ATP is released in many cell types upon mechanical strain, the physiological function of extracellular ATP is largely unknown, however. Here we report that ATP released upon hypotonic stress stimulated prostate cancer cell proliferation, activated purinergic receptors, increased intracellular \([\text{Ca}^{2+}]\), and initiated downstream signaling cascades that involved MAPKs ERK1/2 and p38 as well as phosphatidylinositol 3-kinase (PI3K). MAPK activation, the calcium response as well as induction of cell proliferation upon hypotonic stress were inhibited by preincubation with the ATP scavenger apyrase, indicating that hypotonic stress-induced signaling pathways are elicited by released ATP. Hypotonic stress increased prostaglandin \(E_2\) (\(PGE_2\)) synthesis. Consequently, ATP release was inhibited by antagonists of PI3K (LY294002 and wortmannin), phospholipase \(A_2\) (methyl arachidonyl fluorophosphonate (MAPF)), cyclooxygenase-2 (COX-2) (indomethacin, etodolac, NS398) and 5,8,11,14-eicosatetraynoic acid (ETYA), which are involved in arachidonic acid metabolism. Furthermore, ATP release was abolished in the presence of the adenylate cyclase (AC) inhibitor MDL-12,330A, indicating regulation of ATP-release by cAMP. The hypotonic stress-induced ATP release was significantly blunted when the ATP-mediated signal transduction cascade was inhibited on different levels, i.e. purinergic receptors were blocked by suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), the \(\text{Ca}^{2+}\) response was inhibited upon chelation of intracellular \(\text{Ca}^{2+}\) by 1,2-bis(\(\alpha\)-aminophenoxo)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), and ERK1,2 as well as p38 were inhibited by U0126 and SB203580, respectively. In summary our data demonstrate that hypotonic stress initiates a feed forward cycle of ATP release and purinergic receptor signaling resulting in proliferation of prostate cancer cells.

Cytosolic concentrations of ATP typically amount to \(\sim 3–5 \text{ mM}\), whereas the extracellular concentration is very low (0.1–1 \(\mu\text{M}\)) (1). Despite the huge concentration gradient, ATP and other nucleotides cannot permeate cell membranes due to their negative charge. However, during recent years it has become evident that in many different cell types distinct physiological mechanisms for ATP release exist (2–4). The nucleotides released to the extracellular medium may stimulate purinergic receptors in autocrine and paracrine manner and activate downstream signaling cascades (5). ATP can be released to the extracellular milieu under pathophysiological conditions such as hypoxia, mechanical and osmotic strain, as well as following cytolysis (4). The lifetime of released ATP is short, however, due to the presence of ecto-ATPase, ecto-apyrase, and 5′-nucleotidase. This short half-life-time of extracellular ATP has led to the suggestion that ATP and other purinergic agonists are only biologically active in the close microenvironment of the tissues (4). It has been previously shown that already concentrations as low as 0.1–10 \(\mu\text{M}\) are active, which means that the cells need only release <0.1% of their cellular ATP content to achieve a biological signal that is transduced in autocrine and paracrine manner to more distant areas (1).

Several mechanisms for the controlled release of ATP from cells have been reported previously: (i) in platelets (6), neuronal cells (7), and pancreatic \(\beta\) cells (8) ATP is stored in concentrations up to 1 \(\text{M}\) within intracellular vesicles. It has been discussed that ATP stored in intracellular vesicles is active as neurotransmitter or cotransmitter/coagonist together with classical neurotransmitters or histamine (6). (ii) Non-secretory release of ATP and related nucleotides occurs via adenine-nucleotide transporters, which are driven by the ATP concentration gradient across the plasma membrane (9). These transporters are expressed in mitochondrial membranes, within the endo- and sarcoplasmic reticulum, as well as in the membranes of chromaffin granula and synaptic vesicles of rat neuronal cells (10). (iii) ATP release has been shown to occur via connexin hemichannels (11, 12). (iv) ATP can be released through anion channels, associated to the ABC transporter family, e.g. the cystic fibrosis transmembrane conductance regulator (CFTR) (13) or the multidrug resistance transporter P-glycoprotein (14, 15). Further ATP-conducting channels are the outward rectifying \(\text{Cl}^-\) channel, the voltage-dependent anion channel of the plasma membrane (porin), or maxi-\(\text{Cl}^-\) channels (10). Recently, an ATP-permeable channel, which conducts ATP under hyperpolarizing conditions, was described in Xenopus oocytes, which was 4000 times more permeable for ATP than \(\text{Cl}^-\) under hyperpolarizing conditions (16).

The molecular mechanisms of regulation of ATP release are currently not sufficiently explored nor is there any consent about the physiological function of released ATP. Previously it has been shown that the arachidonic acid metabolism, which results in the formation of cAMP, is involved in signaling via P2Y purinergic receptors (17), which points toward a cAMP regulation of ATP release mechanisms (5).

The current study was undertaken to investigate signaling pathways which relay the mitogenic effect of ATP on DU-145 prostate cancer cells...
previously reported by us (18) with the signaling cascade that regulates ATP release under mechanical strain (hyposmotic) conditions. Our data point toward a cAMP-regulated pathway of ATP release which results in a feed forward cycle of purinergic receptor stimulation and growth stimulation of prostate cancer cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**SB203580, 5,8,11,14-eicosatetraynoic acid (ETYA), LY294002, 1,2-bis(o-aminophenox)-yethane-N,N,N',N'-tetraacetic acid (BAPTA), wortmannin, methyl arachidonyl fluorophosphate (MAFP), MDL-12,330A, etodolac, indomethacin, and NS398 were from Calbiochem (Bad Soden, Germany). UO126, pyridoxalphosphate-6-(BAPTA), wortmannin, methyl arachidonyl fluorophosphonate (PPADS), and apyrase were purchased from Sigma (Deisenhofen, Germany).

**Culture Technique of DU-145 Prostate Cancer Cells—**The human prostate cancer cell line DU-145 was used throughout the whole study. The cell line was grown routinely in 75-cm² tissue culture flasks (BD Falcon, Heidelberg, Germany) in 5% CO₂, humidified air at 37 °C with Ham’s F-10 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Sigma, 2 mM glutamine, 0.1 mM β-mercaptoethanol, 2 mM minimal essential medium, 10 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany).

**Bioluminescence Experiments—**ATP release from confluent DU-145 cells was determined using a luciferin-luciferase assay (Sigma) in a chemiluminescence apparatus (Boluminiscence Analyzer XP2000, SKAN, Basel, Switzerland) under dim light. For data sampling, the output of the photomultiplier tube of the setup was connected to a multimeter (Voltcraft M-3610D, Conrad Electronics, Hirschau, Germany) and a personal computer. Cells grown to confluence on 20 mm coverslips were immersed in an equal volume of isotonic E1 cell culture medium from which sucrose was omitted, resulting in an osmolality of 150 mosmol/kg H₂O. In control experiments, cells grown to confluence on coverslips were immersed in an equal volume of isotonic medium. For the experiments with inhibitors, cells were preincubated for 2 h in isotonic E1 buffer that was supplemented with the respective inhibitor. Subsequently, cells were immersed in 1 ml of hypotonic E1 buffer supplemented with inhibitors. After different times, during which the cells were gently shaken, a 200-μl aliquot was removed and pressure injected via a light-tight access into a 3-ml glass cuvette containing 50 μl of the ATP assay mix and 1.5 ml of ATP assay mix dilution buffer (Sigma). Calibration measurements with ATP were performed in a concentration range of 0–100 nM. The lowest concentration of ATP that could be detected under the applied experimental conditions was 0.5 nM. The half-life time of extracellular ATP in the presence of cells was determined to ~5 min. From the calibration curves the total picomoles of ATP released per 10⁶ cells were calculated. The chemiluminescence output curve was integrated, and the resulting values were set in relation to the calibration curve. To correlate ATP release to the cell number, the cells from which ATP release had been determined were counted using a Neubauer chamber.

**Ca²⁺ Imaging—**DU-145 prostate cancer cells grown on coverslips were loaded with the fluorescent Ca²⁺ indicator fluo-3-AM (10 μM) (Molecular Probes) dissolved in Me₂SO. Following incubation for 30 min cells were washed in isotonic E1 solution and mounted to a perfusion chamber fixed to the stage of the confocal setup. Fluorescence was excited by the 488-nm band of the argon-ion laser of a confocal laser scanning microscope (Leica TCS SP2, Leica, Bensheim, Germany). Emission was recorded at >515-nm. Fluorescence images (512 × 512 pixels) were taken at 1-s intervals; the fluorescence intensity changes were integrated in single cells by the physiology software of the confocal setup.

**BrdUrd Cell Proliferation Assay—**Cells were cultured on 12-mm glass coverslips in 24-well plates until they were 70% confluent and subsequently incubated in serum-free medium for 8 h. After serum-free medium incubation cells were treated with either isotonic E1 buffer in the absence or presence of ATP or hypotonic E1 (for 20 min) and incubated in cell culture medium with 10 μM BrdUrd substance (Boehringer-Ingelheim, Ingelheim, Germany) for 18 h. Subsequently, cells were fixed in 4% paraformaldehyde for 40 min. Denaturation of DNA was achieved with 2 N HCl for 30 min at 37 °C followed by neutralization with 100 mM boric acid for 10 min. To avoid unspecific binding of antibodies cells were incubated for 1 h in phosphate-buffered saline (PBS) containing 0.01% Triton X-100 (Sigma) supplemented with 100 μg/ml bovine serum albumin and subsequently treated with rat anti-BrdUrd antibody (1:50) (Abcam, Cambridge, UK) for 1 h incubation with Cy5-conjugated rabbit anti-rat secondary antibody (Chemicon International, Temecula, CA). For nuclear counter-staining BrdUrd-labeled cells were incubated with Sytox green nuclear stain (Molecular Probes, Eugene, OR) for 15 min.

**PGE₂ Assay—**Cells were cultured in 6-well plates and treated either with isotonic or hypotonic E1 buffer. Volumes of 50 μl of the incubation buffer were collected following 20 min of incubation and assayed for PGE₂ by using PGE₂ enzyme immunoassay kit monoclonal (Cayman Chemical, Ann Arbor, MI) according to manufacturer’s instructions. The absorbance of the product was read at 405 nm in a microplate reader (Dynatech, MR7000), and data were expressed as picograms of PGE₂ per milliliter. According to the manufacturer, this assay system has a specificity of 100% for PGE₂ and PGE₂ ethanolamide and 43 and 18.7% for PGE₃ and PGE₄, respectively. Other prostanoids have specificities of 0.01–1%.

**Immunohistochemical Techniques and Quantitative Immunohistochemistry—**Monoclonal anti-human-Ki-67 antibody was obtained from Sigma. Polyclonal phospho-specific rabbit antibodies directed against active ERK1,2 (dilution 1:50), active JNK (dilution 1:50), active p38 MAPK (dilution 1:50), and active p13 (dilution 1:50) were obtained from New England Biolabs (Beverly, MA). Cells on coverslips were washed in PBS, fixed in 4% paraformaldehyde (4 °C), and permeabilized from New England Biolabs (Beverly, MA). Cells on coverslips were washed in PBS, fixed in 4% paraformaldehyde (4 °C), and permeabilized in 100% methanol. Subsequently, they were incubated for 1 h in 0.01% PBST (PBS + 0.01% Triton X-100) containing 10% fat-free milk powder to reduce nonspecific binding and over night at 4 °C with primary antibody. After washing three times in 0.1% PBST the cells were incubated for 1 h in 0.01% PBST supplemented with 10% milkpowder and either a Cy5™-conjugated F(ab)₂ fragment goat anti-rabbit IgG (H+L) (concentration 4.6 μg/ml) (Dianova, Hamburg, Germany) or a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Ki-67). Excitation was performed using either a 633-nm helium-neon laser or an argon-ion laser (488 nm band) of the confocal setup. Emission was recorded at >655 nm (Cy5 fluorescence) or at >515 nm (fluorescein isothiocyanate fluorescence). —For quantitative immunohistochemistry confocal images were recorded from cells stained with only secondary antibodies (background fluorescence image) and cells stained with primary and secondary antibodies. After subtraction of background fluorescence, the fluorescence signal (counts) was evaluated by the image analysis software of the confocal setup in single cells and was routinely exported for further analysis to the Sigma Plot graphic software (Erkrath, Germany).
hypotonic stress-induced release of ATP from DU-145 prostate cancer cells. A, time course of hypotonic stress-induced ATP. The curve shows a representative trace of ATP release under isotonic and hypotonic conditions. The inset shows mean values ± S.D. (n = 63) of ATP release after 20 min incubation. B, effects of exogenous apyrase on ATP concentration in the supernatant under either isotonic or hypotonic conditions. *, p < 0.05, significantly different as indicated.

**Statistical Analysis**—Data are given as mean values + S.D., with n denoting the number of experiments. Student’s t test for unpaired data were applied as appropriate. A value of p < 0.05 was considered significant.

**RESULTS**

**Time Course of Hypotonic Stress-induced ATP Release in Prostate Cancer Cells**—Mechanical (hyposmotic) strain is a stimulus for ATP release in many cell types (4) including prostate cancer cells (19). We have previously shown that ATP is released within a few seconds from DU-145 prostate cancer cells via anion channels that are activated by mechanical stimulation (19). The signaling cascades underlying ATP release have not yet been investigated, however.

When DU-145 prostate cancer cells grown to confluence on coverslips were incubated in hypotonic (150 mosm/kg H₂O) solution a transient increase in extracellular ATP concentration was observed, which peaked at 20 min with 72 ± 22 pmol/10⁵ cells (basal release under isotonic conditions, 11 ± 4 pmol ATP/10⁵ cells) and a gradual decline toward the basal level within 120 min (Fig. 1A) (n = 63). The extracellular ATP concentration was significantly reduced under isotonic as well as hypotonic conditions when 6 units/ml apyrase was present in the extracellular medium (n = 4) (Fig. 1B). A comparable release of ATP was observed when cells were seeded on flexible membranes, which were stretched by 10% through application of vacuum (data not shown). ATP release was apparently not due to membrane rupture as investigated by lethal cell staining using the cell membrane-impermeable fluorescence dye Sytox green, which accumulates in the cell nuclei of dead cells. Under conditions of hypotonic stress no increase in numbers of Sytox green-positive cell nuclei were observed, indicating absence of cell lethality (data not shown).

**Stimulation of Cell Proliferation by ATP Released following Hypotonic Stress**—Although it is meanwhile well established that many cells own ATP release mechanisms, the physiological role of extracellular ATP is not well known. In a number of different cell types including coronary artery smooth muscle cells (20), human intestinal epithelial cells (20), rat astrocytes (21), and rat mesangial cells (22) exogenous ATP has been shown to exert mitogenic effects. The role of ATP in the regulation of prostate cancer cell proliferation is controversial, however. Whereas we have previously shown that exogenous added ATP stimulates the growth of DU-145 prostate tumor spheroids (18), others have shown a growth inhibitory effect of ATP (23), which was observed after long term incubation with the nucleotides and repeated application. In the present study cell proliferation following hypotonic stress of cells for 20 min was assessed by immunohistochemical quantification of the proliferation marker Ki-67 (Fig. 2A) as well as BrdUrd incorporation (Fig. 2B) into the cell nuclei of proliferating cells. Hypotonic stress significantly increased Ki-67 expression to 150 ± 16% (untreated control set to 100%), which was in the same magnitude as achieved upon treatment for 1 time with either 10 or 100 μM exogenous ATP (n = 3). Interestingly, the proliferation stimulus obtained with hypotonic stress was completely abolished in the presence of the ATP scavenger apyrase (10 units/ml), which indicates that cell proliferation requests ATP release from the cells. Furthermore, incubation with apyrase under isotonic conditions resulted in an ~20% decrease in Ki-67 expression, suggesting proliferation control of cells by exogenous ATP (data not shown). The data achieved with Ki-67 staining were corroborated by labeling cells with BrdUrd. Upon hypotonic stress significantly increased cell numbers of BrdUrd-positive, proliferating cells were observed (n = 3), which supports a role of released ATP as a mitogen in prostate cancer cells.

**Activation of MAPKs upon Hypotonic Stress of DU-145 Prostate Cancer Cells**—Stimulation of cell proliferation is generally associated with phosphorylation/activation of MAPK members. In DU-145 prostate cancer cells transient activation of ERK1,2 (maximum after 20 min) (Fig. 3A) and p38 (maximum after 10 min) (Fig. 3B) was observed, whereas JNK phosphorylation was not increased upon hypotonic stress (data not shown). Furthermore, hypotonic stress resulted in activation of P38K, which was absent in the presence of the P38K inhibitors LY294002 (100 μM) and wortmannin (1 μM) (data not shown). As expected, activation of ERK1,2 as well as p38 was abolished in the presence of either the ERK1,2 inhibitor UO126 (10 μM) or the p38 inhibitor SB203580 (5 μM) as well as in the presence of the P38K inhibitors wortmannin (1 μM) and LY294002 (100 μM) suggesting upstream activation of P38K (n = 3). Notably, preincubation with apyrase (6 units/ml) significantly inhibited activation of ERK1,2 as well as p38 upon hypotonic stress, which clearly indicates that hypotonic stress does not per se activate MAPK pathways but requires ATP release to the extracellular milieu and activation of signaling pathways presumably via purinergic receptor binding. Likewise, the activation of P38K upon hypotonic stress was abolished in the presence of apyrase (data not shown).

**Effects of ATP Scavenging and Purinergic Receptor Inhibition on Hypotonic Stress-induced Calcium Responses**—Hypotonic stress increases intracellular calcium concentration [Ca²⁺], in a variety of preparations, and the sources of the observed [Ca²⁺], increase as well as...
possible mechanisms of mechano-transduction of Ca²⁺ responses have been extensively studied during recent years (for review, see Refs. 24 and 25). The interrelation between hypotonicity-induced ATP release and subsequent Ca²⁺ responses have, however, so far not been investigated.

In the present study it was hypothesized that the hypotonic stress-induced Ca²⁺ response was dependent on extracellular ATP and purinergic receptor activation. During hypotonic incubation of cells a transient rise in [Ca²⁺]ᵢ was observed, which was still present albeit at reduced duration in Ca²⁺-free solution containing 1 mM EGTA (Fig. 4A and B), suggesting that the Ca²⁺ response was composed of Ca²⁺ release from intracellular stores followed by capacitative Ca²⁺ influx across the cell membrane. When apyrase (6 units/ml) was present in the incubation medium only about 40% of the cells responded toward the hypotonic challenge, and the amplitude of observed [Ca²⁺]ᵢ gradients was significantly reduced (n = 3). Comparable results were observed when cells were treated with the purinergic receptor antagonist suramin (100 μM), which likewise decreased the number of responding cells and the amplitude of the [Ca²⁺]ᵢ response (n = 3). Notably, the Ca²⁺ response was totally absent in all cells when suramin-treated cells were swollen in hypotonic solutions in the absence of extracellular calcium supporting a role of extracellular Ca²⁺ for the hypotonicity-induced Ca²⁺ response.

Taken together these data suggest that the hypotonicity-induced Ca²⁺ response required extracellular ATP and purinergic receptor activation and point toward the notion that hypotonic cell swelling per se is not sufficient to raise [Ca²⁺]ᵢ in DU-145 prostate cancer cells.
### Osmotic Stress-induced ATP Release

**Prostaglandin Synthesis upon Hypotonic Stress of DU-145 Prostate Cancer Cells**—Exogenous ATP (17, 26, 27) as well as mechanical stress (28) and hypotonic stress (29) have been previously shown to activate arachidonic acid metabolism and prostaglandin generation. It is, however, presently not known whether prostaglandins are involved in hypotonic stress-induced ATP release and whether prostaglandin synthesis requires the presence of ATP released upon hypotonic stress of cells. To address this issue, DU-145 prostate cancer cells were incubated either with exogenous ATP (50 nM) or hypotonic solution in the absence or presence of the COX-2 inhibitor NS398 (30 nM). Following 20 min of hypotonic incubation the generation of PGE2 was monitored in the presence of either apyrase or suramin (100 nM), which indicates the requirement of ATP release for activation of the arachidonic acid metabolism. *p < 0.05, significantly different as indicated.

**Inhibition of Hypotonic Stress-induced ATP Release by Purinergic Receptor Antagonist suramin (100 nM) or PPADS (100 nM) (Fig. 6). It was observed that in the presence of purinergic receptor antagonists hypotonic stress-mediated ATP release was totally abolished (n = 8), which strongly suggests a feed forward cycle of ATP release via purinergic receptor activation.**

**ATP Release Is Dependent on Hypotonic Stress-induced [Ca\(^{2+}\)]; Response**—Hypotonic stress induces a transient rise in [Ca\(^{2+}\)], in DU-145 prostate cancer cells, which requires ATP release and subsequent purinergic receptor activation (see Fig. 4). The intracellular Ca\(^{2+}\) response may in turn be involved in the ATP release mechanism following hypotonic challenge. To address this assumption ATP release was assessed in the absence of extracellular Ca\(^{2+}\) as well as after chelation of [Ca\(^{2+}\)], by BAPTA (10 mM) (Fig. 7). Absence of extracellular Ca\(^{2+}\) did not impair the hypotonic stress-mediated ATP release, indicating that the intracellular Ca\(^{2+}\) response was sufficient to regulate ATP release. The increase in extracellular ATP was, however, totally abolished when intracellular [Ca\(^{2+}\)], was chelated with BAPTA (n = 9), which suggests that hypotonicity-induced ATP release in prostate cancer cells is a Ca\(^{2+}\)-dependent effect.

**The Arachidonic Acid Metabolism and cAMP Are Involved in the ATP Release Mechanism**—Upon hypotonic stress PGE\(_2\) is released from DU-145 cells, the physiological function of released PGE\(_2\) is unknown, however. PGEs including PGE\(_2\) have been previously discussed to be involved in mechanisms of ATP release from cells upon mechanical strain (30). Furthermore, PGEs have been shown to activate receptors coupled to the stimulation of adenylate cyclase (AC), which generates cAMP (31). In heart cells it has been previously evidenced that cAMP can regulate ATP-permeable anion channels (32). To assess whether the arachidonic acid metabolism was involved in the mechanisms of ATP release from DU-145 prostate cancer cells, cells were treated with hypotonic solution in the presence of the non-metabolizable arachidonic acid analogue ETYA (50 nM) (n = 3) the COX-2 inhibitors indomethacin (100 nM) (n = 4), etodolac (100 nM) (n = 3), and NS398 (30 nM), (n = 9) as well as in the presence of the PLA\(_2\), inhibitor MAFP (n = 8) and the adenylate cyclase inhibitor MDL-12,330A (10 nM) (n = 8) (Fig. 8). All applied inhibitors significantly blunted the hypotonic stress-induced ATP release, which strongly supports the notion of involvement of the arachidonic acid pathway and generation of cAMP in the mechanism of ATP release.
Hypotonic Stress-induced ATP Release Requires Activation of MAPK/PI3K Pathways—The data of the present study point toward the notion of a cAMP-mediated ATP-release mechanism in DU-145 prostate cancer cells. It was shown that released ATP activated the purinergic receptor signaling cascade which involved activation of MAPK pathways and presumably PI3K. Since the purinergic receptor signaling cascade may provide a feed forward cycle for further ATP extrusion, it was investigated whether inhibition of either ERK1,2, p38 (Fig. 9A), or PI3K (Fig. 9B) would impair hypotonic stress-induced ATP release. Indeed the ERK1,2 inhibitor UO126 (10 μM) (n = 7), the p38 inhibitor SB203580 (10 μM) (n = 7), as well as the PI3K inhibitors wortmannin (1 μM) (n = 11) and LY294002 (100 μM) (n = 7), significantly inhibited ATP extrusion, which indicates that activation of the purinergic receptor signaling cascade is necessary to initiate the ATP release mechanism in DU-145 prostate cancer cells.

**DISCUSSION**

Despite prostate cancer being the second leading cause of cancer death (33), little is known about the regulation of prostate cancer cell proliferation after having acquired an androgen-independent characteristics. Previously it was reported that exogenous added ATP and related nucleotides inhibited the growth of androgen-independent prostate carcinoma cells (23, 34, 35), which led to the suggestion of novel therapeutic approaches based on purinergic receptor stimulation with the aim to initiate apoptotic cell death. However, in these studies rather high concentrations of ATP were applied repetitively and for long times, therefore presumably not representing the situation of physiological (non-lytic) ATP release, which is in the nanomolar to picomolar range and short term, due to the action of ecto-ATPases and other ecto-nucleotidases. It should, however, be mentioned that for short times the extracellular ATP reaches concentrations up to 10–20 μM in the close vicinity of cell membranes as was previously shown by using a cell surface-attached firefly luciferase method (36). In contrast to the studies reporting on growth inhibition of prostate cancer cells by ATP, experiments of our group have previously shown that low concentra-
tures and single treatment of prostate tumor spheroids with ATP stimulated tumor growth (18) corroborating the emerging physiological function of ATP as a potent mitogen that has been observed not only in endothelial cells and vascular smooth muscle cells (37) but comparatively in a variety of other cell types. In the present study it is demonstrated that ATP released from cells by mechanical (hyposmotic) stimulation as well as addition of ATP to the cell culture medium increased cell proliferation of the androgen-insensitive DU-145 cell line grown in monolayer culture. Since prostate epithelial cells are exposed to mechanical strain during prostate gland contraction, our data are of potential clinical importance for the understanding of benign as well as neoplastic prostate cell growth.

The data of the present study demonstrate that mechanical strain induced by hypotonic swelling of DU-145 cells elicited a Ca\(^{2+}\) response, activated ERK1,2, p38, and PI3K, stimulated PGE\(_2\) synthesis, and promoted cell proliferation. All these biological effects were efficiently inhibited upon hydrolysis of extracellular ATP by apyrase, which clearly shows that hypotonic swelling per se is not sufficient to initiate "classical" hypotonicity-mediated signaling pathways, which are known to involve activation of arachidonic acid metabolism, Ca\(^{2+}\) signaling, and MAPK activation (25) but requires active release of ATP to the extracellular compartment, where it apparently activates purinergic receptors of the P2Y or P2X class, which have been previously characterized in a variety of cell types including DU-145 prostate cancer cells (23). Notably, activation of purinergic receptors of the P2Y class involves just the same signaling pathways as are elicited by hypotonic stress, which points toward the notion that hypotonic stress-induced signaling cascades are mediated by purinergic receptor activation. It may be therefore concluded that ATP release represents the key event in the initiation of signaling events during hypotonic cell swelling. In this regard it has been previously shown that even such fundamental cell reactions as is regulatory volume decrease (RVD) are dependent on ATP release and purinergic receptor stimulation (38, 39).

In the present study it was hypothesized that ATP release from prostate cancer cells required the activation of the purinergic signaling cascade thus initiating a feed forward cycle of prolonged ATP extrusion. Indeed our experiments demonstrated that blocking purinergic receptors by either suramin or PPADS significantly reduced ATP release. Furthermore, ATP release was impaired upon interference with any of the downstream steps of purinergic receptor signaling, i.e., upon chelation of intracellular Ca\(^{2+}\), inhibition of PI3K, ERK1,2, and p38, as well as inhibition of the arachidonic acid metabolism by interfering with COX-2, PLA\(_2\), and AC.

Our data suggest that cAMP generated through AC activity is presumably the candidate second messenger responsible for the regulation of ATP permeation pathways in DU-145 prostate cancer cells, thereby corroborating the observations of Insel and co-workers (30), which were obtained with mechanically stimulated Madin-Darby canine kidney epithelial cells. Furthermore, stimulation of cAMP production has been previously described to occur after purinergic receptor stimulation in Madin-Darby canine kidney cells (17, 31), as well as after hypotonic stress in A6 cells (40) and mechanical stimulation in endothelial cells (41). Several cAMP-regulated ATP-conducting ion channels have been described previously. The most well known ATP-permeable chloride channel activated by cAMP is CFTR, which was extensively studied during recent years and was shown to regulate outwardly rectifying chloride channels (42, 43). In previous studies we have, however, demonstrated that hypotonic stress-induced ATP secretion from DU-145 prostate cancer cells was not impaired in the presence of the specific CFTR channel blocker glibenclamide. Furthermore, we demonstrated that ATP release was not impaired in the presence of inhibitors of P-glycoprotein (19), which has been demonstrated to conduct ATP in several preparations (14, 44). Our data therefore point toward a cAMP regulated anion conductance independent of CFTR and P-glycoprotein, which requires further electrophysiological characterization.

In recent years many cell types including DU-145 prostate cancer cells have been demonstrated to express a multitude of different G-protein-coupled P2Y as well as ion channel P2X receptors, which underscores the primordial role of ATP as an extracellular signaling molecule that is utilized in a variety of different signal transduction cascades. Recently, it has been pointed out that ATP is used as a molecule to establish the “set point” for different signaling cascades (30), which may include growth factor and cytokine-mediated pathways that regulate prostate cancer cell growth. Investigations undertaken to define the precise mechanisms involved in ATP release are therefore of primordial importance and will enforce the development of strategies that interfere with purinergic signaling in cancer cells thus aiming to slow down or even inhibit prostate tumor growth. These interventions may intend to treat cancer either with high ATP concentrations applied over long times or by permanent purinergic receptor activation using chemical compounds (45). However, since the data of the present study demonstrate that ATP permeation pathways and very low extracellular ATP elevations are involved in the regulation of prostate cancer cell proliferation, an alternative approach based on the inhibition ATP release and/or prevention of binding of ATP and related nucleotides to their receptors may be likewise efficient and may be exploited in prostate cancer treatment in the future.

REFERENCES
1. Gordon, J. L. (1986) Biochem. J. 233, 309–319
2. Burnstock, G. (1999) J. Anat. 194 (Pt 3), 335–342
3. Schwiebert, E. M. (2000) Am. J. Physiol. Cell Physiol. 279, C281–C283
4. Schwiebert, E. M., and Zsembery, A. (2003) Biochim. Biophys. Acta. 1615, 7–32
5. Insel, P. A., Ostroom, R. S., Zambon, A. C., Hughes, R. J., Balboa, M. A., Shehnaz, D., Gregorion, C., Torres, B., Firestein, B. L., Xing, M., and Post, R. S. (2001) Clin. Exp. Pharmacol. Physiol. 28, 351–354
6. Randriamboavonjy, V., Schrader, J., Busse, R., and Fleming, I. (2004) J. Exp. Med. 199, 347–356
7. Jo, Y. H., and Schlichter, R. (1999) Nat. Neurosci. 2, 241–245
8. Hazama, A., Hayashi, S., and Okada, Y. (1998) Pflugers Arch. 437, 31–35
9. Brustovsky, N., Becker, A., Klingenberg, M., and Bamberg, E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 664–668
10. Schwiebert, E. M. (2001) Clin. Exp. Pharmacol. Physiol. 28, 340–350
11. Stout, C. E., Costantin, J. L., Nau, C. C., and Charles, A. C. (2002) J. Biol. Chem. 277, 10482–10488
12. Cotrina, M. L., Lin, J. H., Alves-Rodrigues, A., Liu, S., Li, J., Azmi-Ghadimi, H., Kang, J., Nau, C. C., and Nedergraaf, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15737–15740
13. Braunstein, G. M., Roman, R. M., Clancy, J. P., Kudlow, B. A., Taylor, A. L., Shlyonsky, V. G., Jovov, B., Peter, K., Jilling, T., Ismailov, I. L., Benos, D. J., Schwiebert, L. M., Fitz, J. G., and Schwiebert, E. M. (2001) J. Biol. Chem. 276, 6621–6630
14. Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arceci, R. J., Kramer, R., Guidotti, G., and Cantiello, H. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 312–316
15. Roman, R. M., Lomri, N., Braunstein, G., Feranchak, A. P., Simeoni, L. A., Davison, A. K., Mechetner, E., Schwiebert, E. M., and Fitz, J. G. (2001) J. Membr. Biol. 183, 165–173
16. Bodas, E., Aleu, J., Pujol, G., Martin-Satue, M., Marsal, J., and Solsona, C. (2000) J. Biol. Chem. 275, 20268–20273
17. Post, S. R., Jacobson, J. P., and Insel, P. A. (1996) J. Biol. Chem. 271, 2029–2032
18. Sauer, H., Klimm, B., Hescheler, J., and Wartenberg, M. (2001) FASEB J. 15, 2539–2549
19. Sauer, H., Hescheler, J., and Wartenberg, M. (2000) Am. J. Physiol. Cell Physiol. 279, C295–C307
20. Coutinho Silva, R., Stahl, L., Cheung, K. K., de Campos, N. E., de Oliveira, S. C., Ouel, D. M., and Burnstock, G. (2005) Am. J. Physiol. Gastrointest. Liver Physiol. 288, G1024–G1035
21. Franke, H., Krugel, U., and Illes, P. (1999) Glia 28, 190–200
22. Schulze-Lohoff, E., Zanner, S., Ogilvie, A., and Sterzel, R. B. (1992) Am. J. Physiol. 263, F374–F383
23. Janssens, R., and Boeynaems, J. M. (2001) *Br. J. Pharmacol.* **132**, 536–546
24. Okada, Y., Maeno, E., Shimizu, T., Dezaki, K., Wang, J., and Morishima, S. (2001) *J. Physiol.* **532**, 3–16
25. Jakab, M., Furst, J., Gschwentner, M., Botta, G., Garavaglia, M. L., Bazzini, C., Rodighiero, S., Meyer, G., Eichmueller, S., Woll, E., Chwatal, S., Ritter, M., and Paulmichl, M. (2002) *Cell Physiol. Biochem.* **12**, 235–258
26. Welch, B. D., Carlson, N. G., Shi, H., Myatt, L., and Kinhore, B. K. (2003) *Am. J. Physiol. Renal Physiol.* **285**, F711–F721
27. Xing, M., Post, S., Ostrom, R. S., Samardzija, M., and Insel, P. A. (1999) *J. Biol. Chem.* **274**, 10035–10038
28. Alexander, L. D., Alagarsamy, S., and Douglas, J. G. (2004) *Kidney Int.* **65**, 551–563
29. Lundgren, D. W., Moore, R. M., Collins, P. L., and Moore, J. J. (1997) *J. Biol. Chem.* **272**, 20118–20124
30. Ostrom, R. S., Gregorian, C., and Insel, P. A. (2000) *J. Biol. Chem.* **275**, 11735–11739
31. Xing, M., Firestein, B. L., Shen, G. H., and Insel, P. A. (1997) *J. Clin. Invest.* **99**, 805–814
32. Lader, A. S., Xiao, Y. F., O’Riordan, C. R., Prat, A. G., Jackson, G. R., Jr., and Cantiello, H. F. (2000) *Am. J. Physiol. Cell Physiol.* **279**, C173–C187
33. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. (1997) *CA Cancer J. Clin.* **47**, 5–27
34. Fang, W. G., Pirnia, F., Bang, Y. J., Myers, C. E., and Trepel, J. B. (1992) *J. Clin. Invest.* **89**, 191–196
35. Calvert, R. C., Shabir, M., Thompson, C. S., Mikhailidis, D. P., Morgan, R. J., and Burnstock, G. (2004) *Anticancer Res.* **24**, 2853–2859
36. Beigi, R., Kobatake, E., Aizawa, M., and Dubyak, G. R. (1999) *Am. J. Physiol.* **276**, C267–C278
37. Burnstock, G. (2002) *Arterioscler. Thromb. Vasc. Biol.* **22**, 364–373
38. Feranchak, A. P., Fritz, J. G., and Romman, R. M. (2000) *J. Hepatol.* **33**, 174–182
39. Braunstein, G. M., Zsembarya, A., Tucker, T. A., and Schwiebert, E. M. (2004) *J. Cyst. Fibros.* **3**, 99–117
40. Matsumoto, P. S., Mo, L., and Wills, N. K. (1997) *J. Membr. Biol.* **160**, 27–38
41. Cohen, C. R., Mills, I., Du, W., Kamil, K., and Sumpio, B. E. (1997) *Exp. Cell Res.* **231**, 184–189
42. Schwiebert, E. M., Egan, M. E., Hwang, T. H., Fulmer, S. B., Allen, S. S., Cutting, G. R., and Guggino, W. B. (1995) *Cell* **81**, 1063–1073
43. Schwiebert, E. M., Morales, M. M., Devidas, S., Egan, M. E., and Guggino, W. B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2674–2679
44. Naumann, N., Siratska, O., Gahr, M., and Rosen-Wolff, A. (2005) *J. Cyst. Fibros.* **4**, 157–168
45. Burnstock, G. (2002) *Clin. Med.* **2**, 45–53