Pregnancy-enhanced Ca^{2+} responses to ATP in uterine artery endothelial cells is due to greater capacitative Ca^{2+} entry rather than altered receptor coupling

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Abstract

Uterine artery endothelial cells (UAEC) derived from pregnant (P-UAEC) and nonpregnant (NP-UAEC) ewes retain pregnancy-specific differences in cell signaling as well as vasodilator production through passage 4. In particular, when P- and NP-UAEC are stimulated with ATP over a 2·5 min recording period, they exhibit similar initial transient peaks in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), but the P-UAEC show a heightened sustained phase. In order to establish whether this was due to an altered subclass of purinergic receptor (P2), both the dose dependency of $[\text{Ca}^{2+}]_i$ responses to ADP and UTP and the profile of purinergic receptor expression are determined in NP- and P-UAEC. Our findings indicate that while several isoforms of P2X and P2Y receptors are present, it is P2Y2 that is responsible for the ATP-induced initial transient peak in both cell types. We also characterized several key components of the ATP-induced Ca^{2+} signaling cascade, including the inositol 1,4,5-trisphosphate receptor and

G-proteins, but could not confirm any pregnancy-specific variation in the protein expression that correlated with pregnancy-specific differences in prolonged Ca^{2+} signaling. We thus investigated whether such a difference may be inherent to the cell itself rather than specific to the purinergic receptor-signaling pathway. Using thapsigargin (Tg), we were able to demonstrate that the initial Tg-sensitive intracellular pool of Ca^{2+} is nearly identical with the capacity in both cell types, but the P-UAEC is nonetheless capable of greater capacitative Ca^{2+} entry (CCE) than NP-UAEC. Furthermore, CCE induced by Tg could be dramatically inhibited by 2-aminoethoxydiphenyl borate, suggesting a role for store-operated channels in the ATP-induced $[\text{Ca}^{2+}]_i$ response. We conclude that changes at the level of capacitative entry mechanisms rather than switching of receptor subtype or coupling to phospholipase C underlies pregnancy adaptation of UAEC at the level of Ca^{2+} signaling. *Journal of Endocrinology* (2006) **190**, 373–384

Introduction

We have previously established that enhanced vasodilator production by uterine artery endothelial cells (UAEC) involves remapping of cell signaling in a manner that is programmed (Bird *et al.* 2000, 2003, Di *et al.* 2001). Enhanced vasodilator production is an important physiological endpoint to study in UAEC, because augmented vasodilator production is essential for normal pregnancy outcomes. Preeclampsia and intrauterine growth retardation are both associated with impaired vasodilator production (Goodman *et al.* 1982, Fitzgerald *et al.* 1987, Diket *et al.* 1994).

We have recently established a primary cell culture model of uterine artery endothelial cells to passage 4 and have shown that while many pregnancy-specific changes in protein expression reverse by this time, altered cell signaling remains unchanged with clear differences between cells derived from pregnant (P-UAEC) versus nonpregnant (NP-UAEC) ewes. We have further studied ATP in particular as an agonist of cell signaling and associated endothelial nitric oxide synthase

(eNOS) activation, because thus far it has been shown to stimulate both Ca^{2+} and kinase signaling and further we have established that eNOS activity is controlled through both pathways (Bird *et al.* 2000, Di *et al.* 2001, Gifford *et al.* 2003). Typically, endothelial cells respond to agonists such as ATP with a biphasic intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) response (Srinivas *et al.* 1998, Gifford *et al.* 2003, Tanaka *et al.* 2003, reviewed by Zunkler *et al.* 1999, Tran *et al.* 2000). This type of response is most commonly initiated when ATP binds to a P2Y receptor (Kunapuli & Daniel 1998, Murthy & Makhoulf 1998, Ralevic & Burnstock 1998, Strassheim & Williams 2000, von Kgelgen & Wetter 2000). These purinergic receptors are G-protein coupled receptors and typically activate phosphoinositide-specific phospholipase $\text{Fic } \beta$ ($\text{PLC}\beta$) via $\text{Gq}/11$. The activation of $\text{PLC}\beta$ in turn produces inositol 1,4,5-trisphosphate (IP3), which binds to its receptor (IP3R) and so leads to activation of the channel. The rapid release of Ca^{2+} from the intracellular stores not only produces a rapid transient increase in $[\text{Ca}^{2+}]_i$ but also depletes the stores. In many cells, the depletion of the intracellular stores

then stimulates the influx of extracellular Ca^{2+} in a process called capacitative Ca^{2+} entry (CCE), which produces a prolonged sustained phase.

However, the question at hand is does CCE occur in UAEC and is it altered in pregnancy when more sustained $[\text{Ca}^{2+}]_i$ responses are observed? Several lines of evidence have suggested that the ATP-induced $[\text{Ca}^{2+}]_i$ response was mediated through P2Y in both P- and NP-UAEC. First, when the cells are stimulated with ATP in Ca^{2+} -free media, ATP is still capable of initiating the first phase of the biphasic response, or the initial transient peak, but the sustained phase is lost (Di *et al.* 2001). Secondly, we have recently shown that 2-aminoethoxydiphenyl borate (2APB; an IP3R antagonist) and U73122 (a PLC antagonist, thus inhibiting IP3 production) are equally capable of completely inhibiting both phases of the ATP-induced response (Sullivan *et al.* 2006). Thus, the initial peak and a more sustained response require PLC activation and IP3 production, consistent with our prior demonstration of inositol phosphate production (Di *et al.* 2001). These findings are consistent with the initial and sustained ATP-induced responses being mediated by a P2Y receptor (G-protein coupled receptor) rather than a P2X receptor (ligand-gated ion channel) in both cell types, but what is unclear is whether the isoform or coupling alters to produce the longer $[\text{Ca}^{2+}]_i$ sustained phase observed in P-UAEC. These studies investigate the functional purinergic receptor coupled to Ca^{2+} signaling in P- and NP-UAEC as well as evaluate if changes in expression levels of several other key Ca^{2+} signaling proteins may underlie pregnancy adaptation at the level of Ca^{2+} signaling in UAEC. In addition, we study the ability of the cells to allow influx via capacitative entry (CCE) independent of the agonist. We find that it is not a change in receptor subtype or coupling but rather the greater ability of P-UAEC compared to NP-UAEC to exhibit classically defined CCE that underlies pregnancy adaptation of Ca^{2+} signaling in UAEC.

Materials and Methods

Materials

CaCl_2 was purchased from Calbiochem (San Diego, CA, USA). ATP (disodium salt) and all other chemicals were purchased from Sigma unless stated otherwise. 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). All Western blot supplies were purchased from Bio-Rad unless otherwise stated.

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, Gifford *et al.* 2003). 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 endothelial cells (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, Di *et al.* 2001, Gifford *et al.* 2003), and purity for both NP- and P-UAEC was estimated at >98%. Cells at passage 3 from four separate animals each were 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 the test studies the data from these cells were indistinguishable from our previously published data on repassage 4 cells (Bird *et al.* 2000, Di *et al.* 2001).

Ca^{2+} imaging

Passage 4 cells were grown in 35 mm glass-bottom microwell dishes for 1–3 days before being used for experiments. Cells were incubated in 5 μM of Fura2-AM (Molecular Probes Inc., Eugene, OR, USA) with 0.05% Pluronic F127 (Molecular Probes Inc.) dissolved in 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. The cells were washed with Krebs buffer, covered in 2 ml Krebs buffer, and incubated for 30 min to allow complete ester hydrolysis. Then the cells were removed from the incubator and all of the subsequent steps were performed at room temperature. The cells were washed and covered with 1 ml Krebs buffer. The dish was placed in the field of view and Fura2 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 the data were recorded for several individual, nontouching cells simultaneously for a total of 5 (ADP and UTP dose responses) or 50 (thapsigargin (Tg)) min using alternate excitation at 340 and 380 nm at 1 s intervals and measuring emitted light using a 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 $[\text{Ca}^{2+}]$ is calculated in real time using the InCyt Im2

software (Cincinnati, OH, USA). For all $[Ca^{2+}]_i$ imaging experiments, data were recorded for 30 s before agonist stimulation to establish the basal $[Ca^{2+}]_i$. For ADP and UTP dose responses, an initial 5-min recording was performed using 100 μ M 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 dose of the agonist. The dish was once again rinsed with buffer and allowed to recover before one more 5-min recording with 100 μ M ATP plus 100 μ M ADP or 100 μ M UTP to ensure the cells were still viable. 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 μ M EGTA, pH 7.4) and left in 1 ml Ca^{2+} -free Krebs.

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 with ice-cold PBS and the cells were scraped in 500 μ l 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 μ M leupeptin and 5 μ M aprotinin, pH 7.5) and sonicated. Solubilized cell lysates were then clarified at 500g for 10 min and the supernatant was used for Western blot analysis. Protein concentrations of all samples were determined using the bicinchoninic acid assay (Sigma).

Table 1 Antibodies used for Western blot analysis

Primary Antibody	Company	Catalogue number	Dilution	Secondary Antibody	Company	Catalogue number	Dilution
P2X1	Alomone Labs	APR-001	1:500	Donkey α Rabbit	Amersham	NA9340	1:2000
P2X2	Alomone Labs	APR-003	1:500	Donkey α Rabbit	Amersham	NA9340	1:2000
P2X7	Alomone Labs	APR-002	1:500	Donkey α Rabbit	Amersham	NA9340	1:2000
P2Y1	Alomone Labs	APR-004	1:500	Donkey α Rabbit	Amersham	NA9340	1:2000
P2Y2	Alomone Labs	APR-010	1:500	Donkey α Rabbit	Amersham	NA9340	1:2000
P2Y4	Alomone Labs	APR-006	1:500	Donkey α Rabbit	Amersham	NA9340	1:2000
P2Y6	Alomone Labs	APR-011	1:400	Donkey α Rabbit	Amersham	NA9340	1:2000
P2Y11	Alomone Labs	APR-015	1:100	Donkey α Rabbit	Amersham	NA9340	1:4000
G α -1	Calbiochem	371720	1:2000	Donkey α Rabbit	Amersham	NA9340	1:2000
G α i-1/2	Calbiochem	371723	1:1000	Donkey α Rabbit	Amersham	NA9340	1:2000
G α i-3	Calbiochem	371729	1:1000	Donkey α Rabbit	Amersham	NA9340	1:2000
G α i-3/o	Calbiochem	371726	1:2000	Donkey α Rabbit	Amersham	NA9340	1:2000
G α i-o	Santa Cruz	sc-387	1:200	Donkey α Rabbit	Amersham	NA9340	1:2000
G α q	Calbiochem	371752	1:2000	Donkey α Rabbit	Amersham	NA9340	1:2000
G α s	Calbiochem	371732	1:1000	Donkey α Rabbit	Amersham	NA9340	1:2000
G α z	Calbiochem	371741	1:2000	Donkey α Rabbit	Amersham	NA9340	1:2000
IP3R1	Alomone Labs	ACC-019	1:1000	Donkey α Rabbit	Amersham	NA9340	1:4000
IP3R2	Santa Cruz	Sc-7278	1:1000	Donkey α Goat	Santa Cruz	sc-2020	1:20 000
IP3R3	Beckton Dickinson	610312	1:1000	Sheep α Mouse	Amersham	NA9310	1:2500

Alomone Labs, Jeusalem, Israel, Santa Cruz Biotechnology, Santa Cruz, CA, USA; Beckton Dickinson, San Jose, CA, USA.

SDS-PAGE

A broad range 'rainbow' molecular weight marker (Amersham), seven NP-UAEC samples and nine P-UAEC samples 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 primary antibodies, shown in Table 1, for 2 h at room temperature. The membrane was then incubated with horse radish peroxidase-conjugated secondary antibodies (Table 1) 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 densitometry from all immunoneutralizable bands was combined for analysis.

Statistical analysis

Each treatment in $[Ca^{2+}]_i$ imaging of Fura2 loaded cells was replicated on at least three separate occasions, where multiple individual cells were monitored simultaneously. Student's *t*-test or one-way ANOVA was used to analyze the data as appropriate. $P < 0.05$ was considered significant.

Results

To functionally determine which purinergic receptor was responsible for the ATP-induced initial transient peak, we first examined the dose dependency of the initial $[Ca^{2+}]_i$ response to ADP and UTP. When the cells were treated with

Figure 1 ADP- and UTP-induced $[Ca^{2+}]_i$ responses. Cells were loaded with Fura2 and imaged as described. ADP or UTP was added 30 s after recording commenced and data were collected for 5 min. The maximal $[Ca^{2+}]_i$ over the 5-min recording period was used as a measure of activation. (A) Dose dependency of ADP-induced $[Ca^{2+}]_i$ response in P- and NP-UAEC. *Significant difference from previous, lower dose, $P \leq 0.05$. (B) Comparison of the 100 μM ATP alone $[Ca^{2+}]_i$ response with 100 μM ADP alone or the combination treatment of 100 μM ATP and 100 μM ADP. *Significant difference from ATP alone treatments, $P \leq 0.05$. (C) Dose dependency of UTP-induced $[Ca^{2+}]_i$ response in P- and NP-UAEC. *Significant difference from previous, lower dose, $P \leq 0.05$. (D) Comparison of the 100 μM ATP alone $[Ca^{2+}]_i$ response with 100 μM UTP alone or the combination treatment of 100 μM ATP and 100 μM UTP. Changes not significant at $P \leq 0.05$.

1, 3, 10, 30, 100 and 300 μM ADP in random order, there was a dose-dependent rise in $[Ca^{2+}]_i$ as measured by the maximal amplitude of the initial transient peak between 3 and 100 μM in the P-UAEC and between 3 and 300 μM in the NP-UAEC (Fig. 1A). The response to 100 μM ADP was significantly lower than the response to 100 μM ATP in both the P- and NP-UAEC (Fig. 1B). The rise in $[Ca^{2+}]_i$ after 100 μM ADP was added simultaneously with 100 μM ATP was not additive in P-UAEC, but the combined treatment was significantly increased above 100 μM ATP alone for the NP-UAEC (Fig. 1B). Also, only 32.3% of the cells that responded to 100 μM ATP responded to 100 μM ADP.

The Ca^{2+} mobilization induced by UTP was nearly identical in P- and NP-UAEC such that 3 μM UTP caused a significant rise in $[Ca^{2+}]_i$ and there was a dose-dependent increase through 100 μM (Fig. 1C). Figure 1D shows that the response to 100 μM UTP, or 100 μM UTP plus 100 μM ATP was not significantly different from the 100 μM ATP alone responses. In addition, the individual cells that responded to ATP also responded to UTP.

In addition to the functional study, we independently evaluated the expression levels of purinoceptors for which

antibodies are commercially available including P2X1, P2X2, P2X7, P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 (Fig. 2A). In some cases, multiple bands were detected, but this was not unexpected and in each case the bands indicated by molecular weight in figures were also immunoneutralized by the appropriate peptide (data not shown). All of these receptors were detected in both the P- and NP-UAEC whole cell lysates with the exception of P2Y4. However, we were able to use the same antibody to detect an immunoneutralizable band for P2Y4 in COS-7 cells. Therefore, we believe that this negative result is valid (data not shown). While there was some variation in the expression levels of the proteins in cells isolated from different animals, there was no overall significant difference in the expression levels of these proteins between cell preparations from pregnant and nonpregnant ewes (Fig. 2B).

Subsequently, Western blot analysis was performed for other key proteins, which may be involved in the series of events leading to the ATP-induced $[Ca^{2+}]_i$ response. Of the G-protein α subunits that were evaluated ($G\alpha i-1$, $G\alpha i-1/2$, $G\alpha i-3$, $G\alpha i-3/o$, $G\alpha i-o$, $G\alpha q$, $G\alpha s$, $G\alpha z$), the data show that both the P- and NP-UAEC equally expressed $G\alpha i-1$ and possibly $G\alpha i-2$ (Fig. 3).

Figure 2 Equal expression of purinergic receptors. Cell preparations from pregnant ewes and nonpregnant ewes were lysed, run on a 7.5% gel and probed for various purinoceptors. (A) Western blots showing the expression levels of each protein in the individual preparations of P- and NP-UAEC. For P2X1 and P2Y6 all the bands indicated with a molecular weight were immunoneutralizable (data not shown). (B) Densitometry was performed on the Western blots shown. There was animal variation, but no consistent or significant difference between P- and NP-UAEC preparations at the $P < 0.05$ significance level. $n = 9$ for P-UAEC and $n = 7$ for NP-UAEC. Mean $\pm$ S.E.M.

Both cell types also showed similar levels of $G\alpha i-3$ expression, while $G\alpha i-o$ was lacking in both (Fig. 3). $G\alpha i-o$ was detected using the same antibody in a G-protein standard (bovine brain extract) as well as in whole cell lysates from HUVEC-C and

COS-7 cells (data not shown). $G\alpha s$, $G\alpha z$ and $G\alpha q$ were also expressed in both cell types. $G\alpha z$ was uniformly expressed in all of the cell preparations regardless of pregnancy status. All of the other G-proteins tested showed some degree of variation

Figure 3 Equal expression of G-protein subunits by Western blot analysis. Cell preparations from pregnant ewes and nonpregnant ewes were lysed, run on 12% gels and probed for the various G-proteins. (A) Western blots showing the expression levels of each protein in the individual preparations of P- and NP-UAEC. Both the P-UAEC and NP-UAEC express Gαi-1 and possibly Gαi-2. These cells also express Gαi-3, but not Gαi-o. In addition, the cells contain Gαq, Gαs, and Gαz. (B) Densitometry was performed on the Western blots shown. $n=9$ for P-UAEC and $n=7$ for NP-UAEC. Mean $\pm$ S.E.M. No significant differences were seen between the P- and NP-UAEC preparations at the $P<0.05$ significance level.

between animals, but as was noted for the purinergic receptors, this did not correlate to one group over the other (Fig. 3B). The expression of all three known isoforms of the IP3R was also assessed. All three isoforms of the IP3R were present in the P- and NP-UAEC (Fig. 4A). The expression levels of IP3R1 and IP3R2 were relatively uniform between P- and NP-UAEC. The expression of IP3R3 did vary between animals; however, this overall variation did not show consistent or significant differences between P- and NP-UAEC (Fig. 4B). We then sought to evaluate classical CCE in these cells by functional assay using Tg. Cells were stimulated with Tg in media containing 2 mM Ca^{2+} in order to ascertain the typical response of each cell type (Fig. 5A). Stimulation by Tg in Krebs buffer containing 2 mM Ca^{2+} resulted in $[\text{Ca}^{2+}]_i$ mobilization in 100% of P- and NP-UAEC with an increase

in $[\text{Ca}^{2+}]_i$ clearly distinguishable from basal. Figure 5A shows the average response of all the cells tested and that the resulting sustained phase was both unusually long and higher in P- than NP-UAEC. The experiments were then repeated in Ca^{2+} -free media. While not every cell responded to Tg in Ca^{2+} -free Krebs buffer, the majority ($>93\%$) of both P- and NP-UAEC did. Figure 5C shows that the first portion of the average response to Tg in Ca^{2+} -free media was almost identical in P- and NP-UAEC but the response in the P-UAEC came down more quickly, causing a significant difference in the area under the curve. Next, in order to demonstrate that CCE occurs in UAEC, the cells were initially stimulated with Tg in Ca^{2+} -free media and when the $[\text{Ca}^{2+}]_i$ fell back to the basal level, Ca^{2+} was reintroduced. The average induction of CCE was clearly greater in the P- than NP-UAEC (Fig. 5E and F). Likewise, more P-UAEC

Figure 4 No difference in the expression of IP3R subtypes. Distinct cell preparations from pregnant ewes and nonpregnant ewes were lysed, run on 7.5% gels and probed for each of the IP3R. (A) Western blots showing all three isoforms of the IP3R present in the P- and NP-UAEC. (B) Densitometry was performed on the Western blots shown. The expression levels of IP3R1 and IP3R2 were consistent between the P- and NP-UAEC. The expression levels of IP3R3 varied from cell preparation to cell preparation, yet no differences were detected between P- and NP-UAEC at the $P < 0.05$ significance level. $n = 9$ for P-UAEC and $n = 7$ for NP-UAEC. Mean $\pm$ S.E.M.

exhibit CCE than did NP-UAEC (83.5 vs 72.6% respectively).

In order to determine if there was a role for IP3R in initiating CCE, 2APB (an IP3R antagonist and a putative store operated channel (SOC) inhibitor) was used in conjunction with Tg. When the cells were treated with 2APB before Tg, the subsequent response for the majority of the cells (78.5% P-UAEC, 84.0% NP-UAEC) appeared similar to that of the Tg response in Ca^{2+} -free media, even though Ca^{2+} was present in the 2APB experiments (Fig. 6). In comparison with the Tg response in Ca^{2+} -free media, the response after pretreatment with 2APB in the P-UAEC was further suppressed (Fig. 6B), while in the NP-UAEC it was not significantly different (Fig. 6D). In the remaining cells, either the cells did not respond to Tg or the suppression of Tg-induced response was released. Only 6% of NP-UAEC was able to overcome 2APB inhibition compared with 21.5% of P-UAEC.

Effects of 2APB were subsequently further tested by its application shortly before or after Ca^{2+} replacement. In both P- and NP-UAEC, 2APB caused immediate inhibition of CCE when added after the Ca^{2+} was reintroduced (Fig. 7). For these experiments, the graph represents the cells in which CCE was initiated and the suppression by 2APB was not

overcome (60.6% of P-UAEC and 70.9% of NP-UAEC). Cells that did not show CCE were obviously excluded because our aim was to study the differential mechanism of CCE regulation in the two cell types. As in the last set of experiments, more P-UAEC were able to overcome 2APB inhibition than NP-UAEC, 33.3 and 5.3% respectively.

Discussion

The UAEC model is a primary culture model of uterine artery endothelial cell function that retains pregnancy-specific differences, including vasodilator production, kinase activation and Ca^{2+} signaling, through passage 4 (Bird *et al.* 2000, Di *et al.* 2001, Gifford *et al.* 2003). Agonists are typically more capable of inducing nitric oxide and prostacyclin production in P-UAEC than in NP-UAEC. Similar results were also seen regarding kinase activation, wherein all of the agonists tested (TPA, AII, ATP, bFGF, EGF and VEGF) could generally induce ERK-1/2 phosphorylation in the P-UAEC, while AII was unable to stimulate a significant increase in ERK-1/2 phosphorylation in the NP-UAEC (Bird *et al.* 2000, Gifford *et al.* 2003). Moreover, the amount of ERK-1/2

Figure 5 Verification that the P- and NP-UAEC are capable of CCE. Changes in $[Ca^{2+}]_i$ were recorded in P-UAEC (solid squares or black bar) and NP-UAEC (open circles or white bar) for up to 60 min. The scatter plots represent every tenth data point of the average $[Ca^{2+}]_i$ response. The bar graphs represent the area under the curve (arbitrary units). *Significant difference from NP-UAEC, $P \leq 0.05$. (A) Cells were treated with $10 \mu M$ Tg 30 s after the experiment began and the response was recorded for 60 min. Note that in P-UAEC, $[Ca^{2+}]_i$ does not fall back to the basal level during the entire hour of recording while in NP-UAEC $[Ca^{2+}]_i$ fell back to basal at approximately 45 min. (B) Area under the curve since Tg addition as shown in (A). (P-UAEC: $n=81$ over three experiments, NP-UAEC: $n=51$ over three experiments). (C) Cells were treated with $10 \mu M$ Tg 30 s after recording was initiated in Ca^{2+} -free Krebs and the response was recorded for 30 min. The response in the P- and NP-UAEC is very similar. (D) Area under the curve since Tg addition for (C) (P-UAEC: $n=124$ over seven experiments, NP-UAEC: $n=79$ over five experiments). (E) Cells were treated with $10 \mu M$ Tg in Ca^{2+} -free media at 30 s 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 1000 s (16.67 min). (F) Area under the curve since Ca^{2+} addition in (E) (P-UAEC $n=84$ over six experiments, NP-UAEC: $n=46$ over four experiments). Mean $\pm$ S.E.M.

phosphorylation increased more in P-UAEC as compared with NP-UAEC, with one exception. EGF induced more ERK-1/2 phosphorylation in NP-UAEC than in P-UAEC. U0126 (a MEK inhibitor) was able to block this phosphorylation. Another difference involved Ca^{2+} signaling in 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 $[Ca^{2+}]_i$, and marked the beginning of a prolonged sustained phase. NP-UAEC exhibits the initial peak, but the beginning of the sustained phase was consistently lower

Figure 6 The effects of 2APB on the Tg-induced $[Ca^{2+}]_i$ response. The black line or bar represents the results for P- or NP-UAEC copied from Fig. 5E and F for reference purposes. Open circles or white bar represent the mean $\pm$ S.E.M. for the response to 10 μ M Tg in media containing 2 mM Ca^{2+} after a 3 min pretreatment with 50 μ M 2APB. The bar graph shows the area under the curve since Tg stimulation. An asterisk (*) denotes significant difference from Tg in Ca^{2+} -free media treatment, $P \leq 0.05$. (A) and (B) are the mean responses in P-UAEC. (C) and (D) are the mean responses in NP-UAEC (P-UAEC: $n=51$ over three experiments, NP-UAEC: $n=42$ over four experiments).

(Di *et al.* 2001). This cell model and the acute $[Ca^{2+}]_i$ response in the cell model has more recently been further validated by direct comparison with freshly isolated uterine artery endothelial cells derived from pregnant and non-pregnant ewes (Gifford *et al.* 2003). In this study, we investigate further the underlying basis for the more sustained $[Ca^{2+}]_i$ response in cells from pregnant ewes by considering the possibility of altered coupling of different classes of purinergic receptor and associated signaling proteins.

Our immediate questions were which receptor functionally controls the ATP-induced Ca^{2+} mobilization in the P- and NP-UAEC, do differences in protein expression levels account for the differences in Ca^{2+} signaling, and do the cells exhibit CCE? Numerous functional studies have been performed and have demonstrated that the various isoforms of purinergic receptors have different selectivity for adenine versus uracil nucleotides, or indeed for mono-, di-, or triphosphate forms of these nucleotides. Based on this, we initially compared the well-characterized ATP-induced $[Ca^{2+}]_i$ response of the P- and NP-UAEC (Gifford *et al.* 2003) to the ADP and UTP dose responses in order to further identify the purinoceptor responsible for the ATP-induced $[Ca^{2+}]_i$ response (reviewed by Kunapuli & Daniel 1998, Ralevic & Burnstock 1998, Vassort 2001).

Each experiment included a 100 μ M ATP stimulation so that a direct comparison could be made between the ADP (or UTP) response and ATP response in an individual cell and to reveal whether the same population of cells responded to ADP (or UTP) as to ATP. The data show that the amplitude of the 100 μ M ADP-induced initial peak response was $\sim 25\%$ of that of the 100 μ M ATP-induced $[Ca^{2+}]_i$ response in both P- and NP-UAEC (Fig. 1B). As such we conclude that P2Y1 is not involved in the ATP-induced response because P2Y1 receptors are known to be much more responsive to ADP than they are to ATP. Likewise, while P2Y4 and P2Y6 are considered uracil selective, P2Y4 is equally responsive to ATP and ADP, and P2Y6 is more responsive to ADP than ATP. Therefore, they are unlikely to be functional in the ATP-induced $[Ca^{2+}]_i$ response. On the other hand, both P2Y2 and P2Y11 are more responsive to ATP than to ADP. As a result, by simply comparing the 100 μ M ADP response to the 100 μ M ATP response, P2Y2 and P2Y11 emerge on the shortlist of the most likely candidates to be mediators of the ATP-induced $[Ca^{2+}]_i$ response.

In both the P- and NP-UAEC, fewer cells responded to ADP than to ATP, but those cells that did respond to ADP also responded to ATP. Thus, the cells that responded to ADP were not a distinct group of cells compared with those that responded to ATP. Each experiment also contained a

Figure 7 Effects of 2APB on the Tg-induced $[\text{Ca}^{2+}]_i$ response. (A)–(D) the black line or bar represents the average response of the P- or NP-UAEC, to 10 μM Tg in Ca^{2+} -free Krebs followed by the reintroduction of Ca^{2+} copied from Fig. 5E and F. The light gray line or bar represents the response of the cells to Tg in Ca^{2+} -free media, followed by 2APB treatment as soon as the $[\text{Ca}^{2+}]_i$ fell back to basal and then Ca^{2+} introduction 3 min later. The dark gray line or bar represents the average response of the cells to Tg in Ca^{2+} media, followed by Ca^{2+} addition 3 min after the $[\text{Ca}^{2+}]_i$ fell back to basal and then 2APB inhibition 2 min later. The bar graphs represent the area under the curve since Ca^{2+} addition. An asterisk (*) denotes significant difference from the Tg Ca^{2+} -free, Ca^{2+} results while a plus sign (+) denotes significant difference from Tg Ca^{2+} -free, 2APB, Ca^{2+} , $P \leq 0.05$. (A and B). Graphs of the mean Ca^{2+} response for each type of experiment in P-UAEC. (C and D) Graphs of the mean Ca^{2+} response for each type of experiment in NP-UAEC (P-UAEC: Tg Ca^{2+} -free, 2APB, Ca^{2+} $n=86$ over three experiments; Tg Ca^{2+} -free, Ca^{2+} , 2APB $n=83$ over five experiments/NP-UAEC: Tg Ca^{2+} -free, 2APB, Ca^{2+} $n=103$ over six experiments; Tg Ca^{2+} -free, Ca^{2+} , 2APB $n=66$ over six experiments). Mean $\pm$ S.E.M.

combined treatment of 100 μM ADP (or 100 μM UTP) plus 100 μM ATP because we hypothesized that if the ADP (or UTP) and ATP responses were additive, the data would imply that two distinct receptors were functioning. In P-UAEC, when the combined ATP and ADP treatment was compared with ATP alone, results were very straightforward. The combined treatment was not significantly different from the ATP alone treatment, indicating that the same receptors on the same cells were being bound by ADP and ATP (Fig. 1B). In NP-UAEC, the response to combined treatment of ATP plus ADP did appear to be additive, compared to ADP alone and ATP alone treatments (Fig. 1B). However, these results may be deceiving and in reality may not indicate that there are distinct sets of receptors for ADP and ATP on the NP-UAEC. Rather, referring back to the ADP dose response (Fig. 1A), the response to 300 μM ADP is significantly higher than the response to 100 μM ADP for NP-UAEC. Thus, this increased response to the combined treatment of ATP plus ADP is likely indicative of the higher molarity of the combined treatment, rather than distinct groups of receptors. This is further supported by the fact that the combined ATP

plus ADP treatment in the P-UAEC is lower than the ATP alone treatment, consistent with the dose response, wherein the rise in $[\text{Ca}^{2+}]_i$ after 300 μM ATP stimulation is lower, albeit not significantly lower, than the 100 μM dose.

The same type of $[\text{Ca}^{2+}]_i$ imaging experiment was performed using UTP as the agonist in order to continue isolating which purinergic receptor was functional in these cells. This time, the dose response was nearly identical for P- and NP-UAEC, and the same cells that responded to ATP responded to UTP (Fig. 1C). In addition, the combined ATP plus UTP treatment did not cause a rise in $[\text{Ca}^{2+}]_i$ that was significantly different from the ATP alone response (Fig. 1D). Thus, the same cells and the same receptors respond to ATP and UTP. Moreover, the peak rise in $[\text{Ca}^{2+}]_i$ in response to 100 μM UTP was not significantly different from the 100 μM ATP-induced response (Fig. 1D). Since P2Y₁₁ is adenine nucleotide selective and the $[\text{Ca}^{2+}]_i$ response to UTP should have been much lower than the ATP response if it were mediating Ca^{2+} mobilization, P2Y₁₁ is unlikely to be the mediator. On the other hand, P2Y₂ is equally responsive to ATP and UTP and more responsive to tri-phosphate

nucleotides than to di-phosphate nucleotides, which is consistent with the lower response to ADP than ATP. Therefore, these data combined with the ADP dose-response data, strongly imply that P2Y2 is the functional purinergic receptor that is predominantly responsible for ATP-induced Ca^{2+} mobilization in both P- and NP-UAEC.

Independent Western blot analysis and immunoneutralization studies confirmed the presence of P2Y2 (and several other purinergic receptors) in P- and NP-UAEC preparations from many sheep (Fig. 2). Together with our pharmacological data, this expression data, and previous data (Di *et al.* 2001) imply that the P2Y2 receptor is indeed the receptor functionally coupled to Ca^{2+} mobilization in the UAEC model. It is interesting nonetheless that the level of P2Y2 or indeed other classes of purinergic receptors were unaltered by pregnancy so that it seems unlikely that more sustained $[\text{Ca}^{2+}]_i$ responses in P-UAEC are due to changes in purinergic receptor expression. It is also noteworthy to point out that P2Y11 was expressed in many P-UAEC and NP-UAEC preparations and we believe that this is the first description of P2Y11 in ovine tissue (Fig. 2). P2Y11 was expressed at the same molecular weight as in a HUVEC-CS positive control and the band was immunoneutralizable. Furthermore, this band resolved at the same molecular weight as was shown for P2Y11 in human umbilical vein endothelial cells by Wang *et al.* (2002).

Having established the likely receptor subtype candidates for coupling to PLC, we further considered whether variation in expression level of one of the downstream signaling proteins required for Ca^{2+} mobilization might provide an alternate explanation for the difference in the sustained $[\text{Ca}^{2+}]_i$ response seen between the P- and NP-UAEC. The expression levels of several heteromeric G-protein α subunits were evaluated and this revealed that while most G-proteins tested showed some degree of variation between animals, this did not correlate to one group over the other (Fig. 3). The equal expression of $\text{G}\alpha_q$ was significant because when ATP binds to a P2Y receptor in many cells, it activates PLC through $\text{G}\alpha_q$, and this in turn leads to the production of IP3 (Ralevic & Burnstock 1998, Communi *et al.* 2000, Costanzi *et al.* 2004). Thus, $\text{G}\alpha_q$ likely played a critical role in the induction of the initial transient peak in $[\text{Ca}^{2+}]_i$ in UAEC but its role was similar for both cell types. This is indeed consistent with our previous findings that there is no significant difference in PLC activation between P- and NP-UAEC (Di *et al.* 2001). Therefore, we also evaluated the possible pregnancy-specific alterations in expression of IP3R isoforms. All three isoforms of the IP3R were present in both the P- and NP-UAEC and variation in expression was not between P- and NP-UAEC and could not explain the previously described differences in sustained phase of the $[\text{Ca}^{2+}]_i$ response (Fig. 4).

The aforementioned experiments allowed the determination of the functional purinoceptor responsible for the initial $[\text{Ca}^{2+}]_i$ response, but did not explain the differential sustained phase observed in the P- versus NP-UAEC; therefore,

another set of experiments was designed to examine at a more fundamental level the nature of the cells responsiveness independently of receptor class. Treatment with Tg, an inhibitor of the Ca^{2+} -ATPase on the ER which prevents reuptake of Ca^{2+} resulting in a rise in $[\text{Ca}^{2+}]_i$, as the ER is depleted of Ca^{2+} , is classically used to identify CCE. Cells are stimulated with Tg in Ca^{2+} -free media and when the $[\text{Ca}^{2+}]_i$ falls back to basal levels, extracellular Ca^{2+} is re-introduced. If there is a resulting increase in $[\text{Ca}^{2+}]_i$, it is indicative of CCE (Putney 1986). Initial experiments in media containing 2 mM Ca^{2+} immediately suggested there was a difference in CCE between NP- and P-UAEC since $[\text{Ca}^{2+}]_i$ did not fall back to the basal level during the entire hour of recording in the P-UAEC, while the $[\text{Ca}^{2+}]_i$ in the NP-UAEC returned to the basal level at approximately 45 min (Fig. 5A). The experiments were then performed in Ca^{2+} -free media and revealed that only a small portion of the Tg-induced response was due to release of Ca^{2+} from the intracellular stores but the amount of Ca^{2+} mobilization was relatively uniform in P- and NP-UAEC (Fig. 5C and D). Thus, we would conclude it is not a difference in initial intracellular pool size that is different but a difference in subsequent Ca^{2+} entry in P-UAEC when Tg is applied with extracellular Ca^{2+} present. Subsequently, the classical test (Fig. 5E) revealed that both P- and NP-UAEC were capable of exhibiting true CCE but, consistent with the initial two experiments, the amount of CCE exhibited by P-UAEC on re-addition of Ca^{2+} was significantly higher than in NP-UAEC (Fig. 5F). Therefore, these data confirm that the pregnancy-specific difference in Ca^{2+} signaling at the level of more sustained Ca^{2+} influx is due to the enhanced ability of P-UAEC to exhibit CCE.

In order to further elucidate the mechanism underlying the differential CCE, Tg was then used in conjunction with 2APB. Originally, 2APB was defined as a membrane permeant IP3R antagonist (Maruyama *et al.* 1997) but since that time it has become apparent that 2APB not only inhibits IP3Rs but also inhibits SOC function as well as altering function of other proteins and channels in a number of different cell types (reviewed by Bootman *et al.* 2002). Thus, experiments using 2APB must be planned carefully and data analyzed cautiously in order to determine what exactly occurs. Previous work in our laboratory verified that when UAEC were treated with 2APB prior to ATP stimulation, 2APB alone did not cause Ca^{2+} mobilization at any dose tested (0.1–100 μM , data not shown), so some of the specificity problems cited elsewhere do not apply to UAEC. To verify that 2APB was inhibiting SOC function independent of agonist-stimulated IP3, the cells were pretreated with 2APB and then stimulated with Tg in the presence of extracellular Ca^{2+} . The results of this set of experiments appeared very similar to those seen when the cells were treated with Tg in Ca^{2+} -free media, implying that Tg treatment caused the depletion of the intracellular pool of Ca^{2+} but 2APB inhibited CCE by directly or more likely indirectly (at the level of IP3R) inhibiting the activation of SOC (Fig. 6). Further experiments also showed that when the

cells were treated with Tg in Ca^{2+} -free media and then treated with 2APB just before Ca^{2+} addition, no CCE was seen (Fig. 7). Likewise, treatment with 2APB after Ca^{2+} addition caused an immediate decrease in $[\text{Ca}^{2+}]_i$ (Fig. 7). Thus, in the UAEC model, 2APB is at least capable of inhibition of SOC and thus CCE.

In summary, ADP and UTP dose responses, protein expression data, along with our previous data, demonstrated that P2Y2 is likely the functional purinergic receptor in both P- and NP-UAEC. Furthermore, we were able to demonstrate that P- and NP-UAEC express P2X1, P2X2, P2X7, P2Y1, P2Y2, P2Y6, P2Y11, $\text{G}\alpha\text{i}-1$, $\text{G}\alpha\text{i}-3$, $\text{G}\alpha\text{s}$, $\text{G}\alpha\text{q}$, $\text{G}\alpha\text{z}$, IP3R1, IP3R2 and IP3R3. The expression levels of these proteins did not vary significantly between P- and NP-UAEC, therefore the variation in Ca^{2+} signaling cannot be easily explained by pregnancy-induced changes in protein expression level. Yet, the variation in Ca^{2+} signaling could be explained by an intrinsic difference in the ability of P- vs NP-UAEC to exhibit CCE. Furthermore, the fact that 2APB was capable of inhibiting CCE under these conditions suggests that SOC, perhaps under the control of IP3R, plays a role in the variation in Ca^{2+} signaling and further, it is a difference in SOC protein expression levels or functional coupling that underlies pregnancy adaptation at the level of sustained phase of the $[\text{Ca}^{2+}]_i$ response.

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