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 \((\text{[Ca}^{2+}]_i)\) and barrier function in porcine aortic endothelial monolayers. ATP \((0.01-100 \mu M)\) induced a transient rise of \([\text{Ca}^{2+}]_i\) and reduced permeability in a concentration-dependent manner. In contrast, the \([\text{Ca}^{2+}]_i\) ionophore ionomycin \((1 \mu M)\) elicited a rise in \([\text{Ca}^{2+}]_i\), comparable to that induced by ATP \((10 \mu M)\), but it increased permeability. For the reduction of permeability, nucleotides were found to be in the following order of potency: ATP = ATPγS > ADP = UTP. Blockade of adenosine receptors by 8-phenyltheophylline \((10 \mu M)\) did not affect ATP \((10 \mu M)\)-induced reduction of permeability. ATP reduced permeability even in endothelial monolayers that had been loaded with the \(\text{Ca}^{2+}\) chelator BAPTA to prevent the rise in \([\text{Ca}^{2+}]_i\). U-73122 \((1 \mu M)\), an inhibitor of phospholipase C (PLC), completely abolished the effect of ATP \((10 \mu M)\) 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 Go-6976 \((100 nM)\) or bisindolylmaleimide I \((1 \mu M)\). In the presence of PKC inhibitors, however, the permeability effect of ATP was not affected. The presence of inhibitors of adenylate or guanylate cyclase \((50 \mu M \text{ SQ-22536 or } 20 \mu M \text{ ODQ})\) prevented changes in cydic 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.

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 \((6)\) 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 \(P_2Y\) 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-Ca\(^{2+}\) signaling cascade, protein kinase C (PKC), and, directly or indirectly, activation of soluble guanylate cyclase or adenylyl 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 \([\text{Ca}^{2+}]_i\) 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 microvessels 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 \([\text{Ca}^{2+}]_i\) 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 cultures were trypsinized in phosphate-buffered saline (PBS; composed of \((\text{in mM})\) 137 NaCl, 2.7 KCl, 1.5 KH\(_2\)PO\(_4\), and 8.0 Na\(_2\)HPO\(_4\); 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 coverslips, 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 constantly stirred. Trypan blue-labeled albumin (60 µM) was added to the luminal compartment. The appearance of labeled albumin in the blue-labeled albumin (60 µM) was added to the luminal compartment was determined after an initial equilibration period of 15 s. The concentration 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 \( (d[A]1/dt) \) in the abluminal compartment (volume \( V \)) as follows:

\[
F = \frac{(d[A]1/dt \times V)}{S}
\]

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:

\[
\]

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-aminoethoxy)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), Gö-6976, ionomycin, 1H-2,4oxadiozo[4,3-a]quinolinoxalin-1-one (ODQ), SQ-22536, U-37122, U-37343, and thapsigargin were prepared with dimethyl sulfoxide (DMSO). A stock solution of 1-O-octadecyl-2-0-methyl-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⁺] was determined using the fluorescent Ca²⁺ indicator fura 2. Confluent endothelial monolayers cultured on 5 × 20-mm glass coverslips were incubated in medium 199 supplemented with 5% (vol/vol) heat-inactivated NCS and the addition of 5 µM fura 2-AM (acetoxymethyl 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 (Rₘₐₓ) and minimum (Rₐₜ) of the ratio of fluorescence (R), respectively. [Ca²⁺], was calculated according to the equation

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

where \( K_d \) is the dissociation constant of fura 2 (10) and \( \beta \) 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 coverslips were incubated in medium 199 supplemented with 5% (vol/vol) heat-inactivated NCS and the addition of 10 µM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-AM (acetoxymethyl 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 restarted.

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 Na₂VO₄, 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 myristilated alanine-rich C kinase substrate (MARCKS) peptide (acrylodan-C-KKKRRSFKSKFSKL-GFSFKKNK) 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 10^6 pM orthophosphate incorporation into the acrylodan-labeled MARCKS peptide of 580 ± 97 pmol · min⁻¹ · 10 µg membrane protein⁻¹.

**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 x 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 x 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 x 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 x 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 x 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 ATP\(_{\gamma}S\) 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 ATP\(_{\gamma}S\). To test whether the ATP effect is transmitted through adenosine receptors, endothelial cells were preincubated for 10 min with 10 \(\mu\)M 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 \(\mu\)M ATP, but it significantly inhibited the effect of 10 \(\mu\)M AMP or 100 nM NECA, a stable adenosine analog, on albumin permeability.

ATP-induced increase in \([Ca^{2+}]_i\), and permeability. Exposure of endothelial cells to 10 \(\mu\)M ATP, a concentration that caused a marked reduction of albumin permeability, elicited a transient rise of \([Ca^{2+}]_i\) with a maximum of 503 \(\pm\) 47 nM (Fig. 5). The effect of ATP on \([Ca^{2+}]_i\) was comparable to that induced by 1 \(\mu\)M ionomycin. As shown in Table 2, ATP\(_{\gamma}S\), ADP, and UTP also transiently increased \([Ca^{2+}]_i\). Significant differences in peak \([Ca^{2+}]_i\) among ATP, ATP\(_{\gamma}S\), ADP, or UTP were not found. AMP and NECA did not increase \([Ca^{2+}]_i\).

To analyze the relationships between the ATP-induced changes in \([Ca^{2+}]_i\) and albumin permeability, endothelial cells were loaded with BAPTA-AM, a membrane-permeant \(Ca^{2+}\) chelator. Although the macromolecule permeability was reduced to the same extent as in nonloaded cells (Fig. 6A) when BAPTA-loaded cells were exposed to 10 \(\mu\)M ATP at a normal extracellular \(Ca^{2+}\) concentration, the rise in \([Ca^{2+}]_i\) 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 \times 10^{-6} \text{ cm/s} (Fig. 7A) and remained constant at that elevated level during the whole observation period. The addition of 10 \mu M ATP under Ca\(^{2+}\)-free extracellular conditions still caused a reduction of albumin permeability to 6.0 ± 0.7 \times 10^{-6} \text{ 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 \mu M 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.

### ATP-induced effects on PKC, PLC, and permeability.

To analyze whether ATP causes activation of PKC, we isolated the membrane fraction of ATP-stimulated endothelial cells and determined the PKC activity in the native membrane-associated state. Exposure of 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 \mu M 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>
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<tbody>
<tr>
<td>Control</td>
<td>67 ± 7</td>
</tr>
<tr>
<td>Ionomycin (1 \mu M)</td>
<td>490 ± 56*</td>
</tr>
<tr>
<td>ATP (10 \mu M)</td>
<td>503 ± 47*</td>
</tr>
<tr>
<td>ATP(_gS) (10 \mu M)</td>
<td>530 ± 72*</td>
</tr>
<tr>
<td>ADP (10 \mu M)</td>
<td>435 ± 63*</td>
</tr>
<tr>
<td>UTP (10 \mu M)</td>
<td>467 ± 82*</td>
</tr>
<tr>
<td>AMP (10 \mu M)</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\(_gS\), 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\(_gS\), 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 Gö-6976, a selective inhibitor of Ca²⁺-dependent PKC isoenzymes (Table 3). Exposure of endothelial cells to 1 µM BIM or 100 nM Gö-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 Gö-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, 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).

Fig. 7. Influence of external and internal Ca²⁺ deprivation on ATP-induced permeability (A) and [Ca²⁺]ᵢ (B). Control cells (○) were incubated in Ca²⁺-free incubation medium and were treated with neither ATP nor thapsigargin. Cells were treated with thapsigargin (300 nM; ▲) to empty endogenous Ca²⁺ stores (external and internal Ca²⁺ deprivation) and after addition of ATP (10 µM; ▼). Results were compared with effects of ATP alone (10 µM; ■) under extracellular Ca²⁺-free conditions of endothelial cells not treated with thapsigargin. Data are means ± SD of 6 separate experiments of independent cell preparations.

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>Gö-6976</td>
<td>5.7 ± 0.6</td>
<td>89 ± 17</td>
</tr>
<tr>
<td>Gö-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), Gö-6976 (100 nM), U-73122 (1 µM), U-73343 (1 µM), or 1-O-octadecyl-2-0-methyl-rac-glyceryl-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 percentiles of control, where 100% corresponds to a fluorescence decrease of acrylodan-labeled myristolated alanine-rich C kinase substrate (MARCKS) peptide of 5.6 ± 1.3% 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 
\([\text{Ca}^{2+}]_i\), 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\(_{\gamma}S\), 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\(_{\gamma}S\) > 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 a further set of experiments, we tested the question of whether the reduction of permeability in the presence of ATP depends on the concomitant rise in \([\text{Ca}^{2+}]_i\). Two different experimental maneuvers were used. In the first maneuver, \([\text{Ca}^{2+}]_i\) was kept at low intracellular levels during ATP stimulation by loading cells with the chelator BAPTA. In the second maneuver, endothelial cells were incubated in \(\text{Ca}^{2+}\)-free medium and endogenous \(\text{Ca}^{2+}\) stores were preempted by thapsigargin. It has been shown previously (24) that, with the latter protocol, the possibility of a rise in \([\text{Ca}^{2+}]_i\), via extra- or intracellular causes is abolished. In the present study it was found that the loading of endothelial cells with BAPTA as well as extra- and intracellular \(\text{Ca}^{2+}\) deprivation completely precluded the ATP-evoked rise in \([\text{Ca}^{2+}]_i\), but these maneuvers did not affect the ATP-induced reduction of permeability. The experiments with the \(\text{Ca}^{2+}\)-ionophore ionomycin show, from a different point of view, the dissociation between \([\text{Ca}^{2+}]_i\), rise and reduction of permeability evoked by ATP. At the applied concentration, ionomycin causes a transient increase in \([\text{Ca}^{2+}]_i\), that is comparable to the \([\text{Ca}^{2+}]_i\) rise in the presence of ATP. The action of ionomycin circumvents the signal transduction activated by ATP that causes \([\text{Ca}^{2+}]_i\) rise and a multitude of other effects (6). Experiments with ionomycin show that a sole rise in \([\text{Ca}^{2+}]_i\), without the other mechanisms also activated by ATP, causes a spontaneous increase in endothelial permeability. This is in accordance with many other observations (14–16, 18, 24). The contrasting effect of ATP must therefore be due to an intracellular mechanism activated in endothelial cells in response to ATP that overrides the \(\text{Ca}^{2+}\)-activated increase in permeability.

It has been described previously (7, 27) that stimulation of endothelial cells with ATP causes activation of PLC. Here, we studied whether the activation of PLC is involved in the effects of ATP on permeability by using an inhibitor approach. The specific inhibitor of PLC, U-73122, was applied and compared with U-73343, an inactive analog of U-73122. The PLC inhibitor U-73122, but not its inactive analog, abolished the ATP-induced reduction of permeability. This indicates that the effect of ATP on permeability is mediated through activation of PLC. This conclusion is supported by the observation that ET-18-OCH₃, a PLC inhibitor chemically distinct from U-73122, can also attenuate the ATP-induced reduction of permeability. Apart from its effect on permeability, U-73122 also abolished the increase in \([\text{Ca}^{2+}]_i\). This shows that the ATP-induced increase in \([\text{Ca}^{2+}]_i\), even though unrelated to permeability changes, is another PLC-dependent effect. Taken together, the experiments using the PLC inhibitors indicate that the permeability-reducing mechanism observed in the presence of ATP is activated on a level downstream of PLC but upstream of PLC-mediated \([\text{Ca}^{2+}]_i\) rise.

PLC activation can lead to activation of PKC. A parameter of PKC activation is translocation of PKC activity into cell membranes. In the presence of ATP such an increase in membranous PKC activity was indeed observed. This translocation was found to be abolished by pretreatment of cells with the PKC inhibitor U-73122, indicating that it occurs secondarily to PLC activation. PLC translocation was also inhibited in the presence of the nonselective PKC inhibitor BIM or in the presence of Gö-6976, an inhibitor of the \(\text{Ca}^{2+}\)-dependent isofoms of PKC. This suggests that only \(\text{Ca}^{2+}\)-dependent isofoms are translocated in response to stimulation with ATP. Endothelial cells are known to express the \(\text{Ca}^{2+}\)-dependent PKC isoforms \(\alpha\) and \(\beta\) (2).

In contrast to their effects on PKC translocation into the membrane fraction, the PKC inhibitors did not affect the ATP-induced rise in monolayer permeability. This indicates that the latter is independent of an activation of classic PKC isoforms that would all be inhibited by BIM. It leaves open the possibility that nonclassic isofoms of PKC are involved.

We found that the presence of ATP causes a transient rise in cGMP and cAMP in the cells. Processes that stimulate cGMP or cAMP synthesis may cause a reduction of macromolecule permeability of endothelial monolayers (17, 18). The changes in cyclic nucleotides, observed in the presence of ATP, could be blocked in the presence of specific inhibitors, ODQ for soluble guanylate cyclase and SQ-22536 for adenylyl cyclase. The effects of the inhibitors indicate that the rises in cyclic nucleotides are due to activation of synthesis. We did not analyze this signaling of ATP stimulation toward cyclic nucleotide synthesis. Instead, we analyzed whether the changes in cAMP or cGMP contents affected the ATP-lowering effect on permeability. This was not the case. The ATP effect on permeability is thus independent of the changes in cyclic nucleotides.

In conclusion, ATP can stimulate both a rise in \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) seems to increase endothelial permeability primarily by activation of the second type of mechanism (8). One may speculate that ATP overrides these \([\text{Ca}^{2+}]_i\)-mediated effects on cell adhesion by the activation of specific \(\text{Ca}^{2+}\)-independent signal transduction pathways. These have yet to be identified.

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