Extracellular ATP signaling and P2X nucleotide receptors in monolayers of primary human vascular endothelial cells

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Schwiebert, Lisa M., William C. Rice, Brian A. Kudlow, Amanda L. Taylor, and Erik M. Schwiebert. Extracellular ATP signaling and P2X nucleotide receptors in monolayers of primary human vascular endothelial cells. Am J Physiol Cell Physiol 282: C289–C301, 2002. First published September 5, 2001; 10.1152/ajpcell.01387.2000.—ATP and its metabolites regulate vascular tone; however, the sources of the ATP released in vascular beds are ill defined. As such, we tested the hypothesis that all limbs of an extracellular purinergic signaling system are present in vascular endothelial cells: ATP release, ATP receptors, and ATP receptor-triggered signal transduction. Primary cultures of human endothelial cells derived from multiple blood vessels were grown as monolayers and studied using a bioluminescence detection assay for ATP released into the medium. ATP is released constitutively and exclusively across the apical membrane under basal conditions. Hypotonic challenge or the calcium agonists ionomycin and thapsigargin stimulate ATP release in a reversible and regulated manner. To assess expression of P2X purinergic receptor channel subtypes (P2XRs), we performed degenerate RT-PCR, sequencing of the degenerate P2XR product, and immunoblotting with P2XR subtype-specific antibodies. Results revealed that P2X1 and P2X4 are expressed abundantly by endothelial cell primary cultures derived from multiple blood vessels. Together, these results suggest that components of an autocrine purinergic signaling loop exist in the endothelial cell microvasculature that may allow for “self-regulation” of endothelial cell function and modulation of vascular tone.

PURINERGIC SIGNALING REGULATES circulatory function (4, 15, 20, 21, 27). Nowhere has this signaling been studied more extensively in the cardiovascular system than in adenosine modulation of cardiac function (2, 12, 17). G protein-coupled P2Y purinergic receptors are expressed on vascular smooth muscle cells, platelets, and endothelial cells (4, 15, 20, 21, 27, 29, 33). In endothelial cells derived from multiple vessels, extensive study has shown the molecular and functional expression of P2Y1 and P2Y2 receptors (4, 15, 20, 21, 27, 29, 33). A specific isoform of the P2X purinergic receptor channel family, P2X1, a protein that binds ATP in its extracellular domain and also acts as a Ca2+-permeable, nonselective cation channel, was cloned from vascular smooth muscle (32). Expression of P2X1 has been documented in human umbilical and human renal vasculature (3, 9). More recently, expression of P2X4 as well as weaker expression of other P2XR subtypes was documented by Northern blot analysis and competitive, specific RT-PCR in endothelial cells (35). Immunoblotting for P2XR proteins as well as comprehensive studies of ATP release from these same human vascular endothelial cells was not performed (35). Only a few additional studies have hinted at P2X receptor expression in vascular cells (16, 23).

Despite the fact that, for decades, it has been postulated that ATP and adenosine act locally within vascular beds to regulate tissue vascular tone, the sources of ATP within the vasculature are largely unknown. Moreover, P2XR expression has not been characterized fully in vasculature. In particular, ATP release by endothelial cells is ill defined. As such, we tested the hypothesis that the endothelium, the layer of cells that lines all blood vessels, may provide a source of a physiological purinergic agonist, ATP, locally within vascular beds. Moreover, we tested the hypothesis that endothelial cells, in addition to vascular smooth muscle cells, also may express multiple subtypes of the emerging P2XR family to bind this released ATP and transduce this extracellular ATP signal in an autocrine or paracrine manner. Results demonstrate that human endothelial cell monolayers release ATP exclusively into the apical medium in a regulated manner and that endothelial cells express multiple P2XR subtypes.

MATERIALS AND METHODS

Endothelial cell primary culture. Human endothelial cells were isolated into primary culture from different blood vessels by Clonetics and purchased as proliferating cells. The cultures were grown on 2% gelatin-coated or 6% collagen-coated tissue culture flasks, 35-mm dishes, or 12-mm-diameter filter supports (Millicell CM) in endothelial basal medium (Clonetics) supplemented with 5% fetal bovine serum.
(FBS) and a BulletKit of additional additives including (per 500 ml) human epidermal growth factor (0.5 ml), hydrocortisone (0.5 ml), gentamicin (0.5 ml), bovine pituitary extract (2.0 ml), 1× fungizone, and 1× penicillin-streptomycin (31). Filters were bathed in medium on both sides of the filter until a monolayer formed that was tight to fluid for at least 12 h. Monolayers formed after 4–6 days in culture. The endothelial cell primary cultures prepared are as follows: human umbilical vein endothelial cells (HUVEC), human umbilical artery endothelial cells (HUAEC), human coronary artery endothelial cells (HCAEC), human pulmonary artery endothelial cells (HPAEC), human aortic endothelial cells (HAEC), and human lung microvascular endothelial cells (HLMVEC).

**Bioluminescence detection of ATP released from endothelial cell cultures and monolayers.** These methods and materials have been published previously (30) in detail for experiments on epithelial monolayers. Similar methods were followed for endothelial monolayers. Briefly, Opti-MEM-I medium ( Gibco-BRL) with 2 μg/ml luciferin-luciferase reagent (Calbiochem) was added to the apical or basolateral side of the monolayer or of a culture grown in 35-mm dishes. Basal levels of ATP release were measured for at least 2 min in 15-s, nonintegrated photon collection periods in a TD-20/20 Luminometer (Turner Designs). Distilled water with 2 μg/ml luciferin-luciferase was added to dilute the osmotic strength of the Opti-MEM, and luminescence was measured for at least 2 min as described above. 4,4′-Diisothiocyano stilbene-2,2′-disulfonic acid (DIDS) and NaCl were added acutely during these luminescence measurements, since they had no effect on luciferase enzyme activity (7, 30). Apyrase or hexokinase was added at the end of the time courses to eliminate any ATP left in the medium. All assays were performed at room temperature unless otherwise specified. Specifics on data analysis and statistics have been published previously (30) or are described in figure and table legends.

Some important additional points must be emphasized that were not pointed out in our original paper on this assay (30). Different batches of luciferin-luciferase reagent were needed to complete these studies, giving rise to some differences in luminescence detection (for example, see Table 1 vs. Table 2). Unfortunately, we could not perform an ATP standard curve on every vial of detection reagent because we would have run out of detection reagent for the actual ATP release studies on endothelial cell monolayers. We also did not inhibit ecto-ATPases in any pharmacological way in these assays (besides cooling of the monolayers). It also is important to note that the medium used in the release detection assay included 140 mM chloride and that chloride inhibited luciferase activity. As such, together with ecto-ATPase activity uninhibited and luciferase inhibited by physiologically amounts of extracellular chloride, we may be underestimating ATP levels in the extracellular milieu. Therefore, luciferase acts as an “ATP sensor” in this bioassay, not a consumer of ATP.

**Degenerate P2X receptor RT-PCR.** Endothelial primary cultures were grown to confluence on collagen-coated flasks, and total RNA was extracted with Trizol reagent (Life Technologies). Total RNA was treated with DNase and reverse transcribed by standard methods. Each PCR reaction contained 1 μM dNTP mix, 2 μM forward primer, 2 μM reverse primer, 0.5 μM Taq polymerase (Perkin-Elmer), 10× PCR buffer (1.5 mM MgCl₂), and cDNA template. The PCR cycle began with a 5-min, 94°C “hot start” and was followed by 40 cycles of 30 s at 94°C, 60 s at 52°C, and 60 s at 72°C. A 10-min, 72°C elongation ended the reaction. PCR products were run on a 1.5% agarose gel beside a 100 base pair (bp) DNA ladder. Primers were as follows: β-actin forward primer, 5′-TGA CGG GGT CAC CAC TGT GCC CAT CTA-3′; β-actin reverse primer, 5′-CTA GAA GCA TTD CCG TGG ACG ATG GAG GG-3′; P2XR degenerate forward primer, 5′-TTC ACC MTT YTC ATC AAC AGC ATC-3′; and P2XR degenerate reverse primer, 5′-TGG CAA AYC TGA AGT TGW AGC C-3′.

PCR products were rescued from agarose gel slices using the Qiagen gel extraction kit (Qiagen). The purified PCR product was ligated into the pGEM-T vector (Promega). The ligations were transformed into JM109 competent cells (Promega), and the cells were plated onto Luria broth (LB) agar plates containing ampicillin (100 μg/ml), X-Gal (40 μM/l), and isopropyl β-D-thiogalactopyranoside (100 μg/ml). White colonies (those with ampicillin resistance and an interrupted β-galactosidase gene) were picked and grown in 6 ml of LB-ampicillin. The plasmids were isolated from the bacteria using a PerfectPrep miniprep kit (5 Prime-3 Prime). The purified DNA was denatured in 0.2 N NaOH and precipitated in 7.5 M NH₄Cl and 100% ethanol. The Sequenase dyeoxy terminator sequencing kit (Amersham) and the sequencing primer, mono β-D-35S-labeled dATP (NEN-Dupont) was used to sequence the purified DNA product. The DNA sequence was read and screened with the BLAST algorithm to determine the identity of the PCR product (1). In this and other studies on epithelial cells, sequences for every P2XR subtype were amplified with the exception of P2X₆, a brain-specific subtype (data not shown).

**Immunoblotting with P2X receptor channel subtype-specific antibodies.** Cells were lysed in a buffer containing 10 mM Tris, 0.5 mM NaCl, 0.5% Triton X-100, 50 μg/ml aprotinin (Sigma, St. Louis, MO), 100 μg/ml leupeptin (Sigma), and 100 μg/ml pepstatin A (Sigma) adjusted to pH 7.2–7.4. Protein (20 μg/lane) was run and separated on an 8% SDS polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (Osmonics, Westminster, MA). Immunoblotting was performed with rabbit polyclonal antibody to P2X₁, P2X₂, P2X₄, and P2X₇ at a dilution of 1:500 (Alomone Laboratories, Jerusalem, Israel) or with antibody to P2X₆ at a dilution of 1:1,000 (generous gift of Drs. Mark Voigt and Terry Egan, St. Louis University, St. Louis, MO). Reactivity was detected by horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:3,000 dilution) (New England BioLabs, Beverly, MA). Enhanced chemiluminescence was used to visualize the secondary antibody.

**Fura 2-AM imaging of intracellular Ca²⁺.** Cytosolic intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in human vascular endothelial cells was measured with dual-excitation wavelength fluorescence microscopy (Deltascan, Photon Technologies, Princeton, NJ) using the permeant form of the fluorochrome, fura 2-AM (Teflabs, Austin, TX). Fura 2 fluorescence was measured at an emission wavelength of 510 nm in response to excitation wavelengths of 340 and 380 nm, alternated at a rate of 25 Hz by a computer-controlled chopper assembly. Autofluorescence-corrected ratios (340 nm/380 nm) were calculated at a rate of 5 points/s using PTI software. Cells were grown on collagen-coated coverslips (1:30 dilution of Vitrogen 100 in PBS) cut to fit the circulating cuvette and were incubated in media containing 5 μM fura 2-AM and 1 mg/ml Pluronific F-127 dissolved in dimethyl sulfoxide (DMSO) for 2 h to allow loading of the dye into the cells. After loading, a coverslip was rinsed in Ringer solution to remove extracellular fura 2-AM and was positioned in the cuvette at a 45° angle from the excitation light. Two glass capillary tubes were inserted into the cuvette. One of these tubes was extended to the bottom of the cuvette and connected by way of polyethylene tubing to an infusion pump. The other capillary tube was positioned at the circulating
cuvette and served to remove fluid from the cuvette. Flow rate through the cuvette was \(-5\) nL/min. A Ringer solution was used containing (in mM) \(148\) NaCl, \(5\) KCl, \(1\) MgSO\(_4\), \(1.6\) Na\(_2\)HPO\(_4\), \(0.4\) NaH\(_2\)PO\(_4\), \(5\) glucose, \(1.5\) CaCl\(_2\), and \(10\) HEPES at pH \(7.4\) and at room temperature. After 20 min of incubation in the control Ringer solution, fluorescence intensities of both wavelengths stabilized. Once values were stable, purinergic agonists were added to the circulating cuvette for testing. The \(340/380\) ratios (\(R\)) were converted into \([\text{Ca}^{2+}]_i\), values as follows: \([\text{Ca}^{2+}]_i = K_d \times (R - R_{\text{min}})/(R_{\text{max}} - R) \times (S_{380}/S_{380})\), where \(R_{\text{max}}\) and \(R_{\text{min}}\) are \(R\) values under saturating and \(\text{Ca}^{2+}\)-free conditions, respectively, and \(S_{380}\) and \(S_{380}\) are the fluorescent signals (\(S\)) emitted by the \(\text{Ca}^{2+}\)-free (f) and \(\text{Ca}^{2+}\)-bound (b) forms of fura 2 at the 380-nm wavelength. This was accomplished after the cells were permeabilized with \(5\) \(\mu\)M ionomycin and fluorescence ratios and signals were measured under \(\text{Ca}^{2+}\)-free (2 mM EGTA) or \(\text{Ca}^{2+}\)-saturating (1.5 mM CaCl\(_2\)) conditions using fura 2 calibration solution (PBS containing 10 mM MgCl\(_2\), 2 mM EGTA). The dissociation constant (\(K_d\)) of fura 2 for \(\text{Ca}^{2+}\) was taken as 224 nM.

Data analysis and statistics. A \(P\) value of <0.05 was considered significant, whether determined by a paired Students’ \(t\)-test for paired experiments or by ANOVA with a Bonferroni ad hoc test for unpaired data. Most of the experiments involved paired analysis. The figure legends indicate the tests used and the \(P\) values calculated.

Materials. All chemicals were obtained from Sigma unless otherwise noted.

RESULTS

Endothelial cells in primary culture form resistive monolayers in vitro. Human vascular endothelial cells were grown on 12-mm-diameter Millicell PC permeable filters to establish endothelial monolayers. This maneuver was necessary to study the sidedness of ATP release (see data below). Routinely, within 4-7 days, the endothelial monolayers were tight to fluid for a minimum of 1 h, which was significantly longer than the duration of the ATP release assays. For the HUVEC endothelial monolayers, transendothelial resistance (\(R_{\text{Te}}\)) was measured with a Voltohmmeter. \(R_{\text{Te}}\) was measured 2 days after seeding of the filters. After only 2 days, \(R_{\text{Te}}\) was \(154 \pm 2\) \(\Omega\cdot\text{cm}^2\) (\(n = 48\)). Because the resistance of the Millicell PC filter is \(50\) \(\Omega\cdot\text{cm}^2\), significant \(R_{\text{Te}}\) was achieved after only 48 h. \(R_{\text{Te}}\) increased during the next 48-h period and was \(197 \pm 2\) \(\Omega\cdot\text{cm}^2\) at day 4. On day 5, \(R_{\text{Te}}\) was similar \((193 \pm 5\) \(\Omega\cdot\text{cm}^2\); \(n = 48\)), and monolayers were used for ATP release assays on days 5–7. These data show that human vascular endothelial cells grown in primary cultures under these conditions formed endothelial monolayers.

ATP is released predominantly across the apical membrane of human endothelial cell monolayers. Bio-luminescence detection of ATP released from human endothelial cells grown in primary culture as monolayers on permeable supports was performed to assess the magnitude and polarity of ATP release. Primary endothelial cell cultures from six different blood vessel preparations were compared. Significant apically directed ATP release was detected from all endothelial monolayers under basal conditions (Fig. 1). In sharp contrast, little ATP release was measurable in the basolateral medium under basal conditions, although these values were above background (Fig. 1). Although this is a microassay that estimates ATP release from monolayers, correlation of luminescence values with a standard curve of [ATP] indicated that apically directed ATP release elaborated nanomolar quantities of ATP, whereas basolaterally directed ATP release produced only picomolar quantities (Table 1). In particular, HUVEC and HPAEC monolayers released the most ATP across the apical membrane, whereas lesser amounts were measured in the endothelial monolayers derived from other blood vessel sources. Table 1 also shows that luciferase is present in sufficient quantity in the assay to “sense” extracellular ATP but not to consume it rapidly over time. However, because this is a microassay and because ecto-ATPases compete with luciferase for the released ATP (see Fig. 6), the amount of released ATP being detected was likely underestimated. Nevertheless, a second y-axis (to the right of

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**Fig. 1.** Sidedness of constitutive ATP release from polarized vascular endothelial monolayers under basal or unstimulated conditions. A numerical comparison of apical (APM) and basolateral medium (BLM) ATP release is shown in the context of a circulatory system schematic. Values in arbitrary light units (ALU) show that ATP is released mainly into the apical medium; no. of experiments (\(n\)) is shown in parentheses. Note that all endothelial monolayers release significant quantities of ATP apically above background (≤0.1 ALU). Release of ATP from human umbilical vein endothelial cell (HUVEC) cultures grown in 35-mm dishes is also shown. HPAEC, human pulmonary artery endothelial cells; HLMVEC, human lung microvascular endothelial cells; HAEC, human aortic endothelial cells; HCAEC, human coronary artery endothelial cells; HUAEC, human umbilical artery endothelial cells.

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**Table 1.** ATP release from human endothelial cell monolayers

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>APM (ALU)</th>
<th>BLM (ALU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>3.751 +/- 0.871</td>
<td>0.095 +/- 0.006</td>
</tr>
<tr>
<td>HLMVEC</td>
<td>1.396 +/- 1.987</td>
<td>0.943 +/- 0.079</td>
</tr>
<tr>
<td>HAEC</td>
<td>1.596 +/- 0.177</td>
<td>0.529 +/- 0.043</td>
</tr>
<tr>
<td>HPAEC</td>
<td>2.510 +/- 2.255</td>
<td>0.820 +/- 0.167</td>
</tr>
<tr>
<td>HCAEC</td>
<td>16.06 +/- 3.908</td>
<td>0.625 +/- 0.176</td>
</tr>
<tr>
<td>HUAEC</td>
<td>29.57 +/- 3.255</td>
<td>0.820 +/- 0.167</td>
</tr>
</tbody>
</table>

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**Fig. 2.** ATP release from polarized vascular endothelial monolayers under basal or unstimulated conditions. A numerical comparison of apical (APM) and basolateral medium (BLM) ATP release is shown in the context of a circulatory system schematic. Values in arbitrary light units (ALU) show that ATP is released mainly into the apical medium; no. of experiments (\(n\)) is shown in parentheses. Note that all endothelial monolayers release significant quantities of ATP apically above background (≤0.1 ALU). Release of ATP from human umbilical vein endothelial cell (HUVEC) cultures grown in 35-mm dishes is also shown. HPAEC, human pulmonary artery endothelial cells; HLMVEC, human lung microvascular endothelial cells; HAEC, human aortic endothelial cells; HCAEC, human coronary artery endothelial cells; HUAEC, human umbilical artery endothelial cells.
Table 1. Luminescence vs. absolute concentration of ATP vs. rate of ATP efflux, and ATP standard curve and consumption of ATP by luciferase-luciferin reagent over time

<table>
<thead>
<tr>
<th>Condition</th>
<th>Luminescence (ALU)</th>
<th>Absolute [ATP], nM</th>
<th>Rate of ATP Appearance, pM/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>29.57 ± 3.255</td>
<td>20</td>
<td>9.5</td>
</tr>
<tr>
<td>Hypotonicity</td>
<td>87.89 ± 11.56</td>
<td>50</td>
<td>25.0</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>49.15 ± 3.10</td>
<td>30</td>
<td>14.2</td>
</tr>
<tr>
<td>Detergent</td>
<td>&gt;9,999</td>
<td>&gt;10</td>
<td></td>
</tr>
</tbody>
</table>

Values provide a “ballpark” as to how luminescence relates to absolute [ATP] and the rate of appearance of ATP in the medium. Nanomolar amounts are being released constitutively that are up-regulated to higher nanomolar amounts by agonists. This assay only underestimates what may be happening in vivo, because this is a microassay on a small dish or cells or a blood vessel) and because ecto-ATPases compete with luciferase for the released ATP (see Fig. 6). A spiked amount of ATP is cleared slowly over time by the luciferase reagent, showing that it is present in sufficient amount as a detection reagent that is an ATP sensor but does outcompete ecto-ATPases. The assay was designed to be a “real biology” assay in addition to being a real-time assay. Pharmacological inhibitors of ecto-ATPases also inhibit luciferase enzyme activity. ALU, arbitrary light units.

Each luminescence data plot shows absolute [ATP] calculated from the standard curve data provided in Table 1. Together, these results show that endothelial cells release ATP under basal conditions constitutively and that this ATP release is directed predominantly across the apical membrane.

In one long-lived HUVEC primary culture, ATP release was measured across the apical membrane as a function of passage number (Fig. 2). Apically directed ATP release under basal conditions was robust in early passages (Fig. 2). However, by passages 9 and 10, the magnitude of ATP release waned (Fig. 2). In contrast, basolaterally directed ATP release under basal conditions followed a Gaussian-like distribution (Fig. 2). The peak value of basolaterally directed ATP release was measured at passage 8, whereas it was lower at earlier passages (Fig. 2). As in apically directed ATP release measurements, basolaterally directed ATP release waned at passages 9 and 10. This culture dedifferentiated at passage 11. All data from HUVEC primary cultures are included in Fig. 1. These data show that ATP release was dependent on the age of primary culture. Because apically directed ATP release was much greater than basolateral ATP release, apically directed ATP release results are the focus of all subsequent discussion.

ATP release is potentiated by hypotonic challenge. Previous studies by our laboratory alone or in collaboration with other groups have shown that normal hepatocytes (13), normal airway epithelial cells (7, 30), and normal and cystic renal epithelia (34) release ATP profoundly under hypotonic conditions. Dilutions of the medium osmolality with increasing volumes of distilled water were compared with medium controls (similar added volumes of isotonic medium) in bioluminescence detection assays of ATP released from HUVEC monolayers. As little as 13% dilution stimulated a transient increase in ATP release, whereas more robust dilutions of the medium osmolality produced sustained increases in ATP release (Fig. 3). Reconstitution of the medium osmolality by addition of a bolus of 50 mM NaCl (100 mosmol) reversed hypotonicity-induced ATP release across the apical membrane to levels measured under basal conditions (Fig. 3). Further additions of NaCl, which made the medium hypertonic, inhibited basal ATP release in both water dilution and medium control experiments (Fig. 3). In fact, addition of 150 mM NaCl (300 mosmol) inhibited apically directed ATP release under basal conditions by at least 75% (Fig. 3). Note that significant volumes of isotonic medium added to the apical side of HUVEC monolayers had no effect on ATP release (Fig. 3), showing that a dilution of osmolality and not a mechanical perturbation was stimulating ATP release across the apical membrane of HUVEC monolayers. Together, these re-

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sults show that hypotonicity potentiates ATP release across the apical membrane of HUVEC monolayers. Moreover, hypertonicity (addition of osmoles during isotonic medium controls) attenuates basal ATP release in medium controls (Fig. 3). Previously, we showed partial attenuation of ATP release in epithelial monolayers with the broad-spectrum, mechanosensitive ion channel blocker GdCl3 (7, 28, 30). In HUVEC monolayers, GdCl3 inhibited apically directed basal ATP release partially and in a dose-dependent manner (Fig. 4, see legend for summarized data). In this study, we also discovered a second ATP release inhibitor, DIDS, a broad-spectrum anion channel-blocking drug. Figure 4 also shows a representative time course illustrating complete block of basal ATP release with DIDS (see legend for summarized data). Similar blockade of hypotonicity-induced ATP release across the apical membrane was observed with GdCl3 and DIDS (Fig. 4). As in Fig. 3, graded dilutions of the medium osmolality stimulated ATP release. GdCl3 inhibited the potentiated ATP release partially, whereas DIDS inhibited ATP release fully (Fig. 4; see legend for summarized data). Together, these data show that ATP release is inhibited by ion channel blocking drugs and suggest that an ATP-permeable conductive transport pathway may mediate ATP release across the apical membrane of endothelial cell monolayers.

ATP release is potentiated by the Ca^{2+} agonists ionomycin and thapsigargin. Because agonists that increase cytosolic Ca^{2+} have profound effects on endothelial cell function, signaling, and production and on release of vasoactive mediators such as nitric oxide (22, 24), we examined the effect of Ca^{2+} agonists on ATP release across the apical membrane of HUVEC monolayers. Ionomycin (2 μM), a Ca^{2+} ionophore that promotes Ca^{2+} influx into the cell from extracellular stores, elicited an immediate increase in ATP release (Fig. 5). Unlike the hypotonicity-induced ATP release phenotype where luminescence increased and remained elevated for a few minutes before decaying over time, ionomycin-induced ATP release was immediate and continued to increase over the 8-min exposure period.

Fig. 3. Hypotonic challenge stimulates ATP release across the apical membrane of HUVEC endothelial cell monolayers. Raw luminescence values for HUVEC monolayers exposed to hypotonic challenge (Hypo) or to equal volumes of isotonic medium (a similar amount of luciferin-luciferase reagent was present in all stages of the experiment; n = 6 each). Reconstitution of medium osmolality with NaCl reversed hypotonicity-induced ATP release. Note that hyperosmolality in medium controlled and, after the third addition of 50 mM NaCl to water dilutions, inhibited basal ATP release to low levels. The y-axis at right shows a correlation with absolute [ATP] that is an approximation by correlation with the standard curve shown in Table 1.

Fig. 4. Basal and hypotonicity-induced ATP release are inhibited by ion channel blocking drugs. A: representative experiments showing DIDS and GdCl3 inhibition of basal ATP release across the apical membrane of HUVEC monolayers. At the 200 μM dose (data grouped among HUVEC and HCAEC monolayers), GdCl3 inhibited basal ATP release by 47.75 ± 4.92% (n = 4), whereas DIDS inhibited basal ATP release by 93.51 ± 2.50% (n = 4). The y-axis at right shows a correlation to absolute [ATP] that is an approximation by correlation with the standard curve shown in Table 1. B: representative experiments showing DIDS and GdCl3 inhibition of hypotonicity-induced ATP release across the apical membrane of HPAEC monolayers. At the 200 μM dose (data grouped among HAEC and HPAEC monolayers), GdCl3 inhibited hypotonicity-induced ATP release by 47.75 ± 4.92% (n = 4), whereas DIDS inhibited hypotonicity-induced ATP release by 93.51 ± 2.50% (n = 4). All inhibition by the 2 ion channel blockers was significant (P < 0.05). The y-axis at right shows a correlation to absolute [ATP] that is an approximation by correlation with the standard curve shown in Table 1.
DIDS-sensitive ATP release mechanism under basal conditions may require intracellular Ca^{2+}. Importantly, pretreatment of endothelial monolayers with 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid (BAPTA)-AM (10 μM, 30-60 min pretreatment), the permeable form of the Ca^{2+}-chelating agent, abolished ionomycin-induced ATP release. In the absence of BAPTA-AM, ionomycin increased ATP release acutely from 6.79 ± 1.36 to 21.82 ± 3.99 arbitrary light units (ALU) [change (Δ): 15.03 ± 2.64 ALU; n = 5] within 15 s and to a sustained value 8 min after addition of 50.01 ± 3.99 ALU (Δ38.79 ± 1.90 ALU; n = 5). In the presence of the Ca^{2+} chelator, ionomycin failed to stimulate ATP release acutely (Δ14.0 ± 0.80 ALU; n = 5) and chronically (Δ2.02 ± 0.76 ALU; n = 5).

Together, these data show that intracellular Ca^{2+} regulates the release of ATP across the apical membrane of human endothelia and promotes extracellular ATP signaling in the endothelial microenvironment in vitro.

Temperature affects dynamics and magnitude of constitutive, hypotonicity-induced, and Ca^{2+}-agonist-stimulated ATP release assay: a role for exocytosis and ecto-ATPases. To address the possible role of exocytosis in the release of ATP via ATP-filled vesicles, we performed our standard ATP release assay in temperature-controlled rooms at 4, 25, 30, and 37°C. Fortunately, these temperatures do not affect the luciferase detection assay significantly (Table 2). Incubation of cells or monolayers at 4°C slows vesicle trafficking along the secretory pathway, even preformed vesicles that are poised to fuse with the plasma membrane. Figure 6A shows that constitutive release was not affected in its magnitude by performing the experiment at 4°C vs. 25°C. In sharp contrast, however, ~40% of the Ca^{2+}-agonist- and hypotonicity-induced ATP release phenotype was abolished by reduced temperature incubation and assay, suggesting that exocytosis may play a partial role as a mechanism of ATP release.

To our surprise, assay temperatures higher than room temperature produced no increase in bioluminescence. At 37°C, we observed a reduced signal at all stages of the assay (Fig. 6B). At the end of the first stage of the assay monitoring basal release, the 37°C signal was less than room temperature (Fig. 6B). The

<table>
<thead>
<tr>
<th>[ATP], M</th>
<th>4°C</th>
<th>25°C</th>
<th>30°C</th>
<th>37°C</th>
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<td>10^{-11}</td>
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<td>95.87</td>
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<td>256.9</td>
<td>188.4</td>
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<td>≥10^{-5}</td>
<td>&gt;9.999</td>
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<td>&gt;9.999</td>
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</table>

Values are averaged ALU from 3 separate standard curves performed over the course of the fixed temperature assays. Standard error was <10% for all values at all concentrations of ATP.
Ca\(^{2+}\) agonist- and hypotonicity-induced ATP release signal also was reduced at higher temperature (Fig. 6B). Upon more careful review of the data, we found that the decay in bioluminescence in all stages of the experiment was most pronounced at 37°C. This is shown best when comparing basal or constitutive ATP release at 4°C vs. 37°C (Fig. 6C); completely different phenotypes are observed. A gradual rise in bioluminescence was observed at 4°C, suggesting that ecto-ATPases no longer compete for the released ATP. In sharp contrast, at 37°C, the signal peaked within 1 min and decayed by almost 50% by 6 min. In these assays, constitutive ATP release was measured for 6 min, followed by ionomycin-induced release for 8 min and hypotonicity-induced release for 6 min. Blockade with DIDS or GdCl\(_3\) and hexokinase was then carried out. Together, these data show that ecto-ATPase activity, attenuated at 25°C and inhibited at 4°C, is maximally active at 37°C and competes successfully with luciferase for the released ATP. Moreover, the loss of signal at 37°C suggests that ecto-ATPase activity outcompetes luciferase, whereas the attenuated signal at 4°C for Ca\(^{2+}\) agonist- and hypotonicity-induced ATP release suggests a component of ATP release that relies on exocytosis of ATP-filled vesicles.

**P2X receptor channels are expressed by human endothelial cells.** To test the hypothesis that subtypes of a subclass of purinergic receptors, the P2X receptor channels (P2XRs), are expressed in endothelial cells, we performed degenerate RT-PCR. To confirm the authenticity of the cDNA before P2XR RT-PCR, we performed RT-PCR with primers for the housekeeping gene β-actin (Fig. 7). The same cDNA was amplified with primers designed from an alignment of the first three rat P2XR cDNA sequences (rP2XR 1–3) in a PCR reaction. Figure 7 shows bands of the expected size (330 bp) in all endothelial cell samples. Negative controls (no cDNA) lacked the 330-bp band (Fig. 7). There are no bands in RNA samples amplified without pretreatment with reverse transcriptase (data not shown). As a positive control, amplification of the human P2Y\(_2\) gene from HUVEC total RNA also was performed. A band of the expected size (483 bp) was amplified that was confirmed to be P2Y\(_2\) (Fig. 7, inset). Together, these results show that endothelial cells derived from multiple vascular beds express P2X receptor channels. Confirmatory results for a P2Y\(_2\) G protein-coupled receptor also are shown.

**Human endothelial cells express multiple P2X receptor channel isoforms.** Although it is clear that endothelial cell primary cultures express P2X receptors, it is unclear from RT-PCR alone which P2XR isoforms are expressed. To determine which isotypes are present,
we performed a large-scale cloning and sequencing analysis. Multiple colonies containing pGEM-T plasmid with P2XR PCR insert were sequenced from each cell type in an effort to determine whether P2X receptor channel isoforms were expressed and which P2X isotypes were expressed most abundantly. Sequences were subjected to BLASTN analysis (1) to determine which P2X receptor isoforms were present in each endothelial cell primary culture. Table 3 shows the results of this differential DNA sequencing analysis for the five endothelial cell primary cultures analyzed. Readily apparent from this analysis, P2X4 and P2X5 were present in all samples (Table 3). These two isoforms were the only isoforms found in HPAEC (Table 3). P2X1 expression was unique to HCAEC, whereas P2X7 was found only in HAEC and HCAEC (Table 3). Novel sequences were found rarely in both primary cultures derived from umbilical vasculature in HAEC, HUAEC, and HUEVC (Table 3). Interestingly, these novel sequences were most identical to splice variants or isolated regions of P2XR isoforms. In these stretches of homology, sequence identity was virtually 100%, although this stretch only encompassed <50% of the PCR product. More work is being performed with these clones that is beyond the scope of this study; similar novel P2XR-like sequences have been identified in epithelial cell models from lung and liver (Taylor AL and Schwiebert EM, unpublished observations).

Table 3. Incidence of P2X receptor channel isoform sequences determined by degenerate RT-PCR

<table>
<thead>
<tr>
<th>P2X4</th>
<th>P2X5</th>
<th>P2X7</th>
<th>Other</th>
<th>Total Minipreps</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>0</td>
<td>18</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>HAEC</td>
<td>0</td>
<td>6</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>HCAEC</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>HPAEC</td>
<td>0</td>
<td>5</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>HUAEC</td>
<td>0</td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are based on minipreps sequenced and subjected to BLASTN analysis. Human lung microvascular endothelial cell (HLMVEC) PCR products were not processed in this manner, although a band of the expected size was amplified from these primary cultures as well (data not shown). “Other” reflects sequences with partial homology to a portion of a P2X receptor subtype sequence that may be a novel type of P2X receptor channel or an orphan receptor channel. These primers detect each P2XR subtype; however, we have never detected P2X6, a brain-specific subtype, in any endothelial or epithelial mRNA sample to date (over 300 DNA sequences).

HAEC, human aortic endothelial cells; HCAEC, human coronary endothelial cells; HPAEC, human pulmonary artery endothelial cell; HUAEC, human umbilical artery endothelial cells.
could be competed away by the peptide immunogen used to generate the antibody (data not shown). No consistent signals were observed for P2X1, P2X2, or P2X7 (data not shown). Together, these data show that P2X4 and P2X5 receptor channel subtypes are expressed as membrane proteins poised to receive extracellular ATP signals. These data agree with degenerate RT-PCR analysis showing abundant expression of the same two P2XR subtypes.

Nucleotide agonists increase cytosolic Ca\(^{2+}\) in human vascular endothelial cells. In HAEC and HUVEC cells loaded with fura 2-AM, the effect of individual nucleotide agonists and cocktails of P2Y- and P2X-selective agonists on cytosolic Ca\(^{2+}\) was assessed. As this fura 2 analysis was being performed in addition to the ATP release assays and receptor expression studies, Yamamoto et al. (35) published results showing ATP-induced increases in [Ca\(^{2+}\)]\(_i\) and argued a role for P2X receptors in this response. Unfortunately, Yamamoto et al. used only ATP itself, which binds to and stimulates both P2Y and P2X receptor subtypes, as an agonist. Antisense blockade of P2X4 mRNA attenuated [Ca\(^{2+}\)]\(_i\) marginally, suggesting that other P2X receptor subtypes were still functional and/or that P2Y receptors also were mediating ATP-induced increases in intracellular [Ca\(^{2+}\)].
Basal $[\text{Ca}^{2+}]_i$ in endothelial cell primary cultures was $60 \pm 6$ nM ($n = 9$). Addition of a cocktail of P2Y receptor-selective agonists (UDP, ADP, and UTP; 100 μM each) triggered an increase in $[\text{Ca}^{2+}]_i$ that had transient ($\Delta 93 \pm 14$ nM for peak responses; $n = 9$) and sustained ($\Delta 45 \pm 6$ nM before washout; $n = 9$) components. An example of data calibrated to calculate free $[\text{Ca}^{2+}]_i$ shows that the P2Y receptor agonist cocktail increased $[\text{Ca}^{2+}]_i$, which showed only limited desensitization (Fig. 10A). Similar addition of a cocktail of P2X receptor-selective agonists (benzoyl-benzoyl ATP and α,β-methylene-ATP, 100 μM each) failed to increase $[\text{Ca}^{2+}]_i$ ($n = 9$; see Fig. 10A for example). UDP (100 μM) and ADP (100 μM) produced weak transient responses ($\Delta 30 \pm 5$ nM) that were inconsistent from culture to culture, suggesting that weak expression of P2Y4, P2Y6, and/or P2Y11 may be present in these cultures. Addition of UTP (100 μM) elicited a response that mirrored the P2Y receptor cocktail response with transient ($\Delta 55 \pm 9$ nM for peak responses; $n = 4$) and sustained ($\Delta 30 \pm 5$ nM before washout; $n = 4$) components. An example of a UTP stimulation is shown in Fig. 10B. This trace in Fig. 10B shows the fluorescence ratio data for comparison with the calibrated data in

![Image](http://ajpcell.physiology.org/)

**Fig. 10.** P2 receptor agonists increase cytosolic $\text{Ca}^{2+}$ and ATP scavengers and receptor antagonists lower basal cytosolic $\text{Ca}^{2+}$ in HUVEC endothelial cell primary cultures. A: a cocktail of P2Y-selective agonists (UTP, ADP, and UDP, 100 μM each) was added in 3 separate boluses to reproducibly increase cytosolic $\text{Ca}^{2+}$. In sharp contrast, a cocktail of 2 P2X-selective agonists failed to increase $\text{Ca}^{2+}$. Calibrated data for actual free cytosolic $\text{Ca}^{2+}$ is shown. B: in a separate experiment, the effect of UTP alone (100 μM) is shown in a longer-lived time course. In this plot, fluorescence ratio of the dual-wavelength fura 2 dye is shown. C: in a third experiment plotting fluorescence ratio, a cocktail of ATP, ADP, and adenosine scavengers (designed to eliminate any and all active purinergic agonists that would be released endogenously into the system) lowered basal cytosolic $\text{Ca}^{2+}$. Suramin, a P2 receptor antagonist, was without effect at the 1 μM dose; however, 10 and 100 μM doses reversibly lowered basal cytosolic $\text{Ca}^{2+}$. This experiment was representative of 3 such time courses that produced similar results. All agonists, antagonists, and inhibitors were screened against all wavelengths that detect the fura 2-AM fluorescent dye to ensure that none affected fura 2 fluorescence. For example, pyridoxal phosphate 6-azophenyl-2',4'-disulphonic acid tetrasodium (PPADS), another P2 receptor antagonist, is auto fluorescent and cannot be used for these experiments.
agonist cocktail, increased 

nists vs. UTP alone. Because ATP, but not the P2X

butions of other P2Y subtypes may explain the larger

basal Ca\(^{2+}\). Additional contributions of other P2Y subtypes may explain the larger

magnitudes of the response to the cocktail of P2Y agonists vs. UTP alone. Because ATP, but not the P2X agonist cocktail, increased [Ca\(^{2+}\)]\(_i\) in a phenotype that was similar to that of UTP and P2Y agonists, we conclude that ATP increases [Ca\(^{2+}\)]\(_i\) via P2Y receptors.

In endothelial cells, P2X receptor channels may affect signal transduction by changing the resting membrane voltage or by other mechanisms.

A nucleotide/nucleoside scavenger cocktail and a nonselective P2 receptor antagonist, suramin, lower basal cytosolic Ca\(^{2+}\) reversibly. To provide further evidence for autocrine/paracrine signaling by nucleotide agonists in the endothelial cell microenvironment as well as to tie together the ATP release and ATP receptor limbs of this autocrine/paracrine signaling system, we assessed the effects of antagonism of extracellular ATP signaling on cytosolic Ca\(^{2+}\). Figure 10C shows that a scavenger cocktail designed to eliminate ATP, ADP, AMP, and adenosine that included 0.1 U/ml hexokinase (converts ATP to ADP), 0.1 U/ml apyrase (converts ATP and ADP to AMP), and adenosine deaminase (converts adenosine to inosine) lowered basal Ca\(^{2+}\) significantly and in a manner that was reversible with a wash. Moreover, in the same experiment, suramin, an antagonist that blocks P2Y and P2X receptor subtypes, lowered basal cytosolic Ca\(^{2+}\) in a dose-dependent and reversible manner. Together with findings for exogenous nucleotide agonists delivered to the fura 2-loaded endothelial cells, these data suggest that endogenous ATP, released into the circulating imaging system, is sufficient in amount to maintain basal cytosolic Ca\(^{2+}\) levels in an autocrine/paracrine manner.

**DISCUSSION**

The strength of this study is the integrative analysis of all limbs (release, receptors, and receptor-driven signaling) of autocrine purinergic signaling. These studies were performed on several different human endothelial cell primary cultures grown as monolayers. To our knowledge, there are several novel aspects of the study. First, we have performed real-time bioluminescence detection of released ATP on endothelial monolayers. Burnstock’s group has documented ATP-stimulated ATP release, lipopolysaccharide (LPS)-driven ATP release, and shear stress-induced ATP release from HUVEC primary cultures (5, 6, 36). However, these were nonpolarized cultures, whereas our studies were performed on human primary endothelial cell monolayers. Second, we have assessed the sidedness of ATP release from endothelial monolayers. Third, systematic analysis of constitutive, Ca\(^{2+}\)-agonist-induced, and hypotonicity-induced ATP release as well as the temperature dependence of basal and stimulated ATP release has not all been performed in this integrative way. Fourth, through the use of ion transport inhibitors such as DIDS or GdCl\(_3\) and inhibition of exocytosis with 4°C cooling, we have uncovered roles for transport and exocytosis in ATP release from biological cells. Similar data have been shown in epithelial and heterologous cells (7, 13, 28, 30, 34). Although the blockade with DIDS, Gd\(^{3+}\), and NaCl is rapid, ATP standard curves in the absence and presence of these substances do not affect luciferase activity (7). We cannot rule out the fact that they might somehow speed the buffering capacity of the cell or enhance ecto-ATPase activity; however, the fact that DIDS or Gd\(^{3+}\) (data not shown) was ineffective in blocking Ca\(^{2+}\)-agonist-induced ATP release suggests a different cellular mechanism. Moreover, the fact that we were dealing with a Cl\(^{-}\)-containing medium in all stages of the experiment shows that we were dealing with a luciferase that has very different biochemical properties from that of a luciferase lyophilized and prepared in Cl\(^{-}\)-free solutions. Thus reversal of hypotonicity-induced ATP release with NaCl suggests an osmotic mechanism. Fifth, along with extensive documentation of P2Y receptor expression on endothelial cells, we have provided degenerate RT-PCR and biochemical evidence for expression of multiple P2X receptors (P2X\(_4\) and P2X\(_5\)) as well as P2Y\(_2\) in positive controls. These data, together with the effect of ATP scavengers and ATP receptor antagonists on basal Ca\(^{2+}\), suggest that an autocrine and paracrine signaling loop exists in the circulation. Sixth, we have provided new evidence that P2X receptors may not mediate Ca\(^{2+}\) influx directly but, rather, may stimulate voltage-dependent Ca\(^{2+}\) channels through changing membrane potential.

At the center of this system, endothelial cells are a rich source of released ATP under basal or stimulated conditions. Because endothelial cells also express multiple purinergic receptors, endothelial cells could undergo autocrine “self-regulation” or modulate the activity of neighboring cells (circulating cells, vascular smooth muscle cells) that also express P2X receptor channels and P2Y G protein-coupled receptors and could transduce this extracellular ATP signal.

How might ATP, in an autocrine or paracrine manner, affect vascular function? P2Y G protein-coupled receptors coupled to phospholipases stimulate signal transduction within most cells (21, 27). Phospholipase C-β (PLC-β) is the major signal transduction enzyme coupled to P2Y receptors (21); however, evidence also exists for coupling to PLD and PLA\(_2\) in specific cell types such as Madin-Darby canine kidney renal epithelial cells (14). As such, P2Y receptors trigger phosphoinositide signaling and increases in intracellular Ca\(^{2+}\). P2X receptor channels, either by themselves or by depolarizing the membrane to open voltage-gated Ca\(^{2+}\) channels, may mediate capacitive Ca\(^{2+}\) entry and signaling (8, 11). Our work suggests that P2X receptor channels may not mediate Ca\(^{2+}\) influx from extracellular stores, as was suggested by Yamamoto et al. (35).
The work on Ca\(^{2+}\) permeability has been performed exclusively in neurons, oocytes, and heterologous cells. Ca\(^{2+}\) permeability through the P2X receptor channels in patch-clamp experiments does not suggest independently that P2XRs increase cytosolic Ca\(^{2+}\) significantly. One also cannot rule out P2X receptor stimulation of other signal transduction pathways that are, as yet, undiscovered. One also cannot rule out the possibility that P2X receptor channels may depolarize the membrane potential in some cell systems to allow Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels that are expressed concomitantly.

Adenosine, a metabolite of ATP, has been studied extensively in the coronary circulation and in other vascular beds (2, 12, 17). Adenosine affects vascular tone and cardiac function (2, 12, 17). It is tempting to speculate that much of the adenosine in the circulation is created via metabolism of ATP. Indeed, ecto-ATPases and ecto-ATPases are expressed by endothelial cells to metabolize ATP (15, 36). In our data in assays performed at 37°C, the ATP release signal was dampened or decayed rapidly over time. Our assay detected ATP released by an endothelial monolayer by using a sufficient quantity of luciferase-luciferin detection reagent in the medium to compete with ecto-ATPases and ecto-ATPases. ATP was consumed within seconds in the assay; that is why sharp increases and decreases were observed when ATP release was potentiated and inhibited. However, ecto-ATPases compete for ATP in this assay, raising the possibility that circulating adenosine may be derived, at least in part, from released ATP.

How might extracellular purinergic signaling be important in vascular pathophysiology? Extracellular purinergic signaling plays a central role in platelet function at the clotting zone (20). Extracellular nucleotides and nucleosides may be detrimental or therapeutic, respectively, in ischemia-reperfusion injury (2, 10, 12, 17, 18, 25, 26). Indeed, anoxia or ischemia of cardiac tissue may cause cell swelling that would, in turn, augment ATP release. Extracellular purinergic signaling by ATP and adenosine has been hypothesized to play a central role in modulation of skeletal muscle and renal blood flow and glomerular filtration rate in the kidney (19). Recent evidence also has suggested that “cross talk” may exist between purinergic signaling and nitric oxide signaling in vascular beds (22, 24, 29). These issues require further investigation. It is possible that inhibitors of ATP release, ATP scavengers, and/or purinergic receptor antagonists may be of benefit in hypertension or other circulatory syndromes and treatments.

In conclusion, these data initiate a new extracellular autocrine and paracrine signaling cascade that may have profound implications for vascular physiology and pathophysiology. The mechanisms of ATP release, the regulation of ATP release, purinergic receptor signaling, and their physiological roles in endothelial cell biology are future considerations.

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