Pregnancy-enhanced store-operated Ca^{2+} channel function in uterine artery endothelial cells is associated with enhanced agonist-specific transient receptor potential channel 3-inositol 1,4,5-trisphosphate receptor 2 interaction

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Abstract

We have previously shown that endothelial cells (EC) derived from the uterine artery (UA) of both pregnant (P-UAEC) and nonpregnant (NP-UAEC) ewes show a biphasic intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) response after ATP stimulation. In each case, the initial transient peak, caused by the release of Ca^{2+} from the intracellular Ca^{2+} stores, is mediated by purinergic receptor-Y2 and is very similar in both cell types. However, the sustained phase in particular, caused by the influx of extracellular Ca^{2+} , is heightened in the P-UAEC, and associates with an increased ability of the cells to demonstrate enhanced capacitative Ca^{2+} entry (CCE) via store-operated channels (SOCs). Herein we demonstrated that the difference in the sustained $[\text{Ca}^{2+}]_i$ response is maintained for at least 30 min. When 2-aminoethoxydiphenyl borate (2APB) (an inhibitor of the inositol 1,4,5-trisphosphate receptor (IP3R) and possibly SOC) was used in conjunction with ATP, it was capable of completely inhibiting CCE. Since 2APB can inhibit SOC in some cell types and 2APB was capable of inhibiting CCE in the UAEC model, the role of SOC in

CCE was first evaluated using the classical inhibitor La^{3+} . The ATP-induced sustained phase was inhibited by 10 μM La^{3+} , implying a role for SOC in the $[\text{Ca}^{2+}]_i$ response. Since canonical transient receptor potential channels (TRPCs) have recently been identified as putative SOCs in many cell types, including EC, the expression levels of several isoforms were evaluated in UAEC. Expression of TRPC3 and TRPC6 channels in particular was detected, but no significant difference in expression level was found between NP- and P-UAEC. Nonetheless, we were able to show that IP3R2 interacts with TRPC3 in UAEC, forming a protein complex, and that this interaction is considerably enhanced in an agonist sensitive manner by pregnancy. Thus, while IP3R and TRPC isoforms are not altered in their expression by pregnancy, enhanced functional interaction of TRPC3 with IP3R2 may underlie pregnancy-enhanced CCE in the UAEC model and so explain the prolonged $[\text{Ca}^{2+}]_i$ sustained phase seen in response to ATP.

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Introduction

Endothelial cells (EC) function at many levels to maintain a balance between angiogenesis and apoptosis (Schriffin 2001), vasodilation and vasoconstriction (Sullivan *et al.* 2000), and coagulation and fibrinolysis (Baumgartner-Parzer & Waldhauser 2001, Vallet & Wiel 2001, Levi *et al.* 2002). In addition, the endothelium serves as a selective permeability barrier between the blood and tissue, including the brain (Albert *et al.* 1997), and produces a variety of growth factors and peptide hormones (Miller 1999, Vapaatalo & Mervaala 2001). While multiple signaling pathways impact on these diverse end points, many of these diverse cell functions including nitric oxide (NO) production by endothelial NO synthase (eNOS) and prostanoid production in response to cytosolic

phospholipase A2 activation are thought to be mediated by Ca^{2+} signaling.

Pregnancy is a time of increasing needs and demands by the growing fetus and these are met in part by redistribution of maternal blood flow. In particular, uterine artery blood flow increases dramatically and uterine artery endothelium function is altered to enhance vasodilator production in particular (Bird *et al.* 2003). Uterine artery (UA) EC derived from pregnant (P-UAEC) and nonpregnant (NP-UAEC) ewes have recently been isolated and show differences in function and vasodilator production through passage 4 (Bird *et al.* 2000, Di *et al.* 2001, Gifford *et al.* 2003). While these cells at passage 4 no longer demonstrate the difference in eNOS expression observed *in vivo*, pregnancy-enhanced production of NO is still observed along with a number of alterations in associated cell signaling

(Bird *et al.* 2003). One important difference involves Ca^{2+} signaling, wherein P-UAEC show a greater sustained phase than NP-UAEC. This may have great physiological significance because several studies showed that the sustained phase of the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) response may have functions separate from the initial transient peak. For example, several studies in a variety of cell types have emphasized the importance of extracellular Ca^{2+} influx, which is typically required for the sustained phase but not the initial transient peak in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), for mitogenesis, indirect ERK 1/2 activation, and nitric oxide production (Fleming & Busse 1999, Mulvaney *et al.* 1999, Taniguchi *et al.* 1999, Lin *et al.* 2000, Sjöholm *et al.* 2000, Kawanabe *et al.* 2001, 2002, Mathov *et al.* 2001, Tahara *et al.* 2001, Gomez *et al.* 2002). Thus, during pregnancy, when blood flow must increase to the uterus to provide the exchange of nutrients and waste for the fetus, EC exhibit a greater sustained phase which in turn support increased blood flow to the uterus through effects on angiogenesis and vasodilation (eNOS activation).

Since Ca^{2+} signaling controls many aspects of EC function, it is no surprise that impairment of EC Ca^{2+} signaling is associated with cardiovascular diseases. More specifically, many ECs normally respond to agonist stimulation in a biphasic manner consisting of an initial transient peak rise in $[\text{Ca}^{2+}]_i$ followed by a prolonged sustained phase, but in many cardiovascular disease states the sustained phase may be lost (Salameh & Dhein 1998, Kimura *et al.* 2001, Kuroda *et al.* 2001, Tran *et al.* 2001). Furthermore, Steinert *et al.* (2002) and Mahdy *et al.* (1998) demonstrated that human fetal venous EC and maternal human hand vein EC show a loss of the sustained phase in subjects with preeclampsia, a condition that is characterized by hypertension (Mahdy *et al.* 1998, Steinert *et al.* 2002). This data along with our previous data on UAEC led us to believe that the sustained phase of the $[\text{Ca}^{2+}]_i$ response in NP-UAEC is relatively unresponsive, while P-UAEC mobilize Ca^{2+} more like calf pulmonary artery EC, bovine aortic EC, and rat aortic EC (Chu *et al.* 2000, Bishara *et al.* 2002, Wilkinson & Jacob 2003).

The UAEC model is a unique and valuable tool that was developed to allow investigation of the underlying molecular mechanisms by which pregnancy alters endothelial function and how endothelial dysfunction may relate to pregnancy-induced hypertension (preeclampsia) in particular. Nonetheless, such knowledge also impacts upon cardiovascular disease and hypertension in general. In order to achieve this understanding, we herein investigate the question: what is the underlying adaptive mechanism that augments the sustained $[\text{Ca}^{2+}]_i$ phase in P-UAEC but not NP-UAEC?

Materials and Methods

Materials

Thapsigargin and CaCl_2 were purchased from Calbiochem (San Diego, CA, USA). ATP (disodium salt) and all other

chemicals were purchased from Sigma unless stated otherwise. Minimal essential medium (MEM) D-Val and all other cell culture reagents were purchased from Invitrogen. Glass-bottom microwell dishes (35 mm) for $[\text{Ca}^{2+}]_i$ imaging studies were from MatTek Corporation (Ashland, MA, USA) and BD Falcon T75 flasks were purchased from Fisher Scientific (Itasca, IL, USA).

Isolation of UAEC

Uterine arteries were obtained from Polypay and mixed Western breed nonpregnant sheep ($n=7$) and pregnant ewes at 120–130 days of gestation ($n=9$) during nonsurvival surgery, as described previously (Bird *et al.* 2000, Di *et al.* 2001). Procedures for animal handling and protocols for experimental procedures were approved by the University of Wisconsin-Madison Research Animal Care Committees of both the Medical School and the College of Agriculture and Life Sciences and follow the recommended American Veterinary Medical Association guidelines for humane treatment and euthanasia of laboratory farm animals. Briefly, primary uterine arteries were flushed free of blood and digested with collagenase. Freshly isolated EC (passage 0) were plated to 35 mm dishes in MEM. Cells were then grown and passaged to approximately 70% confluence in T75 flasks, at which point they were passaged once more (passage 3) to medium containing 10% dimethylsulfoxide and frozen in liquid nitrogen for long-term storage. Cells prepared in this way have been shown previously to be uniformly eNOS positive and to take up exogenous low-density lipoprotein (Bird *et al.* 2000, 2003, Gifford *et al.* 2003), and purity was estimated to be similar for NP- and P-UAEC at >98%. Cells at passage 3 from four separate animals were each thawed and grown to passage 4, combined, and then split 1:8 before freezing with 10% dimethylsulfoxide (Sigma) for later plating at lower density as required. Note that in test studies, the data from these cells were indistinguishable from our previously published data on prepassage 4 cells (Vallet & Wiel 2001, Bird *et al.* 2000).

Fura-2 imaging

Passage 4 cells were grown in 35 mm glass-bottom microwell dishes for 1–3 (ATP experiments) or 7–10 days (La^{3+} experiments) before being used for experiments. Cells were then loaded with Fura-2 by adding 5 μM AM (Molecular Probes Inc., Eugene, OR, USA) in the presence of 0.05% Pluronic F127 (Molecular Probes Inc.) in 1 ml Krebs buffer (125 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM KH_2PO_4 , 6 mM glucose, 25 mM HEPES, 2 mM CaCl_2 , pH 7.4) for 45 min at 37 °C. Cells were washed with Krebs buffer, covered in 2 ml Krebs buffer, and incubated for 30 min to allow complete ester hydrolysis. The cells were then removed from the incubator and all of the subsequent steps were performed at room temperature. Cells were again washed and covered with 1 ml Krebs buffer, and the dish was placed in the field of view. Fura-2 loading was verified by

viewing at 380 nm UV excitation on a Nikon inverted microscope (InCyt Im2, Intracellular Imaging, Inc., Cincinnati, OH, USA). The cells were incubated with the appropriate agonist and/or antagonist and the data were recorded for several individual, nontouching cells simultaneously (for all experiments except La^{3+} experiments which were performed at 100% confluence) for a total of 5–60 min using alternate excitation at 340 and 380 nm at 1-s intervals and measuring emitted light using a PixelFly video camera. From the ratio of emission at 510 nm detected at the two excitation wavelengths and by comparison with a standard curve established for the same settings using buffers of known free $[\text{Ca}^{2+}]$, the intracellular free $[\text{Ca}^{2+}]_i$ was calculated in real time using the InCyt Im2 software (Cincinnati, OH, USA). For all $[\text{Ca}^{2+}]_i$ imaging experiments, data were recorded for 30 s before agonist stimulation or antagonist addition to establish the basal $[\text{Ca}^{2+}]_i$. If the experiment was to be performed in Ca^{2+} free media, immediately before beginning the experiment, the dish was washed twice with 2 ml Ca^{2+} free Krebs buffer (125 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM KH_2PO_4 , 6 mM glucose, 25 mM HEPES, 50 μM EGTA, pH 7.4) and left in 1 ml Ca^{2+} free Krebs. Specific times of agonist and antagonist additions for each experiment are listed in the figure legends. For ATP plus 2-aminoethoxydiphenyl borate (2APB) or ATP plus La^{3+} , $[\text{Ca}^{2+}]_i$ imaging experiments, an initial 5-min recording was performed using 100 μM ATP in 2 mM Ca^{2+} and this was used to verify that the cells responded to ATP. Following that recording, the cells were washed twice in Krebs buffer and allowed to recover for 20 min. The dish was then treated with the appropriate agonist/antagonist. The dish was once again rinsed with buffer and allowed to recover before one more 5-min recording with 100 μM ATP to ensure the cells were still viable.

Preparation of whole cell lysates for expression analysis

Passage 4 P- and NP-UAEC were each plated to a T75 flask. Once the cells reached 70% confluence, the flasks were rinsed free of serum using ice-cold PBS and the cells were scraped in lysis buffer (4 mM sodium pyrophosphate, 50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 10 mM sodium fluoride, 2 mM sodium orthovanadate, pH 7.5 with added 1 mM PMSE, 1% Triton X-100, 5 μM leupeptin, and 5 μM aprotinin) and sonicated. Solubilized cell lysates were then clarified at 500g for 10 min and the supernatant used for Western analysis. Protein concentrations of all samples were determined using the bicinchoninic acid assay (Sigma).

SDS-PAGE

A broad range 'rainbow' molecular weight marker (Amersham), $n=7$ NP-UAEC samples, and $n=9$ P-UAEC samples or a full range rainbow molecular weight marker (Amersham), agonist-stimulated whole cell lysate, and 25 μl of first and second elution (for immunoprecipitation experiments) were separated by size

on 7.5 or 12% gels (as appropriate) (1 h, 200 V) using the Criterion electrophoresis system (Bio-Rad). The proteins were then transferred to PVDF membrane (Millipore, Billerica, MA, USA) (1 h, 100 V). Blots were blocked in 0.5% fat-free milk and probed with the following primary antibodies: inositol 1,4,5-triphosphate receptor 1 (IP3R1); 1:1000, transient receptor potential channel (TRPC)1 (Alomone Labs, Jerusalem, Israel) (Alomone Labs; 1:200), TRPC3 (Alomone Labs; 1:200), TRPC4 (Alomone Labs; 1:100), TRPC5 (Alomone Labs; 1:200), and TRPC6 (Alomone Labs; 1:100), for 2 h at room temperature. The membrane was then incubated with horse radish peroxidase-conjugated secondary antibodies (Amersham) for 1 h before final washing and detection of signal using the enhanced chemiluminescence reagent system and HyperFilm (Amersham). Densitometry was utilized to compare expression levels of each protein between P- and NP-UAEC. When multiple bands were present, the bands indicated in the figures were bands that were immunoneutralizable. In addition, the densitometry from all immunoneutralizable bands was combined for analysis. If a blot was to be reprobed (as in the immunoprecipitation experiments), the membrane was frozen for a minimum of overnight. The membrane was then blocked again for 2 h at room temperature before reprobing with the appropriate antibody.

Immunoprecipitation studies

Passage 4 P- or NP-UAEC were plated to two T75 flasks. Once the cells reached confluence, the cells were trypsinized and 2.2 million cells were plated to five separate 60 mm dishes. The cells were allowed to attach overnight before beginning serum starvation for 4 h. Following serum withdrawal, the appropriate dishes were treated with 100 μM ATP or 10 ng/ml vascular endothelial growth factor (VEGF) for 8 or 19 min as appropriate. The reaction was stopped with the addition of ice-cold PBS (pH 7.4). The dishes were rinsed three times with PBS, the PBS was completely removed and the dishes were snap frozen in liquid nitrogen. The cells were scraped in 100 μl lysis buffer (4 mM sodium pyrophosphate, 50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM PMSE, 1% Triton X-100, 5 μM leupeptin, and 5 μM aprotinin, pH 7.5) and sonicated. Solubilized cell lysates were then clarified at 500g for 10 min and the supernatant used for immunoprecipitation. Protein concentrations of all samples were determined using the bicinchoninic acid assay (Sigma).

Agonist-stimulated whole cell lysates (250 μg , ~60–100 μl) were diluted 1:1 with modified PBS (0.14 M NaCl, 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.01 M KCl, pH 7.4) and then combined with 2.5 μg (12.5 μl) IP3R2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-7278) or 2.5 μg (6.25 μl) normal goat IgG (Santa Cruz) and incubated overnight with end-over-end rotation at 4 °C. The following day 10 μl immobilized protein G (Pierce Biotechnology, Rockford, IL, USA) were added to the lysate/antibody mixture and allowed to mix overnight with

end-over-end rotation at 4 °C in a Handee spin cup column (Pierce Biotechnology). The following morning the samples were centrifuged, leaving the protein G complex in the column and collecting the flow through. The protein G complex was washed with modified PBS four times prior to eluting the protein off the protein G with 60 µl boiling 1× sample buffer (0.1 M Tris (pH 6.8), 2% SDS, 10% glycerol, 2 mM EDTA, Coomassie Blue). Three elutions were collected. After running the whole cell lysates (20 µg) and elutions 1 and 2 (25 µl each) on an SDS-PAGE, the blots were first probed for TRPC3, and after freezing, the membranes were reprobed for IP3R2 (see SDS-PAGE). Densitometry was performed on the films for TRPC3 and the data were analyzed by comparing the ratio of total cell TRPC3, i.e. in whole cell lysate to TRPC3 in the elutions, i.e. recovered using IP3R2 IP. Experiments were considered successful if IP3R2 and/or TRPC3 did not appear in the elutions of the IgG control, but were correspondingly enriched in the IP fraction.

Statistical analysis

Each treatment in $[Ca^{2+}]_i$ imaging of Fura-2 loaded cells was replicated on multiple cells simultaneously per dish on at least three separate occasions. Student's *t*-test or one-way (ANOVA) were used to analyze the data as appropriate. $P < 0.05$ was considered significant.

Results

In order to get a better understanding of ATP-induced Ca^{2+} mobilization in the UAEC model, the previously employed length of the recording period was increased from 2.5 to 30 min to determine how long the differential $[Ca^{2+}]_i$ response was maintained. Figure 1A shows the average response of P- and NP-UAEC to 100 µM ATP in 2 mM Ca^{2+} over 30 min. When the same experiment was repeated in Ca^{2+} -free media, the initial transient peak fell to a common level and the sustained phase was lost for both cell types (Fig. 1C). In the following experiment, the cells were stimulated with ATP in Ca^{2+} -free Krebs and when $[Ca^{2+}]_i$ fell back to the basal level, the extracellular Ca^{2+} was reintroduced. Under these conditions, both the P- and NP-UAEC were equally capable of initiating capacitative Ca^{2+} entry (CCE) (Fig. 1E and F). Furthermore, 67.0% of NP-UAEC and 68.4% of P-UAEC responded to ATP, and of those cells, 64.4 and 65.4% of NP- and P-UAEC showed CCE, expressing the data as the percent of total cells that were recorded; 43.2% of NP-UAEC and 44.7% of P-UAEC responded to ATP and exhibited CCE.

Even though no difference was seen in CCE after the cells had been stimulated with ATP in Ca^{2+} -free media, we wanted to determine if 2APB could inhibit CCE in response to a physiological agonist. When 2APB was added after Ca^{2+} application, the amount of CCE was diminished to a

common level in the P- and NP-UAEC, but the inhibition was evident earlier in the P-UAEC (Fig. 2). Furthermore, as noted above, of the cells that did respond to ATP, about 65% of those also exhibited CCE; however, when the cells were treated with 2APB before the Ca^{2+} was reintroduced to the media, 65.6% of NP-UAEC and 80% of P-UAEC did not exhibit any CCE.

Since 2APB could apparently inhibit the sustained phase of the $[Ca^{2+}]_i$ response, even when added after the initial transient peak, it seemed possible that 2APB was inhibiting store-operated channels (SOC) as observed in other cells. Therefore, to demonstrate if there was a role for classical SOC in the ATP-induced response, a dose response for La^{3+} inhibition on the ATP response was performed using $[Ca^{2+}]_i$ imaging; 10 µM La^{3+} caused significant inhibition of both the initial transient peak and the sustained phase (Fig. 3).

In view of the finding that classical SOC activation is the basis of the sustained phase, the cells were evaluated for the presence of a group of recently identified SOC, namely TRPC channels. Both TRPC3 and TRPC6 were readily detected as immunoneutralizable bands in whole cell lysate and showed equal expression in P- and NP-UAEC (Fig. 4). The expression of TRPC1, TRPC4, and TRPC5 was also tested, but they were undetectable in whole cell lysates. However, these proteins were detectable and found to be of equal concentrations in membrane-enriched preparations (data not shown).

To further assess the role of TRPC channels in CCE of the UAEC, P- and NP-UAEC at confluence were stimulated with VEGF (control agonist functioning independently of heptahelical, heterotrimeric G-protein coupled receptor) or ATP for 8 or 19 min and these lysates were used from immunoprecipitation of the possible IP3R2/TRPC complex using IP3R2 antisera. Times of 8 and 19 min were chosen, since they represent the times of greatest difference in sustained phase for P-UAEC versus NP-UAEC after the initial peak had subsided. Effects of ATP were compared with VEGF, since VEGF is also a stimulator of eNOS activation, but does not stimulate UAEC via a heptahelical receptor nor illicit a rapid or substantial $[Ca^{2+}]_i$ response (Bird *et al.* 2000, Gifford *et al.* 2003). The immunoprecipitation procedure routinely recovered an enriched fraction of IP3R2 that was otherwise not seen in IgG controls, and where IP enrichment occurred, the flow through showed that there was a corresponding quantitative or even complete removal of detectable IP3R2 from the whole cell lysate (data not shown). We also routinely found that IP3R2 associated with TRPC3 even in the basal state, but more specifically that there was a significant increase in TRPC3/IP3R2 interaction in response to ATP but not VEGF at 8 min in P-UAEC that was not observed in NP-UAEC (Fig. 5). Treatment of P- or NP-UAEC with ATP or VEGF for 19 min, however, failed to increase association and actually caused a significant decrease in association compared with basal levels (Fig. 5).

Figure 1 ATP induces capacitative Ca^{2+} entry (CCE) that leads to the sustained phase of the ATP-induced $[Ca^{2+}]_i$ response. The $[Ca^{2+}]_i$ concentration was recorded in P-UAEC (solid squares or black bars) and NP-UAEC (open circles or white bars). The scatter plots show every tenth data point for the average response. The bar graphs represent the area under the curve, since ATP stimulation. An asterisk (*) denotes significant difference from NP-UAEC, $P \leq 0.05$. (A) Cells were treated with 100 μ M ATP in 2 mM Ca^{2+} and the response was recorded for 30 min. (B) The data from (A) expressed as the area under the curve since ATP addition (P-UAEC; $n=6$ over six experiments, NP-UAEC; $n=22$ over eight experiments). (C) Cells were treated with 100 μ M ATP in Ca^{2+} -free Krebs and the response was recorded for 30 min. (D) Data from (C) expressed as area under the curve since ATP addition. Response in P- and NP-UAEC was very similar (P-UAEC; $n=23$ over nine experiments, NP-UAEC; $n=47$ over five experiments). (E) Cells were treated with 100 μ M ATP in Ca^{2+} -free media and 3 min after the $[Ca^{2+}]_i$ fell back to the basal level, extracellular Ca^{2+} was reintroduced to the media. Since the time to fall to basal varied slightly, for data analysis, the time Ca^{2+} was added was arbitrarily set to 500 s (8.33 min). When Ca^{2+} was reintroduced, both the NP- and P-UAEC were capable of initiating CCE. (F) Data from (E) expressed as area under the curve since Ca^{2+} addition (P-UAEC; $n=26$ over eight experiments, NP-UAEC; $n=59$ over five experiments). Mean $\pm$ S.E.M.

Figure 2 The effects of 2-aminoethoxydiphenyl borate (2APB) on the ATP-induced sustained phase. (A) and (B) The black line or bar is a reproduction of the response of the P-UAEC shown in Fig. 1E and F. (C) and (D) The black line or bar is a reproduction of the response of NP-UAEC shown in Fig. 1E and F. The dark gray line or bar shows the response of the cells to ATP treatment in Ca^{2+} -free media, 3 min after the $[Ca^{2+}]_i$ level fell back to basal Ca^{2+} was reintroduced, and 2 min later 50 μ M 2APB were added. The light gray line or bar represents the experiment wherein the cells were treated with 100 μ M ATP in Ca^{2+} -free media, once the $[Ca^{2+}]_i$ fell back to basal 2APB was added, and then Ca^{2+} was reintroduced 3 min later. The scatter plots show every tenth data point for the average response. The bar graphs represent the area under the curve since Ca^{2+} addition. An asterisk (*) denotes significant difference from the ATP Ca^{2+} -free, Ca^{2+} results, while a plus sign (+) denotes significant difference from ATP Ca^{2+} -free, 2APB, Ca^{2+} , $P \leq 0.05$. (A) P-UAEC were pretreated with 2APB before the Ca^{2+} was reintroduced, no CCE was observed (light gray), while 2APB added after the Ca^{2+} was resulted almost immediate inhibition of CCE (dark gray). (B) Area under the curves since Ca^{2+} addition for (A). (C) Similar results were obtained in the NP-UAEC; however, the inhibition by 2APB was not as severe. 2APB added prior to Ca^{2+} introduction caused inhibition of CCE and 2APB added after Ca^{2+} led to inhibition of CCE but it was not immediate. (D) Area under the curve since Ca^{2+} addition for (C) (P-UAEC, ATP, Ca^{2+} , 2APB $n=29$ over five experiments; ATP, 2APB, Ca^{2+} $n=10$ over ten experiments; NP-UAEC, ATP, Ca^{2+} , 2APB $n=26$ over three experiments; ATP, 2APB, Ca^{2+} $n=32$ over three experiments).

Discussion

The UAEC model is a primary culture model of UAEC function that retains pregnancy-specific differences in agonist-stimulated vasodilator production, kinase activation, and Ca^{2+} signaling through passages 4–5 (Bird *et al.* 2000, Di *et al.* 2001, Gifford *et al.* 2003). Specific to these acute 2–5-min recordings, P-UAEC respond to ATP with an initial transient peak in $[Ca^{2+}]_i$ followed by a plateau period that was elevated over basal $[Ca^{2+}]_i$ which marked the beginning of the prolonged sustained phase. In contrast, NP-UAEC exhibit a very similar initial peak, but the beginning of the sustained phase was consistently lower and approached baseline more rapidly (Di *et al.* 2001). This difference in Ca^{2+} signaling is physiologically important, since complete inhibition of Ca^{2+} signaling to below basal levels (using

1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) causes complete inhibition of vasodilator production (Di *et al.* 2001). When the $[Ca^{2+}]_i$ is inhibited to a more physiologically relevant basal level with 2APB, it leads to about 60% inhibition of ATP-stimulated eNOS activation (Sullivan *et al.* 2005). Likewise, recent studies on freshly isolated UAEC from pregnant and nonpregnant ewes using simultaneous imaging of $[Ca^{2+}]_i$ and NO production (Yi *et al.* 2005) have confirmed that increases in $[Ca^{2+}]_i$ in response to ATP are paralleled by increases in NO production, and that both the $[Ca^{2+}]_i$ and NO responses are more sustained in pregnancy. In addition, application of sufficient 2APB to prevent any rise in $[Ca^{2+}]_i$ again results in about 60% inhibition of the NO response (Yi *et al.* 2005). Thus, Ca^{2+} mobilization is very important to NO production in UAEC endothelium and the development of a sustained $[Ca^{2+}]_i$.

Figure 3 La^{3+} dose response. P-UAEC were pretreated with various doses of La^{3+} for 1 min before ATP stimulation and the data were collected for 30 min. (A) 10 μM La^{3+} causes significant inhibition of the area under the curve for the initial peak. *Signifies significant difference from ATP control, + denotes significant difference from 0.1 and 1.0 μM doses, $P \leq 0.05$. (B) 10 μM La^{3+} also leads to significant inhibition of the sustained phase as measured by the area under the curve for the last 20 min of the 30-min recording period. *Signifies significant difference from ATP control, ‡ signifies significant difference from 0.1 μM dose, $P \leq 0.05$ ($n = 150$ over three experiments, mean $\pm$ S.E.M.)

response to ATP in pregnancy may be one way in which more sustained vasodilator production and so blood flow can be increased to meet the needs of the growing fetus.

In order to expand upon our previous knowledge of ATP-induced Ca^{2+} signaling in the UAEC model, the recording period for $[Ca^{2+}]_i$ imaging experiments was increased from 2.5 to 30 min. These experiments revealed that the difference in CCE during the sustained phase is maintained for at least 30 min when the cells are stimulated in media containing Ca^{2+} (Fig. 1A). Consistent with our previous findings (from the 2.5-min recordings), the response to ATP in Ca^{2+} -free media fell to the same level in both cell types (Fig. 1C and D). Likewise, treatment with ATP in Ca^{2+} -free media followed by addition of extracellular Ca^{2+} when $[Ca^{2+}]_i$ fell back to

basal produced the same amount of CCE in P- and NP-UAEC (Fig. 1E and F). Based on our observations with thapsigargin (see parallel submission), we had anticipated that there would be different amounts of CCE seen between the two cell types but this was not the case. Nonetheless, it must be remembered that thapsigargin more completely/supra-physiologically empties the endoplasmic reticulum (ER) of Ca^{2+} so it may be the effect of ATP in releasing Ca^{2+} from the ER was not sufficiently strong to be apparent in this manner. We therefore continued with another set of experiments utilizing 2APB (an IP3R antagonist and a putative SOC inhibitor).

Effects of 2APB were tested by its application shortly before or after Ca^{2+} replacement. This antagonist proved capable of inhibiting CCE in both NP- and P-UAEC (Fig. 2). Nonetheless, it is not entirely clear if 2APB actually directly inhibits SOC function or indirectly by acting solely as an IP3R antagonist. Our previous observation that the structurally and pharmacologically distinct compound U73122 (a phospholipase C (PLC) inhibitor) can also cause complete inhibition of the initial transient peak and sustained phase (Sullivan *et al.* 2005) suggests that IP3 generation is essential to both phases of the $[Ca^{2+}]_i$ response and 2APB is probably in part inhibiting SOC opening by its action as an IP3R inhibitor. The previous finding that 2APB can inhibit thapsigargin-induced SOC function (parallel submission) also suggests that such inhibition of IP3R does not require IP3 to be present.

A further question arises concerning the identity of the SOC and indeed if it is similar to that seen in other cells. Treatment with a well-characterized classical inhibitor of extracellular Ca^{2+} influx at the plasma membrane, La^{3+} , successfully inhibited both phases of the ATP-induced response in UAEC, further suggesting the SOC involved in the ATP-induced $[Ca^{2+}]_i$ response is indeed similar to that classically observed in other cell types (Fig. 3). Such previously identified SOC candidates include L-type channels, plasma membrane IP3R, and TRPC channels. Microarray analysis has demonstrated that UAEC express L-type channels (Gifford *et al.* 2003) and some EC do contain functional L-type channels (Nilius & Droogmans 2001), but we have shown that the highly specific L-type channel antagonist, nifedipine, did not inhibit either phase of the ATP-induced response, and BAYK8644 (L-type channel agonist) did not cause a rise in the $[Ca^{2+}]_i$ (Di *et al.* 2001 and data not shown). We must conclude therefore that L-type channels are not involved in agonist-stimulated Ca^{2+} influx in UAEC, consistent with the majority of other EC types.

Some recent studies in other cells have also suggested that there may be IP3R or IP3R-like proteins associated with the plasma membrane (Fujimoto *et al.* 1992, Putney 1997, Kiselyov *et al.* 1999b), and so may directly mediate Ca^{2+} influx (Putney 1997, Kiselyov *et al.* 1999b). However, it seems more likely that there are distinct SOC proteins located in the plasma membrane which couple to IP3R on the ER. One such candidate family of proteins is TRPC channels, recently identified as putative SOCs in many cell types (Boulay *et al.*

Figure 4 TRPC3 and TRPC6 expression in UAEC. Distinct cell preparations from pregnant ewes and nonpregnant ewes were lysed, run on 7.5% gels and then probed for TRPC3 or TRPC6. (A) The top panel shows a Western blot for TRPC3 and TRPC6. There were four bands (see arrows) for TRPC3 that were immunoneutralized with the control peptide (data not shown). The lower panel shows a Western blot for TRPC6. (B) Densitometry showing that both TRPC3 and TRPC6 showed equal expression in the P- and NP-UAEC. $n=9$ for P-UAEC and $n=7$ for NP-UAEC. Mean $\pm$ s.e.m. No significant differences were seen at the level $P<0.05$ between the P- and NP-UAEC preparations, as shown by the densitometry.

1999, Kamouchi *et al.* 1999, Kiselyov *et al.* 1999a, Birnbaumer *et al.* 2000, Lockwich *et al.* 2001, Vazquez *et al.* 2001, Beech *et al.* 2003). These channels are activated by PLC-mediated events, but it is still a matter of debate which products of PLC directly or indirectly activate the channels. It is generally accepted that IP₃, diacylglycerol (DAG), and polyunsaturated fatty acids can activate TRPC3/6/7, while TRPC1/4/5 can be activated by IP₃. Furthermore, all of the TRPC channels can bind IP₃R (reviewed in Vazquez *et al.* 2004a) and have been shown to couple to IP₃R on the ER through protein–protein interactions (conformational coupling) (Boulay *et al.* 1999, Kiselyov *et al.* 1999a, Birnbaumer *et al.* 2000, Lockwich *et al.* 2001, Vazquez *et al.* 2001). The IP₃R on the ER is not only a target for IP₃, but also independently senses intracellular store depletion and communicates this to the TRPC channel in the plasma membrane directly above as a conformational change, so allowing the influx of extracellular Ca^{2+} . TRPC channels

have been described as functional SOC in other EC models such as the bovine aortic endothelial cell and can be inhibited by 2APB (Tanimura *et al.* 2000); it is not clear if this is direct or more likely via inhibition of the IP₃R which otherwise interacts with TRPC. In view of this and our functional data, we considered that any one of these TRPC channels could potentially be the SOC in UAEC, so expression levels of these proteins were evaluated, with the exception of TRPC2 and TRPC7. TRPC2 appears to be a pseudogene in humans and only functional in rats and mice and was therefore not studied. TRPC7 was not included because it has not yet been reported in EC (however, this may simply be due to limited tools for investigation). Identity of these channels was established by Western analysis with immunoneutralization and revealed that TRPC3 and TRPC6 show equally abundant expression in P- and NP-UAEC whole cell lysates (Fig. 4). While the levels of TRPC1, TRPC4, and TRPC5 were too low to detect in whole cell lysates, all three were expressed

Figure 5 IP3R2 interaction with TRPC3 in UAEC. P- and NP-UAEC were stimulated with 100 μ M ATP or 10 ng/ml VEGF for 8 or 19 min, lysed, and then used for immunoprecipitation of IP3R2. Whole cell lysate, flow through, and all three elutions were separated on an SDS-PAGE, transferred to a PVDF membrane and then probed for TRPC3 and IP3R2. (A) represents the typical result. WCL, whole cell lysate; FT, flow through collected after conjugation of antibody with whole cells lysate; E1-3, elutions 1-3. (B) depicts the ratio of TRPC3 in the elutions to TRPC3 in the whole cell lysate. $n=4$ each for P- and NP-UAEC. Mean $\pm$ s.e.m. $P \leq 0.05$. *Denotes significant difference from unstimulated control (con).

in enriched membrane preparations of P-UAEC (data not shown).

The TRPC1 antibody from Alamone Laboratories is known to function as an inhibitor of the TRPC1 channel (Antoniotti *et al.* 2002, Rosado *et al.* 2002); therefore, it was used to determine if TRPC1 was responsible for the difference in Ca^{2+} signaling in the P- and NP-UAEC. Following a 10-min pretreatment with 15 μ g/ml TRPC1 Ab, the ATP response was unaffected (data not shown). Thus, TRPC1 does not appear to function in this response.

Kamouchi *et al.* (1999) showed that in bovine pulmonary EC transfected with TRPC3, Ca^{2+} mobilization was similar to, that in P-UAEC, while wild-type cells show data similar to NP-UAEC (Trebak *et al.* 2004). This, combined with the high expression levels in the UAEC, led us to believe that TRPC3 may be the primary isoform activated by agonist

stimulation in the P-UAEC. Further investigation revealed that conformational coupling does occur in both P- and NP-UAEC, since TRPC3 immunoprecipitates with IP3R2 in the absence of added agonist in both cell types. Furthermore, ATP is capable of increasing the extent of this TRPC3/IP3R2 interaction, but only in P-UAEC and not in NP-UAEC (Fig. 5). Thus, TRPC3 is likely a functional SOC mediating the ATP-stimulated $[\text{Ca}^{2+}]_i$ elevation during the prolonged sustained phase in the UAEC, but more importantly its functional interaction with IP3R2 appears to be specifically enhanced in pregnancy and this may explain the differences in CCE observed between P- and NP-UAEC.

All of this leads us to a new model of the cell signaling events leading to the differential Ca^{2+} signaling in the P- and NP-UAEC. First, for both NP- and P-UAEC, extracellular ATP binds to its P2Y2 receptor on the plasma membrane in both cell types. The binding of ATP to this purinergic receptor activates $\text{G}\alpha_q$, which in turn activates PLC. The PLC can then hydrolyze phosphatidylinositol bisphosphate to IP3 and DAG. The DAG stays in the plasma membrane, perhaps to activate kinases, while IP3 diffuses into the immediately adjacent cytosol where it can bind to its receptors on the surface of the ER. The IP3R is a Ca^{2+} gated ion channel which mediates the release of Ca^{2+} from the ER leading to the initial transient peak in $[\text{Ca}^{2+}]_i$. As the intracellular store is increasingly depleted, it causes a conformational change in IP3R2 allowing it to interact with and so activate the TRPC3 located immediately above in the plasma membrane. When TRPC3 is activated, it permits the influx of extracellular Ca^{2+} that is characteristic of the sustained phase or CCE. All of the molecular components for these events are present in both the P- and NP-UAEC, and to some extent both use this system. It appears, however, that the functional interaction between the IP3R and the TRPC3 channel is specifically enhanced in the P-UAEC, as indicated both by the greater CCE apparent in the P-UAEC when pharmacologic agents such as thapsigargin are used and by the greater sustained phase observed in response to physiologic agonists such as ATP. Coincident with this greater sustained phase, IP3R2/TRPC3 association is increased in response to ATP stimulation specifically in P-UAEC.

In summary, we were able to demonstrate that the increased sustained phase of the $[\text{Ca}^{2+}]_i$ response of the P-UAEC is maintained for 30 min and that ATP-induced CCE can be inhibited by 2APB in P- and NP-UAEC. We were also able to use La^{3+} treatment to demonstrate that the SOC involved in ATP-induced Ca^{2+} influx was similar to that classically described in other cell types. Western analysis further revealed that UAEC expressed TRPC3 and TRPC6 together with traces of TRPC1, TRPC4, and TRPC5. Finally, while TRPC3 associated with IP3R2 in the basal state, an ATP-induced increase in conformational coupling was only observed between TRPC3 and IP3R2, and only in P-UAEC. Our previous data have also demonstrated that, in addition to the altered $[\text{Ca}^{2+}]_i$ responses studied herein,

P-UAEC also exhibits more agonist-induced kinase phosphorylation than NP-UAEC and it is now clear that phosphorylation of IP3R and TRPC play important roles in their regulation (Jayaraman *et al.* 1996, Cui *et al.* 2004, Kwan *et al.* 2004, Trebak *et al.* 2004, Vazquez *et al.* 2004b). Thus, it is quite possible that altered kinase activation may impact upon and perhaps be responsible for pregnancy-induced changes in SOC function independently of SOC (TRPC) or IP3R protein expression levels. Further investigation will be required to determine if any other TRPC and IP3R are involved in Ca^{2+} signaling in the UAEC model and the nature of the mechanism leading to pregnancy-enhanced IP3R2/TRPC3 interaction in the P- versus NP-UAEC.

In closing, since Mahdy *et al.* (1998) showed that hand vein EC from women with preeclampsia exhibit Ca^{2+} signaling similar to that of nonpregnant women (i.e. lacking the sustained phase), elucidation of the exact alterations in Ca^{2+} signaling could have profound effects on the treatment of preeclampsia (Mahdy *et al.* 1998). Likewise, numerous other studies have demonstrated that the sustained phase is altered in many cardiovascular diseases associated with endothelial dysfunction including general systemic hypertension, atherosclerosis, diabetes, and high levels of unsaturated free fatty acids. While our studies investigate mechanisms underlying pregnancy-induced programming/enhancement of Ca^{2+} signaling in UAEC, our findings in this model may well have direct relevance to the vasculature in general and so aid in the future design of treatments for several cardiovascular diseases, as well as those related to pregnancy.

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