ATP reduces macromolecule permeability of endothelial monolayers despite increasing \([\text{Ca}^{2+}]_i\)

T. Noll, H. Hölschermann, K. Koprek, D. Gündüz, W. Haberbosch, H. Tillmanns, and H. M. Piper. ATP reduces macromolecule permeability of endothelial monolayers despite increasing \([\text{Ca}^{2+}]_i\), Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1892–H1901, 1999.—We investigated the relationship between the ATP-evoked rise of cytosolic \([\text{Ca}^{2+}]_i\) concentration and barrier function in porcine aortic endothelial monolayers. ATP (0.01–100 µM) induced a transient rise of \([\text{Ca}^{2+}]_i\) and reduced permeability in a concentration-dependent manner. In contrast, the \([\text{Ca}^{2+}]_i\) rise evoked by ATP (10 µM) did not affect ATP (10 µM)-induced reduction of permeability. ATP reduced permeability even in endothelial monolayers that had been loaded with the \([\text{Ca}^{2+}]_i\) chelator BAPTA to prevent the \([\text{Ca}^{2+}]_i\) rise. U-73122 (1 µM), an inhibitor of phospholipase C (PLC), completely abolished the effect of ATP (10 µM) on permeability. It also abolished the translocation of protein kinase C (PKC) in response to ATP, which could also be achieved by the PKC inhibitors Go6976 (100 nM) or bisindolylmaleimide I (1 µM). In the presence of PKC inhibitors, however, the permeability effect of ATP was not affected. The presence of inhibitors of adenylate or guanylate cyclase (50 µM SQ-22536 or 20 µM ODQ) prevented changes in cyclic nucleotides but did not affect the permeability effects of ATP. The study shows that ATP reduces macromolecule permeability via a PLC-mediated mechanism that is independent of the concomitant effects of ATP on cytosolic \([\text{Ca}^{2+}]_i\), cyclic nucleotides, or PKC.

adrenosine 5'-triphosphate; paracellular permeability; phospholipase C; protein kinase C; cyclic nucleotides

ATP is an important mediator involved in signaling between cardiovascular cells. Extracellular levels of ATP are normally maintained at extremely low levels due to ubiquitous ectonucleotidases, which rapidly hydrolyze nucleotides. However, in the vasculature significant amounts of extracellular ATP may locally accumulate at the site of thrombus formation due to a release from activated platelets or in the hypoxic myocardium (4) when ATP is released together with degradation products such as adenosine from energy-depleting myocardial cells. A systemic increase in ATP and its immediate hydrolytic products up to micromolar concentrations is found in blood plasma under conditions of traumatic shock (9).

ATP may act on endothelial cells through multiple receptors and second messengers. The majority of known effects elicited by nonhydrolyzed ATP are mediated via P2x and P2y receptors (5). These receptors are both coupled to phospholipase C (PLC), but via distinct G proteins (21). Signal transduction events stimulated by ATP include the inositol lipid-Ca2+ signaling cascade, protein kinase C (PKC), and, directly or indirectly, activation of soluble guanylate cyclase or adenylate cyclase (6). ATP may also act on endothelial cells after degradation to adenosine via adenosine receptors (25).

The information on ATP effects on endothelial barrier function is scarce and inconsistent. Depending on the endothelial cell population, ATP can modulate endothelial barrier function in one direction or the other. In the special case of microvascular endothelium contained in venular microvessels from frogs (13–16), ATP was found to increase paracellular permeability. Similar to effects of inflammatory mediators, this rise in permeability in response to ATP coincided with a rise in cytosolic Ca2+ concentration \([\text{Ca}^{2+}]_i\) within the cells. In other populations, ATP was found to decrease paracellular permeability. Examples are bovine aortic endothelial cells (12) or, from preliminary experiments of the present study, cultured endothelial cells from other macrovessels such as the porcine aorta, porcine pulmonary artery, bovine aorta, or human umbilical vein. In this second type of reaction, the role of \([\text{Ca}^{2+}]_i\) is unclear. The present study was undertaken to analyze the role of \([\text{Ca}^{2+}]_i\) changes in endothelial cell preparations in which ATP reduces permeability. The effects of ATP were contrasted with those of ionomycin, a Ca2+ ionophore, which causes a rise in \([\text{Ca}^{2+}]_i\) in a receptor-independent manner. Endothelial barrier function was studied by determining the passage of albumin through confluent monolayers of porcine aortic endothelial cells. Variations of albumin passage in this model are to be attributed to changes in paracellular permeability.

MATERIALS AND METHODS

Cell cultures. Endothelial cells from the bovine aorta, porcine aorta, and pulmonary artery were isolated and cultured as previously described (26). Human endothelial cells from umbilical cords were isolated and cultured according to van Hinsberg et al. (30). Confluent cultures of primary endothelial cell were trypsinized in phosphate-buffered saline [PBS; composed of (in mM) 137 NaCl, 2.7 KCl, 1.5 KH2PO4, and 8.0 Na2HPO4, pH 7.4, supplemented with 0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA] and seeded at a density of
7 × 10^4 cells/cm² on 24-mm round polycarbonate filters (pore size 0.4 µm), 5 × 20-mm glass cover slips, or 30-mm culture dishes for determination of albumin permeability, [Ca²⁺], or cyclic nucleotide contents, respectively. Experiments were performed with confluent monolayers 4 days after seeding.

Macromolecule permeability. The permeability of the endothelial cell monolayer was studied in a system of two compartments separated by a filter membrane (23, 24). Both compartments contained as basal medium modified Tyrode solution [composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.0 CaCl₂, and 30.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); pH 7.4, 37°C] supplemented with 5% (vol/vol) heat-inactivated newborn calf serum (NCS; 10 min at 60°C). There was no hydrostatic pressure gradient between the compartments. The "luminal" compartment containing the monolayer had a volume of 2.5 ml, and the "abluminal" compartment had a volume of 13 ml. The fluid in the abluminal compartment was continuously stirred. Trypan blue-labeled albumin (60 µM) was added to the luminal compartment. The appearance of labeled albumin in the luminal compartment was determined every 10 min during incubation. It did not change significantly in the time frame of the experiments.

The albumin flux [F; expressed in mol/(s·cm²)] across the monolayer with the surface area (S) was determined from the rise of albumin concentration (d[A]₂) during the time interval (dt) in the abluminal compartment (volume V) as follows

\[ F = \frac{(d[A]₂/dt) \times V}{S} \]

To facilitate the comparison of data obtained in the present study with those of other studies, the permeability coefficient (P; expressed in cm/s) of the combined system of monolayer and filter support was calculated from F as follows

\[ P = \frac{F}{[(A]₁ - [A]₂)} \]

where [A]₁ and [A]₂ denote tracer concentrations in the luminal and abluminal compartments, respectively. Because the driving force ([A]₁ - [A]₂) remained virtually unchanged in the course of the described experiments, the relative changes in F correspond to similar changes in P.

Experimental protocols. The basal medium used in incubations was modified Tyrode solution (composition as described in Macromolecule permeability). Macromolecule permeability of the endothelial monolayer, transferred to the incubation chamber, was determined after an initial equilibration period of 20 min. The basal albumin permeability of each monolayer-filter system was then determined during another 20 min of incubation. Agents were added as indicated in RESULTS, and the response of albumin permeability was recorded for another 40–80 min. For the incubations under Ca²⁺-free extracellular conditions, a Ca²⁺-free basal medium (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 0.5 ethylene glycol-bis(β-aminoethvl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 30.0 HEPES; pH 7.4, 37°C) supplemented with heat-inactivated 5% (vol/vol) NCS was used. Stock solutions of bisindolylmaleimide I (BIM), G-6976, ionomycin, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), SQ-22536, U-37122, U-37343, and thapsigargin were prepared with dimethyl sulfoxide (DMSO). A stock solution of 1-O-octadecyl-2-O-methyl-γ-rac-glycero-3-phosphorylcholine (ET-18-OCH₃) was prepared with absolute ethanol. Appropriate volumes of these solutions were added to the cells, yielding final solvent concentrations ≤0.1% (vol/vol). The same final concentrations of DMSO or ethanol were also included in all respective control experiments. Stock solutions of all other substances were prepared in basal medium (composition as described in Macromolecule permeability). Appropriate volumes of these solutions were added to the cells. Identical additions of basal medium were included in all respective control experiments.

Cytosolic Ca²⁺. Free [Ca²⁺], were determined using the fluorescent Ca²⁺ indicator fura 2. Confluent endothelial monolayers cultured on 5 × 20-mm glass cover slips were incubated in medium 199 supplemented with 5% (vol/vol) heat-inactivated NCS and the addition of 5 µM fura 2-AM (acetoxy-methyl ester of fura 2) at 20°C in the dark. After a 50-min incubation period, the extracellular fura 2-AM was removed by medium change. This was followed by a 20-min incubation period in the same medium before measurements were started. The coverslips were then aligned in a quartz cuvette into the beam of a fluorescence spectrophotometer (LS 50B; Perkin-Elmer, Uberlingen, Germany). During incubations, the excitation wavelength was alternated between 340 and 380 nm (bandwidth 5 nm). Emitted light was detected at 510 nm (bandwidth 2 nm). Fura 2 fluorescence was calibrated according to the method described by Grynkiewicz et al. (10). For this purpose, the cells were exposed to 5 µM ionomycin in modified Tyrode solution containing either 3 mM Ca²⁺ or 5 mM EGTA to obtain the maximum (R_max) and minimum (R_min) of the ratio of fluorescence (R), respectively. [Ca²⁺] was calculated according to the equation

\[ [Ca^{2+}] = K_D \times \beta \times (R - R_{min})/(R_{max} - R) \]

where K_D is the dissociation constant of fura 2 (10) and β is the ratio of the 380-nm excitation signals of ionomycin-treated cells at 5 mM EGTA and 3 mM Ca²⁺.

Loading of BAPTA-AM. Confluent endothelial monolayers cultured on either filter membranes or 5 × 20-mm glass cover slips were incubated in medium 199 supplemented with 5% (vol/vol) heat-inactivated NCS and the addition of 10 µM 1,2-bis(2-aminoethoxy)ethane-N,N',N'-tetraacetic acid-AM (acetoxy-methyl ester of BAPTA) at 37°C. After a 30-min incubation period, the extracellular BAPTA-AM was removed by medium change and the experiments were started.

Determination of PKC activity. The activity of PKC was determined in the membrane fraction of endothelial cells using the method of Chakravarthy et al. (3) that allows measurement of PKC activity in its native membrane-associated state. Cultured endothelial monolayers were incubated for the time indicated in RESULTS, and the cultures were then rinsed twice with ice-cold PBS and subsequently covered with ice-cold hypotonic lysis buffer (composition in mM: 1 NaHCO₃, 5 MgCl₂, and 0.1 PMSF; pH 7.4). The cells were scraped off the dish and vigorously mixed at room temperature for 2 min. The lysates were centrifuged at 1,000 g for 5 min at 4°C to sediment nonlysed cells and nuclei. The postnuclear supernatants were centrifuged at 4°C for 10 min at 425,000 g. The sedimented endothelial membrane fractions were resuspended in 200 µl of assay buffer (composition in mM: 0.002 CaCl₂, 10 MgCl₂, 0.2 PMSF, 2 NaF, 0.2 Na₃P₂O₇, 0.2 Na₂VO₃, and 50 Tris-HCl buffer; pH 7.4).

The activity of PKC in the membrane fraction was determined by a continuous fluorescence assay using an acrylodan-labeled myristoylated alanine-rich C kinase substrate (MARCKS) peptide (acyclo-dan-C-KKKKRRFSKKSKFSLGFSFKKNNKK) as PKC substrate (20). Fluorescence studies were performed at 22°C in the fluorescence spectrophotometer (LS 50B). The reaction mixture (total volume 0.7 ml) contained assay buffer, 10–50 µg protein of endothelial
membrane fraction, and 75 nM acrylodan-labeled MARCKS peptide. The reaction was started by 0.5 mM ATP. The fluorescence decrease during phosphorylation was monitored for 15 min at the 480-nm emission maximum of the acrylodan-labeled MARCKS peptide with excitation at 370 nm. PKC activity was determined from the initial slope of the fluorescence signal (fluorescence decrease per minute per 10 µg of membrane protein) and is expressed as the percentage of a defined control condition.

To validate the continuous fluorescence assay, phosphate incorporation into the acrylodan-labeled MARCKS peptide was determined as described previously (22). Under defined control conditions, a decrease of fluorescence of 5.6 ± 0.9% per minute per 10 µg of membrane protein corresponded to a [32P]orthophosphate incorporation into the acrylodan-labeled MARCKS peptide of 580 ± 60.0 pmol·min⁻¹·10 µg membrane protein⁻¹.

Cellular contents of cyclic nucleotides. Cultured endothelial monolayers were incubated for the time indicated in RESULTS. At the end of the incubations, the incubation medium was aspirated, ice-cold ethanol was added, and the culture dishes were stored at −80°C. To determine the intracellular cyclic nucleotide contents, the ethanol was evaporated at 60°C, and the samples were suspended in double-distilled water, transferred into Eppendorf reaction tubes, and centrifuged for 5 min at 14,000 g. Cyclic nucleotide concentrations of the supernatants were determined by using radioimmunoassays (Amersham, Braunschweig, Germany). The protein contents of the samples were determined according to Bradford (1) using bovine serum albumin as the standard.

Materials. ODQ was from Alexis Biochemicals (Grünewald, Germany); Falcon plastic tissue culture dishes were from Becton Dickinson (Heidelberg, Germany); acrylodan-labeled MARCKS peptide (as PKC fluorescence substrate), ATP, adenosine 5’-O-(3-thiotriphosphate) (ATPγS), ADP, AMP, and UTP were from Boehringer Mannheim (Mannheim, Germany); BIM, ET-18-OCH₃, G6-6976, ionomycin, SQ-22536, U-37122, U-37343, and thapsigargin were from Calbiochem (Bad Soden, Germany); Transwell polycarbonate filter inserts (24-mm diameter, 0.4-µm pore size) were from Costar (Bodenheim, Germany); NCS, medium 199, penicillin-streptomycin, and trypsin-EDTA were from GIBCO Life Technologies (Egggenstein, Germany); and 5’-(N-ethylcarboxamido)adenosine (NECA) was from Sigma (Deisenhofen, Germany). All other chemicals were of the best available quality, usually analytic grade.

Statistical analysis. Data are given as means ± SD of n = 6 experiments using independent cell preparations. Statistical analysis of data was performed according to Student’s unpaired t-test. Probability (P) values of <0.05 were considered significant.

RESULTS

Macromolecule permeability. Exposure of aortic endothelial monolayers to 10 µM ATP reduced their albumin permeability from a control level of 5.8 ± 0.7 to 3.5 ± 0.9 ¥ 10⁻⁶ cm/s after 20 min (Fig. 1). Albumin permeability remained that low for a further 60 min. This observation was not restricted to porcine aortic endothelial monolayers but was also made in endothelial monolayers derived from the porcine pulmonary artery, bovine aorta, and human umbilical vein (Table 1). For the remainder of this study, endothelial cells from the porcine aorta were used. In contrast to the effect on permeability induced by ATP, the addition of ionomycin (1 µM) increased permeability rapidly to a peak value of 8.9 ± 0.5 ¥ 10⁻⁶ cm/s within 10 min. Afterward, permeability decreased but remained significantly elevated during the time of observation. The effects of ATP and ionomycin on permeability were concentration dependent. Both agents were tested in a concentration range between 10⁻⁸ and 10⁻⁵ M (Fig. 2). The permeability experiments were performed in the presence of 5% heat-inactivated NCS. This small amount of serum was included in the incubation medium because basal permeability of endothelial monolayers was then stable up to 2 h. Under serum-free conditions, permeability was also reduced by 10 µM ATP (from a control value of 6.7 ± 0.5 to 4.1 ± 0.5 ¥ 10⁻⁶ cm/s after 20 min) or increased by 1 µM ionomycin (from a control value of 6.7 ± 0.5 to 9.7 ± 0.8 ¥ 10⁻⁶ cm/s after 10 min).

Table 1. Effect of ATP on albumin permeability of endothelial monolayers derived from porcine aorta, porcine pulmonary artery, bovine aorta, and human umbilical vein

<table>
<thead>
<tr>
<th>Endothelial Monolayers</th>
<th>Permeability, %control</th>
</tr>
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<tbody>
<tr>
<td>Porcine aorta</td>
<td>63.2 ± 3.5*</td>
</tr>
<tr>
<td>Porcine pulmonary artery</td>
<td>67.5 ± 5.1*</td>
</tr>
<tr>
<td>Bovine aorta</td>
<td>71.3 ± 4.3*</td>
</tr>
<tr>
<td>Human umbilical vein</td>
<td>76.8 ± 6.5*</td>
</tr>
</tbody>
</table>

Data are means ± SD of n = 6 separate experiments of independent cell preparations. Endothelial monolayers were incubated in presence of 10 µM ATP for 20 min. Data are percentages of control, where 100% corresponds to an albumin permeability of 5.8 ± 0.6, 6.3 ± 0.5, 7.6 ± 0.7, or 8.4 ± 0.7 ¥ 10⁻⁶ cm/s for endothelial monolayers derived from porcine aorta, porcine pulmonary artery, bovine aorta, or human umbilical vein, respectively. *P < 0.05, significantly different from control.
The dose-dependent effect of ATP on albumin permeability was compared with that of other nucleotides (Fig. 3). The poorly hydrolyzable ATP analog ATPγS reduced permeability to the same extent as ATP. ADP as well as UTP also reduced albumin permeability in a concentration-dependent manner, but these nucleotides were less potent than ATP or ATPγS. To test whether the ATP effect is transmitted through adenosine receptors, endothelial cells were preincubated for 10 min with 10 µM 8-phenyltheophylline (8-PT), an inhibitor of adenosine receptors. As shown in Fig. 4, this pretreatment did not affect the reduction of permeability induced by 10 µM ATP, but it significantly inhibited the effect of 10 µM AMP or 100 nM NECA, a stable adenosine analog, on albumin permeability.

ATP-induced increase in [Ca²⁺]i and permeability. Exposure of endothelial cells to 10 µM ATP, a concentration that caused a marked reduction of albumin permeability, elicited a transient rise of [Ca²⁺]i with a maximum of 503 ± 47 nM (Fig. 5). The effect of ATP on [Ca²⁺]i was comparable to that induced by 1 µM ionomycin. As shown in Table 2, ATPγS, ADP, and UTP also transiently increased [Ca²⁺]i. Significant differences in peak [Ca²⁺]i among ATP, ATPγS, ADP, or UTP were not found. AMP and NECA did not increase [Ca²⁺]i.

To analyze the relationships between the ATP-induced changes in [Ca²⁺]i and albumin permeability, endothelial cells were loaded with BAPTA-AM, a membrane-permeant Ca²⁺ chelator. Although the macromolecular permeability was reduced to the same extent as in nonloaded cells (Fig. 6A) when BAPTA-loaded cells were exposed to 10 µM ATP at a normal extracellular Ca²⁺ concentration, the rise in [Ca²⁺]i remained absent (Fig. 6B).

To validate this last result, another approach was also used. In this protocol endothelial cells were extra-
and intracellularly deprived of Ca\(^{2+}\) before the addition of ATP. In Ca\(^{2+}\)-free medium the control permeability was increased from 5.8 ± 0.7 to 9.7 ± 0.8 × 10\(^{-6}\) cm/s (Fig. 7A) and remained constant at that elevated level during the whole observation period. The addition of 10 µM ATP under Ca\(^{2+}\)-free extracellular conditions still caused a reduction of albumin permeability to 6.0 ± 0.7 × 10\(^{-6}\) cm/s and evoked a transient rise of [Ca\(^{2+}\)]\(_i\), to a maximum of 500 nM (Fig. 7B). Exposure of the endothelial cells to 300 nM thapsigargin in Ca\(^{2+}\)-free extracellular medium discharged their endogenous Ca\(^{2+}\) stores (18, 22, 24) and was accompanied by a transitory small rise of [Ca\(^{2+}\)]\(_i\), and permeability that returned to basal level within 20 min. When endothelial cells were exposed to 10 µM ATP after this maneuver was completed, ATP no longer induced a rise in [Ca\(^{2+}\)]. Albumin permeability decreased nevertheless at the same velocity and to the same extent as under control conditions in Ca\(^{2+}\)-free medium.

### Table 2. Maximum cytosolic Ca\(^{2+}\) concentration of porcine aortic endothelial monolayers within the first minute after addition of agents

<table>
<thead>
<tr>
<th>Agents</th>
<th>[Ca(^{2+})] (_i), nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67 ± 7</td>
</tr>
<tr>
<td>Ionomycin (1 µM)</td>
<td>490 ± 56*</td>
</tr>
<tr>
<td>ATP (10 µM)</td>
<td>503 ± 47*</td>
</tr>
<tr>
<td>ATP(_\gamma)S (10 µM)</td>
<td>530 ± 72*</td>
</tr>
<tr>
<td>ADP (10 µM)</td>
<td>435 ± 63*</td>
</tr>
<tr>
<td>UTP (10 µM)</td>
<td>467 ± 82*</td>
</tr>
<tr>
<td>AMP (10 µM)</td>
<td>59 ± 5 NS</td>
</tr>
<tr>
<td>NECA (100 nM)</td>
<td>63 ± 8 NS</td>
</tr>
</tbody>
</table>

Data are means ± SD of \(n = 6\) separate experiments of independent cell preparations. ATP\(_\gamma\)S, adenosine 5′-O-(3-thiotriphosphate; NECA, 5′-(N-ethylcarboxamido)adenosine. *P < 0.05, significantly different from control; NS, not significantly different from control. There is no significant difference among effects of ATP, ATP\(_\gamma\)S, ADP, and UTP on cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)).
lial cells to 10 µM ATP for 10 min increased PKC activity in the membrane fraction by 90% above the control level (Table 3). The increase in PKC activity in the membrane fraction was virtually abolished when cells had been pretreated for 20 min with 1 µM BIM, a panspecific inhibitor of PKC, or 100 nM Go6-6976, a selective inhibitor of Ca2+-dependent PKC isoenzymes (Table 3). Exposure of endothelial cells to 1 µM BIM or 100 nM Go6-6976 alone had no effect on basal PKC activity in the membrane fraction.

In contrast to their ability to suppress ATP-induced activation of membranous PKC, neither BIM nor Go6-6976 prevented the ATP-induced reduction of macromolecule permeability (Table 3).

In the next step we tested the role of PLC in the ATP-stimulated increase in membranous PKC activity and cytosolic Ca2+ and the ATP-induced reduction of macromolecule permeability. Endothelial monolayers were pretreated for 20 min with either 1 µM U-73122, an inhibitor of PLC (11), or its inactive analog, 1 µM U-73343, used as a negative control (11). Pretreatment of the monolayers with U-73122 completely abolished the ATP-stimulated increase in membranous PKC activity (Table 3), but its inactive analog, U-73343, had no effect.

Pretreatment of endothelial monolayers with 1 µM U-73122 completely blocked the ATP-induced reduction of permeability, whereas pretreatment with U-73343, the inactive analog, had no effect on permeability (Fig. 8A). We tested whether U-73122 also affects the ATP-induced rise in cytosolic Ca2+. Pretreatment with U-73122 completely abolished the [Ca2+]i response, but pretreatment with U-73343 had no effect (Fig. 8B). A second inhibitor of PLC, ET-18-OCH3 (28), which is chemically different from U-73122, was also tested at a dosage of 50 µM. It significantly reduced the ATP effect on macromolecule permeability (Table 3).

Table 3. Effect of inhibitors of PKC and PLC on permeability and membranous PKC activity in porcine aortic endothelial cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Permeability, ×10^-6 cm/s</th>
<th>PKC Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>5.8 ± 0.7</td>
<td>100 ± 21</td>
</tr>
<tr>
<td>ATP alone</td>
<td>3.6 ± 0.4*</td>
<td>193 ± 31*</td>
</tr>
<tr>
<td>BIM</td>
<td>6.4 ± 0.7</td>
<td>94 ± 19</td>
</tr>
<tr>
<td>BIM + ATP</td>
<td>4.3 ± 0.4*</td>
<td>103 ± 12 NS</td>
</tr>
<tr>
<td>Go6-6976</td>
<td>5.7 ± 0.6</td>
<td>89 ± 17</td>
</tr>
<tr>
<td>Go6-6976 + ATP</td>
<td>3.9 ± 0.3*</td>
<td>116 ± 23 NS</td>
</tr>
<tr>
<td>U-73122</td>
<td>6.2 ± 0.4</td>
<td>97 ± 19</td>
</tr>
<tr>
<td>U-73122 + ATP</td>
<td>5.9 ± 0.5 NS</td>
<td>105 ± 18 NS</td>
</tr>
<tr>
<td>U-73343</td>
<td>5.7 ± 0.5</td>
<td>115 ± 27</td>
</tr>
<tr>
<td>U-73343 + ATP</td>
<td>3.2 ± 0.3*</td>
<td>217 ± 35*</td>
</tr>
<tr>
<td>ET-18-OCH3</td>
<td>6.1 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>ET-18-OCH3 + ATP</td>
<td>4.7 ± 0.4*</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are means ± SD of at least 5 separate experiments of independent cell preparations. Endothelial monolayers were first incubated for 20 min in absence or presence of inhibitors bisindolylmaleimide I (BIM; 1 µM), Go6-6976 (100 nM), U-73122 (1 µM), U-73343 (1 µM), or 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphorylcholine (ET-18-OCH3; 50 µM). ATP (10 µM) was then added, and monolayer permeability or protein kinase C (PKC) activity was determined after 10 min. Data for PKC activity are given in percentages of control, where 100% corresponds to a fluorescence decrease of acrylodan-labeled myristolated alanine-rich C kinase substrate (MARCKS) peptide of 5.6 ± 1.6% per minute per 10 µg of membrane protein. PLC, phospholipase C.*P < 0.05, significantly different from respective control; NS, not significantly different from respective control; ND, not determined.
ATP-induced effects on cyclic nucleotides and permeability. We analyzed whether ATP stimulates cyclic nucleotide synthesis in endothelial cells and whether this synthesis is related to the ATP-induced reduction of macromolecule permeability.

Addition of 10 µM ATP to endothelial monolayers induced a rise in the cellular cGMP content within 5 min (Fig. 9B). Pretreatment of the endothelial cells for 10 min with 20 µM ODQ (29), a specific inhibitor of soluble guanylate cyclase, completely abolished the ATP-stimulated increase in cellular cGMP content. However, the ATP-induced reduction of macromolecule permeability was not changed in the presence of ODQ (Fig. 9A).
Addition of 10 µM ATP to endothelial monolayers induced also an increase in cellular cAMP content within 5 min (Fig. 10B). Pretreatment of the endothelial cells for 10 min with 50 µM SQ-22536 (19), a selective inhibitor of adenylate cyclase, completely abolished the ATP-stimulated increase in cellular cAMP content. However, it had no effect on the ATP-induced reduction of macromolecule permeability (Fig. 10A).

**DISCUSSION**

The most prominent findings of the present study are that ATP induced a reduction of albumin permeability even though it provoked transient rises of \([Ca^{2+}]_i\), cGMP, cAMP, and translocation of PKC in porcine aortic endothelial monolayers. The ATP-induced rises of \([Ca^{2+}]_i\), cGMP, cAMP, and translocation of PKC could be prevented without abolishing the effect of ATP on permeability. The effect of ATP on permeability, however, was sensitive to inhibitors of PLC. These data show that ATP reduces macromolecule permeability via a PLC-mediated signaling pathway that is independent of concomitant effects on \([Ca^{2+}]_i\), cyclic nucleotides, or PKC.

Exposure of endothelial cells from the porcine aorta to ATP caused a sustained reduction of albumin permeability. Similar effects were observed in endothelial monolayers derived from the porcine pulmonary artery, bovine aorta, and human umbilical vein. A detailed analysis was carried out on endothelial cells from the porcine aorta. The effect of ATP on barrier function was concentration dependent. ATP\(_{GS}\), which is much less hydrolyzable than ATP, reduced permeability in the same manner as ATP, indicating that ATP, but not its degradation products, caused the reduction of permeability. It has been reported (5, 21) that ATP can stimulate endothelial cells via purinergic receptors, in particular \(P_{2y}\) and \(P_{2u}\). The latter of these receptors has a high affinity for UTP. In endothelial monolayers UTP also caused a reduction of permeability in a concentration-dependent manner, but this reduction was less potent than that induced by ATP. The fact that the action of ATP can be mimicked by other nucleotides suggests that ATP exerts its effect on permeability by acting on purinergic receptors. The tested nucleotides were in the following rank order of potency: ATP > ATP\(_{GS}\) > ADP > UTP. The order of potency is not identical to that known for either \(P_{2y}\) or \(P_{2u}\). It may therefore correspond to that of yet another one of the multitude of purinergic receptors identified in cloning experiments.

To validate the conclusion that ATP and not a derivative is responsible for its effect on permeability, the actions of ATP, AMP, and the stable adenosine analog NECA were compared. We reported in a previous study (31) that NECA reduces endothelial albumin permeability via stimulation of endothelial adenosine receptors in the same model used in the present study. It has now been confirmed that NECA reduces endothelial permeability and that AMP has a comparable effect. Both effects could be fully antagonized by 8-PT, an adenosine-receptor antagonist. In contrast, 8-PT failed to antagonize the reduction of permeability obtained with ATP. These data therefore show independently that the effect of ATP on permeability is not due to the actions of adenosine.
In conclusion, ATP can stimulate both a rise in [Ca$^{2+}$]i and a reduction of macromolecule permeability in the types of endothelial monolayers tested here. ATP seems to be the first mediator described that can exert such apparently contrasting effects. In general, rapid effects on endothelial permeability, as investigated here, seem to be due to two kinds of mechanisms: 1) a modulation of tension of the endothelial contractile machinery and 2) changes in cell-cell or cell-matrix adhesion. A transient rise of [Ca$^{2+}$]i seems to increase endothelial permeability primarily by activation of the second type of mechanism (8). One may speculate that ATP overides these [Ca$^{2+}$]i-mediated effects on cell adhesion by the activation of specific Ca$^{2+}$-independent signal transduction pathways. These have yet to be identified.

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REFERENCES


13.  
He, P., and F. E. Curry.  

12.  

15.  
He, P., B. Liu, and F. E. Curry.  

14.  
He, P., and F. E. Curry.  

10.  
Grynkiewicz, G., M. Poenie, and R. Y. Tsien.  

17.  
Hemptel, A., T. Noll, A. Muhs, and H. M. Piper.  

18.  

19.  

20.  
McIlroy, B. K., J. D. Walters, and J. D. Johnson.  

Camussi, and A. Bosia.  


Communi, D., E. Raspe, S. Pirotton, and J. M. Boeynaems.  


Difool, T. A. Hempel, and H. M. Piper.  

Motto, S., S. Pirotton, and J. M. Boeynaems.  

Communi, D., E. Raspe, S. Pirotton, and J. M. Boeynaems.  

Coexpression of P2Y and P2U receptors on aortic endothelial cells.  

Comparison of cell localization and signaling pathways.  


Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides.  


Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacyclin formation in adrenal mediolary endothelial cells by means of P2-purinergic receptors.  


Mechanisms of ionomycin-induced endothelial cell barrier dysfunction.  


Gordon, J. L.  

Extracellular ATP: effects, sources and fate.  


Gryniewicz, G., M. Poenie, and R. Y. Tsien.  

A new generation of Ca2+ indicators with greatly improved fluorescence properties.  


Hansen, M., S. Boitano, K. E. Dirksen, and M. J. Sanderson.  

A role for phospholipase C activity but not ryanodine receptors in the initiation and propagation of intercellular calcium waves.  


Adenosine decreases permeability of in vitro endothelial monolayers.  


He, P., and F. E. Curry.  

Differential actions of cAMP on endothelial [Ca2+]i and permeability in microvessels exposed to ATP.  


He, P., and F. E. Curry.  

Measurement of membrane potential of endothelial cells in single perfused microvessels.  


He, P., B. Liu, and F. E. Curry.  

Effect of nitric oxide synthase inhibitors on endothelial [Ca2+]i and microvesSEL permeability.  


He, P., X. Zhang, and F. E. Curry.  

Ca2+ entry through conductive pathway mediates receptor-mediated increase in microvesSEL permeability.  


Hemptel, A., T. Noll, A. Muhs, and H. M. Piper.  

Functional antagonism between cAMP and cGMP on permeability of coronary endothelial monolayers.  


Dual role of cGMP in modulation of macromolecule permeability of aortic endothelial cells.  


Mechanical strain-enhanced fetal lung cell proliferation is mediated by phospholipases C and D and protein kinase C.  


McIlroy, B. K., J. D. Walters, and J. D. Johnson.  

A continuous fluorescence assay for protein kinase C.  


Motto, S., S. Pirotton, and J. M. Boeynaems.  

Heterogeneity of ATP receptors in aortic endothelial cells.  

Involvement of P2Y and P2U receptors in inositol phosphate response.  


Muhs, A., T. Noll, and H. M. Piper.  

Vinculin phosphorylation and barrier failure of coronary endothelial monolayers under energy depletion.  


Noll, T., A. Hempel, and H. M. Piper.  

Neuropeptide Y reduces macromolecule permeability of coronary endothelial monolayers.  


Noll, T., A. Muhs, M. Besselmann, H. Watanabe, and H. M. Piper.  

Initiation of hyperpermeability in energy-depleted coronary endothelial monolayers.  


Cardiovascular purinocceptors.  


Microvascular endothelial cells from heart.  

In: Cell Culture Techniques in Heart and Vessel Research, edited by H. M. Piper.  


Pirotton, S., E. Raspe, D. Demolle, C. Erneux, and J. M. Boeynaems.  

Involvement of inositol 1,4,5-trisphosphate and calcium in the action of adenine nucleotides on aortic endothelial cells.  


Selective inhibition of phosphatidylinositol phospholipase C by cytotoxic ether lipid analogues.  


Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase.  


Van Hinsberg, V. W., M. A. Scheffer, and E. G. Langelur.  

Macro- and microvascular endothelial cells from human tissue.  

In: Cell Culture Techniques in Heart and Vessel Research, edited by H. M. Piper.  


Watanabe, H., W. Kuhn, P. Schwartz, and H. M. Piper.  

A2-adenosine receptor stimulation increases macromolecule permeability of coronary endothelial cells.  


Downloaded from http://ajpheart.physiology.org/ by 10.220.33.5 on October 23, 2017