Investigation of G protein-initiated, Ca^{2+}-dependent release of ATP from endothelial cells

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Abstract

We investigated G protein-stimulated release of ATP from human umbilical vein endothelial cells (HUVECs) using the G protein stimulant compound 48/80. Application of compound 48/80 resulted in dose-dependent ATP evolution from cultured HUVECs. This release was not cytotoxic as demonstrated by a lactate dehydrogenase assay and the ability of the cells to load and retain the viability dye calcein following stimulation. Mastoparan also stimulated release of ATP, further suggesting the process was G-protein initiated. This G protein was insensitive to pertussis toxin and appeared to be of the Gq-subtype. The ATP efflux was completely abolished in the presence of EGTA and thapsigargin signifying a strict Ca^{2+} dependence. Furthermore, compound 48/80-induced release was significantly decreased in cells pretreated with the phospholipase C inhibitor U73122. Thus, the release pathway appears to proceed through an increase in intracellular Ca^{2+} via PLC activation. Additionally, the G protein-initiated release was attenuated by pretreatment of the cells with either phorbol ester or indolactam V, both activators of protein kinase C. Finally, ATP release was not affected by treating HUVECs with nitric oxide synthase (NOS) inhibitors or glybenclamide.

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1. Introduction

The role of ATP as an intercellular messenger has attracted widespread interest over the past 10 years. The binding of ATP to purinergic receptors on the surface of cells initiates receptor-mediated responses. These purinergic receptors are classified into two categories: ion channel-coupled P2X type and G protein-coupled P2Y type. P2 receptors are further classified by their pharmacological and genetic properties [1,2]. Their activation initiates several different second messenger pathways in many types of cells, including neurons, smooth muscle cells, mast cells, and epithelial cells [3]. The most thoroughly investigated of these pathways is the elevation of intracellular Ca^{2+} levels via activation of PLC and subsequent production of inositol [1,4,5] triphosphate (IP_{3}). IP_{3} binds to receptors on the endoplasmic reticulum releasing stored Ca^{2+}. Increasing intracellular Ca^{2+} initiates/-regulates numerous cellular responses, such as insulin secretion in β-cells [4], synaptic transmission in neurons [5,6], mast cell degranulation [7], and platelet aggregation at injury sites [8].

ATP signaling in endothelial cells appears to be important for proper endothelium function. Activation of endothelial cells by ATP in vitro has been shown to increase cytosolic Ca^{2+} levels and consequently to cause production of nitric oxide (NO) [9]. NO regulates endothelium vascular tone, inhibits platelet aggregation, and reduces vascular smooth muscle proliferation in the cardiovascular system [10]. Ca^{2+} signaling also activates secretion of von Willebrand factor from the Weibel-Palade bodies, the main exocytotic vesicle in endothelial cells [11]. A number of other important effects occur in
endothelial cells upon increase of intracellular Ca$^{2+}$, including release of prostacyclin and endothelin-1, and an enhancement in microvascular permeability (see Ref. [12] for a review).

While much is known about the stimulatory effects of ATP on endothelial cells, the source of this ATP is unclear. One possibility is secretion of ATP stored in exocytotic vesicles. A number of researchers have reported ATP being copackaged with either norepinephrine or acetylcholine in neuronal synaptic vesicles where it acts as a fast excitatory neurotransmitter [13–15]. Shear stress has been reported to induce release of ATP in endothelial cells via excytosis [35]. The cytoplasm is the other obvious source for release. Under extreme circumstances, ATP may be liberated from a cell’s cytoplasm following lysis. However, recent evidence suggests that release of ATP from the cytosolic pool may also occur in a regulated, non-cytotoxic manner. ATP is reported to be released from many cell types via ATP binding cassette proteins, such as the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel [16]. The concept of this ion channel being able to pass a large highly charged molecule like ATP is at this time highly controversial, and numerous researchers have reported conflicting results on the matter [15,17].

Mechanical stimulation also results in release of ATP in numerous cell lines [18–20]. Stout et al. [21] have recently postulated that ATP is released through connexin proteins in mechanically stimulated astrocytes. Connexins commonly form gap junctions between cells, but can also form channels to the extracellular matrix termed hemichannels [22]. The presence of hemichannels and their ability to release small dye molecules has been known for some time. Cotrina et al. [19] previously discovered that C6 glioma cells with increased connexin43 expression release larger amounts of ATP than do control cells. A recent report by Arcuino et al. [23] lends support to this proposed release mechanism. They note that release of ATP from the cytoplasmic pool represents a viable method for cellular communication in nonexcitable cells. Furthermore, numerous surrounding cells can be influenced by the release of ATP in this manner, because the concentration of ATP in the cytoplasm is ~2 mM [23].

In this study, we performed experiments on human umbilical vein endothelial cells (HUVECs), a popular model for the investigation of endothelial function. The G protein agonist compound 48/80 was employed to stimulate release of ATP from HUVECs. Chemiluminescence produced by the reaction of luciferin and ATP in the presence of luciferase was monitored in real time on a microscope equipped with an intensified CCD camera. Ca$^{2+}$ imaging was conducted on the same setup and served in both experimental and control capacities. Several of the key enzymes and second messengers involved in this ATP release mechanism were identified, and release in HUVECs was compared to other proposed pathways.

2. Experimental procedures

2.1. Chemicals

Compound 48/80, thapsigargin, and EGTA were purchased from Sigma (St. Louis, MO). Firefly luciferase (from Photinus pyralis) and D-luciferin were obtained from both Sigma and R & D Systems (Minneapolis, MN). BAPTA AM, Fluo-3 AM, calcein AM, and Pluronic F-127 were from Molecular Probes (Eugene, OR). U73122 (1-[6-((17β)-3-methoxy-1,3,5(10)-trien-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-[6-((17β)-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl)-2,5-pyrrolidinedione) were purchased from both Calbiochem (La Jolla, CA) and Sigma. All other drugs were from Calbiochem. U73122, U73343, phorbol-12-myristate-13-acetate (PMA), Go6893, bisindolylmaleimide (BIM), indolactam V, thapsigargin, BAPTA AM, Fluo-3 AM, and calcein AM were dissolved in dimethylsulfoxide (DMSO, Sigma) as stock solutions. BAPTA AM, Fluo-3 AM, and calcein AM were diluted 1000-fold in buffer containing 0.1% Pluronic F-127 (20% in DMSO) to aid in solubilization of the dye. All other drugs were diluted to the pretreatment concentration in imaging buffer. The concentration of DMSO in pretreatment solutions never exceeded 0.3%. In control experiments, all drug compounds were tested for and displayed no interference with the ATP-luciferin-luciferase bioluminescent reaction. Other drug compounds, such as suramin, reactive blue 2 and gadolinium ion, interfered with bioluminescent imaging and thus were not employed in this study. For the specific case of compound 48/80, injection of 20 nM ATP yielded 98.9% of the chemiluminescence signal for luciferase containing 1 mg/ml compound 48/80 as luciferase solution alone.

2.2. Cell Cultures

HUVECs were purchased from American Type Culture Collection (Manassas, VA). These cells were in passage 14 when ordered. Experiments were performed only on cultures between 15 and 25 total passages to ensure minimal chance of change in cellular phenotype. Cells were grown in Medium 199 (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated on 22×22-mm square coverslips coated with poly-L-lysine (Sigma) substrate. Teflon O-rings (Small Parts, Inc., Logansport, IN) were attached to coverslips with Sylgard 184 (Dow Corning, Midland, MI) prior to coating with poly-L-lysine and acting as solution chambers. Cells were grown at 37 °C in an atmosphere of 95% air-5% CO₂. Prior to stimulation, cells were rinsed with buffer (135 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazinenulesulfonic acid) and 10 mM glucose (pH=7.3)). In experiments performed with buffers containing different concentrations of Ca$^{2+}$, the ionic strength of the solution was maintained by varying the
sodium concentration accordingly. Experiments were conducted only on confluent cultures.

2.3. ATP Imaging

ATP imaging was performed on the stage of an inverted microscope (Axiovert 100 TV, Zeiss, Germany). Chemiluminescence signal was collected with a Zeiss Apochromat 20× microscope objective (NA=0.75) and detected with an intensified charge-coupled device (iCCD; EEV 576×384 pixels CCD chip, Roper Scientific, Trenton, NJ) attached to the camera mount of the microscope. Cells were incubated in 100-μl buffer containing 100 μg/ml firefly luciferase and 205 μM D-luciferin. Images were collected at a frequency of 1.33 Hz with 500-ms exposure times. All experiments were performed at room temperature. Stimulant was applied to the cells via a 10-μl Hamilton syringe within the first 10 frames of the collected images. The camera offset was removed by subtracting an image file collected with the camera shutter closed.

2.4. Ca2+ imaging

The same experimental setup was utilized for Ca2+ imaging as was used for ATP imaging. HUVECs were loaded for 50 min at room temperature with 8.85 μM Fluo-3 AM in buffer with reduced Ca2+ (200 μM). The cells were then incubated, also at room temperature, for an additional 30 min in buffer to allow for complete de-esterification of the Fluo-3. Excitation was accomplished using a Zeiss Atto Arc HBO 100-W mercury lamp with a 475±20-nm bandpass filter. Fluorescence emission was collected through a 505-nm dichroic mirror and isolated with a 530±18-nm bandpass filter. No autofluorescence was detected at the wavelengths utilized. The exposure time of the iCCD and the power of the mercury lamp were varied based on the efficiency of the Fluo-3 loading of each individual coverslip.

2.5. Cytotoxicity assay

LDH assays (Sigma) were performed at room temperature on a UV–VIS spectrometer (HP 8452A Diode Array Spectrophotometer, Hewlett Packard, Palo Alto, CA). Coverslips were rinsed with buffer three times at 15-min intervals. The final rinse from each coverslip was collected as a background sample. Compound 48/80, A23187, or buffer was added to each coverslip and then sampled after 10 min. The LDH content was determined by adding the assay solution (50 μl) to each sample (100 μl) and allowing the assay reaction to proceed for 25 min. The reaction was halted with 1 N HCl (15 μl), and the peak absorbance of each sample was evaluated from 480 to 500 nm to quantitate LDH. For control coverslips (buffer as stimulant), an additional step was performed. After the background and experimental samples were obtained, 20 μM digitonin was added to the coverslip to achieve total lysis of the cells. Complete cell lysis was estimated by optically observing the morphological change of digitonin-stimulated cells. The digitonin-treated sample was diluted with buffer until its absorbance neared that of the compound 48/80 and A23187-treated samples. This sample was assayed identically to the others and marked the maximum releasable LDH.

2.6. Calcein loading efficiency

Calcein loading efficiency was assessed on the same fluorescence microscope setup as was used for Ca2+ imaging. Dishes containing HUVECs were stimulated with compound 48/80 or control (buffer). After 15 min, the cells were rinsed and incubated in buffer containing 1 μM calcein AM for 45 min at room temperature and in buffer for 20 min at room temperature to allow for loading and complete de-esterification of calcein, respectively. Cell viability was expressed as the percent of cells able to load calcein.

2.7. Calcein leakage

Again the same instrumental setup was employed for calcein leakage experiments as was for Ca2+ imaging. Dishes of HUVECs were loaded with 1 μM calcein AM in buffer for 45 min at room temperature. After incubation in buffer for 20 min to allow de-esterification of the dye, compound 48/80 or buffer was added to the dishes. The number of cells exhibiting fluorescence was monitored prior to and following stimulation. The percent of calcein-loaded

Table 1

Cytotoxicity tests for compound 48/80 on HUVECs

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>LDH release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.62±0.11</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>1.15±0.13</td>
</tr>
<tr>
<td>A23187</td>
<td>0.97±0.51</td>
</tr>
<tr>
<td>Digitonin</td>
<td>100±6.16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Number of cells loading calcein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>129.2±5.1</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>136.0±5.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>% of cells leaking calcein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1.7±1.7</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>48.9±9.8</td>
</tr>
</tbody>
</table>

(A) Quantities of LDH released from HUVECs stimulated with buffer (N=5), 25 μg/ml compound 48/80 (N=7), 5 μM A23187 (N=7), or 40 μM digitonin (N=7). (B) Number of cells able to load and maintain calcein AM after stimulation with buffer (N=13) or 16.8 μg/ml compound 48/80 (N=19). (C) Percent of calcein-loaded HUVECs that lost fluorescence 30 min after addition of either buffer (N=3) or 15 μg/ml compound 48/80 (N=6).
Fig. 1. Compound 48/80-induced release of ATP in HUVECs. ATP signal was monitored from cultured HUVECs stimulated with 15 μg/ml compound 48/80. (A) Single frames of data are shown at various times after stimulation. (B) Concentration of ATP efflux following stimulation is plotted versus time after stimulation. (C) The dose dependence of ATP release is depicted in a plot of the maximum ATP release versus compound 48/80 concentration. The overall curve is the combined data from five separate dose response curves obtained from HUVECs in different passages. All data points represent the combined data from at least five independent compound 48/80 injections, except for [compound 48/80]=0 and 5 μg/ml, for which only three injections were performed.
cells maintaining fluorescence was reported for both compound 48/80 and buffer-stimulated dishes.

2.8. Data presentation/analysis

Chemiluminescence and fluorescence signal were obtained and processed with Winview32 software (Roper Scientific). The mean chemiluminescence signal was calculated for each frame of data. The net ATP signal was calculated by subtracting the pre-stimulation average signal from the maximum ATP signal. Concentrations of ATP release were calculated from a calibration curve obtained from imaging solution and ATP standards. Other ATP data are reported as a percentage (of counts) of the experimental versus the control experiments (compound 48/80 stimulation of untreated dishes, unless otherwise noted). For temporal responses, the initiation and maximal times represent the 10% and 90% maximal signals, respectively. Calcium fluorescence images were analyzed by subtracting a prestimulation image from all poststimulation images. A cell was deemed as having signal only if its fluorescence after stimulation was at least 10 times the background standard deviation. Identical processing was performed for calcine images except that the poststimulation image was subtracted from a prestimulation image for calcine leakage experiments. Standard errors were determined for each condition based on the number of samples analyzed. A $P$ value of $<0.05$ was considered significant as calculated by the Student’s $t$-test for paired experiments.

3. Results

The toxicity of compound 48/80 to the HUVECs was first investigated. For this purpose, an LDH assay was employed. LDH is a large cytoplasmic protein whose release is a marker of the loss of cell membrane integrity. HUVEC plates were sampled prior to and following activation with compound 48/80, buffer, or the Ca$^{2+}$ ionophore A23187. A23187 was utilized as a control stimulant since it activates HUVECs by increasing the ionophore A23187. A23187 was utilized as a control

![Graph showing G protein dependence of compound 48/80-induced release of ATP](image)

Fig. 2. G protein dependence of compound 48/80-induced release of ATP from HUVECs. HUVECs were stimulated with 10 µM mastoparan. HUVECs pretreated overnight with 250 ng/ml PTX or buffer were stimulated with 15 µg/ml compound 48/80. Representative time courses of ATP release are shown.

When 15 µg/ml compound 48/80 was added to calcine-loaded HUVECs, 48.9±9.8% of the cells failed to maintain calcine fluorescence (Table 1C). Taken together, the three assays suggest that upon stimulation with compound 48/80 the HUVEC membrane is permeable to calcine (622 Da) but not LDH (134 kDa). This permeability to calcine is not permanent and ceases upon removal of compound 48/80.

Furthermore, the LDH levels obtained for compound 48/80 the HUVEC membrane is permeable to calcine (622 Da) but not LDH (134 kDa). This permeability to calcine is not permanent and ceases upon removal of compound 48/80.

Addition of the G protein stimulant compound 48/80 to confluent coverslips of HUVECs caused release of cellular ATP, as depicted in Fig. 1 (A and B). ATP release was initiated 41.1±1.8 s ($N$=30) after addition of 15 µg/ml compound 48/80. The ATP signal reached a maximum at 126.2±5.8 s and slowly decreased back to basal levels (10–15 min). For the injection displayed in Fig. 1 (A and B), basal release of ATP was below our detection limits (<5 nM) while the maximal release was at approximately 600 nM ATP. Since a perfusion system was not utilized in these experiments, the slow decrease to basal levels could be a result of the continued presence of compound 48/80 and/or from ATP-induced ATP release from HUVECs. The dose dependence of compound 48/80-stimulated release of ATP is shown in Fig. 1C. ATP efflux was not detected at compound 48/80 concentrations lower than 5 µg/ml and plateaued at ~60 µg/ml compound 48/80 at around 4 µM ATP.

Next we attempted to confirm that a G protein was indeed the initiation site for compound 48/80-induced ATP release. Direct stimulation of HUVECs with another G protein stimulant, mastoparan, also produced significant release of ATP. As shown in Fig. 2, mastoparan induced as much ATP release as did compound 48/80 (67.2±10.8% ($N$=3)). The kinetics of ATP release (38.4±5.8 s and 104.1±8.1 s, $N$=4) was also similar to that of compound 48/80-induced release. PTX, an inhibitor of $G_i$ and $G_o$, but not...
Fig. 3. Compound 48/80-induced increases in intracellular Ca\(^{2+}\) and Ca\(^{2+}\)-dependent release of ATP from HUVECs. Intracellular stores of Ca\(^{2+}\) were depleted by incubation of HUVECs with 250 nM thapsigargin for 20 min, and extracellular Ca\(^{2+}\) was removed by bathing cells in buffer with Ca\(^{2+}\) removed and 100 \(\mu\)M EGTA added to remove any residual Ca\(^{2+}\) ions (Tg/EGTA). Compound 48/80 induced intracellular Ca\(^{2+}\) increases in HUVECs loaded with Fluo-3. Single frames of Ca\(^{2+}\) imaging data are depicted at various times after stimulation with 15 \(\mu\)g/ml compound 48/80 (A). Representative time courses of ATP release (B) and Ca\(^{2+}\) signaling (C) are depicted for HUVECs stimulated with 15 \(\mu\)g/ml compound 48/80 (Tg/EGTA and 48/80), 10 \(\mu\)M Ca\(^{2+}\) ionophore (A23187), 5 \(\mu\)M thapsigargin (Tg) or buffer. Inset for B: Zoomed-in look at the lack of ATP release caused by thapsigargin, A23187, and buffer.
Gq proteins, was utilized to further investigate the involvement of G proteins in initiating release of ATP. As shown in Fig. 2, overnight incubation of HUVECs with PTX did not alter the magnitude of compound 48/80-evoked ATP efflux (95.2±13.9% (N=22) of buffer-pretreated HUVECs). Furthermore, PTX treatment did not significantly affect the temporal onset (43.8±3.0 s, N=22) of G protein-initiated release of ATP. Thus, a Gq protein is the probable initiation site for compound 48/80-induced ATP efflux.

Addition of compound 48/80 to HUVECs also stimulated increases in intracellular Ca2+ as depicted in Fig. 3A. 83.7±3.3% of cells responded with increased Ca2+ levels upon addition of 15 μg/ml compound 48/80 (N=15). The temporal onset and maxima of Ca2+ increase were at 37.3±1.5 and 51.2±1.9 s (N=42), respectively. The temporal onsets of both Ca2+ and ATP release were quite similar. Thus, the ATP and Ca2+ responses were further investigated to determine whether: (1) the Ca2+ increase was the result of ATP release, (2) the Ca2+ increase was necessary for the release of ATP, or (3) the two were simply two parallel results of the same stimulation. Thapsigargin, an inhibitor of Ca2+ ATPases, was employed to deplete intracellular Ca2+ stored within the ER, and 100 μM EGTA in Ca2+-free buffer was used to remove the extracellular Ca2+ source. As depicted in Fig. 3B, compound 48/80-stimulated cells pretreated for 20 min with Tg an Ca2+-free buffer did not release significant amounts of ATP (5.9±2.7% of control, N=6). Also, the temporal onset of ATP release under these conditions was significantly delayed (initiation at 84.6±10.7 s) for HUVECs that did exhibit ATP release. Ca2+ imaging confirmed that under Ca2+-free conditions, compound 48/80 did not cause significant Ca2+ increases (3.2±2.3% of cells responded, N=9) in HUVECs as shown in Fig. 3C. This data suggests
that G protein-initiated release of ATP from HUVECs is strictly Ca\textsuperscript{2+}-dependent.

Based on these results, the possibility that increasing intracellular Ca\textsuperscript{2+} levels alone was sufficient for initiating ATP efflux from HUVECs was investigated. Exposure of cells to the Ca\textsuperscript{2+} ionophore A23187 (10 \(\mu\)M) in buffer containing 4 mM CaCl\textsubscript{2} (double that of the control imaging buffer) failed to stimulate significant release of ATP without the addition of compound 48/80. As shown in Fig. 3B, A23187 caused 5.2\(\pm\)0.3\% \((N=3)\) of the ATP release found with 15 \(\mu\)g/ml compound 48/80, while buffer alone produced 4.5\(\pm\)1.7\% \((N=3)\). Ca\textsuperscript{2+} imaging demonstrated that intracellular Ca\textsuperscript{2+} was increased in 89.6\(\pm\)0.6\% \((N=3)\) of cells, as depicted in Fig. 3C. Likewise, direct stimulation of HUVECs with 5 \(\mu\)M thapsigargin increased intracellular Ca\textsuperscript{2+} levels in 92.1\(\pm\)4.2\% \((N=3)\) of cells (Fig. 3C), but was unable to induce release of ATP (4.8\(\pm\)0.7\% \((N=3)\) versus release caused by compound 48/80), as illustrated in Fig. 3B. These experiments provide evidence that ATP release from HUVECs was dependent on increased intracellular Ca\textsuperscript{2+} levels, but that increasing intracellular Ca\textsuperscript{2+} levels was not sufficient to cause release.

Next, experiments were conducted to determine whether the Ca\textsuperscript{2+}-based control of G protein-initiated ATP release in HUVECs was exerted through the well-established phospholipase C/IP\textsubscript{3} pathway. Prior to stimulation, cells were incubated for 30 min in imaging buffer containing either U73122 or U73343, an inhibitor of phospholipase C and its inactive analog, respectively. When stimulated with compound 48/80 under extracellular Ca\textsuperscript{2+}-free conditions, ATP release from HUVECs was significantly inhibited in cells pretreated with U73122 (12.7\(\pm\)3.4\% of cells compared to those pretreated with U73343, \(N=6)\), as shown in Fig. 4A. When
extracellular Ca\(^{2+}\) was restored and EGTA was removed, U73122 still attenuated stimulated ATP release but to a lesser degree (20.9±8.9% compared to U73343-treated cells, N=4). Ca\(^{2+}\) imaging confirmed that intracellular Ca\(^{2+}\) signaling was reduced in compound 48/80-stimulated HUVECs pretreated with U73122 with and without extracellular Ca\(^{2+}\) present. As shown in Fig. 4B, 64.6±10.6% (N=4) of cells responded with increased Ca\(^{2+}\) when cells were pretreated with U73122 compared to 81.4±4.1% (N=8) of cells pretreated with U73343. Additionally, when Ca\(^{2+}\) was removed, only 25.0±10.6% (N=12) of U73122 treated cells responded with increased Ca\(^{2+}\) versus 67.7±16.0% (N=4) for U73343. The temporal initiation of ATP release was extended slightly in cells pretreated with U73122 (49.5±9.1 and 49.4±12.3 s under Ca\(^{2+}\)-free conditions and Ca\(^{2+}\)-present conditions, respectively), for those cells in which release was detected (three of seven for U73122 under Ca\(^{2+}\)-free conditions and five of seven for U73122 under Ca\(^{2+}\)-present conditions). Moreover, U73343 did not appear to affect ATP or Ca\(^{2+}\) signaling. The temporal response of compound 48/80-stimulated cells pretreated with U73343 with and without Ca\(^{2+}\) present was not significantly different than those obtained for either buffer or EGTA treatment (data not shown). Together with the earlier experiments, these results suggest that G protein-activated release of ATP proceeds via activation of phospholipase C and subsequent increases in intracellular Ca\(^{2+}\).

The protein kinase C dependence of G protein-initiated ATP release was also examined. Phospholipase C, which produces IP\(_3\), additionally generates diacylglycerol (DAG). When activated by DAG, protein kinase C modulates Ca\(^{2+}\) signaling in HUVECs [24] and thus could be involved in ATP release. As illustrated in Fig. 5A, pretreatment with neither BIM nor Go6893, both inhibitors of PKC, altered the magnitude of compound 48/80-stimulated release of ATP (102.8% ± 4.9, N=7 and 95.1% ± 8.9, N=6 of controls, respectively). Likewise, pretreatment with BIM or Go6893 did not significantly affect the temporal onset of ATP release. Hence, activation of PKC did not appear to be necessary for compound 48/80-induced release of ATP.

Next, the effect of PKC activation on ATP efflux was studied using the PKC activators PMA and indolactam V. Fig. 5B shows the ATP release for compound 48/80-stimulated HUVECs pretreated with PMA and indolactam V. As depicted, treatment with the PKC activators reduced the magnitude of stimulated ATP release from HUVECs (14.2±3.4, N=7 and 56.8±9.9%, N=7 of control, respectively). The temporal initiation of ATP release was delayed in compound 48/80-stimulated HUVECs pretreated with PKC activators (59.9±8.6 s, N=10 and 49.7±3.1 s, N=7 for PMA and indolactam V, respectively) versus buffer only (41.2±1.8 s).

We then examined whether the production of nitric oxide was involved in G protein-initiated release of ATP in HUVECs. As mentioned earlier, increases in intracellular Ca\(^{2+}\) activate nitric oxide synthase (NOS). The results of experiments conducted with the NOS inhibitors L-NAME and L-NMMA are depicted in Fig. 6. The magnitude of compound 48/80-stimulated release of ATP was not significantly altered in either L-NAME- or L-NMMA-pretreated cells (96.0±6.7%, N=8 and 95.7±8.3%, N=7 of untreated cells, respectively). Additionally, the temporal onset of ATP release for inhibitor-treated cells was not significantly different from that of control cells. Thus, it appears that nitric oxide production is not necessary for G protein-initiated release of ATP.

Finally, we wanted to determine if compound 48/80-induced release of ATP in HUVECs occurred via a CFTR-transmembrane ion channel. Glybenclamide blocks CFTR channels and is commonly employed to identify CFTR-coupled release. In HUVECs treated with 100 μM glybenclamide, compound 48/80-induced release of ATP was not significantly modified. As shown in Fig. 7, the magnitude of release was 96.3±10.3% (N=6) of control dishes and its onset occurred at 39.3±2.9 s. These results generally
suggest that ATP release in this G protein-initiated pathway was not occurring through a CFTR channel.

4. Discussion

The above observations provide insights into the pathway through which HUVECs release ATP when stimulated with the mast cell degranulator compound 48/80. Compound 48/80 likely initiates the release pathway by activating a G
t protein which has been previously identified in HUVECs [25]. In general, Gq proteins are not pertussis toxin-sensitive [26], which supports this hypothesis. However, both compound 48/80 and mastoparan must permeate the cell membrane in order to activate a G protein. This permeation could make the membrane permeable to ATP as well. However, the release of ATP in this case was eliminated with the removal of cell Ca2+ sources. The possibility remains that the permeability of the membrane to compound 48/80, and therefore ATP, is decreased in the absence of Ca2+. However, the inhibition of PLC also attenuated ATP release without the elimination of the cellular Ca2+ sources. These results suggest that the release mechanism was physiologically initiated but do not completely negate the involvement of an increase in membrane permeability. The next step in the release pathway appeared to be the activation of PLC by the Gq protein. PLC then produced IP3 which caused release of Ca2+ from the ER, which was followed by initiation of capacitive Ca2+ entry from the extracellular matrix [27]. Following this increase in intracellular Ca2+, ATP was released from the cell into the extracellular medium. Up-regulation of PKC significantly attenuated ATP release. PKC activation, by treatment of the cells with PKC activators for ~20–30 min, has been reported to inhibit PLC in many cell types, including endothelial cells [28,29]. The rate and magnitude of release under conditions of PKC activation in our experiments were similar to those in U73122-treated HUVECs. This suggested that protein kinase C possibly down-regulated phospholipase C in our system and could act in feedback inhibition of ATP release upon extended stimulation of the release mechanism.

The maximal release induced by addition of compound 48/80 was 4 μM ATP. From calcein imaging data, the observation area of the microscope contained, on average, 134 endothelial cells. Taking an average volume of 1 μl per 106 cells, the cells in the observation area contain 2.68×10−7 μmol of ATP. The imaging volume is ~2.71×10−6 ml, taking into account the depth of field of our microscope objective. Utilizing this volume, the 4 μM ATP detected represents 1.08×10−8 μmol of ATP. Thus, the maximal release of ATP in these experiments represents release of ~4% of the total ATP. This is likely a slight overestimate of the actual release as at least part of the depth of field of the microscope is below the cells, and thus yields no signal. This magnitude of release is significantly higher than that reported in other ATP release studies [Ref. [17,36]]. As demonstrated by Dubyak and colleagues, the release of up to 100 nM ATP in astrocytes can be effectively enzymatically degraded by ecto-ATPases on the outer plasma membranes. This likely limits the actions of released ATP to cells immediately neighboring the releasing cells. Additionally, the ecto-ATPases could also act in eliminating the effect of low level ATP release, such as from basal release across the cell membrane. For Gq protein-induced ATP release from endothelial cells, the level of ATP release can likely overcome the action of the ecto-ATPases and have more profound effects in cellular systems.

As observed above, increasing Ca2+ alone is not sufficient to induce release of ATP. Many possibilities exist as to what other metabolic pathways/enzymes are necessary for inducing release of ATP from HUVECs upon G protein stimulation. For instance, photoliberation of IP3 can induce release of ATP in astrocytes [37], and could be adequate in HUVECs, as well. Alternatively, the simultaneous activation of another cellular pathway by compound 48/80 may be necessary for release to occur. Activation of an alternative pathway by the G protein (either the α- or βγ-subunit) could feasibly be involved. Platelet-activating factor stimulation of Goq has been shown to stimulate numerous second messenger pathways in HUVECs, including both PLC activation and increased cAMP levels [38]. In mast cells, G protein βγ-subunits are implicated in controlling exocytosis while they are not sufficient by themselves to trigger exocytosis. Instead, Gomberts et al. suggest that activation of Rho-related GTPases is likely necessary for induction of exocytosis [39]. This mechanism is further postulated in the action of both compound 48/80 and mastoparan activation of mast cells.

A number of recent studies have been published which are especially interesting in light of the results obtained in this work. Stout et al. [21] reported ATP release through connexin hemichannels in mechanically stimulated astrocytes. They noted that cells loaded with calcein blue dye (MW=400 Da) released the dye upon mechanical stimulation. However, neither Oregon Green (MW=1100 Da) nor LDH (MW=134 kDa) was released under these same conditions. Moreover, the connexin channel inhibitor flufenamic acid attenuated the release of both calcein blue and ATP. Arcuino et al. [23] undertook a similar study. They noted that ATP was released in astrocytes upon lowering extracellular Ca2+ levels. Fluorescence imaging revealed propidium (MW=563 Da) and dicarboxy-dichlorofluorescein (MW=445 Da) uptake also occurred upon stimulation. However, fluorescein–dextran conjugates (MW=1.5–2000 kDa) were unable to enter the astrocytes under identical conditions. Furthermore, connexin-deficient C6 glioma did not release ATP when stimulated. Like Stout and coworkers, Arcuino and colleagues hypothesized that connexin hemichannels may be the channel through which ATP efflux occurs. Finally, they also reported release of ATP from HUVECs and epithelial cells stimulated identically to the
astrocytes. To date, this mechanism of ATP release in astrocytes has not been identified. However, ATP release in both of these reports would correlate well with the pathway investigated here. We noted that HUVECs stimulated with compound 48/80 were still able to load and maintain calcein after compound 48/80 washout. However, we also observed that calcein preloaded into HUVECs was released upon stimulation with compound 48/80 while LDH was not. Moreover, compound 48/80 was able to stimulate release of ATP from astrocytes, and like in HUVECs, this release of ATP was found to be Ca\(^{2+}\)-dependent (Gruenhagen, J.A. and Yeung, E.S., unpublished data).

Exocytosis of ATP is another possible mechanism for release. Both compound 48/80 and mastoparan are commonly used to study secretion of histamine from mast cells through activation of G\(_i\) proteins. As is typically the case in exocytosis, we observed a strict dependence on Ca\(^{2+}\) in our experiments. However, unless calcein was confined to exocytotic vesicles, the observed calcein leak experiments are difficult to explain. Calcein fluorescence, in fact, was found to be very uniform across the cell, suggesting this was not the case. Furthermore, the involvement of a G\(_i\) protein in the release mechanism was widely negated as treatment with pertussis toxin was without effect. Nonetheless, it is still possible that exocytosis of ATP and release of ATP from the cytoplasm occur simultaneously upon introduction of compound 48/80. We found no data to suggest the release of ATP occurred through the ATP binding cassette (ABC) protein, CFTR. ATP release stimulated by compound 48/80 was not inhibited by glybenclamide, which is in contrast to the majority of proposed CFTR-permeable ATP release mechanisms [17]. Furthermore, these results also suggest against the involvement of a sulfonylurea receptor in ATP release. Several other mechanisms for release of ATP have been proposed. These include release through large-conductance and volume-regulated anion channels [30,31]. Unfortunately, the luciferase imaging reaction was inhibited in the presence of DIDS. Thus, we could not negate the involvement of these channels. The involvement of other ABC proteins possibly involved in release, including P-glycoprotein, was not examined in this report and deserves future scrutiny. Identification of the true release mechanism in HUVECs will necessitate eliminating all of the other proposed ATP release schemes through the use of appropriate activators and inhibitors and an in-depth investigation of each mechanism.

The physiological role of ATP release in endothelial cells in vivo has not been thoroughly elucidated but many possibilities exist. As discussed earlier, released ATP causes the activation of several pathways in numerous cell types. In addition, endothelial cells interact with many different cell types in the human body. These include smooth muscle cells around the blood vessels, glial cells in the brain, platelets and neutrophils in the blood, and other endothelial cells [32]. One possible physiological effect of release of ATP from endothelial cells would be in regulating vasodilation/vasoconstriction of blood vessels. Stimulants either carried in the blood or released by cells carried in the blood could stimulate G\(_q\) protein-coupled receptors on the endothelial cells. The stimulated endothelial cells would then release ATP onto neighboring smooth muscle cells and onto other unstimulated endothelial cells. This ATP would modulate vasoconstriction via activation of P2X receptor ion channels. Additionally, ATP has been shown to induce the synthesis of nitric oxide, a major modulator of vascular smooth muscle tone, in endothelial cells [9]. Therefore, release of ATP from endothelial cells might regulate smooth muscle function through a balance between activation of ionotropic purinergic receptors on the smooth muscle cells and stimulation of nitric oxide production by neighboring endothelial cells [10]. Glial cell/endothelial cell interactions are responsible for communication across the blood brain barrier [32]. Activation of G\(_q\) protein-coupled receptors on endothelial cells by either the glial cells or compounds carried in the blood could induce ATP efflux. ATP would then act on neighboring endothelial cell purinergic receptors regulating the microvascular permeability via control of intracellular \(\text{Ca}^{2+}\) signaling and cAMP levels [33]. Perhaps more importantly, released ATP would activate Ca\(^{2+}\) signaling in glial cells, which has been implicated in the modulation of neuronal synaptic transmission [5,6]. ATP release from endothelial cells also appears to be exceedingly important in inflammation and wound healing. For instance, ATP released by endothelial cells can cause degranulation in mast cells, releasing histamine and additional ATP. This ATP would then stimulate degranulation of additional neighboring mast cells. ATP released by endothelial cells also initiates \(\text{Ca}^{2+}\) signaling in platelets and neutrophils, inducing their aggregation as wound sites [34]. The balance between release of ATP and NO, which inhibits platelet aggregation, could regulate inflammatory response in the vascular system. Whether G\(_q\) protein-induced release of ATP participates in any of these or other physiological systems remains to be fully clarified. Additionally, further research is necessary to identify the actual physiological stimulants that induce G\(_q\) protein-initiated ATP release in HUVECs and possibly in other cell types.

In summary, the activation of G proteins in HUVECs by compound 48/80 was investigated. It was discovered that ATP is released upon stimulation of the cells. This release, which was seemingly initiated via a G\(_q\) protein, was modulated by PLC and subsequent \(\text{Ca}^{2+}\) signaling. It did not appear to occur through the known CFTR ion channel or exocytosis mechanisms but instead through an as of yet unidentified cytoplasmic channel.

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