The effect of cAMP on ion transport in Fallopian tube epithelial cells in vitro

Tariq Mahmood1, Ovrang Djahanbakhch1,4, David Burleigh2, John R. Puddefoot3 and Gavin P. Vinson3

1Academic Department of Obstetrics and Gynaecology, St Bartholomew’s and The Royal London School of Medicine, 4th Floor, Holland Wing, The Royal London Hospital, Whitechapel, London E1 1BB, 2The Department of Pharmacology and 3The Department of Biochemistry, Queen Mary College, London E1 4NS, UK

4To whom correspondence should be addressed. E-mail: O.B.Djahanbakhch@mds.qmw.ac.uk

The coupled movement of ions and water across epithelia determines the composition and volume of fluid present in the lumen of organs. The second messenger cAMP is important in effecting electrolyte and water transport in many transporting epithelia; however, its role in Fallopian tube transport is uncertain. We have conducted electrophysiological studies on Fallopian tube epithelial cell monolayers in Ussing chambers and have demonstrated that exogenously added cAMP and agents that generate its intracellular production results in an increase in short-circuit current consistent with the transport of net electrical charge from a basal to mucosal direction. In contrast to the known effects of ATP in this tissue, the increase in short-circuit current was not explicable in terms of electrogenic chloride secretion as it was not affected by the chloride channel inhibitors, 4-acetamido-4'-isothiocyanostilbene-2,2-disulphonic acid 1 mmol/l (SITS) and frusemide. Instead the current was reduced by the sodium channel inhibitor, amiloride, and was therefore, in part, explicable in terms of electrogenic Na+ absorption. These findings will enhance our understanding of the physiological mechanisms responsible for human Fallopian tubal fluid formation and composition.

Key words: cAMP/Fallopian tube/ion transport

Introduction

The Fallopian tube is the site of gamete transport and conditioning, fertilization, and preimplantation embryonic development. Not surprisingly, anatomical tubal disease accounts for a significant proportion of all cases of female infertility and ectopic pregnancy. Furthermore, tubal dysfunction with normal patency may be responsible for a proportion of cases of ‘idiopathic’ infertility. One important aspect of tubal physiology that could be subject to dysfunction and therefore result in infertility is tubal fluid production and composition. A better understanding of normal tubal fluid production and composition would improve our understanding of clinical tubal disease and allow the formulation of more physiological culture media for the culture of gametes in vitro.

Studies in the oviduct (Brunton and Brinster, 1971; Gott et al., 1988; Dickens et al., 1993) and the Fallopian tube (Dickens et al., 1995; Downing et al., 1997) have implicated a role for the nucleotide ATP, acting via purinergic receptors and increases in intracellular calcium, in stimulating chloride secretion. Water then follows the osmotic gradient created by chloride transport.

In addition to this mechanism of ion transport, epithelia can also transport ions in relation to increases in intracellular concentrations of the second messenger cAMP (Wong, 1988; Leung et al., 1995; Hwang et al., 1996; Chan et al., 1997). In electrophysiological studies using cultured rabbit oviductal cells under short-circuit conditions, cAMP and agents increasing intracellular cAMP, such as adrenergic agonists, have been shown to increase short-circuit current (SCC) implying electrogenic ion transport (Brunton, 1972). Similarly Leung et al., using mouse oviductal cells under short-circuit conditions, demonstrated an increase in SCC after stimulation with forskolin, due to an increase in cAMP-related chloride secretion (Leung et al., 1995). This chloride secretion is thought to occur via a chloride channel known as the cystic fibrosis transmembrane regulator (CFTR). A similar increase in SCC due to chloride transport was demonstrated after addition of ATP (Downing et al., 1997). In airway epithelia too, cAMP, forskolin and ATP increase SCC through electrogenic chloride secretion (Hwang et al., 1996). In contrast, Gott et al., using an in-situ vascular perfusion model of whole rabbit oviduct, demonstrated that cAMP and agents that increase its intracellular concentration result in a reduction in chloride secretion and fluid production (Gott et al., 1988). The authors suggested...
T. Mahmood et al.

therefore that the oviduct was unique amongst chloride-secreting epithelia. Subsequent studies with vascular perfusion models of rabbit oviduct (Dickens and Leese, 1994) and human Fallopian tubes (Tay et al., 1997) confirmed that cAMP decreased tubal fluid production. Paradoxically, however, both these studies demonstrated an increase in tubal fluid production after stimulation with isoprenaline and isoproterenol despite the fact that these agents increase intracellular cAMP concentrations. Furthermore, another study (Dickens and Leese, 1994) could not confirm the finding of Gott et al. (1988) that an increase in intracellular cAMP causes a reduction in chloride transport. Because of these uncertainties, we have studied the effect of forskolin and cAMP on ion transport across polarized human Fallopian tube epithelial cell monolayers under short-circuit conditions.

Materials and methods

Tissue collection
Healthy-looking Fallopian tubes were collected at various stages of reproductive life from women undergoing hysterectomy for benign conditions, after obtaining written consent and appropriate local ethical committee approval.

Fallopian tubes were collected into ice-cold minimum essential medium (MEM) with Earle’s salts and l-glutamine and supplemented with heparin (1.8 IU/ml; CP Pharmaceuticals, Wrexham, UK), streptomycin (50 µg/ml; Evans Medical, Surrey, UK), penicillin (100 IU/ml; Glaxo Laboratories, Middlesex, UK) and HEPES (10 mmol/l; Life Technologies, Paisley, UK) and processed immediately for cell culture.

Epithelial cell culture
The method of Fallopian tube epithelial cell culture and characterization was similar to that previously described (Dickens et al., 1993; Kervancioglu et al., 1995). The Fallopian tube was thoroughly rinsed in MEM with Earle’s salts and l-glutamine and supplemented with heparin, streptomycin, penicillin and HEPES and cut longitudinally along the anti-mesenteric border. Healthy 5 mm portions of mucosa from epithelial folds were excised from along the length of the Fallopian tube. Tissue explants were digested in Ca- and Mg-free Hanks’ balanced salt solution (Life Technologies) in tissue culture flasks containing 0.5% trypsin and 2.7% pancreatin (Sigma Chemical Co., Poole, Dorset, UK) for 1 h at 4°C followed by 1 h at room temperature. The enzyme medium was removed, MEM added and the culture flask shaken to release epithelial cells into suspension. The procedure was repeated and the suspensions centrifuged at 500 g for 5 min. The cells were washed in MEM and after centrifugation re-suspended in culture medium at a concentration of 10³ cells/ml.

The culture medium consisted of a 1:1 mix of Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient mix (Life Technologies) with streptomycin (Evans Medical; 50 µg/ml), penicillin (100 IU/ml; Glaxo Laboratories), 10% fetal bovine serum (Sigma) and 15 µg/ml of fungizone (Life Technologies). 250 µl of cell suspension was plated onto Snapwell clear polyester filters (12 mm diameter, effective insert growth area 1 cm², 0.4 µm pore size) which had previously been coated with type IV collagen (Sigma) and sterilized by overnight exposure to UV light, and cultured at 37°C, 100% humidity and 5% CO₂/95% air in 6-well plates (Corning Costar, High Wycombe, Bucks, UK). Cell growth was observed periodically under a phase contrast inverted microscope until confluence was attained and cultures were then processed for electrophysiological studies. The culture medium was changed after 24 h and thereafter every 48 h until confluence was attained between 4 and 9 days.

Immunofluorescence for cytokeratin and vimentin
Immunofluorescence was performed on parallel cultures grown on glass slides, with cytokeratin and vimentin antibodies, to determine the percentage of epithelial cells in cultures. These cells were subjected to the same culture conditions as cells grown on filters.

Cells were fixed in a fresh mixture of equal parts of methanol and acetone for 2 min and washed in Tris-buffered saline (TBS; 50 mmol/l Tris, 150 mmol/l NaCl, pH 7.6). Non-specific sites were blocked with normal rabbit serum (1:5 dilution in TBS for 20 min; Dako). After a 60 min incubation with mouse monoclonal anticytokeratin or antivimentin antibodies (1:100 dilution in TBS; Amersham International plc., Bucks, UK) followed by washing for 5 min in TBS, the slides were incubated with fluorescein isothiocyanate (FITC)- conjugated antimouse IgG antiserum (1:20 dilution in TBS; Dako) for 30 min. Excess antibody was removed as above and the cells were examined by fluorescence microscopy. All experiments included control dishes in which the primary antibody was omitted.

Electrophysiological studies
Transepithelial electrophysiological properties of cultured Fallopian tube cells were studied by mounting Snapwell inserts containing confluent monolayers of cells between two modified perspex Ussing half-chambers (chamber volume, 1 ml) (Ussing and Zerahn, 1950). The principle of this electrophysiological assessment of ion transport is to eliminate any chemical gradient across the cell cultures by adding equal concentrations of Krebs–Henseleit solution to either side of the culture and to eliminate any electrical gradient (potential difference) by voltage clamping the cell culture to zero (also known as short-circuiting) so that no potential difference now exists across it. In this state, the current that flows (the short-circuit current, SCC) can only be the net result of all the active transport processes occurring across the cell culture. Stimulation of the cell monolayer with the test substance under these conditions will result in a change in the SCC only if the test substance induces the net transport of charged ions in one direction. This stimulated current is called the increased SCC or ISC. It must be noted that transport of electro-neutral species or of equally charged ions moving in opposite directions will not be detected as there is no net transfer of charge. An assessment of the potential difference between the apical and basal aspects of the cell culture can be made before voltage clamping (the open-circuit potential difference).

After mounting Snapwell inserts containing confluent monolayers of cells into the Ussing chambers, the cells were bathed on both sides with 10 ml of equal concentrations of modified Krebs–Henseleit solution with the following composition: NaCl 118 mmol/l, NaHCO₃ 25 mmol/l, glucose 11.5 mmol/l, KCl 4.7 mmol/l, MgCl₂ 2.56 mmol/l, KH₂PO₄ 1.19 mmol/l, CaCl₂ 2.53 mmol/l. The Krebs–Henseleit solution was gassed with 95% O₂/5% CO₂ and maintained at 37°C.

Prior to any electrical measurements and addition of test substances, the Krebs’ solution bathing either side of the epithelial cell layer was changed several times to remove any cell debris and culture medium and the current was allowed to reach a stable reading. At this point a record of the open-circuit potential difference (mV) was made. The tissue was then voltage-clamped to zero with a DVC-1000 dual voltage high impedance clamp (World Precision Instruments, Sarasota, FL, USA) and the baseline SCC was measured in µA/cm² and continuously recorded using a Linseis flat bed 4 channel recorder (Linseis, Cambridge, UK). The voltage clamp was intermittently
raised to 1 mV every 5 min for 20 s and the change in current used to calculate the resistance (Ω) using Ohm’s law.

All chemicals used were dissolved in either fresh Krebs’ solution or dimethyl sulphoxide (DMSO). In preliminary experiments, neither DMSO nor Krebs’ solution alone affected baseline SCC or baseline resistance at the volumes and concentrations used.

Concentrated solutions of the test substances forskolin (10 µmol/l) and dibutyryl cAMP (1 mmol/l) were added in small volumes (maximum, 1% of chamber volume) to the basal aspect of the polarized cell preparation and their effect on the active transport of ions was inferred from the associated change in SCC (ISC). To determine the actual current-carrying species, inhibitors of specific membrane-bound transporters were used. If the SCC induced by the test substance (due to net ion flow) is eliminated by pretreatment with a particular ion transport inhibitor, then the identity of that ion can be indirectly inferred. The direct determination of the current carrying species, however, requires measurement of ion flux, for example by using radioisotopes. The following specific membrane ion channel inhibitors (Sigma) were used: amiloride 100 µmol/l (inhibitor of the Na+/H+ antiport), 4-acetamidostilbene-2,2-disulphonic acid 1 mmol/l (SITS, inhibitor of the Cl-/HCO3- exchanger), tetraethylammonium chloride 25 mmol/l (TEA, non-specific K+ channel blocker), frusemide 100 µmol/l (Na+/K+/Cl- co-transport inhibitor). In separate experiments, cell monolayers were pretreated (apical and basal) with these inhibitors for at least 30 min before forskolin or dibutyryl cAMP and the effect on SCC recorded.

Statistics
Results are expressed as means ± SEM. *P < 0.05 was considered significant (paired and unpaired Student’s t-tests and analysis of variance).

Results
Fallopian tube cell cultures were confluent after 4–9 days in culture and predominantly (>90%) epithelial in nature. Transmission electron microscopy demonstrated polarized cell growth on collagen-coated filters with the basal side in contact with the collagen layer and apical surface covered with microvilli (Figure 1). Cell cultures were excluded from study if the basal resistance was below 50 Ω, if the resistance deteriorated significantly during experimentation, or if a stable reading of SCC could not be obtained. The baseline SCC, resistance and open-circuit potential difference of 61 confluent Fallopian tube epithelial cell cultures were 7.3 ± 0.7 µA/cm², 234 ± 19.2 Ω and 2.31 ± 0.35 mV respectively.

Effect of forskolin and cAMP on baseline short-circuit current
The addition of forskolin (which generates intracellular cAMP by stimulating the enzyme adenylate cyclase, 10 µmol/l) to the basal aspect of cell cultures produced a rapid increase in SCC of 9.2 ± 2.2 µA/cm². This typically reached a peak within 60 s, quickly returned to a baseline which was well above pretreatment levels, then oscillated around this baseline with decreasing amplitude (Figure 2). Preincubation of cell cultures with amiloride 100 µmol/l for 30 min reduced, but did not completely abolish, the increase in SCC seen after addition of forskolin, whereas preincubation with either 1 mmol/l SITS, 25 mmol/l TEA or 100 µmol/l frusemide did not prevent the increase in SCC (Table I). Of interest was the fact that SITS actually accentuated the increase in SCC seen after addition of forskolin with a 408% increase in SCC as compared to 144% with forskolin alone (*P < 0.05).

To determine if exogenously added cAMP resulted in similar electrophysiological changes, dibutyryl cyclic AMP (1 mmol/l, which is lipid soluble and therefore crosses the cell membrane) was added to five cell cultures with and five cell cultures without amiloride pretreatment. Addition of dibutyryl cyclic AMP to cell cultures resulted in an increase in SCC of similar characteristic and magnitude to that seen after the addition of forskolin. This current was again reduced by amiloride, but not to the extent seen with forskolin (Table II). In amiloride-pretreated cultures, after the addition of cAMP, the SCC was allowed to stabilize and the purinergic receptor agonist ATP (10 µmol/l) added. ATP has been shown to increase SCC of Fallopian tube
cells by stimulating Cl⁻ secretion. This response is not affected by amiloride (Downing et al., 1997). The addition of ATP to the basal side of cell cultures resulted in an immediate and significant increase in SCC that peaked at 31 µA/cm² and returned to the baseline within 1 min. This current flowed in the same direction as that resulting from stimulation with forskolin and dibutyryl cyclic AMP and its magnitude (268%) was similar (180%) to that described by Downing et al. (1997), confirming normal responsiveness of the cell cultures.

**Discussion**

Forskolin, which generates intracellular cAMP by stimulating the enzyme adenylate cyclase, added to the basal aspect of cell cultures resulted in an increase in SCC of 144%. A similar increase occurred after addition of dibutyryl cyclic AMP, confirming the importance of this second messenger in electrolyte and fluid transport in the Fallopian tube. Similarly a study using cultured rabbit oviductal cells under short-circuit conditions demonstrated that cAMP, and agents increasing intracellular cAMP such as adrenergic agonists, increase SCC implying electrogenic ion transport (Brunton, 1972).

The direction of current flow in our study suggested net transport of current (negative charge) in a mucosal direction (Figure 3). This current was not prevented or reduced by preincubation with SITS (an inhibitor of the Cl⁻/HCO₃⁻ exchanger) or frusemide (the Na⁺/K⁺/Cl⁻ co-transport inhibitor) indicating any role for Cl⁻ secretion in the genesis of this current. Likewise, TEA (a non-specific K⁺ channel blocker) did not inhibit the SCC resulting from stimulation with forskolin, thus excluding K⁺ transport as an explanation. Instead the SCC was reduced by amiloride, suggesting that electronic Na⁺ absorption may be responsible for the stimulated SCC.

It is of interest to note that preincubation with SITS accentuated the SCC response to forskolin. This was an unexpected finding and its explanation is not entirely clear. Secretory chloride currents and sodium absorptive currents result in current flowing in the same direction (basal to mucosal). It is possible that by eliminating secretory chloride currents with SITS, a greater proportion of the current flowing in this direction (after stimulation with forskolin) is now the result of sodium absorption, hence the accentuation. Alternatively the effect may be the result of alterations in intracellular pH caused by SITS.

Published studies provide conflicting evidence regarding the role of cAMP in ion transport in the oviduct. Using rabbit oviductal cell cultures under short-circuit conditions, it was demonstrated that cAMP reverses the flux of Cl⁻ from a secretory to an absorptive direction (Dickens et al., 1993). However, this finding was not subsequently confirmed in a study using a perfused preparation of rabbit oviduct, which showed that cAMP does not affect Cl⁻ flux (Dickens and Leese, 1994), as also demonstrated in our study.

Experiments with the perfused rabbit oviduct preparation have suggested that β-adrenergic agonists such as isoprenaline

---

**Table I.** The effect of specific ion channel blockers on forskolin (FSK)-induced increase in short-circuit current

<table>
<thead>
<tr>
<th>Test substance(s)</th>
<th>SCC (µA/cm²)</th>
<th>ISC (µA/cm²)</th>
<th>Percentage increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(before test substance)</td>
<td>(after test substance)</td>
<td></td>
</tr>
<tr>
<td>FSK alone (n = 8)</td>
<td>6.4 ± 1.2</td>
<td>15.6 ± 2.4</td>
<td>144*</td>
</tr>
<tr>
<td>FSK + frusemide (n = 6)</td>
<td>5.3 ± 1.0</td>
<td>14.0 ± 1.4</td>
<td>164*</td>
</tr>
<tr>
<td>FSK + TEA (n = 5)</td>
<td>5.6 ± 1.5</td>
<td>16.4 ± 0.8</td>
<td>193*</td>
</tr>
<tr>
<td>FSK + SITS (n = 10)</td>
<td>5.3 ± 0.9</td>
<td>27.0 ± 1.4</td>
<td>408*</td>
</tr>
<tr>
<td>FSK + amiloride (n = 11)</td>
<td>5.3 ± 0.8</td>
<td>7.7 ± 1.5</td>
<td>46</td>
</tr>
</tbody>
</table>

Values represent means ± SEM; n = number of cell cultures.

*P < 0.05 comparing short-circuit current (SCC) with increased short-circuit current (ISC).

**Table II.** The effect of amiloride on cAMP-induced increase in short-circuit current

<table>
<thead>
<tr>
<th>Test substance(s)</th>
<th>SCC (µA/cm²)</th>
<th>ISC (µA/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(before test substance)</td>
<td>(after test substance)</td>
</tr>
<tr>
<td>cAMP alone (n = 5)</td>
<td>6.2 ± 1.5</td>
<td>18.2 ± 3.6*</td>
</tr>
<tr>
<td>Amiloride + cAMP (n = 5)</td>
<td>6.2 ± 0.9</td>
<td>13.2 ± 3.5</td>
</tr>
</tbody>
</table>

Values represent means ± SEM; n = number of cell cultures.

*P < 0.05 comparing short-circuit current (SCC) with increased short-circuit current (ISC).

---

Figure 3. Diagram showing the possible mechanism of cAMP-induced Na⁺ absorption. Na⁺ is absorbed via the apical amiloride-sensitive Na⁺/H⁺ channel. The Na⁺ that is absorbed into the cell is extruded by active transport by the Na⁺/K⁺ ATPase. Water follows the osmotic gradient created by Na⁺ transport along paracellular pathways.
(which increase intracellular cAMP) result in an increase in Cl⁻ secretion and fluid production, whereas exogenously added cAMP paradoxically inhibits tubal fluid production (Dickens and Leese, 1994; Tay et al., 1997). This discrepancy may be explained by the fact that forskolin is a non-selective stimulator of adenylate cyclase which results in a non-specific increase in intracellular cAMP, whereas β-adrenergic agonists are more selective stimulators. Gott et al. (1988), using whole rabbit oviduct, also demonstrated that forskolin and cAMP inhibited oviductal fluid production. In addition, they determined that cAMP reduces Cl⁻ secretion.

Leung et al. demonstrated an increase in SCC after stimulating mouse oviductal cells with forskolin, in agreement with the findings of our study (Leung et al., 1995). However, the authors stated that this was due to an increase in cAMP-related chloride secretion via the cystic fibrosis transmembrane regulator (CFTR), whereas the findings from our study in humans suggest that Na⁺ absorption may be responsible for the increase in SCC seen upon stimulation with forskolin. The disparity may be the result of species differences; however, it must be noted that Leung et al. did not test the SCC response to cAMP itself and did not confirm the current-carrying species after stimulation with forskolin with the use of ion channel blockers.

The situation is thus complex and may partly reflect species and experimental differences. In the human cells we used, our finding that amiloride (inhibitor of the apical Na⁺/H⁺ antiport) reduces the SCC after stimulation with forskolin or cAMP suggests that these agents induce electronegative Na⁺ absorption. This would be consistent with the inhibition of fluid production (due to water following the Na⁺ ion absorbed). However, ions other than K⁺ and Cl⁻ must also be implicated in the genesis of the SCC. The vascular perfusion studies mentioned above (Gott et al., 1988; Dickens and Leese, 1994; Tay et al., 1997) have also demonstrated that cAMP reduces tubal fluid production, but have not commented on the relationship of this finding to Na⁺ transport.

In the endometrium, Na⁺ absorption plays an important part in the genesis of basal electrophysiological parameters (Matthews et al., 1993; Chan et al., 1997) and Na⁺ absorption has been shown to be an important mechanism regulating salt and water balance in the nephron, colon and excretory ducts of sweat and salivary glands (Hegel et al., 1993). However the role of active Na⁺ transport in tubal fluid production is less well-defined. The original electrophysiological studies (Brunton and Brinster, 1971) suggested that this ion moves passively and is not involved in the genesis of the baseline SCC of rabbit oviduct. Similarly, using vasically perfused rabbit oviducts it was found that basal tubal fluid production is not affected by amiloride (Dickens and Leese, 1994). A study of the electrophysiological basis of human Fallopian tube fluid production used cultured monolayers of human Fallopian tube epithelial cells determined that Na⁺ transport does not contribute in a major way to transepithelial potential difference, but amiloride did affect basal potential difference and SCC (Downing et al., 1997). In addition, analyses of the electrolyte content of tubal fluid have consistently demonstrated an increase in Cl⁻ and K⁺ concentrations relative to plasma (David et al., 1973; Borland et al., 1980) but no difference in Na⁺ concentrations in tubal fluid relative to plasma. However, a closer look at the data of Borland et al. showed that the Na⁺ concentration in the tubal lumina is lower than plasma in most of the patients studied (Borland et al., 1980). Most of these studies analysed basal (unstimulated) Na⁺ transport, and would therefore not be expected to reveal differences in Na⁺ transport that may occur only in relation to stimulation with agents that increase intracellular cAMP.

In summary, forskolin and cAMP added to Fallopian tube cell monolayers under short circuit conditions result in an increase in SCC that may in part be explicable in terms of electrogenic Na⁺ transport. In contrast to the well-defined role of this cation and cAMP in other transporting epithelia, their exact role in the Fallopian tube is still uncertain and requires further study.

References


Received on December 19, 2000; accepted on July 31, 2001