

## RESEARCH PAPER

# Inhibitory effect of caffeine on pacemaker activity in the oviduct is mediated by cAMP-regulated conductances

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## BACKGROUND AND PURPOSE

Spontaneous electrical activity, termed slow waves, drives rhythmic, propulsive contractions in the smooth muscle of the oviduct (myosalpinx). Myosalpinx contractions cause egg transport through the oviduct. Agents that disrupt slow wave pacemaker activity will therefore disrupt myosalpinx contractions and egg transport. Caffeine is commonly used as a ryanodine receptor agonist and has been previously associated with delayed conception. Here we assessed the effects of caffeine on pacemaker activity in the murine myosalpinx.

## EXPERIMENTAL APPROACH

The effects of caffeine on electrical pacemaker activity were studied using intracellular microelectrode and isometric force measurements on intact oviduct muscle preparations. Responses to caffeine were compared with responses caused by 3-isobutyl-1-methylxanthine (IBMX) and forskolin.

## KEY RESULTS

Caffeine caused hyperpolarization of membrane potential and inhibited slow wave generation and myosalpinx contractions. The effects of caffeine could be mimicked by the  $K_{ATP}$  channel agonist pinacidil and antagonized by the  $K_{ATP}$  channel antagonist glibenclamide. Caffeine is known to inhibit cyclic nucleotide phosphodiesterases (PDEs), leading to an increase in cytosolic cAMP and stimulation of downstream cAMP-dependent mechanisms. The effects of caffeine were mimicked by the PDE inhibitor, IBMX, and the adenylyl cyclase activator forskolin. These effects were also reversed by glibenclamide.

## CONCLUSIONS AND IMPLICATIONS

These results suggest that caffeine activates  $K_{ATP}$  channels in oviduct myosalpinx. Since caffeine abolishes slow waves and associated contractions of the myosalpinx, it would have a negative effect on egg transport through the oviduct and may contribute to the documented delayed conception in women consuming caffeinated beverages.

## Abbreviations

2-APB, 2-aminoethyl diphenylborinate; IBMX, 3-isobutyl-1-methylxanthine; ICC-OVI, oviduct interstitial cells of Cajal; IP<sub>3</sub>, inositol 1,4,5 trisphosphate;  $K_{ATP}$ , ATP-sensitive potassium channel; Kir, inward rectifier potassium channel; KRB, Krebs-Ringer bicarbonate; PDE, phosphodiesterase; PKA, cAMP dependent protein kinase; RMP, resting membrane potential; RyR, ryanodine receptor; SUR2A/2B, sulphonylurea receptor 2A/2B

## Introduction

Caffeine has been reported to be 'the most frequently ingested pharmacologically active substance in the world'

(Nawrot *et al.*, 2003). Dietary sources of caffeine include coffee, tea, soft drinks and chocolate as well as several over-the-counter medications. A number of epidemiological studies have linked caffeine consumption to delayed

conception (Hatch and Bracken, 1993; Stanton and Gray, 1995; Bolumar *et al.*, 1997). A European multicentre study on women aged 25–44 years found that those who consumed  $\geq 501$  mg of caffeine daily while trying to conceive had an increase in the waiting time until their first pregnancy of 11% (Bolumar *et al.*, 1997). A USA conducted study found that women who drank over 300 mg of caffeine a day had a 27% lower chance of conceiving for each cycle compared with women who did not consume caffeine (Hatch and Bracken, 1993). A statistically significant progressive decrease in fertility has also been reported with increasing caffeine consumption in nonsmoking women, suggesting a negative dose-dependent effect of caffeine on conception (Jensen *et al.*, 1998). To provide some perspective, the caffeine content of brewed coffee is 56–100 mg·100 mL<sup>-1</sup>, instant coffee and tea contains 20–73 mg·100 mL<sup>-1</sup> and cola contains 9–19 mg·100 mL<sup>-1</sup> (Nawrot *et al.*, 2003).

A factor linked to infertility is compromised transport of oocytes through the fallopian tubes (Croxatto, 2002). In the mouse, oviducts display spontaneous electrical activity termed slow waves (Dixon *et al.*, 2009). This activity has been recorded from oviducts of several species including guinea pigs (Talo and Hodgson, 1978; Parkington, 1983), baboons (Talo and Hodgson, 1978) and humans (Kishikawa and Kuriyama, 1981). Recently, we demonstrated that slow waves drive the rhythmic contractions of the oviduct smooth muscle termed the myosalpinx, which are essential for egg transport through the oviduct (Dixon *et al.*, 2009). In the same study, we showed that slow waves are generated by a specialized population of pacemaker cells, referred to as oviduct interstitial cells of Cajal (ICC-OVI). Thus, any agent that reduces pacemaker activity can negatively affect contractions of the myosalpinx, delay egg transport through the oviduct and reduce fertility.

Caffeine is a widely used drug for studies of smooth muscle tissues and is known to have, at a minimum, the following three major effects: (i) stimulation of Ca<sup>2+</sup> release from ryanodine receptor (RyR)-operated stores (Berridge, 1993); (ii) inhibition of IP<sub>3</sub> receptors (Parker and Ivorra, 1991) and (iii) inhibition of cyclic nucleotide phosphodiesterases (PDEs), resulting in increased cAMP and enhanced downstream cAMP-dependent mechanisms (Butcher and Sutherland, 1962; Beavo, 1995). We have found that the effects of caffeine on oviduct muscles are unaffected by ryanodine, suggesting that these effects are not mainly due to the stimulant effects of the drug on Ca<sup>2+</sup> release from RyR-operated stores (Dixon *et al.*, unpubl. obs.). Furthermore, in contrast to the hyperpolarization caused by caffeine, the IP<sub>3</sub> receptor antagonist 2-aminoethyl diphenylborinate (2-APB) caused depolarization (Dixon *et al.*, unpubl. obs.). Thus, the effects of caffeine are unlikely to be due to inhibition of IP<sub>3</sub> receptors. In the present study, we tested whether the effects of caffeine on the oviduct are consistent with its action as an inhibitor of PDEs in smooth muscles.

In several previous studies of visceral smooth muscles, increased cAMP resulted in hyperpolarization and inhibition of spontaneous electrical and mechanical activity (von der Weid, 1998; von der Weid and van Helden, 1996; Morales *et al.*, 2004). The adenylyl cyclase activator forskolin and other substances known to elevate cAMP inhibit spontaneous contractions in canine colon and antral smooth muscle

(Huizinga *et al.*, 1991; Ozaki *et al.*, 1992; Smith *et al.*, 1993), hyperpolarize membrane potential and reduce frequency and amplitude of slow waves in murine small intestine (Koh *et al.*, 2000). Similarly, the cAMP analogue 8-bromo-cAMP, forskolin, caffeine and cyclic nucleotide PDE inhibitors have all been shown to reduce slow wave frequency in guinea pig antrum (Tsugeno *et al.*, 1995). Studies on individual smooth muscle cells from gastrointestinal muscles have shown that increased cAMP leads to activation of glibenclamide-sensitive K<sub>ATP</sub> channels via cAMP-dependent protein kinase A (PKA) phosphorylation of the channel (Zhang *et al.*, 1994a,b). We tested the hypothesis that caffeine might cause hyperpolarization of oviduct muscles and inhibit slow waves and contractions by activation of K<sub>ATP</sub> channels.

## Methods

### Ethical approval

An established in-house colony of BALB/c mice, originally set up using breeder pairs purchased from the Jackson Laboratory (Bar Harbor, MN, USA) was used for the present study. Female mice, 8–16 weeks old, were selected from the colony and humanely killed using isoflurane (Baxter, Deerfield, IL, USA) anaesthetic inhalation prior to cervical dislocation. The animals were maintained, and experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The Institutional Animal Use and Care Committee at the University of Nevada approved all procedures used.

### Tissue preparation

Immediately after cervical dislocation, the abdomen was cut open, and the oviducts were removed and placed in Krebs-Ringer bicarbonate solution (KRB). The oviducts were then uncoiled by sharp microdissection and cut free of the ovaries. Care was taken to dissect the intramural segment free of the uterine horn. The final preparations used for electrophysiology experiments were intact (infundibulum to uterotubal junction), uncoiled oviducts.

For reverse transcription PCR (RT-PCR), the above procedure was performed, and uncoiled oviducts were then cut open along their length to reveal the mucosal surface. The mucosa was removed and discarded. The remaining oviduct myosalpinx was then cut into three pieces, which were considered to approximate (i) the infundibulum and ampulla, (ii) the isthmus and (iii) the intramural segment. This approximation was necessary as the ampullary isthmus junction (AIJ) cannot be structurally distinguished in mice. Oviduct myosalpinx segments were then frozen in liquid nitrogen and stored at –80°C until needed.

### Drugs and solutions

Electrophysiology recording chambers were constantly perfused (at a rate of 3 mL·min<sup>-1</sup>) with oxygenated KRB containing (mM): NaCl, 120.35; KCl, 5.9; NaHCO<sub>3</sub>, 15.5; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; MgCl<sub>2</sub>, 1.2; CaCl<sub>2</sub>, 2.5; and glucose, 11.5. KRB was bubbled with 97% O<sub>2</sub>-3% CO<sub>2</sub> at 37 ± 0.5°C to maintain pH at 7.3–7.4. Glibenclamide, caffeine, pinacidil and IBMX (3-isobutyl-1-methylxanthine) were purchased from

Sigma-Aldrich (St Louis, MO, USA). Forskolin was purchased from Calbiochem (San Diego, CA, USA). To make stock solutions, drugs were dissolved in H<sub>2</sub>O, ethanol or dimethyl sulphoxide as per manufacturer's recommendations. To achieve the desired final concentration, appropriate volumes of stock solution were diluted in KRB before being perfused at a rate of 3 mL·min<sup>-1</sup>.

### Electrophysiological and isometric force measurements

For electrophysiology experiments, oviducts were pinned to the Sylgard-elastomer (Dow Corning, Midland, MI, USA) lined base of a recording chamber with fine tungsten pins (120 µm diameter) and left to equilibrate for at least 1 h prior to commencement of experiments. Pinning facilitated intracellular impalements as it stabilized the tissue and minimized movements of the muscle. Impalements of smooth muscle cells were made using sharp glass microelectrodes filled with 3 M KCl with tip resistances of 80–120 MΩ. All impalements were made at locations ~50% along the oviduct length (isthmus segment). Transmembrane potentials were recorded with a high impedance electrometer (Axoclamp 2B, Axon Instruments/Molecular Devices, Sunnyvale, CA, USA). Data were recorded onto a PC via a Digidata 1322A system and Axoscope 9.2 (Axon Instruments). In other experiments, the myosalpinx was isolated using tungsten pins, and the opposite end of the oviduct was attached to an isometric force transducer (Gould-Statham UC3, Gould-Statham, Oxnard, CA, USA) for the simultaneous measurement of intracellular electrical activity and/or phasic contractile activity.

### Statistical analysis

Data analysis and measurements of resting membrane potential (RMP), slow wave amplitude, frequency, 1/2 maximal duration and rate of rise (dV/dt max) were performed using Clampfit 9.0 (Axon Instruments). Statistical analyses and data summary were performed using SigmaStat and SigmaPlot

(Systat Software Inc., Chicago, IL, USA) software. Student's paired *t*-tests or one-way ANOVAs with *post hoc* Tukey tests were used to calculate statistical significance. *P*-values < 0.05 were considered significant. In the results section, all data are expressed as means ± SEM, and *n* numbers refer to the number of oviducts on which experiments were performed, with each oviduct from a different animal. Figures were constructed in Corel Draw 13.0 (Corel Inc., Mountain View, CA, USA).

### RT-PCR

Total RNA from BALB/c oviduct and brain was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA was treated with 1 U·µL<sup>-1</sup> DNase I (Promega, Madison, WI, USA). First-strand cDNA was prepared from 1 µg RNA using SuperScript II<sup>®</sup> reverse transcriptase (Invitrogen) in a 20 µL reaction containing 25 ng oligo dT<sub>(12-18)</sub> primer, 1 µL 10 mM dNTP, 5X first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>) and 0.1 M dithiothreitol. PCR was performed with specific primers for *Kcnj8* and *Kcnj11* encoding Kir 6.1 and Kir 6.2 potassium channels and *Abcc9* encoding the modulatory subunits for the sulphonylurea receptor (SUR) 2A and 2B, on 2 µL cDNA using AmpliTaq Gold<sup>®</sup> PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The PCR primers used are described in Table 1. Primers were designed to span intronic sequences to eliminate amplification of contaminating genomic DNA in the source RNA. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for cDNA integrity. No-template PCR reactions served as controls for primer contamination. PCR reactions were performed in a GeneAmp 2700 thermal cycler (PE Applied Biosystems). The amplification profile was: 95°C for 10 min to activate the AmpliTaq polymerase, then 35 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 30 s, followed by a final step of 72°C for 5 min. RT-PCR amplification fragments were analysed by size analysis on 2% agarose gels alongside a 100-base pair (bp) marker.

**Table 1**

RT-PCR primers used in this study

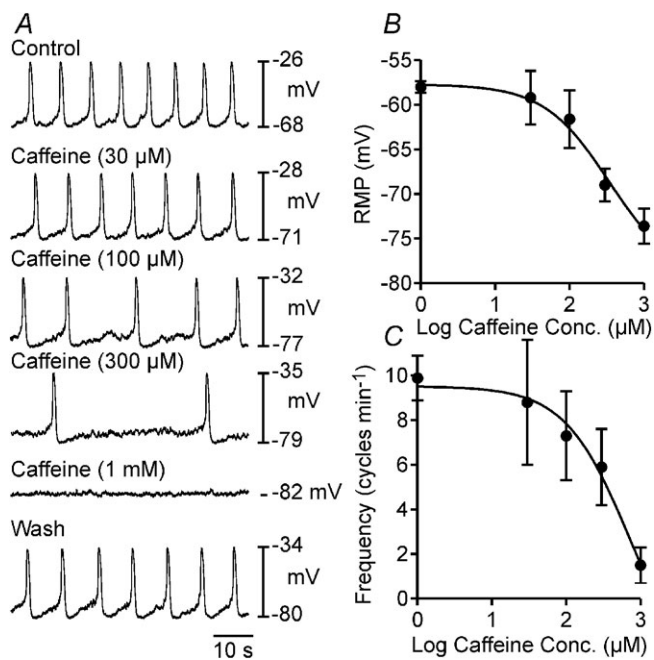
Target transcript	Accession number	Oligonucleotide sequences (5'-3') (+): sense (-): antisense	Fragment length (bp)
Gapdh	NM_008084	GTCTTCACCACCATGGAGA (+) AAGCAGTTGGTGGTGCAG (-)	190
<i>Kcnj8</i> (Kir6.1)	NM_008428	CTCTCCCTCATGGTCCCAAGGTG (+) CGGAACTCAATGAAGTGTCTCCGGA (-)	190
<i>Kcnj11</i> (Kir6.2)	NM_010602	TCTGCTTTCAGCTCCCATGGGCAG (+) TGGAGTTCTGGGCAAGGACACCACA (-)	200
<i>Abcc9</i> (SUR2A)	NM_021041	CCAGCGTGATCTGCTCCGACAAGTG (+) CTGCACGGACAAACGAGGCAAACAC (-)	167
<i>Abcc9</i> (SUR2B)	NM_021041	AGTCATGACAGCCTTTGCGGATCGC (+) TGCACGGACAAACGAGGCAAACACT (-)	172

All primers were designed against *Mus musculus* GenBank sequences. *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase).

## Results

### Effects of caffeine on slow wave activity and associated myosalpinx contractions

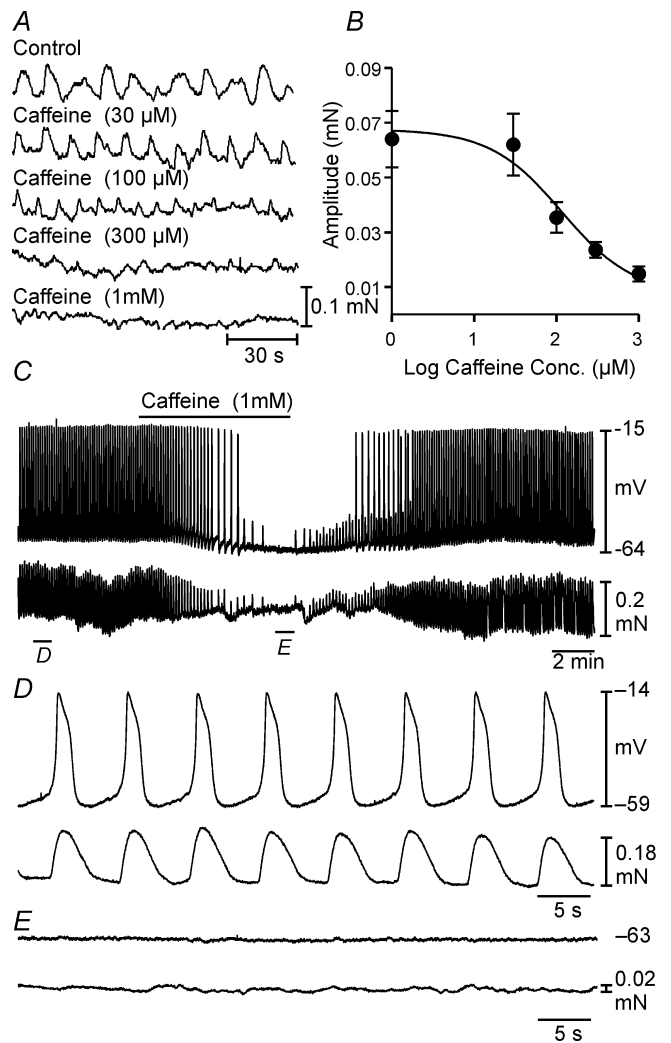
Application of caffeine (10  $\mu\text{M}$ –1 mM) to the myosalpinx caused a dose-dependent membrane hyperpolarization and a reduction in oviduct myosalpinx pacemaker frequency (Figure 1). For example, 300  $\mu\text{M}$  and 1 mM caffeine caused membrane hyperpolarization from an average of  $-58 \pm 2$  mV under control conditions ( $n = 13$ ) to  $-69 \pm 4$  mV ( $n = 6$ ; 300  $\mu\text{M}$ ) and to  $-74 \pm 4$  mV ( $n = 5$ , 1 mM; one-way ANOVA,  $P = 0.025$ ). Slow wave frequency decreased from  $9.9 \pm 1$  under control conditions to  $5.9 \pm 1.7$  cycles $\cdot\text{min}^{-1}$  in 300  $\mu\text{M}$  and to  $1.5 \pm 0.8$  cycles $\cdot\text{min}^{-1}$  in 1 mM caffeine ( $n = 5$ ;  $P < 0.001$ ). At concentrations higher than 1 mM (i.e. 5 mM), caffeine caused membrane hyperpolarization from  $-55 \pm 3$  mV to  $-68 \pm 2$  mV ( $n = 7$ ;  $P < 0.001$ ) and completely inhibited pacemaker activity. We therefore chose concentrations of 1 and 5 mM to test the mechanisms underlying the caffeine inhibition of myosalpinx slow waves in additional experiments. The effects of caffeine on pacemaker activity were completely reversible upon washout of the drug. Caffeine also



**Figure 1**

Effects of caffeine on myosalpinx pacemaker activity. (A) Shows the dose-dependent effects of caffeine on oviduct slow wave activity. Caffeine at concentrations between 30 and 300  $\mu\text{M}$  hyperpolarized RMP and reduced the frequency of slow waves. Application of 1 mM caffeine produced further hyperpolarization of membrane potential and abolished slow wave activity. Upon washout of caffeine, slow wave activity returned. (B and C) A summary of data on the effects of caffeine on RMP (B) and slow wave frequency (C) as a function of caffeine concentration ( $n = 5$ ). The  $\text{EC}_{50}$  for caffeine on oviduct RMP and slow wave frequency was 337 and 713  $\mu\text{M}$  respectively. RMP, resting membrane potential.

caused dose-dependent inhibition of phasic myosalpinx contractions (Figure 2A,  $n = 5$ ); these effects are summarized in Figure 2B. Simultaneous intracellular microelectrode and isometric force recordings revealed that reduction and inhibition in electrical slow waves produced by caffeine caused the reduction and inhibition in phasic contractile activity (Figure 2C–E).



**Figure 2**

Effects of caffeine on myosalpinx contractions. (A) The dose-dependent effects of caffeine on oviduct phasic contractions. (B) A summary of the effects of caffeine on contraction amplitude as a function of caffeine concentration ( $n = 5$ ). The  $\text{EC}_{50}$  for caffeine on phasic contractile amplitude was 116  $\mu\text{M}$ . (C) The intracellular microelectrode (top trace) and myosalpinx contractions in the bottom trace before, during and after the application of caffeine (1 mM). (D) Slow waves were coupled on a 1:1 basis with myosalpinx contractions. Caffeine (1 mM) slowed slow wave frequency and hyperpolarized membrane potential (C; top trace). The reduction in slow wave frequency was accompanied by an associated reduction in the frequency of myosalpinx contractions (C; bottom trace). (E) After several minutes of exposure to caffeine, slow wave and contractile activity was completely abolished and returned upon washout.



### ATP-sensitive $K^+$ channel and sulphonylurea receptor subunit transcriptional expression in oviduct myosalpinx

The hyperpolarization observed upon application of caffeine suggested that either a depolarizing conductance (inward current) was inhibited or a hyperpolarizing conductance (outward current) was activated. We hypothesized that the inhibitory effects of caffeine might activate  $K_{ATP}$  channels because these channels are known to be activated by cAMP-dependent mechanisms in a variety of smooth muscles (Quayle *et al.*, 1994; Zhang *et al.*, 1994a; Wellman *et al.*, 1998).

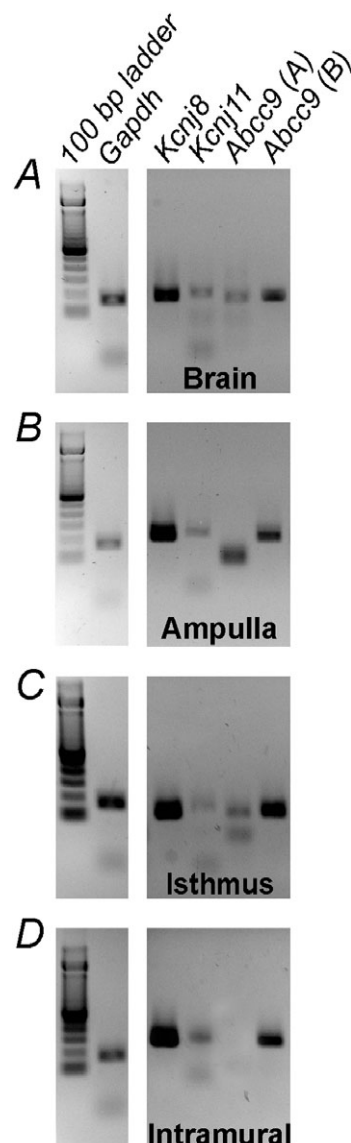
RT-PCR was performed on oviduct myosalpinx to examine the transcriptional expression of *Kcnj8*, *Kcnj11* and *Abcc9*, which encode  $K_{ATP}$  channels Kir6.1, Kir6.2 and sulphonylurea receptors (SUR; Figure 3). Transcripts encoding Kir6.1, Kir6.2 and SUR2B were found in all segments of the oviducts, whereas SUR2A was only present in the isthmus.

### Effects of $K_{ATP}$ channel blockade on slow waves

Glibenclamide (10  $\mu$ M) was used to selectively block  $K_{ATP}$  channels in oviduct preparations ( $n = 15$ ; Figure 4; Sanborn, 1995; Kubo *et al.*, 2005). Glibenclamide significantly depolarized myosalpinx membrane potential from  $-60 \pm 1$  mV under control conditions to  $-55 \pm 2$  mV in glibenclamide ( $P < 0.05$ ; Figure 4A–C). Frequency of slow waves increased from  $8.4 \pm 1.0$  cycles·min<sup>-1</sup> under control conditions to  $10.3 \pm 0.9$  cycles·min<sup>-1</sup> in glibenclamide; however, this did not reach statistical significance. Slow wave amplitude was significantly affected by glibenclamide, decreasing from  $41 \pm 1$  mV in control to  $35 \pm 2$  mV ( $P < 0.05$ ). The half-maximal duration of slow waves was unaltered ( $1.7 \pm 0.2$  s in control,  $2.1 \pm 0.4$  s in glibenclamide). The rate of rise of the upstroke was slightly reduced from  $175 \pm 17$  mV·s<sup>-1</sup> to  $143 \pm 11$  mV·s<sup>-1</sup> in glibenclamide, but this did not reach statistical significance ( $P = 0.12$ ). These results suggest that  $K_{ATP}$  channels probably contribute to the maintenance of RMP and may influence the frequency of slow waves in the oviduct myosalpinx.

### Inhibitory effects of caffeine were antagonized by glibenclamide

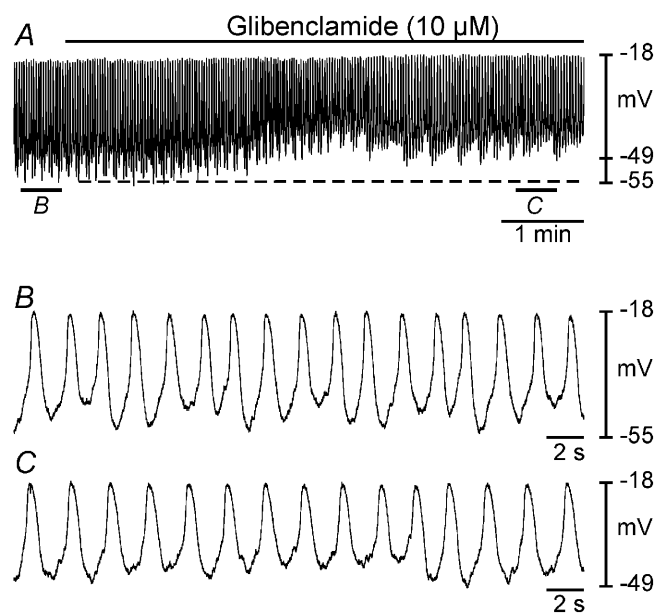
We further tested whether the effects of caffeine (1 and 5 mM) could be antagonized by glibenclamide (10  $\mu$ M). As above, glibenclamide (10  $\mu$ M) caused membrane depolarization from control averaging  $-60 \pm 1$  to  $-58 \pm 2$  mV ( $n = 10$ ; Figure 5A–C). In the continued presence of glibenclamide, caffeine still produced a transient hyperpolarization in membrane potential and reduction in slow wave activity. However, unlike the direct exposure to caffeine, in the presence of glibenclamide, membrane hyperpolarization and inhibition of myosalpinx slow waves often recovered within 5–10 min of exposure to caffeine (1 or 5 mM). For example, in the presence of glibenclamide, caffeine (1 mM) caused a similar membrane hyperpolarization as caffeine alone (i.e.  $-68 \pm 2$  mV (1 mM;  $n = 10$ ;  $P < 0.002$  compared with glibenclamide alone; Figure 5A, D). At 5 mM, caffeine produced an initial transient hyperpolarization to  $-67 \pm 2$  mV ( $P < 0.05$  compared with glibenclamide) and loss of slow waves (Figure 5A, E), but this was always followed by membrane



**Figure 3**

Transcriptional expression of ATP-sensitive  $K^+$  channels and sulphonylurea receptor subunits in the oviduct myosalpinx. RT-PCR showing transcriptional expression of *Kcnj8* and *Kcnj11* encoding the  $K_{ATP}$  channels Kir6.1, 6.2 and *Abcc9*, which encodes the sulphonylurea receptor (SUR) subunits SUR2A and 2B. Brain was used as a positive control for each channel transcript and *Gapdh* was used as a loading control. The gel was part of a complete analysis of potassium channel expression within the myosalpinx. Other potassium channels not relevant to this study have been excluded. NB. The large band in the *Abcc9* (A) lane of the ampulla gel was judged to be nonspecific as it was lower than the predicted fragment length of 167 bp.

depolarization to a membrane potential similar to glibenclamide alone (i.e. to  $-59 \pm 2$  mV;  $n = 10$ ;  $P = 0.776$ ; Figure 5A, F). In the presence of glibenclamide, caffeine reduced the frequency of slow waves from  $10 \pm 1$  to  $7 \pm 2$  cycles·min<sup>-1</sup> (1 mM;  $n = 5$ ;  $P < 0.05$ ) but did not inhibit them (Figure 5A, D). The reduction in frequency was less than with caffeine alone (i.e.  $1.5 \pm 0.8$  cycles·min<sup>-1</sup>). Caffeine (5 mM) caused an



**Figure 4**

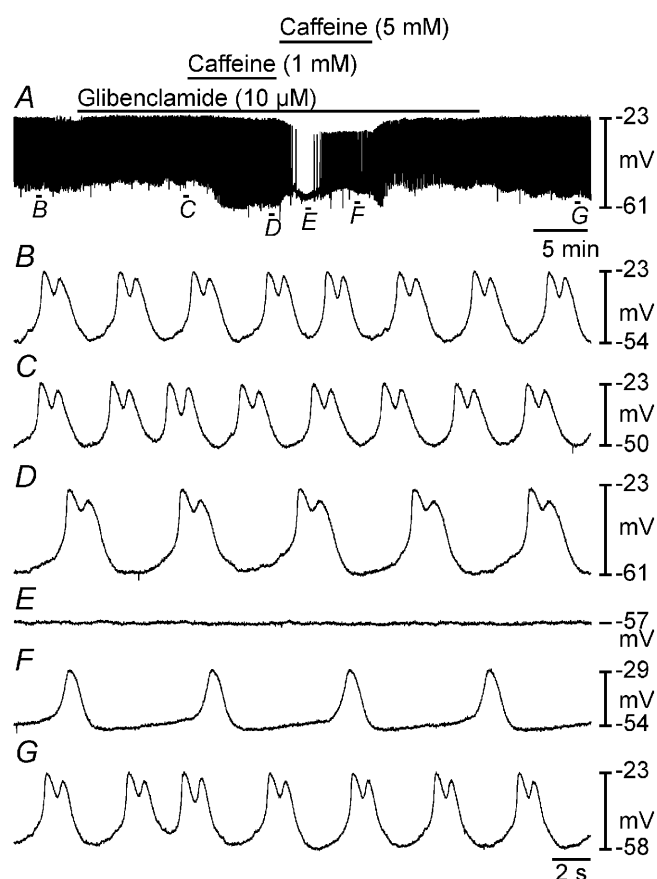
Effects of glibenclamide on slow wave activity. Intracellular microelectrode recordings (A–C) show the effects of the  $K_{ATP}$  channel antagonist glibenclamide ( $10 \mu\text{M}$ ) on oviduct slow wave activity. (A) A period of control slow wave activity followed by application of glibenclamide (black bar). (B and C) Expanded time scales of the sections indicated in (A). Glibenclamide caused membrane depolarization (dashed line), increased slow wave frequency, decreased slow wave amplitude and reduced rate of rise of the upstroke phase of slow waves.

initial inhibition (Figure 5A, E), but slow wave frequency recovered to  $5 \pm 3 \text{ cycles}\cdot\text{min}^{-1}$  in the continued presence of caffeine (Figure 5A, F) compared with caffeine alone ( $n = 4$ ;  $P < 0.05$  compared with glibenclamide alone). Slow wave amplitude recovered to control levels upon washout of caffeine and glibenclamide (Figure 5A, G).

In a separate series of experiments, oviduct myosalpinx was initially exposed to caffeine, and then glibenclamide was added in the continued presence of caffeine. Caffeine ( $5 \text{ mM}$ ) caused myosalpinx hyperpolarization from  $-57 \pm 3 \text{ mV}$  to  $-68 \pm 2 \text{ mV}$ ; ( $n = 11$ ;  $P < 0.002$ ; Figure 6), and spontaneous slow wave activity was abolished. Application of glibenclamide ( $10 \mu\text{M}$ ) in the continued presence of caffeine caused repolarization to  $-55 \pm 2 \text{ mV}$  (not statistically different from control,  $P = 0.45$ ) and restored myosalpinx slow wave activity (Figure 6). These data suggest that the caffeine-induced membrane hyperpolarization and inhibition of slow waves was at least in part due to activation of  $K_{ATP}$  channels.

### Effects of pinacidil on electrical slow waves

Since caffeine appeared to hyperpolarize the membrane by activating  $K_{ATP}$  channels, we examined the effects of the  $K_{ATP}$  channel opener pinacidil to see whether this would mimic the effect of caffeine. Pinacidil ( $10 \mu\text{M}$ ) hyperpolarized the membrane potential from a mean control level of  $-59 \pm 1.5 \text{ mV}$  to  $-71 \pm 2.8 \text{ mV}$  ( $n = 5$ ;  $P < 0.01$ ; Figure 7A–C) and

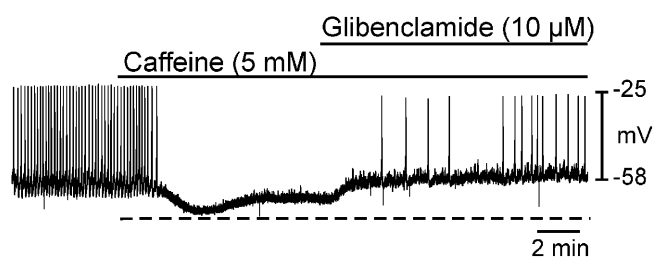


**Figure 5**

Glibenclamide partially antagonized the effects of caffeine on myosalpinx slow waves. (A) A period of control slow waves followed by application of glibenclamide ( $10 \mu\text{M}$ , black bar), which produced membrane depolarization and reduced slow wave amplitude. In the continued presence of glibenclamide, caffeine 1 and 5 mM were exogenously applied (as indicated by black bars). Caffeine ( $1 \text{ mM}$ ) caused membrane hyperpolarization and a reduction in slow wave frequency but did not reduce the amplitude of slow waves. Caffeine ( $5 \text{ mM}$ ) caused an initial transient hyperpolarization and loss of slow waves, but slow wave activity recovered in the continued presence of caffeine. Following washout of caffeine ( $5 \text{ mM}$ ), slow wave frequency recovered, and washout of glibenclamide membrane potential returned to control levels. (B–G) Expanded time scales of the sections indicated in (A).

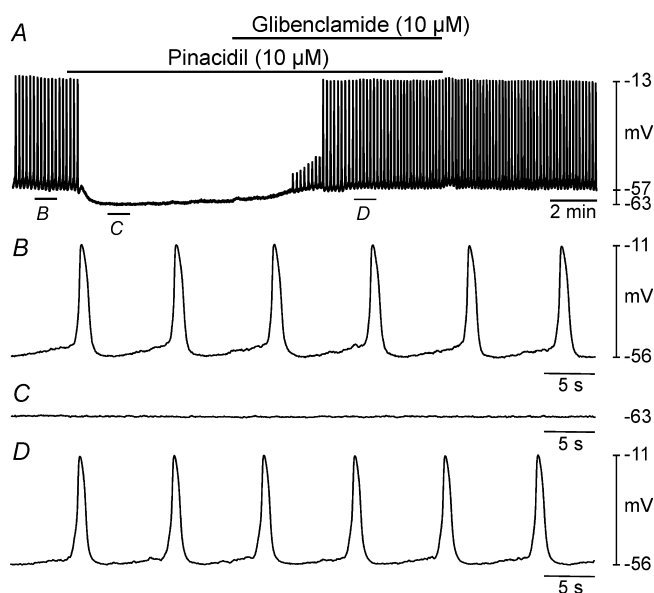
abolished slow wave activity in all tissues (Figure 7A, C). The effect of pinacidil was very similar to that of caffeine ( $-55 \pm 3 \text{ mV}$  in control to  $-68 \pm 2 \text{ mV}$  in  $5 \text{ mM}$  caffeine; i.e.  $12 \text{ mV}$  vs.  $13 \text{ mV}$  hyperpolarization).

If opening of  $K_{ATP}$  channels causes membrane hyperpolarization and abolishes slow wave activity, then closing them again should depolarize the membrane and slow wave generation should resume. To test this, we added the  $K_{ATP}$  channel antagonist glibenclamide ( $10 \mu\text{M}$ ) in the continued presence of pinacidil ( $10 \mu\text{M}$ ). Coapplication of pinacidil and glibenclamide caused the membrane potential to depolarize to  $-61 \pm 1.4 \text{ mV}$  (not statistically different from control RMP,  $P = 0.32$ ), and slow wave activity resumed ( $n = 5$ ; Figure 7A, D).



**Figure 6**

Glibenclamide reversed the inhibitory effects of caffeine on slow wave activity. Intracellular microelectrode recording showing the effects of caffeine and caffeine in the presence of glibenclamide on myosalpinx slow waves. Application of caffeine (5 mM; black bar) caused membrane hyperpolarization and abolished slow wave activity. In the continued presence of caffeine, application of glibenclamide (10  $\mu$ M; black bar) caused membrane depolarization and resumption of slow wave activity.



**Figure 7**

Inhibitory effects of pinacidil on slow waves were antagonized by glibenclamide. (A) A microelectrode recording showing the effects of pinacidil (10  $\mu$ M) on pacemaker activity and in the continued presence of pinacidil application of glibenclamide (10  $\mu$ M). (B–D) Faster sweep speed traces of the regions indicated in (A). Pinacidil caused membrane hyperpolarization and abolished slow waves (C). Upon addition of glibenclamide in the continued presence of pinacidil, membrane potential depolarized towards resting levels and slow wave activity resumed (D).

The depolarized membrane potential observed in experiments in which glibenclamide antagonized the effects of caffeine (i.e. RMP =  $-68 \pm 2$  mV in caffeine, RMP in caffeine and glibenclamide =  $-58 \pm 2$  mV, i.e. 10 mV depolarization) was compared with the membrane potential observed when glibenclamide antagonized the effects of pinacidil (i.e. RMP in pinacidil =  $-71 \pm 2.8$  mV, RMP in glibenclamide and pinacidil =  $-61 \pm 1.4$  mV, i.e. 10 mV depolarization). A Student's *t*-test

revealed that the difference between these groups was not statistically significant.

### Effects of the PDE inhibitor IBMX on slow wave activity

Caffeine and other xanthines inhibit cyclic nucleotide PDEs that mute or terminate cellular signalling mediated by cAMP (Butcher & Sutherland, 1962; Beavo, 1995). Inhibition of PDEs causes accumulation of cAMP and enhanced PKA activity. We hypothesized that caffeine may act via this mechanism because it is known that  $K_{ATP}$  channels are activated by PKA phosphorylation in visceral smooth muscles. We compared the effects of caffeine with the general PDE inhibitor, IBMX (10  $\mu$ M;  $n = 9$ ; Figure 8A). IBMX hyperpolarized cells from  $-66 \pm 2$  mV to  $-72 \pm 1$  mV ( $P < 0.001$ ) and reduced slow wave frequency from control averaging  $9.1 \pm 1.0$  cycles·min $^{-1}$  to  $1.7 \pm 0.5$  cycles·min $^{-1}$  ( $P < 0.001$ ). Slow wave generation was totally abolished by IBMX in three of nine preparations (Figure 8A, C). These effects are the same qualitatively as those observed in response to caffeine and were reversible upon washout of the drug (Figure 8A).

### Inhibitory effects of IBMX were antagonized by glibenclamide

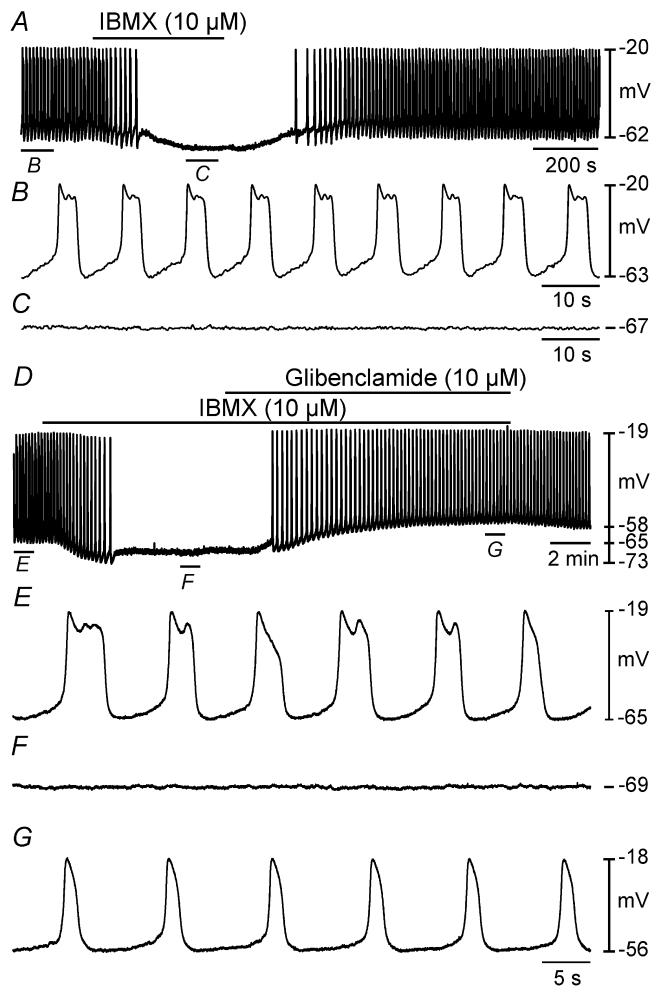
In the next series of experiments, we tested the effects of glibenclamide (10  $\mu$ M) on responses to IBMX. In the continued presence of IBMX, glibenclamide depolarized cells to  $-60 \pm 2$  mV, and slow waves were restored to a frequency not statistically different from control (e.g.  $7.7 \pm 1.4$  cycles·min $^{-1}$  in control,  $6.1 \pm 2.1$  cycles·min $^{-1}$  in IBMX and glibenclamide;  $n = 4$ ;  $P = 0.724$  one-way ANOVA; Figure 8D–G).

### Effects of the adenylyl cyclase stimulator forskolin on slow wave activity

We studied the effects of forskolin on electrical activity as a more direct test of the role of cAMP in the responses to caffeine. Forskolin (100 nM) hyperpolarized cells from  $-64 \pm 2$  mV to  $-74 \pm 1$  mV ( $P = 0.012$ ) and reduced slow wave frequency from  $8.9 \pm 1.1$  cycles·min $^{-1}$  to  $0.4 \pm 0.4$  cycles·min $^{-1}$  ( $n = 5$ ;  $P = 0.004$ ; slow waves were completely abolished in 4 out of 5 preparations; Figure 9A–C). Glibenclamide (10  $\mu$ M), added in the continued presence of forskolin (100 nM), repolarized cells to  $-65 \pm 3$  mV and restored slow waves ( $n = 4$ ; Figure 9D–G).

## Discussion

Data presented in the current study indicate that oviduct  $K_{ATP}$  channels are stimulated by caffeine in a cAMP-dependent manner. Caffeine hyperpolarized membrane potential, abolished slow waves and inhibited myosalpinx contractions. These effects were mimicked by the  $K_{ATP}$  channel opener pinacidil. Furthermore, the effects of caffeine could be reversed by the  $K_{ATP}$  channel inhibitor glibenclamide. The effects of the PDE inhibitor IBMX and the adenylyl cyclase activator forskolin mirrored those of caffeine and were also reversed by glibenclamide. Caffeine is best known as an agent which activates ryanodine receptors (Berridge, 1993), but it has also

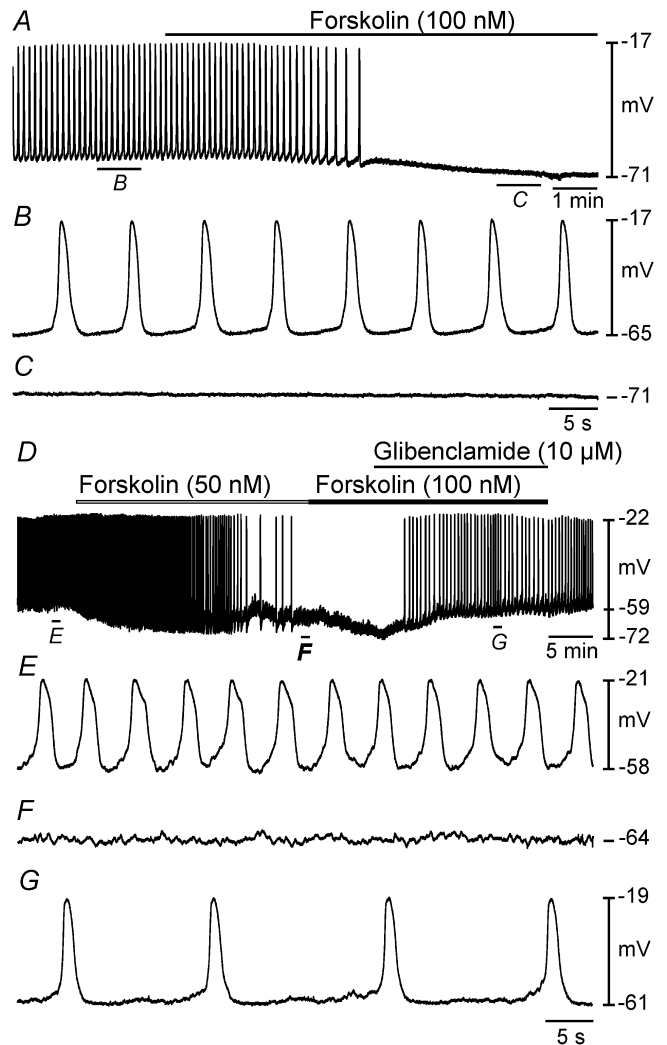


**Figure 8**

Inhibition of myosalpinx slow waves by IBMX was antagonized by glibenclamide. (A) An intracellular microelectrode recording showing the effects of the PDE inhibitor IBMX (10 μM) on slow wave activity. Application of IBMX caused membrane hyperpolarization and inhibited slow wave activity. (B and C) Indicate sections of the trace in (A) at a faster sweep speed. (D) Intracellular recording showing the effects of IBMX and coapplication with glibenclamide on slow waves. Application of IBMX (10 μM) caused significant membrane hyperpolarization and abolished slow waves. In the continued presence of IBMX, application of glibenclamide (10 μM) caused membrane depolarization and resumption of slow wave activity. (E, F, G) Indicate sections of the trace in (D) at a faster sweep speed. IBMX, 3-isobutyl-1-methylxanthine.

been reported to inhibit IP<sub>3</sub> receptors (Parker and Ivorra, 1991) and PDEs (Butcher and Sutherland, 1962; Beavo, 1995). The effects of caffeine are not altered by coapplication of ryanodine and are not mirrored by the IP<sub>3</sub> receptor blocker 2-APB (Dixon *et al.*, unpubl. obs.) and based on the results of the present study, the mechanism of action of caffeine on the oviduct myosalpinx must involve its known inhibitory action on PDEs rather than its action on ryanodine receptors.

Inhibition of PDEs leads to accumulation of cyclic nucleotides including cAMP, while activation of adenylyl cyclase catalyses the conversion of ATP to cAMP. In the present study,



**Figure 9**

Inhibitory effects of the adenylyl cyclase activator forskolin on slow wave activity was blocked by glibenclamide. (A) Intracellular microelectrode recording showing the effects of the forskolin (100 nM) on slow wave activity. Indicated sections of the trace in (A) are shown at a faster sweep speed in (B and C). Application of forskolin caused membrane hyperpolarization and abolished slow waves. (D) An intracellular recording showing the effects of forskolin and coapplication with glibenclamide on myosalpinx slow waves. Indicated sections of the trace in (D) are shown at a faster sweep speed in (E–G). Application of forskolin (50 nM) hyperpolarized membrane potential and reduced slow wave frequency. At a higher concentration, forskolin (100 nM) caused further membrane hyperpolarization and abolished slow wave generation (D). In the continued presence of forskolin (100 nM), application of glibenclamide (10 μM) caused membrane depolarization and restoration of slow wave activity (D, G).

caffeine was found to inhibit slow waves and associated myosalpinx contractions; this may be the result of elevated levels of cytosolic cAMP, consequential to caffeine's inhibitory effect on PDEs. Similarly, the PDE inhibitor IBMX also inhibited slow waves, as did the adenylyl cyclase activator forskolin, again resulting in elevated cAMP. Elevation in cytosolic cAMP can lead to activation of PKA which can phosphorylate



phospholamban, preventing it from inhibiting the SERCA pump (Eggermont *et al.*, 1988). Enhanced store refilling leads to increased frequency of calcium release events which can stimulate  $Ca^{2+}$ -activated  $K^+$  channels ( $K_{Ca}$ ) and promote membrane hyperpolarization and smooth muscle relaxation (Kim *et al.*, 2005). PKA has been reported to directly stimulate  $K_{Ca}$  channels (Meera *et al.*, 1995; Sanborn *et al.* 1998) and can also phosphorylate myosin light chain kinase (MLCK), which decreases its affinity for the calcium–calmodulin complex and inhibits smooth muscle contraction (Price and Bernal, 2001). Finally, in vascular smooth muscle tissues, PKA has been reported to phosphorylate specific serine and threonine residues on  $K_{ATP}$  channels, activating them (Quayle *et al.*, 1994; Wellman *et al.*, 1998; Quinn *et al.*, 2004; Shi *et al.*, 2007; 2008). The resultant hyperpolarization caused by the activation of  $K_{ATP}$  channels, shifts membrane potential out of the activation range for voltage-dependent  $Ca^{2+}$  channels and promotes relaxation. Since the effects of caffeine, IBMX and forskolin were mirrored by pinacidil and could all be reversed by the  $K_{ATP}$  channel inhibitor glibenclamide, this suggests that these agents all activate  $K_{ATP}$  channels and promote relaxation. This mechanism must be dependent on cAMP since all of these substances produce elevations in cytosolic cAMP. It remains to be determined whether cAMP stimulates  $K_{ATP}$  channels directly or whether its stimulation of PKA leads to  $K_{ATP}$  channel phosphorylation and subsequent activation of these channels. It is possible that other  $K^+$  channels, as well as  $K_{ATP}$ , may be activated by caffeine although additional conductances were not investigated in the present study.

It has been previously reported that slow waves provide the electrical basis for rhythmic contractions of the myosalpinx (Dixon *et al.*, 2009). In the present study, this concept has been proven again with an intracellular microelectrode recording and simultaneous isometric force measurement. Slow waves were coupled to phasic myosalpinx contractions on a 1:1 basis. Myosalpinx contractions have been found to be essential effectors of egg transport (Dixon *et al.*, 2009). The loss of slow wave activity and associated loss of contractions observed with caffeine suggests that this agent has the potential to impede egg transport along the oviduct. Several studies have reported a correlation between high consumption of caffeinated beverages and a delay in conception (Hatch and Bracken, 1993; Stanton and Gray, 1995; Bolumar *et al.*, 1997; Jensen *et al.*, 1998). The present study provides a possible explanation as to why elevated caffeine consumption could lead to delayed conception or infertility.

In conclusion, oviduct  $K_{ATP}$  channels are activated upon application of caffeine. This effect is mirrored by IBMX and forskolin, suggesting that it is cAMP-dependent. The hyperpolarization caused by the activation of  $K_{ATP}$  channels leads to inhibition of spontaneous myosalpinx contractions that are essential to egg transport along the oviduct, providing an intriguing explanation as to why women with high caffeine consumption often take longer to conceive than women who do not consume caffeine.

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## Conflicts of Interest

None.

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