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The γ -subunit of the Na–K-ATPase as a potential regulator of apical and basolateral Na⁺-pump isozymes during development of bovine pre-attachment embryos

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Expression and activity of the Na–K-ATPase within the basolateral membrane domains of the trophectoderm epithelium provide the driving force for accumulation of Na⁺ and Cl⁻ across the nascent epithelium, mediating fluid movement into the forming blastocoel. Within the trophectoderm of the bovine blastocyst, multiple isozymes of the Na–K-ATPase are expressed. Immunolocalization has demonstrated that the α 1-isozyme localizes within the basolateral membrane, whereas the α 3-isozyme localizes to the apical cell margins. Gene-specific RT-PCR and wholemount indirect immunofluorescence confocal laser scanning microscopy were used to examine expression of the Na–K-ATPase γ -subunit (a regulatory subunit of the Na–K-ATPase) throughout development of bovine preattachment embryos *in vitro*. Expression of mRNA transcripts for the γ -subunit was detected throughout bovine pre-attachment development from the fertilized

one-cell embryo to the blastocyst stage. A similar pattern of expression was also observed for γ -subunit protein, and immunofluorescence was detected within the membranes of embryonic blastomeres at all stages of development. In contrast to the expression patterns observed for the α -subunits, γ -subunit proteins were detected in both the basolateral and apical cell margins of the trophectoderm, and surrounding all cells of the inner cell mass. Co-localization studies demonstrated that γ -subunit peptides are co-expressed with the α 1-subunit in the basolateral domains of the trophectoderm. These results indicate a role for the γ -subunit of the Na–K-ATPase in modulating Na⁺-pump activity in both apical and basolateral margins of the trophectoderm during formation and expansion of the bovine blastocyst, and adds a further level of complexity to Na⁺-pump regulation of cavitation.

Introduction

A prerequisite for successful completion of the pre-implantation phase of mammalian development is the differentiation of the trophectoderm epithelium after compaction of the morula stage embryo. Cavitation is initiated by the establishment of ion gradients and subsequent fluid transport arising from the polarized distribution of ion transporters and channels in the apical and basolateral membrane domains (for reviews, see Borland, 1977; Wiley, 1984; Biggers *et al.*, 1988; Benos and Balaban, 1990; Watson, 1992; Watson *et al.*, 1992a, 1999; Watson and Barcroft, 2001). Transporting epithelia use the polarized localization of ion transporters and channels within apical and basolateral membrane domains to establish and maintain the transcellular ion gradients necessary for ion and solute transport. Activity of the Na–K-ATPase within the basolateral membranes of epithelial cells is crucial for the absorptive, secretory and ion concentrating capacity of this

tissue (Balaban *et al.*, 1980; Jorgenson, 1986; Horisberger, 1994). Epithelial functions in fluid and ion transport, as well as maintenance of the ion gradients used by other epithelial transporters, are impacted greatly by small changes in ion affinities of the Na⁺-pump under physiological conditions (Herrera *et al.*, 1987; Jewel and Lingrel, 1991; Blanco *et al.*, 1995a,b; Ueno *et al.*, 1997; Blanco and Mercer, 1998; Crambert *et al.*, 2000).

The Na–K-ATPase is considered to consist of two subunits, forming a heterodimer of one of four isoforms of the catalytic α -subunit in combination with one of three isoforms of the glycosylated β -subunit (Jorgenson, 1982; Rossier *et al.*, 1987; McDonough *et al.*, 1993). Epithelial Na⁺-pump activity can be modulated by short-term mechanisms (that is, protein kinase-mediated subunit phosphorylation; Beguin *et al.*, 1996) leading to altered cell surface availability or enzyme kinetics, as well as long-term regulation (that is, by hormones; Ismail-Beigi, 1992; Wang *et al.*, 1994) leading to changes in protein synthesis and expression of Na⁺-pump isozymes (for reviews, see Rossier *et al.*, 1987; Ewart and Klip, 1995). Different combinations of the α - and β -isoforms of the Na–K-ATPase possess different ion affinities and enzyme kinetics (Jewel and Lingrel, 1991; Munzer *et al.*, 1994; Blanco *et al.*, 1995a,b;

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Zahler *et al.*, 1997; Crambert *et al.*, 2000), providing a further avenue for tissue-specific modulation of Na⁺-pump activity. In addition, there is growing evidence that expression and association of a third Na–K-ATPase subunit, the γ -subunit, have a marked effect on enzymatic properties of the Na⁺-pump in some tissues. The presence of the γ -subunit was first demonstrated by Forbush *et al.* (1978), in which a small hydrophobic peptide specific to the Na⁺-pump was specifically labelled along with the α -subunit by a photoactive derivative of ouabain, and was present in approximately equimolar amounts with α - and β -subunits (Hardwicke and Freytag, 1981; Collins *et al.*, 1982). The ' γ -subunit' was subsequently cloned by Mercer *et al.* (1993), and has been shown to copurify and co-immunoprecipitate with the α - and β -subunits (Mercer *et al.*, 1993; Beguin *et al.*, 1997). The γ -subunit is a small (approximately 7 kDa) type I transmembrane protein (N-terminus extracellular; C-terminus cytoplasmic), belonging to the FXYD family of proteins, which are involved in regulating ion channel activity in a variety of tissues (for a review, see Sweadner and Rael, 2000). The γ -subunit of the Na–K-ATPase (FXYD 2) is now the best characterized member of this family of small membrane proteins (Beguin *et al.*, 1997; Therien *et al.*, 1997; Arystarkhova *et al.*, 1999; Sweadner and Rael, 2000; Pu *et al.*, 2001), which includes phospholemman (FXYD 1; Moorman *et al.*, 1995), corticosteroid hormone induced factor (FXYD 4; Attali *et al.*, 1995; Beguin *et al.*, 2001), related to ion channel (FXYD 5 (RIC); Fu and Kamps, 1997), mammary tumour marker 8 (FXYD 3; Morrisson *et al.*, 1995), and two uncharacterized proteins FXYD 6 and 7 (Sweadner and Rael, 2000).

Multiple subtypes of the γ -subunit have been identified (Beguin *et al.*, 1997; Küster *et al.*, 2000; Sweadner *et al.*, 2000; Jones *et al.*, 2001; Pu *et al.*, 2001), which are products of a single gene (Sweadner *et al.*, 2000; Jones *et al.*, 2001), and differ in their N-terminal domain sequences. The γ_a and γ_b variants have a broad tissue distribution at least at the mRNA level (Kuster *et al.*, 2000; Jones *et al.*, 2001; Pu *et al.*, 2001), and have been shown to modulate Na⁺-pump activity by altering Na⁺, K⁺ and ATP affinities of the enzyme (Beguin *et al.*, 1997; Therien *et al.*, 1997, 1999; Arystarkhova *et al.*, 1999). The most recently identified γ_c variant (Jones *et al.*, 2001) is expressed at the mRNA in mouse heart, brain, kidney (medulla and cortex), testis and ovary, as well as in human brain, spleen and testis, although a functional role for this subtype has not been determined.

Establishment of ion gradients across the trophectoderm is mediated largely by expression and activity of the Na–K-ATPase within the basolateral cell margins of the trophectoderm (Watson and Kidder, 1988; Jones *et al.*, 1997; Betts *et al.*, 1998; Watson *et al.*, 1999). Inhibition of Na⁺-pump activity *in vitro*, in developing embryos (Wiley, 1984) or experimentally collapsed blastocysts (Dizio and Tasca, 1977; Betts *et al.*, 1997), blocks blastocyst expansion, indicating a key role for Na⁺-pump activity in the process of cavitation. Previous studies have demonstrated that the mammalian blastocyst expresses multiple isoforms of

Na–K-ATPase α - and β -subunits at both the mRNA (Betts *et al.*, 1997; MacPhee *et al.*, 2000) and protein level (Betts *et al.*, 1998; MacPhee *et al.*, 2000). The α_1 -subunit is the predominant isoform expressed in the basolateral cell margins of murine and bovine blastocysts (Betts *et al.*, 1998; MacPhee *et al.*, 2000). Although α_3 -subunit polypeptides are detected within both murine and bovine blastocysts (Betts *et al.*, 1998; MacPhee *et al.*, 2000), this isozyme demonstrates plasma membrane localization only within the bovine blastocyst, where it is predominantly expressed in the apical trophectoderm margins (Betts *et al.*, 1998). The presence of both basolateral (α_1 -/ α_3 -subunit) and apical (α_3 -subunit) Na⁺-pump isoforms within the bovine blastocyst indicate the potential for both basolateral and apical modulation of the Na⁺-gradients across the trophectoderm and complex Na–K-ATPase-mediated regulation of cavitation in this species. Although the presence of an apical Na⁺-pump isozyme has not been demonstrated within the murine blastocyst, analysis of expression of the γ -subunit has demonstrated expression of this modulator of Na⁺-pump activity in both the basolateral and apical margins of the trophectoderm (Jones *et al.*, 1997). Antisense disruption of the γ -subunit in murine embryos demonstrated a key role in blastocyst active cation transport and regulation of blastocyst formation (Jones *et al.*, 1997). For these reasons, it was important to examine the expression of gene products encoding the γ -isoform of the Na–K-ATPase during bovine early development *in vitro*.

Materials and Methods

Bovine embryo culture

Bovine pre-attachment embryos used for assessment of mRNA and protein expression were obtained by using standard methods for oocyte maturation and fertilization *in vitro* (Sirard *et al.*, 1988; Wiemer *et al.*, 1991; Watson *et al.*, 1994). Cumulus–oocyte complexes (COCs) were collected by follicular aspiration from bovine ovaries that were obtained from an abattoir within 4 h of removal from the animal. Pooled follicular aspirates were allowed to settle and COCs were collected under a dissecting microscope. Collected COCs were washed with oocyte collection medium (Hepes-buffered TCM-199 medium (Gibco BRL, Mississauga, ON), 2% (v/v) newborn calf serum (Gibco BRL) and 50 iu heparin sulphate ml⁻¹ (Heparin Leo; Organon Teknika, Toronto, ON)). COCs were placed into maturation medium, bicarbonate-buffered (Sigma, St Louis, MO) TCM-199 + 10% newborn calf serum, supplemented with 35 μ g sodium pyruvate ml⁻¹ (Sigma), 5 μ g FSH ml⁻¹ (Follitropin; Vetrapharm, London, ON), 1 μ g LH ml⁻¹ (Leutropin; Vetrapharm) and 1 μ g oestradiol ml⁻¹ (Sigma) for 22 h at 38.6°C in a humidified atmosphere of 5% CO₂ in air. Matured oocytes were washed four times in Hepes buffered modified Tyrode's solution (Parrish *et al.*, 1986) and placed into pre-equilibrated fertilization drops (50 COCs per 300 μ l drop under light paraffin oil (BDH Inc.,

Toronto, ON) composed of bicarbonate-buffered modified Tyrode's solution. Oocytes were fertilized with frozen-thawed bovine semen that had been motility enriched by using a 'swim-up' technique (Parrish *et al.*, 1986) using approximately 2.5×10^5 spermatozoa per fertilization drop for 18 h at 38.6°C in a 5% CO₂ in an air humidified environment. After fertilization, cumulus cells were removed from the oocytes using a finely pulled glass Pasteur pipette and 30–40 inseminated oocytes were placed into 20 µl culture drops consisting of citrate-supplemented synthetic oviductal fluid medium (cSOFM; Keskinetepe and Brackett, 1994; Watson *et al.*, 2000) under light paraffin oil. Embryo culture was carried out at 38.6°C in a multi-gas tissue culture incubator (Sanyo, Mississauga, ON) in a humidified atmosphere of 5% CO₂, 7% O₂ and 88% N₂. Embryo cultures were supplemented with 20 µl fresh cSOFMaa on day 3 and day 5 after insemination, maintaining drop volume at 40 µl for the remaining culture interval to sustain development to the blastocyst stage. Pools of 50–100 embryos were collected from cultures at the one-cell zygote (day 1 after insemination), two-cell embryo (day 2 after insemination), one- to five-cell embryo (day 3 after insemination), six- to eight-cell embryo (day 3 after insemination), 16–32-cell embryo (day 5 after insemination), compacted morula (day 6 after insemination) and blastocyst (day 7 after insemination) stages for analysis of mRNA and protein expression.

RNA isolation and reverse transcription

Three replicate embryo developmental series were collected for isolation of total RNA and analysis of γ -subunit mRNA expression profiles using RT-PCR. Pools of 50–100 staged embryos were washed in TCM-199 medium, transferred in a minimal volume (1–5 µl) of wash medium to 0.5 ml Eppendorf tubes and stored at –70°C. Total RNA was isolated from embryo samples using the Qiagen RNeasy™ mini kit for RNA isolation (Qiagen, Mississauga, ON). Total RNA was eluted from each RNeasy™ spin column in 70 µl sterile dH₂O and RNA sample volume was reduced to approximately 10 µl by centrifugation at room temperature for 45 min at 10 000 *g* in a Speed Vac Plus SC110A (Savant, Holbrook, NY). Frozen bovine liver and kidney samples were ground to a powder with a mortar and pestle on dry ice and total RNA was extracted from 1 to 2 mg of tissue by the same method described above. The concentration of total RNA for liver and kidney samples was determined by spectrophotometry, and aliquots of 1 µg total RNA were used for reverse transcription for both tissues.

Total RNA samples from embryos and somatic tissues were reverse transcribed using oligo(dT) priming and Superscript II™ reverse transcriptase (Gibco BRL; Harvey *et al.*, 1995; Betts *et al.*, 1997; Barcroft *et al.*, 1998). Reconstituted RNA samples were incubated with 0.5 µg oligo(dT)_{12–18} primer ml⁻¹ (Gibco BRL) for 10 min at 70°C. After centrifugation for 10 s at 10 000 *g* and cooling of samples on ice, RNA was incubated with first strand buffer

(Gibco BRL; containing 10 mmol Tris–HCl l⁻¹ (pH 8.3), 75.0 mmol KCl l⁻¹, 3.0 mmol MgCl₂ l⁻¹, 10.0 mmol dithiothreitol l⁻¹, 0.5 mmol dNTPs l⁻¹) and 200 U of superscript II™ reverse transcriptase for 1.5 h at 43°C. The reaction was terminated by heating the samples to 95°C for 4 min and flash cooling on ice. Newly synthesized cDNA was diluted to one embryo equivalent per µl by the addition of sterile dH₂O and stored at –20°C. Bovine liver and kidney RTs were diluted to 40 ng cDNA ml⁻¹.

PCR

Primer sequences for RT-PCR were designed using cDNA sequences retrieved from GenBank (GenBank accession number: X70059) and were synthesized by Gibco BRL. Primers for the γ -subunit were designed to bracket a region of the murine and bovine cDNA sequences (5'-primer = 5'-CTCGTCATCATCCTCAGCAA-3'; 3'-primer = 5'-CACTGCTGGCTCTCTCCTTA-3') corresponding to the γ -subunit, and produce a 95 bp amplification product. All cDNA samples used in the present study were tested for genomic DNA contamination before use in gene-specific PCR by using a set of primers designed to span an intron of the β -actin cDNA (Watson *et al.*, 1992b, 1994; Harvey *et al.*, 1995). All cDNA samples used in the present study displayed amplification of the appropriate sized β -actin PCR product (243 bp), demonstrating the absence of genomic contamination within these samples.

RT-PCR analysis of γ -subunit mRNA expression in one-cell zygotes, two-cell embryos, three- to five-cell embryos, six- to eight-cell embryos, 16–32-cell embryos, compacted morulae and blastocysts was carried out using the AmpliTaq Gold™ DNA polymerase (Perkin Elmer Cetus) as described previously (Watson *et al.*, 1999; Offenber *et al.*, 2000). Aliquots of embryo (four embryo equivalents per reaction) and liver or kidney cDNA (2 µl of a 50 µl cDNA sample) were amplified with 1 U AmpliTaq Gold™ DNA polymerase in a final volume of 50 µl in 0.2 ml Eppendorf tubes containing 10 × PCR buffer (200.0 mmol Tris–HCl l⁻¹ (pH 8.8), 500.0 mmol KCl l⁻¹ + 2.0 mmol MgCl₂ l⁻¹, 0.2 mmol of each dNTP l⁻¹ and 2.0 mmol of each gene specific primer l⁻¹). Amplification of cDNA templates was performed for up to 40 cycles in a DNA thermal cycler (Perkin Elmer Cetus 2400), with each cycle consisting of denaturation at 95°C for 1 min, re-annealing of primers to target sequences at 54°C for 30 s, and primer extension at 72°C for 1 min.

RT-PCR products were visualized by separation on 3% (w/v) agarose gels containing 0.5 mg ethidium bromide ml⁻¹ in 1 × TAE (40 mmol Tris acetate l⁻¹, 1 mmol EDTA l⁻¹) run at 100 mV for 50 min. The products were imaged using an ImageMaster VDS (Pharmacia Biotech, Baie d'Urfe, PQ) and recorded in digital format using the Image Capture Kit and ImageMaster VDS software (Pharmacia Biotech). The identity of γ -subunit PCR amplicons in bovine liver and kidney cDNA samples was confirmed by sequence analysis (Sequencing Facility, Robarts Research Institute, London, ON).

Antisera

The primary antibodies used for embryo whole mount indirect immunofluorescence in the present study included: (i) a rabbit polyclonal antibody (Ab-G17 generously provided by R. Mercer (Washington University, St Louis, MO) (Mercer *et al.*, 1993; Jones *et al.*, 1997) that was raised against a synthetic peptide corresponding to amino acids 6–22 of the sheep Na–K-ATPase γ -subunit; and (ii) a mouse monoclonal IgG_{1K} (clone C464.6) raised against the Na–K-ATPase α 1-subunit from dog kidney (Upstate Biotechnology, NY) (Gottardi and Caplan, 1993a,b; Jones *et al.*, 1997; Betts *et al.*, 1998; MacPhee *et al.*, 2000). All primary antisera were used at a dilution of 1:100. Two secondary antibodies were used: (i) a rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Westgrove, PA); and (ii) a fluoresceine isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch); Both secondary antisera were used at a 1:100 dilution.

Whole mount indirect immunofluorescence and confocal laser scanning microscopy

A whole mount indirect immunofluorescence procedure was used (Jones *et al.*, 1997; Barcroft *et al.*, 1998; Betts *et al.*, 1998; MacPhee *et al.*, 2000) to identify expression of Na–K-ATPase γ -subunit polypeptides in bovine pre-attachment embryos. Pooled bovine oocytes and early embryos (two-cell embryo, six- to eight-cell embryo, morula and blastocyst stages) were washed in 1×60 mmol piperazine-N,N'-bis[2-ethansulphonic acid] (PIPES) I⁻¹, 25 mmol Hepes I⁻¹, 10 mmol EGTA I⁻¹, 1 mmol MgCl₂·6H₂O I⁻¹, pH 6.9 (PHEM) (Schliwa and Van Blerkom, 1981) and fixed through a series of methanol (MeOH) on ice consisting of: 2 min in 1:1 MeOH:PHEM and 2 min in 2:1 MeOH:PHEM, followed by transfer into a 2:1 solution of PHEM:blocking buffer (0.01% (v/v) Triton-X100 + 0.1 mol lysine I⁻¹ + 1% normal goat serum (Gibco BRL) in $1 \times$ PHEM buffer) for 5 min to allow the embryos to sink. Bovine blastocysts were transferred from the 2:1 MeOH:PHEM solution into 100% MeOH on ice for 2 min for fixation before transfer to the PHEM:blocking buffer solution. Embryos were then washed once in $1 \times$ PHEM buffer and either used immediately for immunolocalization studies or stored for up to 2 weeks in embryo storage buffer (0.09% (w/v) sodium azide in $1 \times$ PHEM buffer) at 4°C.

Fixed embryos were blocked and permeabilized for 45 min in blocking buffer at room temperature, and washed through two 10 min changes of antibody dilution buffer (0.002% (v/v) Triton-X100, 0.1 mol lysine I⁻¹ and 1.0% normal goat serum in $1 \times$ PHEM buffer). Embryos were transferred to 50 μ l volumes of 1:100 dilution of the γ -subunit primary antibody in glass wellled Pyrex plates (VWR, Mississauga, ON) and incubated overnight in a humid chamber at 4°C. The embryos were then washed through four changes of antibody dilution buffer (3 \times 10 min and 1 \times 4–6 h) at room temperature. After washing, embryos were incubated in a 1:100 dilution of TRITC-conjugated goat anti-rabbit IgG secondary antibody at room temperature in antibody dilu-

tion buffer for 2–3 h. Samples were subsequently washed four times each for 10 min in fresh antibody dilution buffer and once overnight at 4°C. Washed embryos were transferred to 20 μ l FluoroGuard Antifade Reagent (BioRad, Mississauga, ON) mounting medium on glass coverslips under elevated 22 \times 22 glass coverslips and sealed with nailpolish. Slides were stored in the dark at –20°C until immunofluorescence localization patterns were viewed using a BioRad MRC 600 confocal laser scanning microscope. Non-specific staining by the secondary antibody was determined by treating permeabilized embryos with secondary antibody alone.

Co-localization of the γ -subunit with the α 1-subunit of the Na–K-ATPase within bovine blastocysts was performed using the methods described above. Blastocysts permeabilized for 45 min at room temperature in blocking buffer were incubated overnight in one of four treatments at 4°C: (i) 1:100 dilution of α 1-subunit primary antibody in antibody dilution buffer; (ii) 1:100 dilution of γ -subunit primary antibody in antibody dilution buffer; (iii) 1:100 α 1-subunit + 1:100 γ -subunit primary antibodies combined in antibody dilution buffer; or (iv) antibody dilution buffer alone. Embryos from each treatment group were washed as described above and incubated for 2 h at room temperature in a secondary antibody mixture composed of 1:100 dilutions of both TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG in antibody dilution buffer. Embryos were washed and mounted for confocal microscopy as described above. In embryos treated with α 1- or γ -subunit antibodies alone, secondary antibody cross-reactivity and bleed-through of FITC and TRITC on opposite channels was assessed to ensure overlapping fluorescence in dual-labelled embryos was due to colocalization.

Results

Na–K-ATPase γ -subunit mRNA expression during bovine pre-attachment embryo development

The pattern of Na–K-ATPase γ -subunit expression throughout bovine pre-attachment embryo development was examined in three developmental series collected from replicate oocyte collections and embryo cultures. Each RT-PCR reaction was carried out on eight embryo equivalents of cDNA from each embryo stage. The typical pattern of expression of γ -subunit mRNAs in cDNA samples derived from one-cell embryo, two- to four-cell embryo, six- to eight-cell embryo, 16–32-cell embryo, morula and blastocyst stages is shown (Fig. 1a). The γ -subunit PCR primer set designed from bovine and murine mRNA sequences resulted in amplification of a 95 bp fragment in bovine kidney, liver (not shown) and embryo cDNA samples (Fig. 1a) for all embryo stages from one-cell to blastocyst. The identity of bovine kidney and embryo PCR products produced using the γ -subunit PCR primer set was verified by sequence analysis, demonstrating 100% and 73% nucleotide sequence homology, respectively, with bovine (GenBank X70059) and murine (GenBank X70060) Na–K-ATPase γ -subunit mRNA sequences (Fig. 1b).

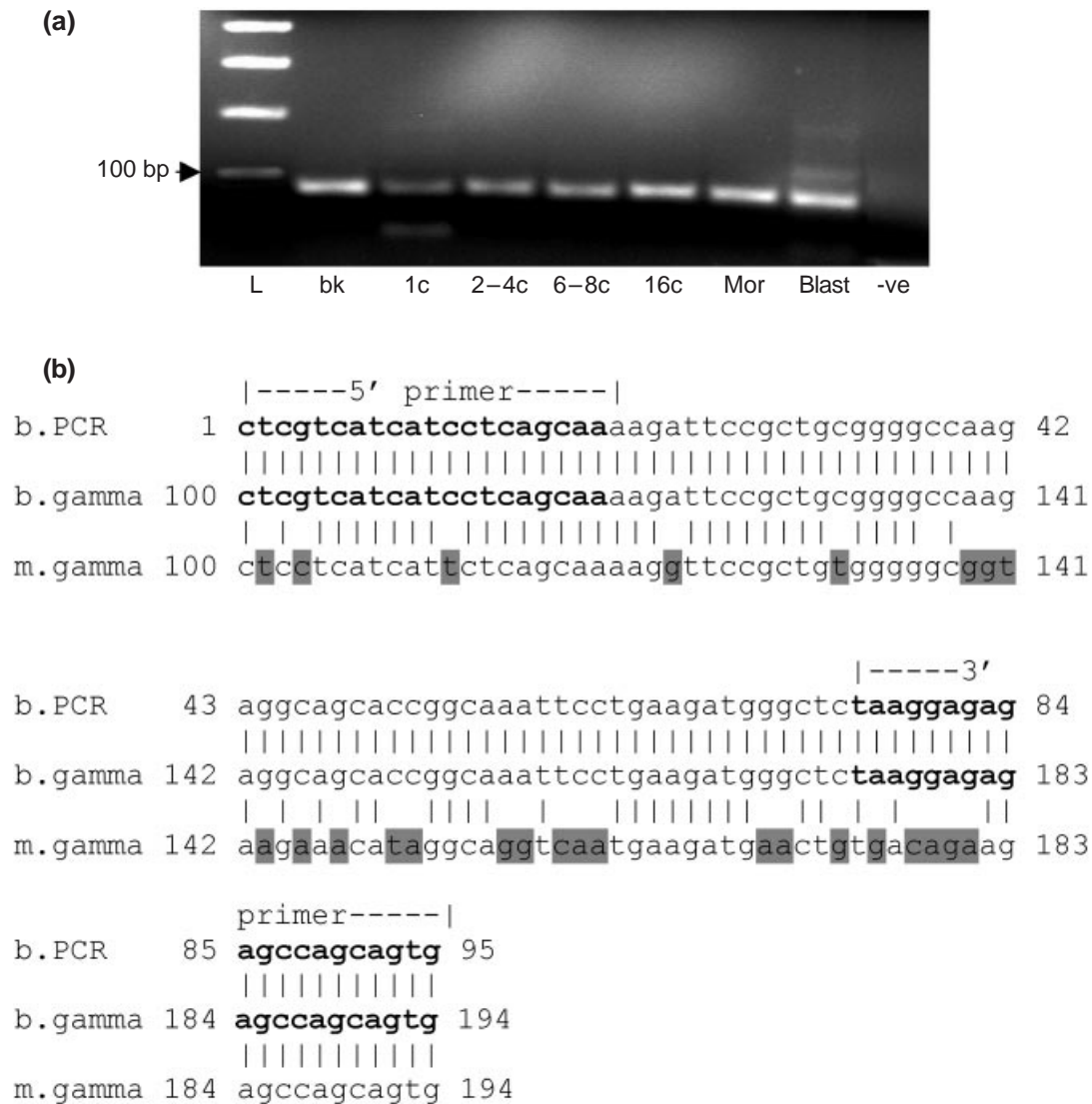


Fig. 1. Detection of transcripts encoding the γ -subunit of the Na-K-ATPase in bovine pre-attachment embryos. Complementary DNA (cDNA) samples were amplified using cDNA from four embryo equivalents for each stage of development throughout 40 cycles of PCR. (a) Detection of PCR amplification products on ethidium bromide-stained 3% (w/v) agarose gels. Lanes are bk: bovine kidney; 1c: one-cell zygote; 2-4c: two- to four-cell stage embryos (day 2 after implantation); 6-8c: six- to eight-cell stage embryos (day 3 after implantation); 16c: 16-32-cell stage embryos (day 5 after implantation); mor: morula stage embryos (day 6 after implantation); blast: blastocyst stage embryos (day 7 after implantation); -ve: negative control. The 100 bp ladder marker (L) is indicated at the left-hand side. In all lanes, an amplification product of approximately 95 bp was observed. (b) Nucleotide sequence homology of bovine embryo RT-PCR amplification products with known bovine (b) (GenBank X70059) and murine (m) (GenBank X70060) Na-K-ATPase γ -subunit mRNA sequences. The 95 bp amplicon shares 100% and 73% sequence similarity with bovine and murine mRNA sequences, respectively. Non-homologous nucleotides are highlighted in the murine γ -subunit sequence.

Timing of appearance and cellular distribution of the Na-K-ATPase γ -subunit protein in bovine pre-attachment embryos

For analysis of Na-K-ATPase γ -subunit protein expression, pools of 15 bovine pre-attachment embryos from each developmental stage (one-cell embryo, two-cell embryo, six- to eight-cell embryo, morula and blastocyst stages)

were collected and fixed in methanol from three replicate oocyte collections. In total, 45 embryos for each developmental stage were examined for immunostaining. The pattern of protein distribution observed in bovine embryos from the one-cell zygote to the six- to eight-cell embryo stage is shown (Fig. 2a-e). In all cleavage stage embryos examined, γ -subunit immunofluorescence was localized to the cell margins of all embryonic blastomeres. This pattern

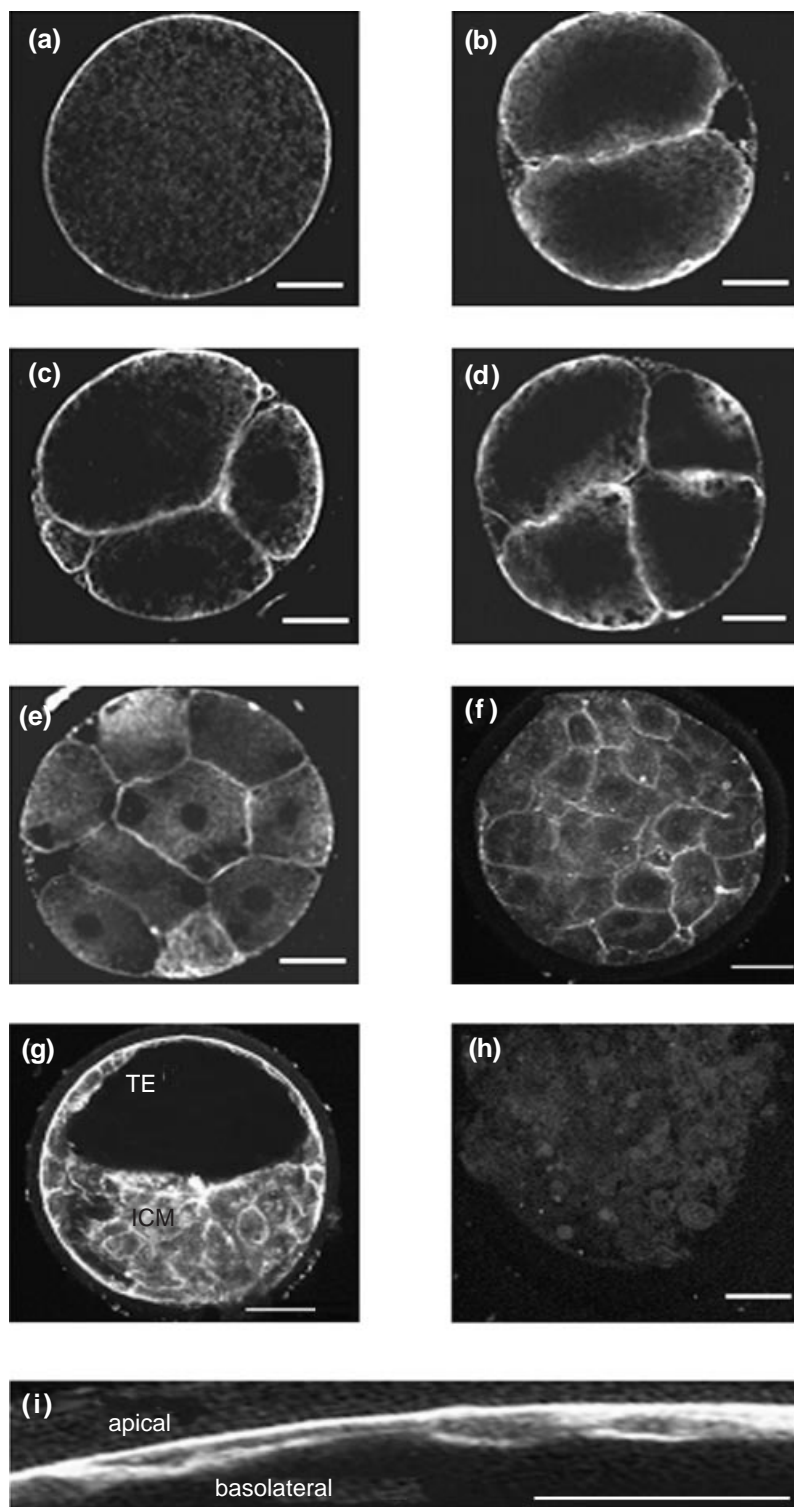


Fig. 2. Immunofluorescence detection of Na-K-ATPase γ -subunit protein in bovine pre-attachment embryos produced *in vitro*. Staining of (a) one-cell, (b) two-cell, (c) four-cell, (d) six-cell and (e) 16-cell stage embryos with γ -subunit antiserum showed immunofluorescence surrounding the cell margins of each blastomere. (f) At the morula stage, γ -subunit immunofluorescence maintains a distribution pattern in which staining is observed around the cell margins of all blastomeres. (g) Within the blastocyst, γ -subunit immunofluorescence was

was maintained within the morula (Fig. 2f) and blastocyst (Fig. 2g) stages. Within the blastocyst, the γ -subunit was localized to the cell margins surrounding cells of the inner cell mass, and adopted an apolar distribution within the trophectoderm of the blastocyst (Fig. 2g,i), although the apparent intensity of staining was greatest in the apical cell margins of the trophectoderm (Fig. 2i). Use of two additional γ -subunit antibodies (antibody 969, polyclonal anti-human synthetic peptide; and antibody GmB, polyclonal anti-sheep γ -subunit from kidney; also supplied by R. Mercer) revealed an identical pattern of immunofluorescence localization in bovine embryos as antibody G-17 (data not shown).

Immunofluorescence co-localization studies for the γ - and α 1-subunits were conducted to determine the potential for γ -subunit regulation of the basolateral α 1-isozyme within the trophectoderm. In total, 75 blastocysts were examined for γ - and α 1-subunit co-localization, representing blastocysts generated from four replicate oocyte collections. In all blastocysts co-incubated with γ - and α 1-subunit primary antibodies, co-localization was observed within the basolateral margins of the trophectoderm (Fig. 3c), the majority of trophectoderm cells demonstrating γ -/ α 1-subunit co-localization within a given blastocyst.

Discussion

Direct evidence that supports a role for the Na-K-ATPase in blastocyst formation has been demonstrated by using the Na⁺-pump specific inhibitor, ouabain, during preimplantation embryo development (Dizio and Tasca, 1977; Biggers *et al.*, 1978; Wiley and Obasaju, 1989; Betts *et al.*, 1997). Blastocyst expansion is restricted in the presence of ouabain, and blastocyst re-expansion following cytochalasin-induced collapse is completely blocked in the presence of ouabain (Dizio and Tasca, 1977; Biggers *et al.*, 1978; Betts *et al.*, 1997). van Winkle and Campione (1991) were able to detect no more than a twofold increase in ouabain-sensitive ⁸⁶Rb⁺ (a congener of K⁺) uptake in murine blastocysts, indicating that ouabain-sensitive enzyme activity may not increase markedly during murine preimplantation development, despite the increase observed in immunoreactive peptides (Gardiner *et al.*, 1990; Watson *et al.*, 1990). However, ouabain inhibition of Na-K-ATPase promotes the exchange of intracellular K⁺ for extracellular Na⁺ in murine embryos (Baltz *et al.*, 1997), demonstrating that Na⁺-pump activity maintains steep Na⁺ and K⁺ gradients in embryonic cells similar to those observed in other cells. In bovine embryos, Betts *et al.* (1998) demonstrated that ouabain-sensitive enzyme activity remains constant up to the morula stage. After compaction, Na⁺-pump mediated ⁸⁶Rb⁺ uptake increases markedly to the blastocyst stage, demonstrating an 18-fold increase in uptake above that observed in six- to eight-cell embryos

(Betts *et al.*, 1998). Intact bovine blastocysts that have not been treated with cytochalasin D also demonstrate a marked level of ouabain-sensitive ⁸⁶Rb⁺ transport (Betts *et al.*, 1998) that is much higher than that observed by van Winkle and Campione (1991) within intact murine blastocysts. These findings indicate that a component of Na-K-ATPase activity may be directed from the apical membranes of the trophectoderm, but that this activity may be much higher in bovine blastocysts. In support of this theory, Betts *et al.* (1998) demonstrated that bovine blastocysts possess apical immunofluorescence for the α 3-isozyme of the Na-K-ATPase in addition to the basolateral α 1-isozyme. Apical localization of the α 3-subunit was not demonstrated within the trophectoderm of murine blastocysts, although western blot analysis has demonstrated the presence of α 3-polypeptides within murine preimplantation embryos (MacPhee *et al.*, 2000). These results indicate species differences in the modulatory role of multiple Na-K-ATPase isozymes in regulating Na⁺-gradients across the trophectoderm during the process of cavitation.

Different isozymes of the Na-K-ATPase display distinct ouabain sensitivities and ion transport kinetics (Jewel and Lingrel, 1991; Munzer *et al.*, 1994; Blanco *et al.*, 1995a,b; Zahler *et al.*, 1997), such that small changes in isozyme expression or ion concentrations can affect physiological processes in a given tissue. Jewel and Lingrel (1991) demonstrated that rat α 1 and α 2 isoforms have similar apparent Na⁺ affinities, although the α 3 isoform demonstrates a fourfold lower affinity for Na⁺ than the other two isoforms (Munzer *et al.*, 1994). Zahler *et al.* (1997) reported that the α 3-isoform may act to regulate high intracellular Na⁺ loads to restore physiological Na⁺ concentrations. Baltz *et al.* (1997) investigated intracellular Na⁺ and K⁺ content within murine cleavage and morula stage embryos, demonstrating a marked increase in basal intracellular Na⁺ by the morula stage. It is not known how high intracellular Na⁺ concentrations are within cells of the trophectoderm during cavitation, as these measurements have not been made. It was hypothesized that increases in intracellular Na⁺ required to drive blastocyst formation must be regulated to maintain cellular function (Betts *et al.*, 1998). An apical α 3-isozyme could perform the role of maintaining trophectoderm cell Na⁺-gradients within physiological limits, without disrupting the establishment and maintenance of α 1-isozyme-mediated trans-trophectodermal ion gradients required for fluid transport.

The γ subunit lowers the apparent Na⁺ and K⁺ affinity of the Na-K-ATPase (Arystarkova *et al.*, 1999), in addition to increasing affinity for ATP (Therien *et al.*, 1997, 1999) and increasing K⁺ antagonism of cytoplasmic Na⁺ activation (Pu *et al.*, 2001). Within the kidney, γ -subunit-Na-K-ATPase associations may be beneficial in segments of the nephron, which show a high rate of Na⁺ reabsorption (Beguín *et al.*,

observed surrounding all cell margins of the inner cell mass (ICM) as well as in both apical and basolateral cell margins of the trophectoderm (TE). Apolar distribution of γ -subunit immunofluorescence is highlighted (i), in which localization of γ -subunit is clearly observed in all cell margins of trophectoderm cells. Scale bars represent (a-f,h) 20 μ m, (g) 50 μ m and (i) 10 μ m.

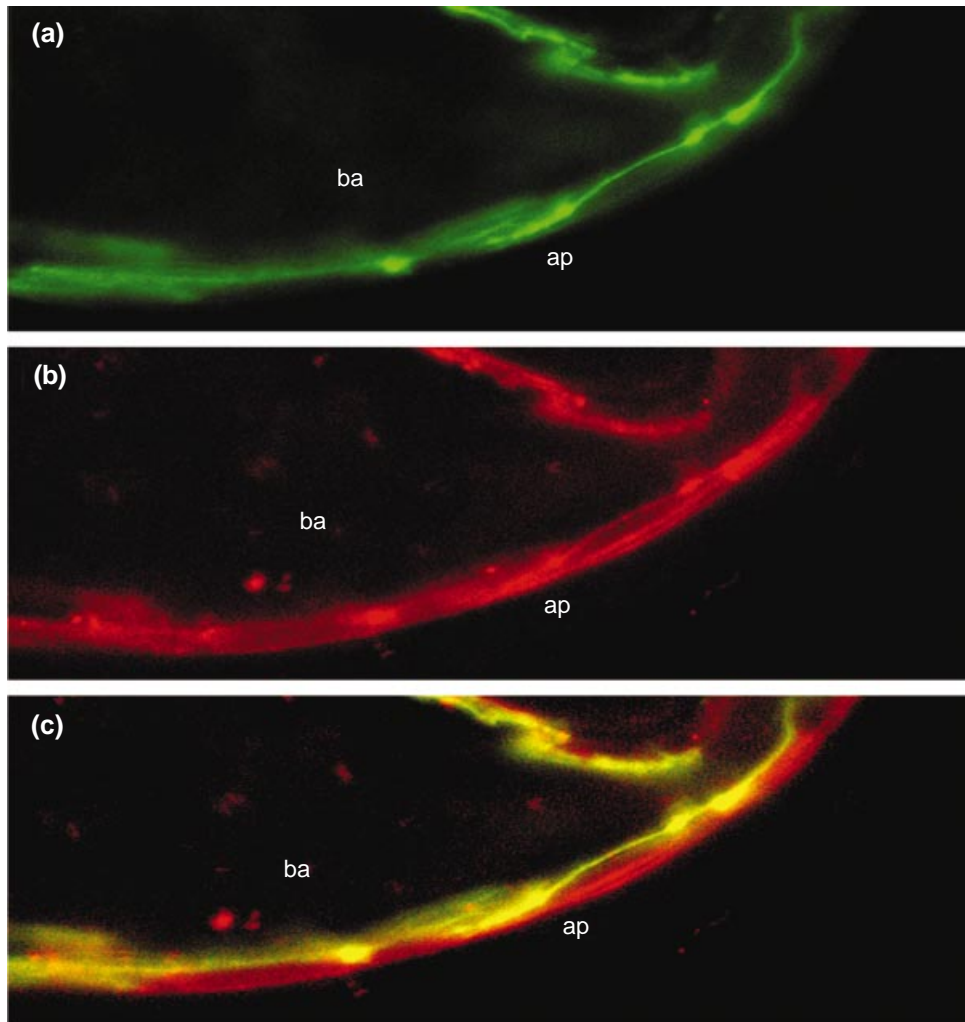


Fig. 3. Co-localization of Na–K-ATPase α 1- and γ -subunit polypeptides in the trophoctoderm of bovine blastocysts produced *in vitro*. Immunofluorescence for the (a; green) α 1-subunit is observed in the basolateral (ba) cell margins of the trophoctoderm, and is absent from the apical (ap) membrane domains. Immunofluorescence for the (b; red) γ subunit is observed in both apical and basolateral cell margins of bovine trophoctoderm. In bovine blastocysts, there is dual labelling with both α 1- and γ -subunit antibodies (c); co-localization (yellow) is observed only within the basolateral membrane domains of the trophoctoderm.

2001) where high density of the Na⁺-pump would be needed. Reduced Na⁺ affinity as a result of association with the γ -subunit results in increased Na⁺-pump efficiency in dealing with and transporting the cellular Na⁺ load (Beguin *et al.*, 2001). Gamma subunit association with Na–K-ATPase isozymes within the mammalian blastocyst may play a similar role in regulating the efficiency of Na⁺-pump-mediated ion concentrating activities across the trophoctoderm epithelium. Jones *et al.* (1997) demonstrated that antisense disruption of γ -subunit expression in murine embryos resulted in a decrease in Na⁺-pump (ouabain-sensitive) ⁸⁶Rb⁺ transport, but was not associated with a decrease in active Na⁺-pump content within the embryo.

Decreased γ -subunit expression was also correlated with a delay in the onset and rate of cavitation (Jones *et al.*, 1997), indicating a key role for γ -subunit modulation of Na⁺-pump activity during preimplantation development. The presence and localization of γ -subunit polypeptides within the basolateral membranes of bovine blastocysts further supports the proposed role for the γ -subunit during blastocyst formation.

The presence of γ -subunit expression within the Na⁺-pump-free apical trophoctoderm membranes of the mouse embryo (Jones *et al.*, 1997) has confounded our understanding of the overall function of the Na⁺-pump regulator during murine blastocyst formation. Beguin *et al.* (1997) demonstrated that α – β -dimer delivery to the plasma

membrane did not require association with the γ -subunit. However, the γ peptide appears to require association with transport-competent α - β complexes to ensure delivery to the plasma membrane (Beguin *et al.*, 1997), a requirement confounded by immunofluorescence data indicating the absence of an apical α -subunit isoform in murine trophectoderm (MacPhee *et al.*, 2000). Apically localized γ -subunits in the bovine trophectoderm observed in the present study correlate with the pattern of α 3-subunit expression demonstrated by Betts *et al.* (1998). Co-localization of α 3- β - γ complexes within the apical trophectoderm membranes of the bovine embryo would address this issue. Co-localization studies for the γ - and α 3-subunits within the apical margins of bovine trophectoderm were also attempted in the present study; however, both the α 3- (previously used by Betts *et al.*, 1998) and γ -subunit antibodies available for this study were rabbit polyclonal antibodies. Although antigen-switching techniques were used, it was not possible to demonstrate specific co-localization due to incomplete masking of the first primary antibody (data not shown), and thus we can only speculate that α 3- and γ -subunits co-localize within the apical trophectoderm cell margins based on their distribution patterns. Apical localization of Na-K-ATPase isozymes has been observed in other epithelia (Masuzawa *et al.*, 1985; Wilson *et al.*, 1991; Wilson, 1997; Villalobos *et al.*, 1997; Burrow *et al.*, 1999; Mobasher *et al.*, 2001); however, γ -subunit expression has not been examined in these tissues. As with the basolateral α 1-isozyme, γ -association with the apical α 3-isoform could also result in increased efficiency of Na⁺ transport during cavitation, thus providing the embryo with another mechanism to regulate intracellular Na⁺ concentrations during cavitation.

In summary, the present study has demonstrated that Na-K-ATPase γ -subunit mRNA and protein expression is maintained throughout bovine pre-attachment embryo development, as was first observed during murine pre-implantation development. These results provide a foundation for the hypothesis that the γ -subunit modulates blastocyst formation in the mammalian embryo. The results from the present study indicate for the first time that γ - α 1 isoforms are co-localized in basolateral trophectoderm membranes. The findings also indicate γ - α 3 co-localization in the apical membranes of bovine trophectoderm. This is the first study indicating co-localization of the γ -subunit with an apically localized Na-K-ATPase isozyme within a transporting epithelium. Regulatory roles for the γ -subunit in conjunction with apical and basolateral trophectoderm membranes of the bovine blastocyst indicate a complex role for the Na-K-ATPase in establishing and maintaining ion gradients required for fluid transport into the nascent blastocoel.

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