure 8A shows that extracellular NH$_4^+$ caused pH$_i$ to rise. This alkalinization is thought to result from the rapid influx of NH$_3$ and the subsequent combination of these molecules with intracellular H$^+$. This was followed by a slow fall in pH$_i$ caused by the entry of NH$_4^+$ and its dissociation into NH$_3$ and H$^+$. The subsequent removal of extracellular NH$_4^+$ causes a fall in pH$_i$ due to the dissociation of NH$_3$ into H$^+$, which remains in the cells, and NH$_3$, which can leave the cytoplasm. This causes an undershoot of the pH$_i$ below the starting value. pH$_i$ subsequently recovered as a result of the activity of acid-extruding systems present in the cells. Recovery was extremely sensitive to 50 μM DMA, an inhibitor of NHE. This inhibitory effect of DMA was reversible (Fig. 8B).

These results lead us to conclude that an amiloride-sensitive NHE is responsible for recovery from an induced acid load under our experimental conditions. Blocking NHE with DMA (50 μM) did not prevent ATP-induced acidification (Fig. 8C, $n = 13$). This result suggests that ATP-induced acidification is not the result of an inhibition of NHE.

**Physiological significance of ATP-induced acidification.** It is now well established that ATP reduces the growth of DU-145 cells (18, 43). This reduction in cell growth is concomitant with a decrease in the amount of releasable Ca$^{2+}$ shown to be able to mediate growth arrest (43). Figure 9 shows that ATP-induced reduction of cell growth (Fig. 9A) correlates with the amplitude of the Ca$^{2+}$ increase (Fig. 9B) and with a decrease in pH$_i$ (Fig. 9C) in a dose-dependent manner. The growth rate inhibition induced by ATP cannot be explained by an increase in apoptosis, because no stimulation of apoptosis was observed in our experiments as determined with Hoechst staining (data not shown).

Because it has been demonstrated that ATP induced a reduction in the releasable Ca$^{2+}$ involved in the decrease in cell growth, we postulated that the decrease in the pH$_i$ induced by ATP could be a part of the mechanism that leads to the reduction in releasable Ca$^{2+}$. Therefore, we tested the effect of artificial acidification induced by an NH$_4^+$ pulse in the presence of DIDS on the ability of TG and ATP to release intracellular Ca$^{2+}$ in DU-145 cells. As shown in Fig. 9D, inset, when cells were submitted to an NH$_4^+$ pulse (20 mM) for 5 min in a DIDS (50 μM)-supplemented medium and kept in the presence of this inhibitor, pH$_i$ was 6.94 ± 0.1 (n = 30). This pH$_i$ was significantly lower than that measured in control cells (7.35 ± 0.1; n = 30). As shown in Fig. 9D, the amount of releasable Ca$^{2+}$ from the TG-sensitive store was about five times lower.
when pH was reduced (by the NH₄⁺ pulse) than under control conditions (in which the pH was not modified). Indeed, the amplitude of the [Ca²⁺]cyt elevation induced by TG (1 µM) in a Ca²⁺-free medium was 103 ± 30 nM (n = 33), corresponding to 481 ± 148 nM (n = 36) in control cells. In the same manner (Fig. 9E), the maximal amount of Ca²⁺ released by ATP (100 µM) by the pHi-lowering treatment reached 36 ± 16 nM (n = 23), corresponding to 15% of the release observed under control conditions (481 ± 148 nM; n = 36). Taken together, these results suggest that the decrease in the amount of releasable Ca²⁺ induced by ATP treatment, already described in this model (43), can result from the pHi decrease generated by ATP. We thus investigated the effect of acidification on Ca²⁺ pool content, measured in Mag-fura-2 AM-loaded cells. The basal level of [Ca²⁺]ER was directly assessed using the fluorescent Ca²⁺ indicator Mag-fura-2 AM. Imaging experiments with Mag-fura-2 AM were conducted on cells permeabilized by mild digitonin treatment. Figure 9F shows the time course of a typical experiment involving measurement of [Ca²⁺]ER in DU-145 prostate cells in response to acidification of the intracellular buffer from 7.2 to 6.8 and to 6.4. Lowering pH of the intracellular buffer from 7.2 to 6.8 did not cause any modification of [Ca²⁺]ER. A pronounced decrease in pH from 7.2 to 6.4 triggered a rapid drop in [Ca²⁺]ER followed by [Ca²⁺]ER recovery due to Ca²⁺ uptake into intracellular stores. This latter result suggests that the decrease in the pool of releasable Ca²⁺ under acidic conditions is not secondary to impairment of Ca²⁺ uptake by the endoplasmic reticulum in our experimental conditions.

We then tested whether pHi decrease alone might be part of the mechanism that leads to ATP-induced growth arrest in DU-145 cells. To test this hypothesis, cells were treated for 48 h with DMA (50 µM) before pHi and proliferation were assessed. As shown in Fig. 10A, 48-h treatment with DMA

Fig. 6. Mitochondrial involvement in the intracellular acidification induced by ATP. A: typical traces showing the time course of change in pH, after an application of 100 µM ATP in the absence (control, open circles) or in the presence of 5 µM FCCP (closed circles). Values are relative to basal pH, designated as 1. B: typical traces showing the time course of change in pH, after an application of 100 µM ATP in the absence (control, open circles) or in the presence of 2 µM oligomycin (shaded circles). Values are relative to basal pH, designated as 1. C: typical traces showing the time course of change in pH, after application of 100 µM ATP in the absence (control, open circles) or in the presence of 30 µM ruthenium red (shaded circles). Values are relative to basal pH, designated as 1. D: mean value of the change in the pH, induced by 100 µM ATP alone (CTRL; n = 64) and in the presence of 5 µM FCCP (n = 62). E: mean values of the changes in the pH, induced by 100 µM ATP alone (CTRL; n = 60) and in the presence of 2 µM oligomycin (n = 60). F: mean values of the change in the pH, induced by 100 µM ATP alone (CTRL; n = 45) and in the presence of 30 µM ruthenium red (RR; n = 44). G: mean value of the change in [Ca²⁺]ER, induced by 100 µM ATP under control condition (CTRL; n = 49), in the presence of 5 µM FCCP (n = 30), in the presence of 2 µM oligomycin (n = 40), and in the presence of ruthenium red (RR; n = 32). Bars represent changes in [Ca²⁺], expressed as means ± SE. **P < 0.01, significantly different from control.
reduced the pH by the same order of magnitude as treatment with ATP (100 μM). pH was 7.11 ± 0.1 (n = 264) and 7.10 ± 0.13 (n = 225), respectively, compared with the control value of 7.32 ± 0.08 (n = 241). A 48-h DMA treatment induced a reduction in cell growth. The cell growth was 59 ± 16% that of control (Fig. 10B). The growth rate inhibition induced by DMA cannot be explained by an increase in apoptosis, because no stimulation of apoptosis was observed in our experiments as determined using Hoechst staining (data not shown). We consequently thought it necessary to examine whether the pH decrease was the initial event leading to cell growth reduction. In these cells treated with DMA, the amount of releasable Ca2+ was reduced by 88% compared with control conditions (Fig. 10C). Indeed, [Ca2+]i increase induced by TG (1 μM) was 36 ± 16 nM (n = 23) in cells treated for 48 h with DMA, whereas it was 295 ± 72 nM (n = 19) for cells cultured in control conditions. These results support the hypothesis that pH decrease may be the initial event leading to DU-145 cells growth reduction and suggests that the reduction in the amount of releasable Ca2+ induced by ATP (Fig. 10C) may be a consequence of pH decrease. Both the cell growth and the releasable Ca2+ reduction induced by DMA were more pronounced than those induced by ATP (Fig. 10, B and E). In effect, cell growth was 86 ± 5% of the control, and the decrease of releasable Ca2+ was ~34%. These results suggest that pH decrease is a part of the mechanism leading to the physiological ATP response.

DISCUSSION

In this study, we have demonstrated that exposure of human androgen-insensitive human prostate cancer DU-145 cells to ATP produces rapid, sustainable, and reversible intracellular acidification. Our data show that ATP-induced acidification results from an increase in [Ca2+]i. Our results also show that the specific effect on mitochondrial function accounts for the ATP-induced acidification in DU-145 cells. Furthermore, inhibition of P2X receptors, which have been described to be activated by ATP in DU-145 cells (18), did not modify the intracellular acidification induced by ATP. We thus
suggest that P2 purinergic receptors are involved in the ATP signaling pathway, causing ATP to induce acidification in DU-145 cells.

\textit{Ca}^{2+} \text{ involvement in ATP-induced acidification.} Because it is well documented that ATP induces \textit{Ca}^{2+} increase in DU-145 cells (18, 43), the link between intracellular \textit{Ca}^{2+} increase and ATP-induced acidification was investigated. 2-APB, known to block \textit{Ca}^{2+} entry in DU-145 cells (43), inhibits ATP-induced acidification, indicating that ATP-induced acidification results from an increase in [\textit{Ca}^{2+}]. In addition, lowering extracellular \textit{Ca}^{2+} or buffering [\textit{Ca}^{2+}], with EGTA-AM also inhibited ATP-induced acidification. Furthermore ATP-induced acidification could be mimicked by an artificial \textit{Ca}^{2+} elevation induced by TG and ionomycin. Intracellular acidification was induced by \textit{Ca}^{2+} influx and not by release from intracellular stores, because it was not observed after TG or ionomycin treatment in the absence of \textit{Ca}^{2+} in the bath solution. Nevertheless, a small \textit{pH}_i decrease was observed when ATP was applied in a \textit{Ca}^{2+}-free medium. This result can be explained, first, by the different kinetics of \textit{Ca}^{2+} release induced by ATP and TG. Indeed, we have demonstrated that metabotropic induced \textit{Ca}^{2+} release is faster than TG- or ionomycin-induced \textit{Ca}^{2+} release as shown in Fig. 9. Second, the decrease in the \textit{pH}_i induced by ATP in a \textit{Ca}^{2+}-free medium could result from an additional \textit{Ca}^{2+}-independent mechanism.

\textit{NHE and Cl}^{-}/\textit{HCO}_3^{-} exchanger involvement in \textit{pH}_i variation induced by ATP.} ATP previously was described as inducing intracellular acidification in a large variety of models (19, 20, 25, 42). It has been reported that the exposure of human bronchial epithelial cells to ATP produced rapid, sustained, and reversible intracellular acidification (42). In human nasal epithelium, rat cardiomyocytes, bovine aortic endothelial cells, and osteoclasts, it was reported that external ATP produced a biphasic variation in \textit{pH}_i. External ATP produced an initial acidification followed by realkalinization equal to or above the steady-state \textit{pH}_i value (19, 20, 25). The differences in \textit{pH}_i profiles observed in different cell lines may result from differences in the mechanisms by which ATP mediates its effect. In human bronchial epithelial cells (in which ATP induces a sustained intracellular acidification), it was suggested that acidification arises from the inhibition of NHE and from the activation of a proton conductance. In osteoclasts and cardiomyocytes (9, 29, 49), ATP also has been shown to induce transient acidification by transient activation of the \textit{Cl}^{-}/\textit{HCO}_3^{-} exchanger. At least four types of acid extrusion mechanisms—NHE, the \textit{Cl}^{-}/\textit{HCO}_3^{-} exchanger, the \textit{Na}^{+}/\textit{HCO}_3^{-} cotransporter, and the \textit{Na}^{+}-dependent \textit{Cl}^{-}/\textit{HCO}_3^{-} exchanger—can be triggered during a \textit{pH}_i decrease (48). The first is DMA sensitive, and the others are DIDS sensitive. We therefore tested whether ATP-induced acidification could result from an inhibition of NHE or from an activation of the \textit{Cl}^{-}/\textit{HCO}_3^{-} exchanger already described in other models. In our cells, the participation of NHE and \textit{Cl}^{-}/\textit{HCO}_3^{-} exchanger in ATP-induced acidification was ruled out. Indeed, NHE inhibition cannot participate in ATP-induced acidification in DU-145 cells, because blockage of NHE (which is functional in our model) by DMA did not reduce ATP-induced acidification. Furthermore, ATP-induced acidification was observed in \textit{Cl}^{-}-free medium or in the presence of DIDS. This eliminated the possibility of activation of the \textit{Cl}^{-}/\textit{HCO}_3^{-} exchanger in the ATP-induced acidification in our model. Rather than being stimulated, the \textit{Cl}^{-}/\textit{HCO}_3^{-} exchanger activity was reduced during ATP exposure.

Cyclic nucleotide-induced acidification previously was reported to be dependent on [\textit{Ca}^{2+}], increase (20), because UTP was able to induce acidification in a \textit{Ca}^{2+}-dependent manner in macrophages. In this model of UTP-induced, \textit{Ca}^{2+}-dependent acidification, lipoxygenase metabolites were suggested as the acidification mediators. The involvement of this pathway in ATP-induced acidification in DU-145 cells remains to be determined.
Mitochondrial involvement in the acidification induced by ATP. The effect of \([\text{Ca}^{2+}]_i\) increase on \(p\text{Hi}\) via the activation of glutamate receptors in neurons was previously described (15, 23). Furthermore, depolarization-induced acidification in vertebrate or invertebrate neurons also occurs through a \([\text{Ca}^{2+}]_i\) influx (1, 40). More recently, the link between the \([\text{Ca}^{2+}]_i\) influx and the depolarization-induced \(p\text{Hi}\) decrease has been described in dendrites (34, 36). A variety of mechanisms have been identified that might generate \(p\text{Hi}\) changes as a consequence of neuronal \([\text{Ca}^{2+}]_i\) increase: the passive binding of \([\text{Ca}^{2+}]_i\) buffers in exchange for protons (17), plasma membrane \(\text{Ca}^{2+}\) extrusion (40), the stimulation of the metabolism (50), and mitochondrial \(\text{Ca}^{2+}\) uptake (45, 46). When \([\text{Ca}^{2+}]_i\) reaches the level at which the rate of \(\text{Ca}^{2+}\) influx into the mitochondria exceeds the rate of \(\text{Ca}^{2+}\) extrusion from the mitochondria, the mitochondria start to accumulate \(\text{Ca}^{2+}\), which depolarizes the inner mitochondrial membrane. To compensate for the mitochondrial membrane potential drop, regulatory mechanisms...
that extrude extra protons from the mitochondrial matrix are activated. In our study using confocal imaging of rhod-2 fluorescence, we have demonstrated that ATP is able to induce Ca\(^{2+}\)/H\(^{+}\) influx in mitochondria. Furthermore, our results show that ATP induced a dissipation of the mitochondrial potential. This depolarization of mitochondria is transient, demonstrating the existence of a regulatory mechanism of the mitochondrial potential. These regulatory mechanisms, which compensate the mitochondrial membrane potential drop, extrude extra protons from the mitochondrial matrix. In our model, we have demonstrated that mitochondrial function accounts for a large part of the mechanism leading to ATP-induced acidification, because mitochondrial inhibitors reduce the effects of ATP on pH\(_{i}\). Indeed, inhibition of the mitochondrial Ca\(^{2+}\) uniporter with ruthenium red reduced the ATP-induced acidification. In addition, we have shown that a reduction in the proton gradient across the inner mitochondrial membrane with rotenone and FCCP strongly reduced ATP-induced acidification, thereby indicating that a specific effect on mitochondrial function accounts for the ATP-induced acidification in DU-145 cells. Furthermore, inhibition of the F\(_{0}\)F\(_{1}\)-ATPase with oligomycin decreased ATP-induced acidification, suggesting that F\(_{0}\)F\(_{1}\)-ATPase may participate in pH\(_{i}\) acidification induced by ATP. Mitochondrial inhibitors block only 60% of the ATP-induced acidification, so we conclude that an additional mechanism that remains to be determined participates in ATP-induced acidification in DU-145 cells. Nevertheless, to our knowledge, this report is the first to clearly demonstrate the involvement of the mitochondrial function in Ca\(^{2+}\)/H\(^{+}\)-induced acidification in non-excitable cells and ATP-induced acidification.

**Involvement of pH\(_{i}\) in the Ca\(^{2+}\) release processes.** Our results show that low pH\(_{i}\) downregulates the ability of Ca\(^{2+}\) to be released by ATP or by TG. Such a reduction in the Ca\(^{2+}\) release process was not due to a reduction in the [Ca\(^{2+}\)]\(_{\text{ER}}\), because direct measurement of the [Ca\(^{2+}\)]\(_{\text{ER}}\) as a function of pH\(_{i}\) did not show any modification of [Ca\(^{2+}\)]\(_{\text{ER}}\) when pH\(_{i}\) fell to 6.8 and did show a transient decrease when pH\(_{i}\) dropped to 6.4. The downregulation of the Ca\(^{2+}\) release after the metabotropic stimulation observed at low pH\(_{i}\) is consistent with previously described results. In effect, the pH dependence of IP\(_{3}\)-induced Ca\(^{2+}\) release has been observed in smooth muscle cells, because intracellular alkalinization enhances the rate of IP\(_{3}\)-induced Ca\(^{2+}\) release (41). Similar results were obtained in human lymphocytes and pancreatic acinar cells (7, 38), and in
neurons the transient increase in \([\text{Ca}^{2+}]\), did not occur for pH below 7.1 (39). Furthermore, because alkalization is able to increase the IP3 level in chondrocytes (5), one might postulate that lowering pH could reduce the IP3 production induced by ATP in our model. Investigators at our laboratory (43) recently demonstrated that the IP3R1-to-IP3R3 ratio in DU-145 cells was 8–92%. Moreover, IP3R3 activity is strongly reduced by low pH compared with IP3R1 (8). Thus the strong pH decrease dependence of ATP-induced Ca2+ release observed in this study of DU-145 cells can be explained by considering the ratio between the different isoforms of the IP3 receptors observed in our model as well as the strong pH dependence of IP3R3.

**Physiological involvement of ATP-induced acidification.**

Relatively small changes in pH can have a profound effect on a variety of cellular functions. For example, pH plays a role in the control of DNA synthesis, cellular proliferation, the rate of protein synthesis, cell fertilization, the regulation of cell volume, muscle contractibility, neurotransmitter reuptake, and apoptosis. Only a few studies have suggested the possible role of intracellular acidification in cancer cell lines. In the present study, we have shown that acidification by itself could be a part of the mechanism that leads to growth inhibition induced by ATP, because artificial acidification induced by DMA can reduce cell growth.

Our results show that pH decrease in DU-145 cells is dose-dependently linked to ATP concentration and is associated with a decrease in the Ca2+ store previously described in this model (43). We have demonstrated that artificial intracellular acidification can induce a decrease in Ca2+ release. In effect, in cells in which pH was reduced (by an NH3 pulse in cells treated with DMA), Ca2+ release induced by TG or ATP also failed. Furthermore, TG-induced Ca2+ release was reduced in cells treated for 48 h with DMA alone, suggesting that pH decrease may be one of the mechanisms that leads to a decrease in \([\text{Ca}^{2+}]\), in DU-145 cells. Taken together, our results suggest that acidification is one of the major mechanisms leading to growth arrest induced by ATP and highlighting cross talk between pH and Ca2+ in prostate cancer cells.

**REFERENCES**


