Effects of *Ureaplasma diversum* on Bovine Oviductal Explants: Quantitative Measurement using a Calmodulin Assay

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ABSTRACT

Calmodulin (CAM) acts as an intracellular regulator of calcium, an important mediator of many cell processes. We used the CAM assay and electron microscopy to investigate the effects of *Ureaplasma diversum* on bovine oviductal explants obtained aseptically from slaughtered cows. A stock suspension of *U. diversum* (treated specimens) and sterile broth (controls) was added to replicates of cultured explants and incubated at 38°C in an atmosphere of 5.5% CO_2 for 48 hours. Explants were examined for ciliary activity, extracellular CAM loss, and for histological and ultrastructural changes. Explants and their culture media were examined for changes in CAM concentration. All experiments were replicated three times. In addition, *U. diversum*, medium and broth were assayed for CAM content.

The concentrations of CAM in explants and media changed significantly ($p < 0.05$) in samples which were inoculated with *U. diversum* when compared to controls. The controls and infected specimens did not differ histologically or ultrastructurally, but *U. diversum* was seen to be closely associated with infected explant tissue. In view of this close affinity it is assumed the loss of CAM from the oviductal cells was causally related, but this was not proven. The failure to show cell membrane injury on light and electron microscopic examination was probably related to the short duration of the experiment and may only point out the sensitivity of the CAM assay in detecting early cell membrane injury. Compromise in characteristics of the medium to support both, the viability of oviductal cells and *U. diversum* limited the experimental time to 48 hours. Further work is necessary to develop a medium which allows for longer explant exposure. Membrane injury in oviductal and uterine cells may be a contributing factor in early reproductive failure associated with *U. diversum* infection in cattle.

RÉSUMÉ

La calmoduline (CAM) agit comme un régulateur intracellulaire du calcium qui est un médiateur important de plusieurs fonctions cellulaires. Le dosage de CAM et la microscopie électronique ont été utilisés afin d'évaluer l'effet d'*Ureaplasma diversum* sur des explants d'oviductes bovins obtenus aseptiquement de vaches à l'abattoir. Une solution-mère d'*U. diversum* (spécimen traité) ou un bouillon stérile (spécimen témoin) était ajouté à des réplicats d'explants en culture et incubés à 38°C dans une atmosphère enrichie de 5,5 % en CO_2 pendant 48 heures. Par la suite, l'activité ciliaire, la perte extracellulaire de CAM, et les changements histologiques et ultrastructuraux des explants ont été mesurés. Les changements dans la concentration de CAM ont été mesurés dans les explants et leur milieu de culture. Chaque expérience a été faite en triplicata et *U. diversum*, le milieu de culture et le bouillon ont été évalués pour leur contenu en CAM.

Des changements significatifs ($p < 0,05$) dans les concentrations de CAM des explants et des milieux furent notés entre les échantillons inoculés et les témoins. Toutefois, aucune différence n'était notée sur le plan histologique et ultrastructural, malgré qu'*U. diversum* semblait étroitement associé avec les tissus infectés. Compte tenu de cette étroite affinité il est présumé, mais non prouvé, qu'il y a une relation de cause à effet avec la perte de CAM par les cellules de l'oviducte. Le fait qu'aucune lésion cellulaire n'ait pu être démontrée suite à l'examen en microscopie électronique et photonique pourrait indiquer la grande sensibilité du dosage de la CAM à détecter de façon précoce des dommages cellulaires. Des compromis quant à la capacité du milieu de culture à assurer la viabilité des cellules de l'oviducte et d'*U. diversum* limitait la durée des expérimentations à 48 heures. Des travaux supplémentaires sont nécessaires afin de développer un milieu assurant une survie prolongée des explants. Un dommage membranaire aux cellules de l'oviducte et de l'utérus pourrait constituer un des facteurs contribuant au problème reproducteur associé aux infections à *U. diversum*. (*Traduit par Dr Serge Messier*)

INTRODUCTION

Calcium is recognized as a primary mediator of many cell processes and calmodulin (CAM) acts as an intracel-

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lular regulator of calcium ions. The role calmodulin plays in ciliary activity of epithelial cells and its specific association with eucaryotic cells has made this molecule a useful measure of cell health and membrane permeability (1). Alterations in concentrations of calmodulin in oviductal explant culture medium and in homogenized samples of oviductal explants has been reported following incubation with *Mycoplasma equigenitalium* (2,3). It was found that a decline in explant concentrations of calmodulin, and increases in medium concentrations were associated with cell membrane damage as viewed by transmission electron microscopy. The membrane damage was believed to be caused by *M. equigenitalium* and resulted in calmodulin leaking from the cell. *Ureaplasma diversum* has been associated with infertility and early pregnancy failure and it is postulated this may be due to injury to the ovulated oocyte, the developing embryo, or to the oviduct and uterine epithelium (4). This injury may result in changes in the oviductal and uterine milieu resulting in failure of embryo attachment and development. The epithelium of the oviduct contributes much to the environment of the oocyte and early embryo (5). In the following experiment the calmodulin assay was used to investigate the effect of *U. diversum* on bovine oviductal explants. Explants were also examined by electron microscopy in an attempt to correlate morphological alterations with biochemical changes.

MATERIALS AND METHODS

1. OVIDUCTAL EXPLANTS

Agarose (0.7% Seakem, FMC Bioproducts, Maine) was added to Hanks' balanced salt solution, autoclaved (121°C, 15 min) and poured into 60 × 15 mm plastic petri dishes (Falcon-1007, Becton Dickinson, New Jersey) in such a way as to leave a 1 cm crescent shaped well at the side of the plate. When solid, the plates were wetted with 1 mL Eagle's Minimum Essential Medium (EMEM, ICN Biomedicals, Ontario) supplemented with 10% fetal calf serum (Cansera, Ontario), 250 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes, Boehringer Mannheim, Quebec), 1500 U/mL potassium penicillin G (Ayerst Laboratories, Quebec), 100 U/mL nystatin, (Gibco Laboratories, New York), and 0.02% urea (Sigma Chemical Co., Missouri). Osmolality ranged from 290–305 mOsm and pH was adjusted to 7.1. Wetted plates were incubated overnight (38°C, 5.5% CO_2) and the following morning, the supplemented EMEM medium was changed.

Oviducts were obtained at slaughter from superovulated, donor cows two days postestrus. The oviduct was transferred to a biological hood and the fimbriae and cranial 1 cm of the infundibulum harvested, washed in sterile phosphate buffered saline containing 500 U/mL nystatin and washed again in two changes of 10 mL supplemented EMEM. Sections of oviductal mucosa (1 × 3 mm) were placed in the plates with supplemented EMEM medium on agarose. Each experiment required one oviduct, from which approximately 40 explants were obtained. Oviducts were cultured for the presence of bacteria and fungi, mycoplasmas and ureaplasmas. All explants were examined for ciliary activity at 0, 8, 24 and 48 h intervals. Plates were incubated at 38°C in 5.5% CO_2 and the supplemented EMEM changed every 24 h.

2. INOCULATION OF OVIDUCTAL EXPLANTS

Each explant was inoculated with 0.2 mL of a stock suspension of *U. diversum* (passage 8, strain 2312) stored in ureaplasma broth (6,7) at −70°C. A sample of the thawed ureaplasma suspension was serially diluted in ureaplasma broth and plated on ureaplasma agar plates to determine the number of colony forming units (CFU). Control cultures of explants were inoculated with 0.2 mL of sterile broth.

3. EXPERIMENTAL DESIGN AND SAMPLING

This experiment was repeated three times, with oviducts from three cows. The following description pertains to each replicate, except where noted otherwise. Each experiment consisted of three infected plates and three control plates. Each of these plates contained eight explants, chosen at random from the trimmed oviduct. Samples were taken for CAM assay, culture and electron microscopy.

Before allocation of explants to plates, three explants from two of the cows were selected as "time = 0" samples, to establish baseline CAM concentration for oviductal mucosa. At 8, 24 and 48 h after inoculation, one aliquot of 0.2 mL incubation medium and one explant were selected from any two randomly chosen plates of each group (treated or control) for CAM assay. All samples were frozen at −70°C.

At 8, 24 and 48 h, 0.2 mL of medium and one explant from each of the three inoculated plates were taken for culture. Explants were ground in sterile sand and 0.2 mL ureaplasma medium. Four serial tenfold dilutions were made of tissue homogenate and media samples and 10 μL of each were plated onto ureaplasma agar and incubated anaerobically at 38°C for two days. The media of control plates were cultured for ureaplasmas and bacterial contaminants.

Samples of explant were taken for electron microscopy at 8, 24 and 48 h. These were fixed in 2.5% glutaraldehyde for 16 h, at 4°C, and rinsed in 0.1 M phosphate buffer, followed by fixation in 1% osmium tetroxide in Sörensen's buffer for 1 h at room temperature (8). After removal of fixative, tissues were dehydrated in ethyl alcohol (scanning electron microscopy, SEM) and acetone (transmission electron microscopy, TEM). Specimens for SEM were coated with 25 nm gold palladium after critical point drying and examined using a SEM Hitachi S-570 electron microscope at 15 kV. Tissue imbedded in EPON and sectioned at 90 nm thickness was examined in a Hitachi HS-9 transmission electron microscope.

4. CAM ASSAY

The test samples included tissue homogenate (TH) of explants and culture medium (MF). All steps were carried out on ice, unless otherwise indicated. Explants weighing approximately 10 mg were homogenized in 80 mM KCl and 5 mM Tris, pH 7.5 using a tissuemizer at 90% output for 2 min, until the suspension was uniform. The homogenates were cen-

trifuged at 13,000 × g at room temperature for 10 min. Medium samples were diluted 1:1 with tissue homogenizing buffer and centrifuged at 110,000 × g for 45 min at 4°C (Beckman Ultracentrifuge TL100).

The supernates of the tissue homogenates (TH) and medium samples (MF) were frozen at −70°C until tested. Before assay, samples were heated to 100°C for 2 min, cooled on ice for 20 min and centrifuged at 13,000 × g, 10 min. Both TH and MF samples were diluted an additional fivefold with homogenizing buffer prior to assaying.

Calmodulin-deficient phosphodiesterase from bovine brain, 5′-nucleotidase from *Crotalus atrox* venom, and cyclic adenosine-3′-5′ monophosphate (sodium salt; cAMP) were obtained from Sigma Chemical Co.). Purified CAM was purchased from Boehringer Mannheim.

A phosphate-liberation end-point assay, (9), was used to measure CAM content. In this assay, activated phosphodiesterase hydrolyzes cAMP into AMP, which is converted by 5′-nucleotidase to adenosine and phosphate. Reactions were terminated and phosphate measured (2–10). The phosphate was reacted with acidic ammonium molybdate, the complex reduced and color intensity determined. Activity was determined at 37°C in a water bath. The reaction volume was 750 μL consisting of: 75 μL of 4 U/mL 5′-nucleotidase, 75 μL of 0.33 U/mL phosphodiesterase, 75 μL of 15 mM cAMP, 375 μL of assay buffer (200 mM Tris, 80 mM imidazole, 10 mM $MgCl_2$, 1 mM $CaCl_2$) and 25 μL of test sample. A standard curve was determined using 5 to 80 μL of purified CAM (1 ng/μL).

Following a 5 min preincubation at 37°C, the reaction was initiated by addition of cAMP. Reaction was terminated after 15 min by adding 750 μL of 10% sodium dodecyl sulfate buffered to pH 7.0 with 20 mM Tris. Tubes were removed from the waterbath and 1.5 mL freshly prepared chromogen, consisting of 5% ferrous sulfate, 10 N sulfuric acid, 10% ammonium molybdate was added to react with liberated phosphate. Light absorbance was read in a spectrophotometer at 720 nm. A negative reference solution contained 750 μL distilled water, 750 μL sodium dodecyl sulfate, and 1.5 mL chromogen. The CAM concentrations in TH and MF samples were calculated using the standard curve.

The CAM content of TH was calculated according to the following formula: CAM in ng/mg wet weight of tissue = CAM [standard] × DF ÷ sample weight, where CAM [standard] is the amount of CAM corresponding to the absorbance of the sample, DF is the dilution factor of 500 volumes diluent per volume of tissue, and sample weight is 25 mg which corresponds to the weight of tissue suspension added to the reaction media. The equation for determining the concentration of CAM in μg/mL of MF was: CAM [standard] × DF × UCF ÷ sample volume, where DF is 10, and UCF is the units conversion factor of 0.001 for converting ng to μg, and sample volume is 25 μL. All assays were performed in duplicate and the mean of the pair used in analysis. Assays were performed in batches of ten.

Infecting inocula, ureaplasma suspension, and ureaplasma culture and sterile broth were assayed for CAM content. *Ureaplasma diversum* in physiological buffered saline, pH 7.4 was stored at −70°C and centrifuged at 100,000 × g, 4°C for 30 min and the PBS supernatant discarded. The pellet was washed in double distilled water and disintegrated ultrasonically. The disintegration process was repeated five times for 1 min duration each time. The sonicate thus prepared was filtered (0.2 μm filter), heated to 100°C for 2 min, cooled on ice for 20 min, and centrifuged 10 min at 13,000 × g.

To demonstrate the specificity of the CAM assay several controls were used: a) The presence of endogenous phosphate in the explants and supplemented EMEM was determined in the absence of cAMP for each sample. b) Inhibition of color development with tissue fractions was controlled by 0.3 to 1 μm of the potent CAM antagonist, calmidazolium, which was vigorously mixed with the preincubation medium. c) For each experiment, the occurrence of spontaneous (nonenzymatic) hydrolysis of ATP was determined in at least one tube that contained all reaction constituents except tissue sample. d) 80 μL of purified CAM was assayed with each batch of samples. e) Activity of phosphodiesterase that was independent of exogenous CAM was determined in the absence of tissue fractions and purified CAM. f) Additive effects of purified CAM to color development with tissue fractions was determined.

Each experiment replicate also included a control sample incubated in the presence of 100 mM sodium azide. This acted as positive control for morphologically recognizable explant cell death and therefore assumed severe leakage of CAM. An explant incubated in the presence of azide was tested after 24 h.

5. STATISTICAL ANALYSIS OF TEST SAMPLES

Tissue homogenate (TH) and medium fraction (MF) concentrations of CAM were analyzed by least squares analysis of variance (general linear model, GLM) using the Statistical Analysis System (11). Regression analysis was performed on the means of the duplicates of each sample, by removing hour from the class statement of GLM, which left two classes, namely treatment and experiment (or replicate). The means of the duplicates of TH and MF, were analyzed to evaluate difference in the quadratic curves of treated and control samples. This analysis included the following effect: experiment; hour; hour by hour; hour by treatment; hour by hour by treatment.

RESULTS

1. MORPHOLOGY OF OVIDUCTAL EXPLANTS

Ciliary activity was visualized with an inverted microscope and appeared as rapid vibrating movement on the edge of explants. Explants with poorly visible or nonbeating cilia were rare and discarded. There was no visual difference between ciliary activity of the oviductal explants from the three experiments, nor between oviductal explants grossly or in terms

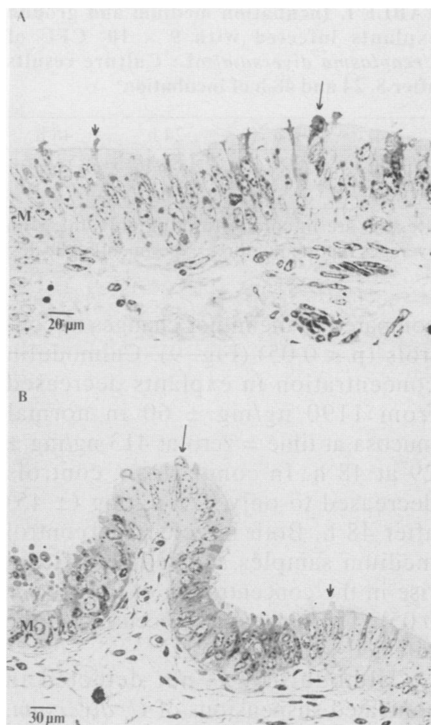


Fig. 1. Bovine oviductal explants, with mucosa showing regular pseudostratified columnar epithelium. Stained with new methylene blue, sectioned at 2 µm.
A. Explant infected with *U. diversum* strain 2312 after 24 hours incubation. Light microscopy, ×560.
B. Control explant after 24 hour incubation. Light microscopy, ×350.
---➤ = Exfoliating epithelial cell.
--➤ = Discharging secretory cell.
M = Mucosal epithelial layer.

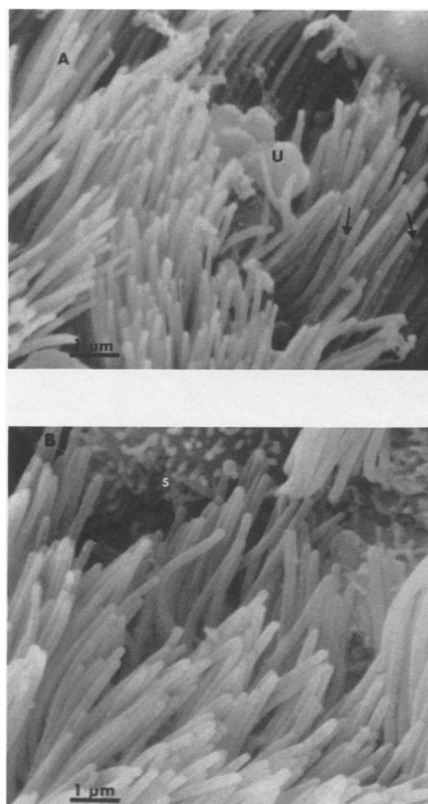


Fig. 2. Bovine oviductal explants after eight hours incubation.
A. Explant infected with *U. diversum* strain 2312. Cilia have randomly distributed multiple blebs. Large, rounded bodies are consistent in size with *U. diversum*. SEM ×12,000.
B. Noninfected control explant with smooth, regular cilia and secretory cells studded with microvilli. SEM ×12,000.
--➤ = Surface blebs on cilia.
S = Secretory cell.
U = Round bodies consistent in size with ureaplasma.

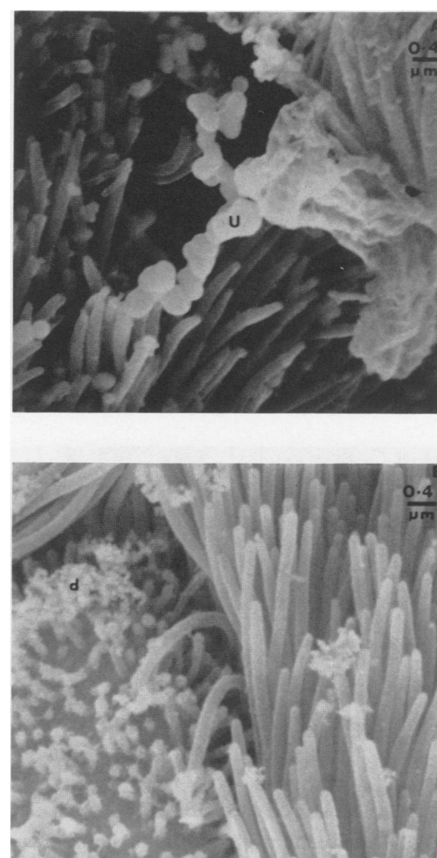


Fig. 3. Oviductal explants incubated for 24 hours.
A. Explant infected with *U. diversum* showing mild, multifocal ciliary blebbing and some terminal bleb formation on cilia tips. Chains of bodies, suggestive of ureaplasma are present. SEM ×17,000.
B. Noninfected control explant with some surface debris, but regular ciliary alignment.
U = Chains of bodies which may be ureaplasmas.
d = Debris.

of activity at 0, 8, 24 or 48 h. The only significant difference in explants viewed by the inverted microscope was in those cultured in 100 mM sodium azide. These showed a decline in ciliary activity at 8 h, fragmented cell clumps at 24 h, and were dead by 48 h.

Figure 1 is a low power view of a histological section of infected and control explants, after 24 h incubation. There were no noticeable differences between the two and mild clumping of the nuclear chromatin in both. Scanning electron microscopy revealed only slight morphological differences between the infected and control tissues. There was mild to moderate blebbing of the sides and tips of the cilia in many areas of both control and treated samples (Figs. 2–4), particularly in the 48 h samples (Fig. 4). Rounded bodies, consistent in size with ureaplasmas (300–500 nm) were identified in the infected explants (compare Figs. 2–4 with Fig. 5).

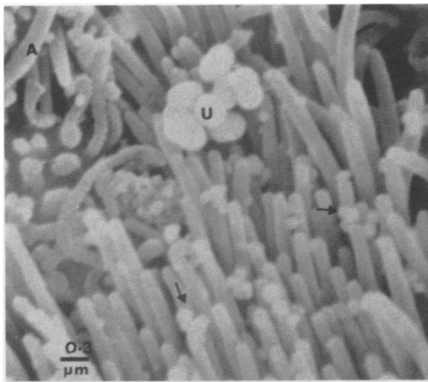
Transmission electron microscopy revealed no difference between infected and control samples of oviductal explants. A representative sample taken at 8 h is shown in Fig. 6. Two explants from each time period were examined. The mild chromatin changes seen in the histological sections were not evident on TEM. The rounded bodies seen on SEM, consistent in size and shape with ureaplasmas could not be identified with confidence on TEM, nor were the cytoplasmic blebs identified. Cilia on both explant groups were uniform.

2. UREAPLASMA IN OVIDUCTAL EXPLANTS

The three oviducts were negative for bacteria (including *Haemophilus somnus*), fungi and mycoplasmas (including ureaplasmas). The inoculum stock suspension of *U. diversum* strain 2312, contained 9×10^6 CFU/mL (equivalent to 1.8×10^6 CFU/mL/0.2 mL). Over the 48 h incubation period, both explants and media samples had decreasing numbers of ureaplasmas in all three experiments (Table I).

3. CALMODULIN

Figure 7 presents the results of stimulation and inhibition tests used to monitor the assay. Calmidazolium markedly reduced absorbance readings in tubes with both purified CAM



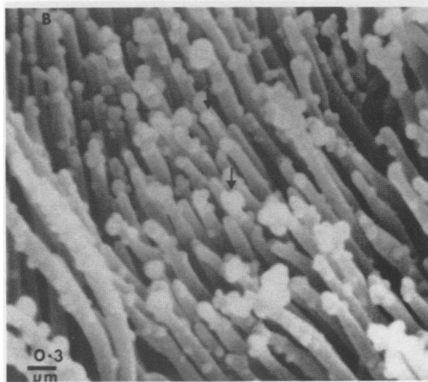


Fig. 4. Oviductal explant after 48 hours incubation.
A. Infected explant showing numerous terminal and lateral ciliary blebs. Round bodies are consistent in size with ureaplasmas. SEM ×25,000.
B. Control explant with numerous lateral terminal blebs. SEM ×25,000.
--> = Ciliary bleb formation.
U = Ureaplasma-like bodies.

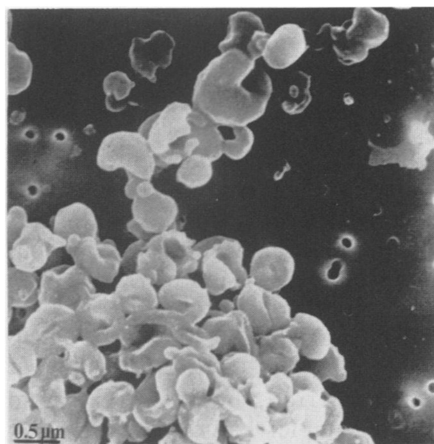
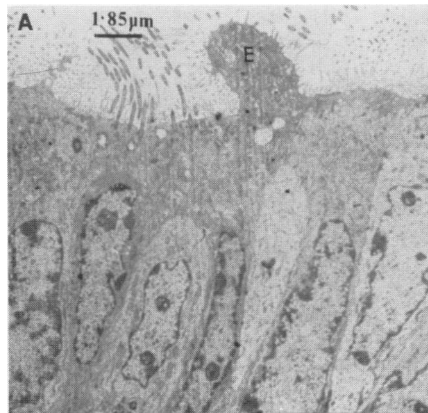


Fig. 5. *Ureaplasma diversum* strain 2312, shown as rounded bodies 0.2–0.5 μm in diameter. The culture was grown in broth, filtered through a 0.22 μm polycarbonate filter, which was then incubated for 18 hours on ureaplasma agar plate. SEM ×19,100.

and test sample CAM. Conversely, addition of purified CAM to test samples increased absorbance. Mean



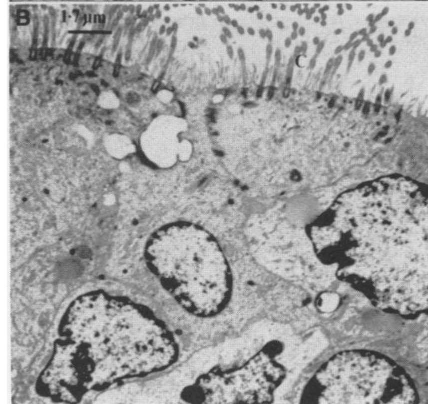


Fig. 6. Oviductal explant after eight hours incubation.
A. Explant infected with *U. diversum* strain 2312, with tangential sections of cilia and extrusion of cell cytoplasm. TEM ×5,625.
B. Noninfected control explant. TEM ×6,000.
E = Extrusion of cell cytoplasm.
C = Cilia.

absorbances for CAM-independent phosphodiesterase activity for three experiments were 0.13, 0.069 and 0.063. Their mean was subtracted from every sample. There was no detectable spontaneous (nonenzymatic) hydrolysis of cAMP, nor any detectable endogenous phosphate. The mean absorbances for control tubes with 80 ng CAM were 0.334, 0.338 and 0.351. Explants tested after 24 h in azide had approximately 1/3 the concentrations of CAM in control expants (353 ng/mg compared to controls of 1090 ng/mg at the same time).

Calmodulin standard curves were produced for each experiment. The curve was linear up to 0.4 absorbance units and 80 ng CAM. One ng of CAM corresponded to an absorbance of 40 (Fig. 8).

Concentration of CAM in explants and in medium fractions with *U. diversum* changed significantly over 48 h

TABLE I. Incubation medium and ground explants infected with 9 × 10^6 CFU of *Ureaplasma diversum*/mL: Culture results after 8, 24 and 48 h of incubation[a]

	8 h	24 h	48 h
Explants	4.0×10^5	2.5×10^5	1.3×10^4
Medium	7.5×10^6	5.0×10^6	1.6×10^5

[a]Results are in colony forming units/mL with averages reported of three experiment replicates

compared to the minor changes in controls ($p < 0.05$) (Fig. 9). Calmodulin concentration in explants decreased from 1190 ng/mg ± 60 in normal mucosa at time = zero at 413 ng/mg ± 29 at 48 h. In comparison, controls decreased to only 975 ng/mg (± 45) after 48 h. Both infected and control medium samples showed significant rise in the concentration of CAM ($p < 0.05$). The biggest rise occurred in the infected samples (Fig. 9).

Calmodulin was not detected in sonicated suspensions of *U. diversum*. Ureaplasma medium gave an insignificant absorbance of 0.04.

DISCUSSION

The objective of this experiment was to develop an organ culture model in which the effects of *U. diversum* on bovine oviductal mucosa could be measured biochemically and visualized ultrastructurally.

The culture model was less than optimal as it failed to support the growth of *U. diversum* adequately and may also have compromised the health of the oviductal explants as evidenced by cilial bleb formation in controls as well as treated samples. Nevertheless the results of the biochemical analysis, the calmodulin assay, on treated samples showed a significant decline in explant concentrations of CAM as compared to the rather subtle decline in concentrations recorded in samples from the control explants. The mechanism by which cellular CAM loss into the medium occurs is unknown. Cell membrane damage and leakage of intracellular cytosolic enzymes is the likely explanation for the loss of calmodulin from the explants. The presence of such large numbers of ureaplasmas in the explants suggests a *U. diversum*/explant interaction and supports the assumption that ureaplasmas were

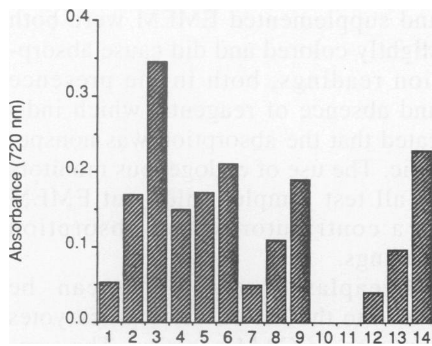


Fig. 7. Results of calmodulin assay controls, test samples stimulated and inhibited versus absorbance.

1 = 10 ng/μL commercial calmodulin
2 = 40 ng/μL commercial calmodulin
3 = 80 ng/μL commercial calmodulin
4 = 80 μL calmidazolium (10 μM) + 80 μL calmodulin (1 ng/μL)
5 = TH fraction from infected explant chosen at random
6 = TH + 25 ng calmodulin
7 = TH + 25 μL calmidazolium (10 μM)
8 = MF from an infected plate
9 = MF + 40 ng calmodulin
10 = MF + 25 μL calmidazolium (10 μM)
11 = *Ureaplasma diversum* sonicate
12 = *Ureaplasma diversum* medium diluted in modified EMEM (0.2 mL in 1 mL)
13 = TH explant cultured in presence of 100 mM sodium azide, sampled 8 h postincubation
14 = Noninfected TH fraction, after 8 h incubation.

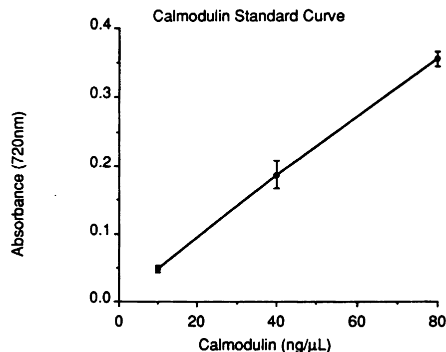


Fig. 8. Calmodulin standard curve measured spectrophotometrically at 720 nm. The average absorbance is represented with the standard error of means from standard curves run with each of the three replicate experiments.

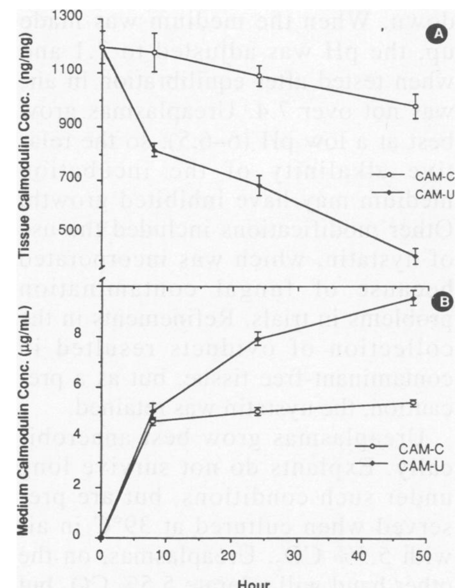


Fig. 9. Changes in concentrations of calmodulin (mean ± standard error) in tissue homogenate (Panel A) and medium fraction (Panel B).

CAM-C = calmodulin concentration of control tissue homogenate or medium fraction samples.

CAM-U = calmodulin concentration of *U. diversum* infected tissue homogenate or medium fraction samples.

responsible for the biochemical changes described.

The counts of *U. diversum* recorded for the ground explants were probably imprecise as explants could not be ground to form a homogenous suspension and sonication would have disrupted the ureaplasmas. The colony forming units recorded for the explants therefore, should have been higher. In spite of this possible inaccuracy, counts of ureaplasma recorded were relatively consistent over the three experiments.

While the CAM concentrations in samples from treated and control explants differed there was not a corresponding significant difference in morphological changes. This differs from the results of a similar experiment where equine oviductal explants were exposed to *Mycoplasma equigenitalium* and morphological injury was evident (2). There are several explanations for the contrasting results in these experiments. It is by no means certain that loss of cellular CAM constitutes an immediately critical biochemical injury which would register as a morphological change: the quantity of CAM leaking from the cell and the time until processing for morphological examination may be determining factors. The duration of the ureaplasma experiment described here was much shorter (48 h) than the experiment conducted using mycoplasma (240 h). In most organs, biochemical changes precede the development of visible morphological lesions and had it been possible to extend the time of the ureaplasma experiment, morphological changes might have become visible. Morphological evidence of cell injury may become apparent a considerable time after a critical intracellular biochemical system has become deranged. Myocardial cells, for example, do not show evidence of light microscopic change until 10–12 hours after total ischemia, yet irreversible injury occurs much earlier (12).

Other differences in the two experiments, which may have contributed to the differences found, include the type of organism (*U. diversum* vs. *M. equigenitalium*), the species of oviductal cell (bovine vs. equine) and the media. The supplemented medium used in this experiment was successful in maintaining ureaplasmas for 48 h, however, failure of the ureaplasmas to replicate indicates a lack of essential ingredients in the culture conditions. This deficiency may have affected the pathogenicity of the organisms, consequently the effects observed may have been less devastating than that of rapidly growing ureaplasmas on the oviduct. The modifications made to the EMEM and the incubation conditions used were the result of a compromise to keep both ureaplasmas and explants together in the best possible conditions. In preliminary trials, explants did not survive well using the same medium as others (13). This was possibly associated with either the low osmolality (250 mOsm/kg) compared to normal physiological osmolality of 280–300 mOsm/kg or the low pH (6.5). The osmolality of the supplemented EMEM used in the experiment was always between 290–305 mOsm/kg.

Ureaplasmas metabolize urea to ammonia, which is capable of causing ciliary damage (14). To prevent this injury, and to keep explants in a healthy state, HEPES was added to EMEM to help stabilize pH. Urea was present in the medium (both added and in fetal calf serum) in slightly elevated concentrations compared to blood urea nitrogen concentrations in normal cows (3.6 mg/mL compared to 2.2 mg/mL). Therefore, ammonia was likely to be produced, resulting in a rise in pH. Incubation of the plates in CO_2 and in the presence of HEPES theoretically helped keep the pH

down. When the medium was made up, the pH was adjusted to 7.1 and when tested after equilibration in air, was not over 7.4. Ureaplasmas grow best at a low pH (6–6.5), so the relative alkalinity of the incubation medium may have inhibited growth. Other modifications included the use of nystatin, which was incorporated because of fungal contamination problems in trials. Refinements in the collection of oviducts resulted in contaminant-free tissue, but as a precaution, the nystatin was retained.

Ureaplasmas grow best anaerobically. Explants do not survive long under such conditions, but are preserved when cultured at 39°C in air with 5.5% CO_2. Ureaplasmas, on the other hand will tolerate 5.5% CO_2 but prefer 38°C. Compromise of the ideal culture environment for tissue and *U. diversum* was required. The use of 10% FCS rather than 20% may also have impeded ureaplasma growth.

Extrapolation of results of experiments conducted on tissue explants to the *in vivo* situation is fraught with difficulties and just how closely this *in vitro* model represents the true state is impossible to say. With this in mind, the ultrastructural changes observed in both the control and treated explants (notably, ciliary blebbing) can perhaps be explained by the *in vitro* conditions in which the tissues were maintained. Such changes have been documented in SEM of normal bovine oviductal mucosa (15). Ciliary blebs were described as "presumably of secretory origin". Alternatively they may be the result of artifact induced by processing. This latter explanation seems the more plausible, since the TEM photos show no equivalent changes. Similarly, the mild chromatin clumping observed in the cells by light microscopy was not seen on the TEM examination. There was no distinct observable difference in morphology between the controls and treated explants.

The CAM assay was laborious to standardize and demanding to run. The rate-limiting enzyme was PDE, which had a bench life, when reconstituted, of about five hours. Vials of PDE were consequently kept frozen (−70°C) and reconstituted with Tris-KCl buffer immediately prior to use.

The monitors which were run parallel with each experiment were very important. The 80 ng commercial CAM tube run with each batch of samples was a continual check that the PDE used was still at full potency. When absorbance readings for this control began to fall, it was evident that PDE was losing activity. It was necessary to have a known positive control for visible explant damage and therefore membrane leakiness, as was provided by the explants incubated in sodium azide. The assay showed that homogenate samples from these explants did indeed have drastically reduced CAM concentrations. Similarly, the stimulation and inhibition tests run on test samples in which commercial CAM or commercial CAM inhibitor, calmidazolium (CAL), were added, showed expected increases and decreases in CAM concentrations. When 10 μM calmidazolium was coincubated with 80 ng CAM there was reduced absorbance due to the inhibitory effects of calmidazolium on CAM, but the absorbance was not reduced to zero. This may be due to use of DMSO as the CAL solvent or inappropriate ratios of CAL:CAM. Calmidazolium used in high concentration can cause nonspecific inhibition of enzymes as well as competitive inhibition of CAM. The quantitites used in this system were reduced to the lowest concentration possible, to prevent this nonspecific effect. A particularly important control was for PDE activity that was independent of the addition of purified CAM or tissue extract. This control was run in multiple with every experiment. It was noticed that coloration and thus absorbance occurred whenever the three reagents PDE, NT and cAMP were coincubated even in the absence of added CAM. However, this was not seen when any two of the three were coincubated. The "activator-deficient PDE" appears not to have been completely activator (i.e. CAM) deficient. The color produced was inhibited by calmidazolium which further supports this explanation.

Ureaplasma medium was tested for absorbance in the same concentration in which it was present in the medium (0.2 mL to 1.0 mL EMEM). Medium and supplemented EMEM were both slightly colored and did cause absorption readings, both in the presence and absence of reagents, which indicated that the absorption was nonspecific. The use of endogenous monitors of all test samples ruled out EMEM as a contributor to test absorption readings.

Ureaplasma organisms can be added to the list of tested procaryotes found to be CAM negative. The sample tested had been pelleted and resuspended in PBS, which likely would have contributed to phosphate present in the test tube, so it was recentrifuged and washed before being tested.

The results of this experiment support the hypothesis that *U. diversum* has an effect on oviductal cell integrity, but in the early stages does not produce a visible morphological change. Whether this biochemical alteration is harmful to the cell remains to be seen, probably a longer incubation time is required to determine this. Further work is necessary to develop a more suitable medium, investigate the effects other strains of *U. diversum* have on explants and to elucidate the mechanism of the biochemical change.

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