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Na/K-ATPase-Mediated $^{86}Rb^{+}$ Uptake and Asymmetrical Trophectoderm Localization of α1 and α3 Na/K-ATPase Isoforms during Bovine Preattachment Development

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This study evaluated Na/K-ATPase α1- and α3-subunit isoform polypeptide expression and localization during bovine preattachment development. Na/K-ATPase cation transport activity from the one-cell to blastocyst stage was also determined by measuring ouabain-sensitive $^{86}Rb^{+}$ uptake. Both α1- and α3-subunit polypeptides were detected by immunofluorescence to encircle the entire cell margins of each blastomere of inseminated zygotes, cleavage stage embryos, and morulae. Immunofluorescent localization of α1-subunit polypeptide in bovine blastocysts revealed an α1 immunofluorescence signal confined to the basolateral membrane margins of the trophectoderm and encircling the cell periphery of each inner cell mass (ICM) cell. In contrast, α3-subunit polypeptide immunofluorescence was localized primarily to the apical cell surfaces of the trophectoderm with a reduced signal present in basolateral trophectoderm regions. There was no apparent α3-subunit signal in the ICM. Analysis of $^{86}Rb^{+}$ transport *in vitro* demonstrated ouabain-sensitive activity throughout development from the one-cell to the six- to eight-cell stage of bovine development. $^{86}Rb^{+}$ uptake by morulae (day 6 postinsemination) did not vary significantly from uptake detected in cleavage stage embryos; however, a significant increase was measured at the blastocyst stage ($P < 0.05$). Treatment of embryos with cytochalasin D (5 μg/ml) did not influence $^{86}Rb^{+}$ uptake in cleavage stage embryos. Cytochalasin D treatment however was associated with a significant rise in ion transport in morulae and blastocysts (13.49 and 61.57 fmol/embryo/min, respectively) compared to untreated controls (2.65 and 22.83 fmol/embryo/min, respectively). Our results, for the first time, demonstrate that multiple Na/K-ATPase α-subunit isoforms are distributed throughout the first week of mammalian development and raise the possibility that multiple isozymes of the Na/K-ATPase contribute to blastocyst formation.

Key Words: **oocyte; blastocyst; gene expression; ion transport.**

INTRODUCTION

Blastocyst formation is an interactive process requiring the coordinated expression of several gene products including Na/K-ATPase subunits, E-cadherin, and tight junction components. The events leading to blastocyst formation are initiated during compaction and include the appearance of focal tight junctions that later expand and divide the plasma membrane of outer blastomeres into apical and basolateral domains (Watson, 1992). Coincident with this event, the cell adhesion molecule E-cadherin (Vestweber *et al.*, 1987; Watson *et al.*, 1990a) and the connexin polypeptides (Kidder, 1993a,b) comprising gap junctions localize to basolateral blastomere surfaces. Polarization continues as tight junctions develop, forming a seal between the outer, differentiated trophectoderm cells (Fleming *et al.*, 1989, 1993; Javed *et al.*, 1993). By the late morula stage, Na/K-ATPase is detectable in all embryonic blastomeres and becomes restricted to the basolateral membrane domain of the outer trophectoderm, at the onset of fluid accumulation (Watson and Kidder, 1988). It is hypothesized that the blastocyst cavity forms as a result of a transtrophectoderm ion gradient established, in part, by a polarized trophectoderm Na/K-ATPase, which facilitates the osmotic movement of water into the extracellular space to form the fluid-filled cavity of the blastocyst (Wiley, 1988; Biggers *et al.*, 1988; Wiley *et al.*, 1990; Watson, 1992). The tight junction permeability seal contributes to blastocyst formation by restricting the leakage of fluid via paracellular routes, thereby ensuring the expansion of the embryo as fluid accumulates (McLaren

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and Smith, 1977; Biggers *et al.*, 1988; Fleming *et al.*, 1989; Watson, 1992). This early developmental program is dependent upon the Ca^{2+}-mediated cell adhesion provided by E-cadherin (uvomorulin) (Watson *et al.*, 1990a). Murine embryos treated with an antibody to uvomorulin do not undergo compaction and do not proceed with normal blastocyst formation; instead, several smaller intracellular fluid-filled cavities form, each encircled by Na/K-ATPase (Watson *et al.*, 1990a). Furthermore, transgenic mouse lines carrying null mutations for E-cadherin fail to form normal blastocysts and never hatch from the zona pellucida (Larue *et al.*, 1994; Riethmacher *et al.*, 1995). Interestingly, the embryos do compact, illustrating the impact of maternal transcripts and proteins on early development (Larue *et al.*, 1994; Riethmacher *et al.*, 1995).

Our efforts have primarily focused upon characterizing the role of Na/K-ATPase in blastocyst formation. Na/K-ATPase is composed of three subunits, a catalytic, nonglycosylated α-subunit; a glycosylated β-subunit; and a γ-subunit (Reeves *et al.*, 1980; Jorgensen, 1982; Collins and Leszyk, 1987; Rossier *et al.*, 1987; Fambrough, 1988). The α-subunit is responsible for the physiological role of the enzyme (Jorgensen, 1986), while the β-subunit may facilitate the processing and insertion of the α-subunit into the plasma membrane (Fambrough, 1988; Geering, 1991a,b). The γ-subunit is a small (10-kDa) hydrophobic peptide whose role is currently under investigation (Mercer, 1993; Beguin *et al.*, 1997; Jones *et al.*, 1997). The α- and β-subunits in mammals are encoded by multigene families, as four α-subunit genes (α1, α2, α3, and α4) and three β-subunit genes (β1, β2, and β3), each displaying distinct temporal and spatial expression patterns having been characterized (Kawakami *et al.*, 1986; Kent *et al.*, 1987; Rose and Valdes, 1994; Schmalzing and Gloor, 1994).

In the preimplantation mouse embryo, transcripts encoding the α1-subunit of Na/K-ATPase are detectable in all stages by Northern hybridization (Watson *et al.*, 1990b; Gardiner *et al.*, 1990b). β1-Subunit transcripts can be detected by RT-PCR in two-, four-, or eight-cell embryos and increase greatly in abundance by the morula and blastocyst stages as determined by Northern blotting (Watson *et al.*, 1990b). Therefore, enhanced expression of the β1 gene is correlated with the onset and timing of murine blastocyst formation. Northern blotting failed to detect other isoform mRNAs (α2, α3, and β2) in preimplantation mouse embryos (Watson *et al.*, 1990b). Recently, we have demonstrated by RT-PCR that transcripts encoding α1, α2, α3, and β2 isoforms of the Na/K-ATPase are expressed throughout the first week of bovine embryonic development (Betts *et al.*, 1997). β1 mRNAs are not detected until the morula stage and are readily observed in bovine blastocysts (Betts *et al.*, 1997). The detection of β1-subunit mRNAs at the morula stage of bovine development parallels the increase in the level of β1-subunit mRNA (Watson *et al.*, 1990b), β1-subunit protein (Gardiner *et al.*, 1990b), and enzyme activity (Vorbrodt *et al.*, 1977; Van Winkle and Campione, 1991; Baltz *et al.*, 1997) observed during murine preimplantation development. All of these results support a central role for this enzyme in blastocyst formation (Watson, 1992). In addition, blastocyst formation and expansion can be blocked by treating murine or bovine embryos with ouabain, a specific inhibitor of Na/K-ATPase activity (Dizio and Tasca, 1977; Manejwala *et al.*, 1989; Betts *et al.*, 1997; Baltz *et al.*, 1997).

The present study was conducted to characterize the expression and localization of Na/K-ATPase α-subunit polypeptides during bovine preattachment development by applying whole-mount immunofluorescence microscopy. In addition, Na/K-ATPase transport was measured throughout this developmental interval by conducting ouabain-sensitive $^{86}Rb^{+}$ uptake experiments. Our results demonstrate that multiple Na/K-ATPase α-subunit isoforms are expressed and that ouabain-sensitive $^{86}Rb^{+}$ uptake increases markedly throughout the first week of mammalian embryonic development.

MATERIALS AND METHODS

Bovine Embryo Culture

The *in vitro* oocyte maturation, fertilization, and embryo culture methods outlined by Sirard *et al.* (1988), Wiemer *et al.* (1991), Xu *et al.* (1992), Watson *et al.* (1994), and Betts *et al.* (1997) were employed for the production of bovine preattachment embryos. Cumulus oocyte complexes (COCs) were collected from slaughterhouse ovaries and washed four times with oocyte collection medium [Hepes-buffered TCM-199 (Gibco, Grand Island, NY) + 2% newborn calf serum]. COCs were placed into maturation medium [TCM-199 medium + 10% (v/v) newborn calf serum (NCS) supplemented with sodium pyruvate (35 mg/ml; Sigma), 5 μg/ml FSH (Follitropin; Vetrapharm, London, Ontario, Canada), 5 μg/ml LH (Vetrapharm), and 1 μg/ml estradiol-17β (Sigma)] for 22 h at 38.6°C in a humidified 5% CO_2 air atmosphere. Matured oocytes were inseminated *in vitro* with frozen–thawed bovine semen (Semex Canada Inc., Guelph, Ontario, Canada) subjected to standard ''swim-up'' procedures as described (Parrish *et al.*, 1986; Wiemer *et al.*, 1991). The sperm/COC cultures were incubated for 18 h at 38.6°C in a humidified 5% CO_2 air atmosphere before removal of the remaining cumulus cell investment. Inseminated oocytes (25–30) were placed into 50-μl culture drops consisting of TCM-199 + 10% NCS under oil with up to 40 nonattached ciliated primary oviduct epithelial cell vesicles (Xu *et al.*, 1992; Xia *et al.*, 1996; Winger *et al.*, 1997). Fifty microliters of TCM-199 + 10% NCS was added to each culture drop following 48 h of culture. All of the collected COCs were utilized in this study and no oocyte selection strategy was employed.

Establishment of Primary Oviductal Epithelial Cell Cultures

Bovine oviductal cell cultures were established as outlined by Xu *et al.* (1992), Harvey *et al.* (1995), Xia *et al.* (1996), and Winger *et al.* (1997). Epithelial cell sheets were isolated from trimmed oviducts prior to dispersal through a syringe with an 18-gauge needle. Following four washes in Hank's balanced salt solution [HBSS, (Gibco)], 60 μl of cell suspension was placed into individual 35-mm petri dishes containing 3 ml of TCM-199 medium supple-

mented with 10% NCS for 24 h under an atmosphere of 5% CO_2 in air at 38.6°C. Up to 40 vesicles were selected and transferred into each embryo culture microdrop to support bovine embryo development through to the blastocyst stage.

Whole-Mount Confocal Immunofluorescence Microscopy

To identify Na/K-ATPase α-subunit isoform polypeptides in bovine preattachment embryos, a whole-mount immunofluorescence procedure was employed (Becker and Davies, 1995; De Sousa *et al.*, 1993). Briefly, pooled bovine inseminated oocytes and early embryos (approximately 100 of each stage for each primary antiserum) were fixed in cold 1:1 methanol (−20°C):PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM $MgCl_2 \cdot 6H_2O$, pH 6.9; Schliwa and Van Blerkom, 1981) for 2 min, then 100% methanol for 1 min and PHEM:methanol (1:2) for 2 min. Bovine blastocysts (n = 100 for each α-subunit primary antiserum) were fixed for 1 min in PHEM:methanol (1:2) and then for 2 min in 100% methanol. Fixed embryos were washed four times in fresh 1× PHEM buffer before permeabilization and blocking employing 1× PHEM buffer with 0.01% Triton X-100, 0.1 M lysine with 1% goat serum for 45 min at room temperature. Permeabilized embryos were washed four times in fresh 1× PHEM buffer and were then incubated in primary antiserum in blocking solution (1× PHEM + 0.002% Triton X-100 + 1% goat serum) overnight at 4°C. The embryos were washed through four changes, the last at least 4–6 h, of 1× PHEM + 0.002% Triton X-100 + 1% goat serum. This was followed by an overnight incubation (4°C) with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG secondary antibody or a FITC-conjugated goat anti-rabbit IgG secondary antibody at a 1:50 dilution with 1× PHEM + 0.002% Triton X-100 + 1% goat serum. The samples were subsequently washed 4× 10 min in fresh 1× PHEM buffer and then left overnight at 4°C in the last wash (1× PHEM + 0.002% Triton X-100 + 1% goat serum). Finally the embryos were placed onto glass microscope slides in approximately 20 μl of FluoroGuard antifade reagent (Bio-Rad) mounting medium. Slides were viewed with a Bio-Rad MRC 600 confocal laser-scanning microscope.

Antisera

The primary antibodies employed in this study included (1) mouse monoclonal IgG_{1k} (clone C464.6) raised against the Na/K-ATPase α1-subunit from dog kidney, provided by Dr. Michael Caplan, Yale University; and (2) rabbit polyclonal antibody raised against the Na/K-ATPase α3-subunit derived from amino acids 2–14 of the rat α3 polypeptide sequence, also provided by Dr. Caplan. Both of these antisera have been extensively characterized by Western blot analysis (Blanco *et al.*, 1994; Cameron *et al.*, 1994; Gottardi and Caplan, 1993a; Koster *et al.*, 1995, 1996; Munzer *et al.*, 1994; Urayama *et al.*, 1989; Zlokovic *et al.*, 1993) and immunofluorescence studies (Gottardi and Caplan, 1993a,b; McGrail *et al.*, 1991; Pietrini *et al.*, 1992) applied to a variety of mammalian tissues and cultured cell lines. Their specificity in recognizing each respective Na/K-ATPase α-isoform is determined. Both antisera were used at a dilution of 1:100 in 1× PHEM buffer.

Paraffin-Embedded Kidney and Brain Tissue

Bovine brain and kidney tissue samples were employed as positive controls to confirm the isoform specificity of each antiserum. Whole kidneys and brains were cut into ~3-mm cubes, washed in PBS, and fixed overnight in 80% methanol, 20% dimethyl sulfoxide (DMSO) or 4% paraformaldehyde. Automated paraffin embedding and tissue sectioning were performed at the Histology/Pathology Department, University Hospital, London, Ontario.

Paraffin sections (5 μm) were rehydrated through 10 min in xylene, 3 min in 100% EtOH, 3 min in 95% EtOH, 10 min in 70% EtOH with 1 mg/ml $NaBH_4$, 3 min in 70% EtOH, 3 min in 50% EtOH, and 10 min in PBS and were blocked in 1% goat serum albumin in PBS for 1 h at room temperature. Sections were placed in primary antisera or blocked and incubated overnight at 4°C. Unbound primary antibody was removed by washing slides 4× 5 min in PBS containing 1% goat serum. Washes were followed by a 1-h incubation with a secondary antibody containing a FITC-conjugated goat anti-rabbit or rabbit anti-mouse IgG (ICN Pharmaceuticals Canada Ltd.) at a 1:50 dilution. Unbound secondary antibody was removed by 4× 5-min washes in PBS. Sections were mounted in FluoroGuard antifade reagent (Bio-Rad) mounting medium and were viewed with a Bio-Rad MRC 600 confocal laser-scanning microscope.

α1-Subunit Immunofluorescence of Cytochalasin D-Treated Blastocysts

Whole-mount immunofluorescence microscopy was applied to cytochalasin D-treated bovine blastocysts to determine influences of cytochalasin D treatment on Na/K-ATPase α1-subunit expression and distribution. Pools of 30-day eight-cell blastocysts were placed in 50-μl culture drops of either TCM-199 supplemented with cytochalasin D (5 μg/ml) or TCM-199 alone for 1.5 h. Half of the cytochalasin D-treated blastocysts were removed from the culture drops, washed three times in fresh medium, and transferred to 50 μl TCM-199 microdrops for recovery (2 h). Embryos in all three groups (cytochalasin D-collapsed, cytochalasin D-recovered, and untreated controls) were fixed and processed for α1-subunit immunofluorescence as described above.

Ouabain-Sensitive $^{86}Rb^+$ Transport in Bovine Preattachment Embryos

General methods and experimental approach. Ouabain-sensitive $^{86}Rb^+$ uptake experiments were conducted by employing methods outlined by Van Winkle and Campione (1991) and Jones *et al.* (1997). Briefly, embryos were collected, washed once in TCM-199 medium, and treated with or without medium supplemented with 5 μg/ml cytochalasin D (Sigma) for 1.5 h in 20-μl drops under paraffin oil. Each embryo group (±cytochalasin D) was split into two additional treatment groups by an additional incubation for 1 h in the presence or absence of 1×10^{-7} M ouabain (a specific inhibitor of Na/K-ATPase; Sigma; Betts *et al.*, 1997). Following ouabain treatment embryos were labeled in 0.4 mM $^{86}Rb^+$ in K^+-depleted (KH_2PO_4 levels adjusted to lower overall K^+ in medium by 0.4 mM) KSOMaa (potassium simplex optimized media; Erbach *et al.*, 1994). The concentration of $^{86}Rb^+$ and K^+ within the labeling medium was equal to the K^+ concentration of nonmodified KSOMaa levels ($[^{86}Rb^+ + K^+]$ = 14.05 mM). Embryos were labeled for 20 min followed by four washes through cold (4°C) TCM-199 (3 ml per wash) and then lysis (five embryos per sample) in 100 μl 2% sodium dodecyl sulfate (SDS). Lysed embryo samples were combined with 3.5 ml of scintillation fluid (5 g/liter 2,5-diphenyloxazole, 3% acetic acid in toluene). Each sample was counted for 20 min employing

a Beckman LS 5000CE liquid scintillation system. Samples containing a small volume (~10 μl, representing the transfer volume of each embryo sample) of the final wash were prepared and counted along with each embryo sample to ensure the efficacy of the washes. Embryo cpms for each experiment were converted to fmol of $^{86}Rb^+$/embryo/min.

Experiment 1: Effect of concentration of cytochalasin D on $^{86}Rb^+$ uptake in blastocysts. To determine optimal cytochalasin D treatment doses for assessing ouabain-sensitive $^{86}Rb^+$ transport, day 8 blastocysts were subjected to a cytochalasin D concentration series (0, 5, 10, 20, or 30 μg/ml) for 1.5 h. Blastocyst samples from each treatment group were split equally and further treated with and without ouabain for 1 h prior to labeling for 20 min in 0.4 mM $^{86}Rb^+$ as described above. This experiment was repeated five times and a total 250 bovine blastocysts were used.

Experiment 2: Time course for $^{86}Rb^+$ uptake by bovine embryos. Bovine inseminated oocytes and early embryos consisting of one cell [day 1 postinsemination (p.i.)] and blastocyst (day 8 p.i.) pools were treated with (collapsed) or without (intact) cytochalasin D (5 μg/ml) for 1.5 h prior to division into 20-μl culture drops of $\pm 1 \times 10^{-7}$ M ouabain for 1 h and transfer into a labeling time-course experiment consisting of 5, 10, 20, and 40 min labeling times (20-μl culture drops). Each experiment for each stage was repeated a minimum of three times and a total 240 inseminated oocytes and 240 blastocysts were employed.

Experiment 3: Developmental profile of ouabain-sensitive $^{86}Rb^+$ transport. The developmental profile of ouabain-sensitive $^{86}Rb^+$ transport was determined using one-cell inseminated oocytes and three- to five- (day 2 p.i.) and six- to eight-cell (day 3 p.i.), morula (day 6 p.i.), and blastocyst stages (day 8 p.i.). Pools of 10 embryos for each developmental stage were treated with (collapsed) or without (intact) cytochalasin D (5 μg/ml) for 1.5 h before embryo pools were divided between 20-μl drops of $\pm 1 \times 10^{-7}$ M ouabain for 1 h. Embryos from each developmental stage (five embryos/sample) were transferred to 20-μl culture drops containing 0.4 mM $^{86}Rb^+$ and labeled for 20 min. The experiment was repeated on five independent developmental series and a total 360 embryos (representing 60 three- to five-cell stages and morulae 80 one-cell inseminated oocytes and six- to eight-cell stages and blastocysts) were utilized.

Statistical Analysis

Experiments were repeated a minimum of three times on embryo collection replicates. Bartlett's test was employed to test for heterogeneity of the variance; when present data were square-root-transformed. Nontransformed (cytochalasin D dose response) and transformed data (developmental series, uptake time-course trials) were subjected to one-way repeated-measures analysis of variance by applying Bonferroni's t test and the Student–Neuman–Kuels test for multiple comparisons. Differences of $P < 0.05$ were considered significant. Linearity of the uptake time-course data was analyzed by analysis of variance with regression. Time courses were considered linear if the regression coefficients were significantly different from zero, as determined by Student's t statistic ($P < 0.05$).

RESULTS

Bovine Kidney and Brain Na/K-ATPase α-Subunit Immunofluorescence

Typical Na/K-ATPase α1-subunit immunofluorescence patterns were observed in paraffin sections of bovine kidney (Fig. 1f). The proximal and distal convoluted tubules are major sites of Na^+ transport (Jorgensen, 1986), and the most intense fluorescence was observed in these tubules. The collecting tubules express Na/K-ATPase at lower levels (Jorgensen, 1986) and it is likely these tubules displayed a reduced immunofluorescence in these control samples. The kidney immunofluorescent staining was localized to the basolateral cell margins, reflecting a polarized distribution. Further proof of the specificity of the Na/K-ATPase α1-subunit fluorescence pattern is found in the failure of glomeruli to display any immunofluorescent staining (Fig. 1f). Bovine brain tissue sections displayed typical immunofluorescent patterns for the Na/K-ATPase α3-subunits (Fig. 3f). This antiserum was also applied to bovine kidney sections. Whereas the α1-specific antiserum brilliantly stained appropriate kidney structures (Fig. 1f), the α3-subunit antiserum did not result in fluorescent signals above secondary antiserum control levels (data not shown). These tissue controls demonstrate that the immunofluorescence methods and antisera employed in this study provided isoform-specific and typical localization patterns in bovine tissues.

Timing of Appearance and Cellular Distribution of the Na/K-ATPase α1-Subunit

Identical procedures were applied to samples from inseminated oocytes through to the blastocyst stage, except that the blastocysts were fixed with 100% methanol while the cleavage stage embryos were fixed with methanol:1× PHEM (2:1) to reduce embryo shrinkage. We examined a minimum of 100 zygotes from each embryo stage and the procedures were repeated four to five times for each embryo stage. We did not observe any variation (i.e., 100% of the embryos in each staged group) in immunofluorescence pattern between experiments or among the individuals composing each embryo group.

Na/K-ATPase α1-subunit immunofluorescence was detected within the outer cellular margins encircling each blastomere in inseminated oocytes through to the 16- to 32-cell morula stage (Figs. 1a–1e). In blastocysts the Na/K-ATPase α1-subunit pattern changed to a polarized distribution, restricted to the basolateral membrane domains of the outer epithelial trophectoderm (Figs. 2d and 2e and Figs. 4a and 4b). The fluorescence pattern encircled the blastocyst cavity and was present around the entire cell periphery of the cells of the inner cell mass.

This transition from an apolar cleavage stage distribution to a polarized trophectoderm distribution was recorded. In late morulae the α1 immunofluorescent signal was detected encircling the cell surface within the membrane domains of both the inner and outer blastomeres (Figs. 1e and 2a). Staining of early blastocysts revealed α1-subunit immunofluorescence in both the apical and basolateral membrane domains of the trophectoderm (Fig. 2b). The α1 immunofluorescence disappeared from the apical membrane regions of the trophectoderm (Fig. 2c) and eventually became restricted to the basolateral membrane domains of the troph-

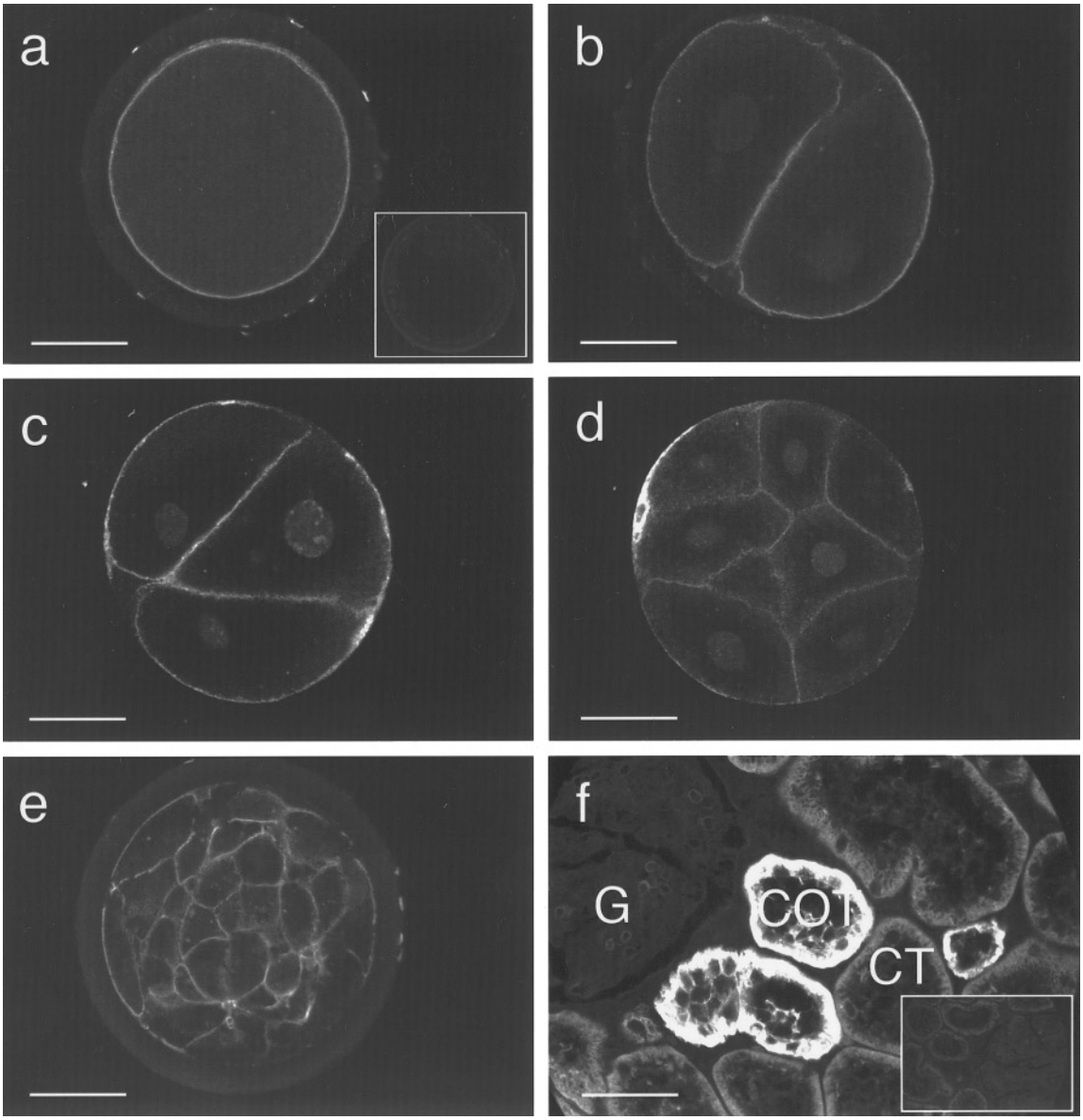

FIG. 1. Immunofluorescent localization of Na/K-ATPase α1-subunit polypeptide in bovine cleavage stages. Inseminated oocytes of 1-cell (a), 2-cell (b), 4-cell (c), and 8-cell (d) embryos and 16- to 32-cell morulae (e) incubated with α1-subunit antiserum revealed α1-subunit immunofluorescence encircling the outer margins of each blastomere. (f) Immunofluorescence confocal micrograph of paraffin-embedded bovine kidney treated with the α1-subunit antiserum. The most intense signal was localized in the convoluted tubules (COT), restricted to the basolateral membrane domains. The signal is less intense in the collecting tubules (CT) but still maintained a polarized basolateral cell distribution. The absence of fluorescence in kidney glomeruli is displayed (G). No α1-subunit immunofluorescence was observed in any of the secondary controls as exemplified by the kidney paraffin section inset in f. All images are 1-μm-thick confocal laser-scanning projections. Scale bars = 50 μm.

ectoderm of later stage blastocysts (Figs. 2d and 2e and 4a and 4b).

Timing of Appearance and Cellular Distribution of Na/K-ATPase α3-Subunit

Identical immunofluorescence methods were applied to whole-mount bovine embryos treated with the α3 antiserum. Once again we examined approximately 100 zygotes from each embryo stage (representing four experimental replicates) and in all cases 100% of the zygotes in each group displayed an identical α3-subunit immunofluorescent pattern.

The Na/K-ATPase α3-subunit polypeptide was detected by immunofluorescence in all stages of early bovine devel-

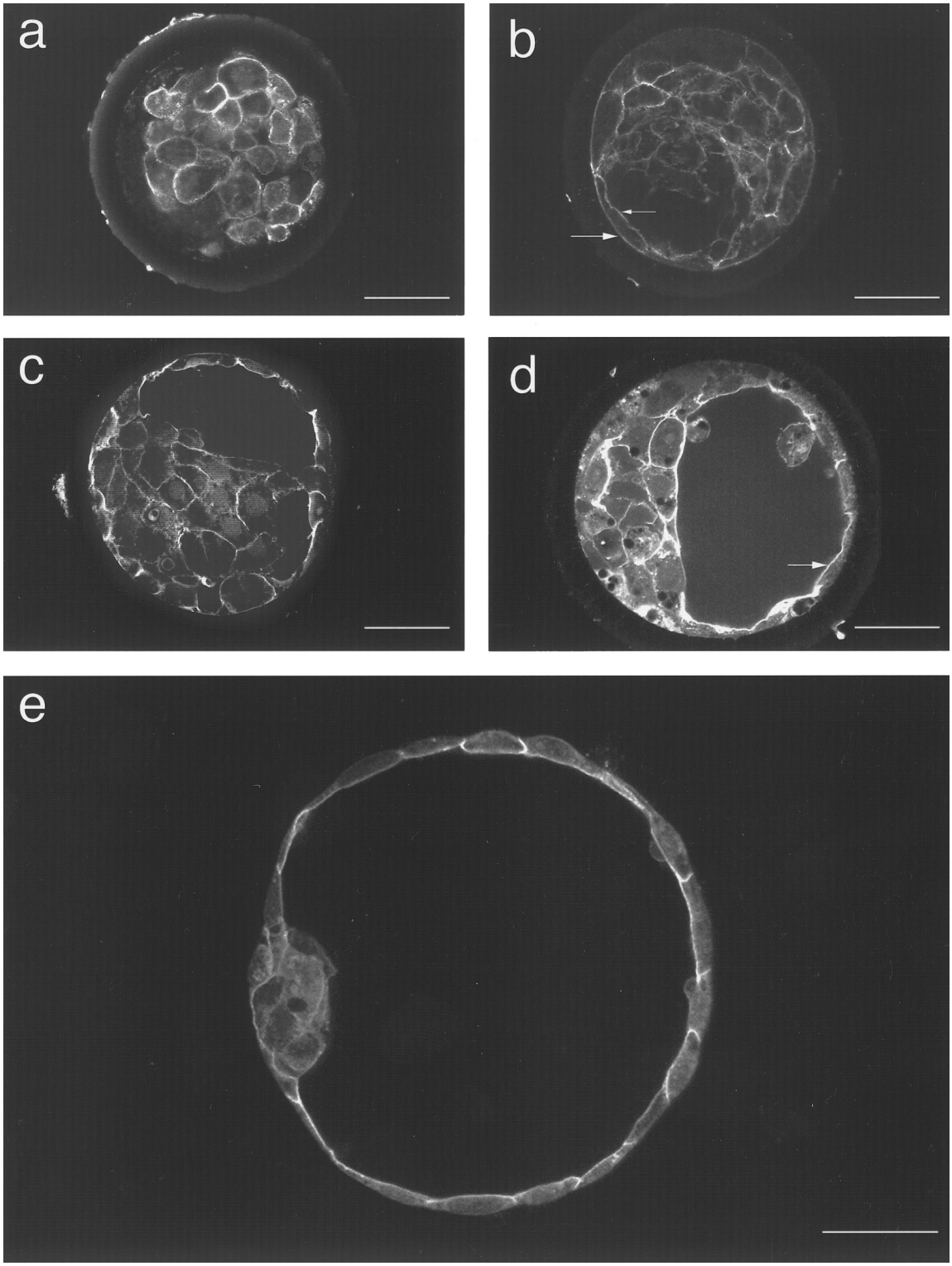

FIG. 2. Localization of α1-subunit during morula–blastocyst transition. Na/K-ATPase α1-subunit was detected encircling the cell surfaces of both the outer and inner blastomeres of 16- to 32-cell morulae (a). In early blastocysts (b) immunofluorescence revealed α1-subunit in both the apical (large arrow) and basolateral (small arrow) surfaces of the trophectoderm. The α1 immunofluorescence progressively disappeared from the apical regions of the trophectoderm (c) and became restricted to the basolateral cell regions (arrow) of the trophectoderm of expanding blastocysts (d). Expanded bovine blastocysts (e) clearly displayed the restricted localization of the α1-subunit polypeptide confined to the basolateral membrane regions of the outer epithelial trophectoderm. Scale bars = 50 μm.

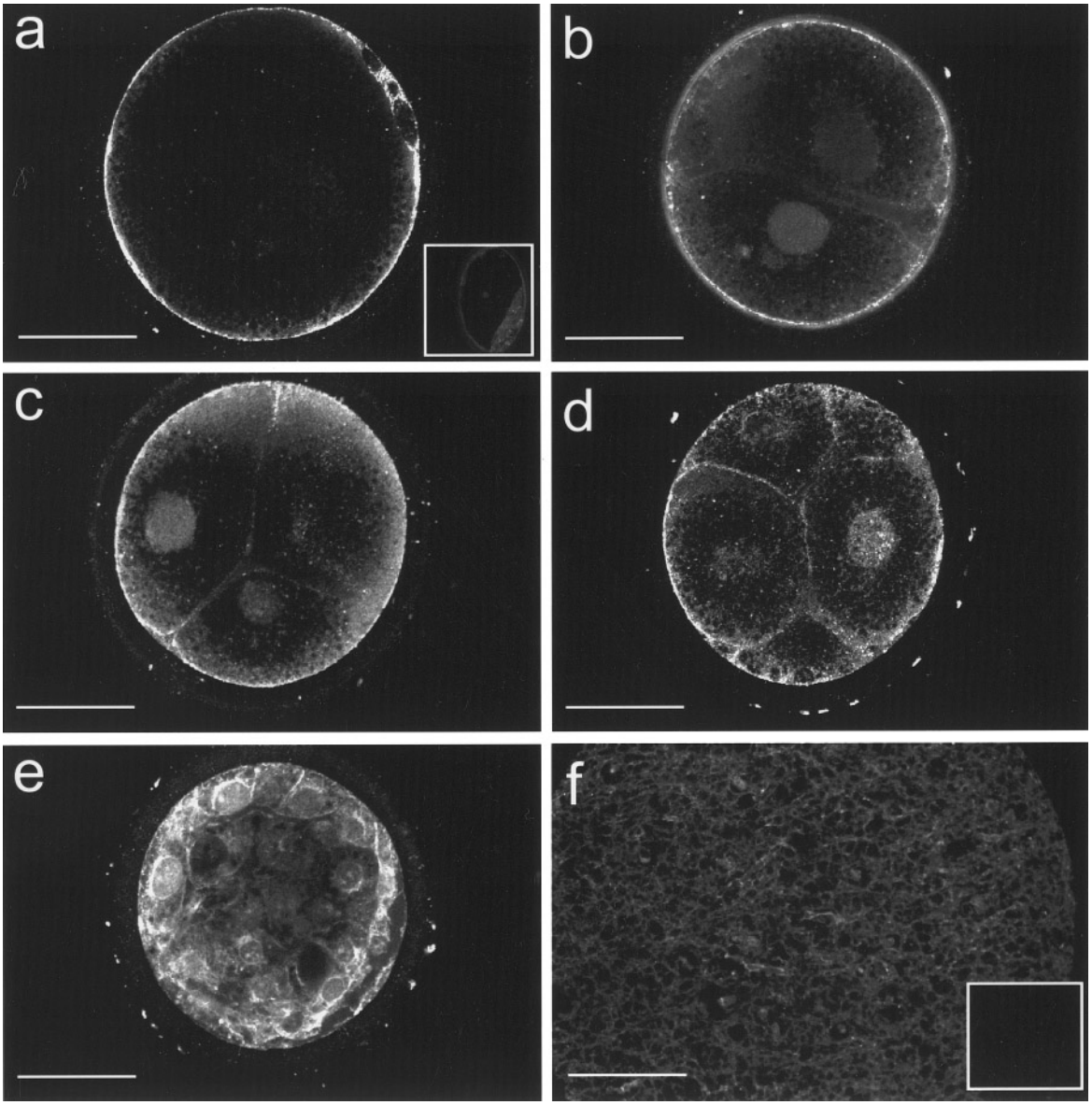

FIG. 3. Detection of Na/K-ATPase α3-subunit polypeptides in bovine cleavage stages. One-cell (a), 2-cell (b), 4-cell (c), and 8-cell (d) embryos and 16- to 32-cell morulae (e) incubated with α3-subunit antiserum revealed α3-subunit immunofluorescence encircling the cell margins of each blastomere. An immunofluorescence confocal image of paraffin-embedded bovine brain treated with the Caplan α3-subunit antibody is shown (f). α3-subunit immunofluorescence is localized to neurons. Secondary controls (no primary antibody) are shown (insets, a and f). Scale bars = 50 μm.

opment, from one-cell zygotes to blastocysts (Figs. 3a–3e and 4c and 4d). Na/K-ATPase α3-subunit immunofluorescence was present within cell margins encircling each blastomere in one-cell zygotes through to the morula stage and in some cases included cortical cytoplasmic regions of each blastomere as well (Figs. 3a–3e). Unexpectedly, the α3 immunofluorescent signal was observed in the apical cell margins of the trophectoderm with a reduced signal present in the basolateral membrane regions (Figs. 4c and 4d). There was no apparent detection of the α3 polypeptide within the ICM. This is in marked contrast to the blastocyst α1 immunofluorescence pattern reported above.

$^{86}Rb^{+}$ Transport in Bovine Embryos and Blastocysts

Cytochalasin D was employed since it specifically disrupts the actin cytoskeleton (required to break the tight

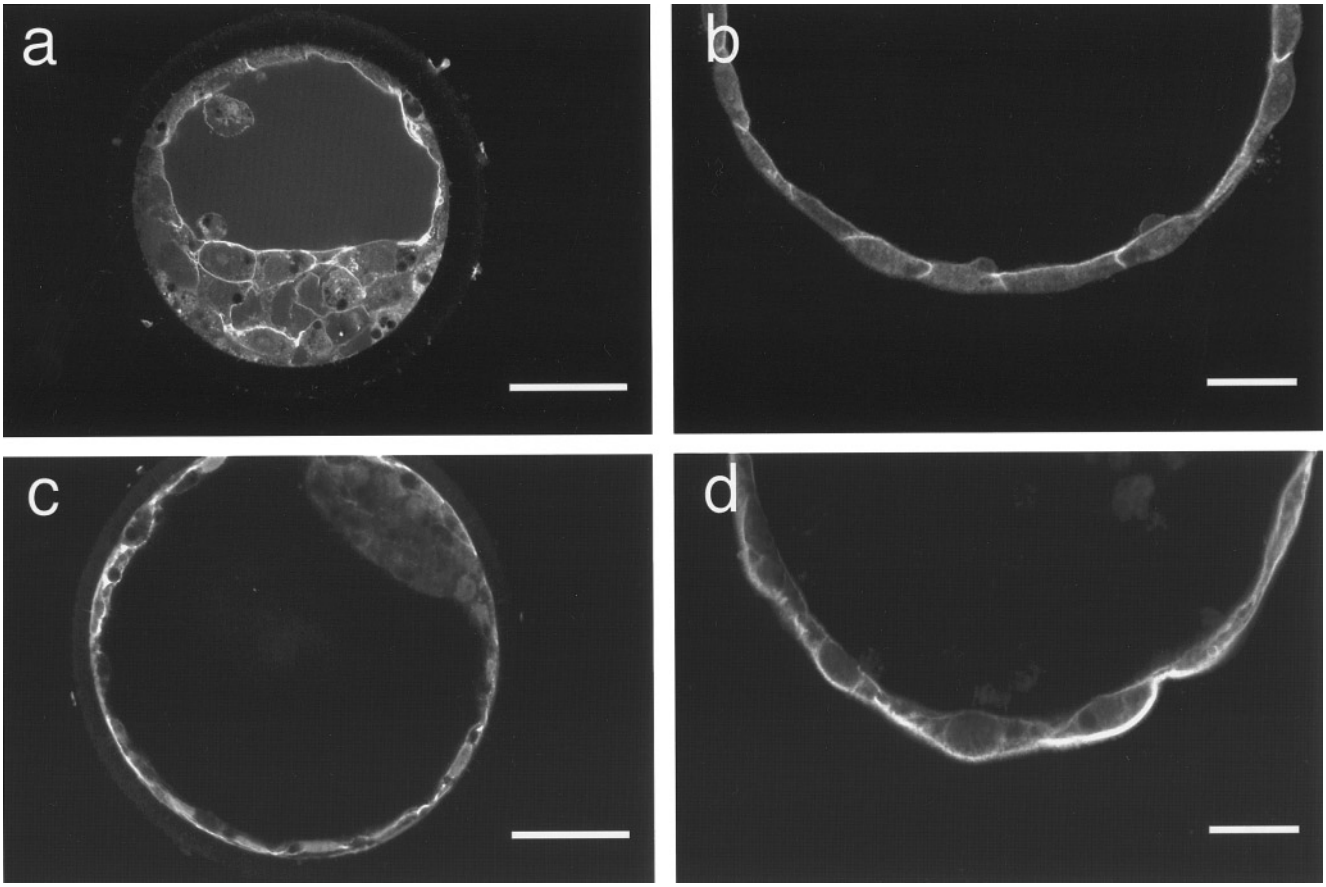

FIG. 4. Localization of Na/K-ATPase α1- and α3-subunit polypeptides in bovine blastocysts. Localization of α1-subunit of Na/K-ATPase in day 8 *in vitro* cultured bovine blastocysts (a). Immunofluorescence was confined to the basolateral cell margins of the trophectoderm, but encircled the entire cell periphery of each inner cell mass cell (ICM). An enlarged image of the trophectoderm highlighting the basolateral distribution of the α1-subunit immunofluorescent signal is shown (b). Localization of the α3-subunit of Na/K-ATPase in day 8 *in vitro* cultured bovine blastocysts is shown (c). The α3-subunit immunofluorescent signal was predominantly confined to the apical cell margins of the polar and mural trophectoderms with a markedly reduced (in comparison to the α1-subunit signal) signal in the basolateral cell margins. There was no apparent detection of the α3 polypeptide within the ICM. Enlarged image of the trophectoderm highlighting the apical and reduced basolateral α3-subunit immunofluorescence (d). Scale bars = 50 μm (a, c) and = 25 μm (b, d).

junctional seal in epithelial tissues) without affecting glucose transport. Treatment of bovine blastocysts with cytochalasin D ranging from 5 to 30 μg/ml significantly increased ($P < 0.05$) ouabain-sensitive $^{86}Rb^{+}$ uptake compared to untreated blastocysts (on average threefold over control embryos; Fig. 5). Increasing cytochalasin D concentration did not significantly influence uptake by blastocysts (Fig. 5). Therefore, all subsequent experiments were performed using the lowest effective concentration of cytochalasin D (5 μg/ml).

$^{86}Rb^{+}$ uptake was linear between 5 and 40 min (Fig. 6) for treated one-cell ($y = 5.14x - 4.57$, $P < 0.05$) and untreated blastocysts ($y = 44.69x - 793.2$, $P < 0.05$). In contrast, uptake in treated blastocysts was linear only over the 5- to 20-min labeling interval ($y = 54.1x + 333.6$, $P < 0.05$).

While uptake was significantly ($P < 0.05$) higher in treated versus untreated blastocysts over this time course, there was no significant difference in uptake between treated and untreated one-cell embryos. We selected a 20-min labeling interval for all subsequent experiments because $^{86}Rb^{+}$ uptake was linear for this interval in all embryo stages. In addition, longer labeling intervals (i.e., 40 min) displayed an increased variability in uptake between experiments.

Analysis of ouabain-sensitive $^{86}Rb^{+}$ uptake data did not reveal any significant differences in Na/K-ATPase-mediated ion transport from the inseminated oocyte to the six- to eight-cell stage in either intact or cytochalasin D-treated groups (Fig. 7). Interestingly, while morulae (untreated day 6 p.i.) did not vary significantly from one-, three- to five-, or six- to eight-cell embryo groups, cytochalasin D-treated morulae displayed a significant ($P < 0.05$) increase in $^{86}Rb^{+}$

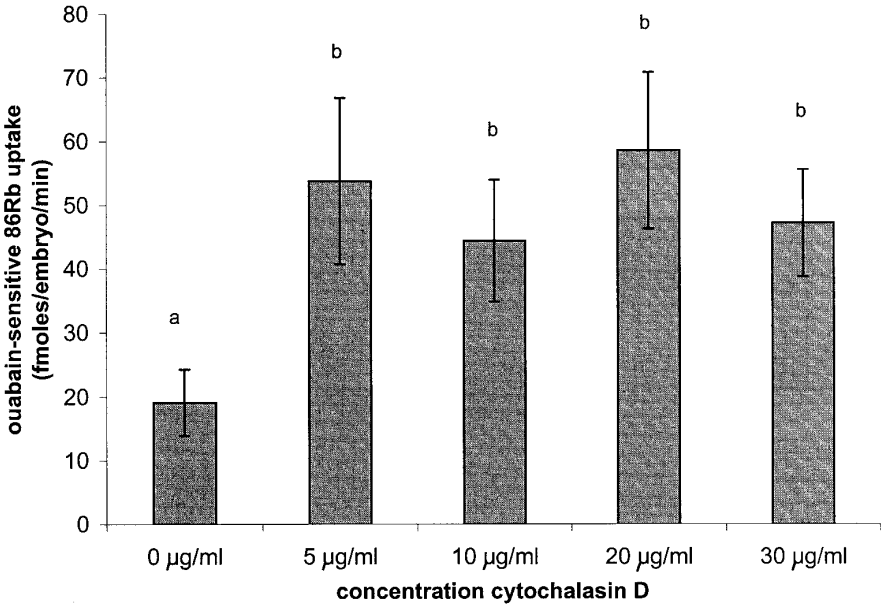

FIG. 5. Effect of cytochalasin D treatment on ouabain-sensitive $^{86}Rb^{+}$ uptake by bovine blastocysts. Day 8 postinsemination blastocysts were treated with 0, 5, 10, 20, or 30 μg/ml cytochalasin D for 1.5 h prior to labeling for 20 min with 0.4 mM $^{86}Rb^{+}$ ($n = 5$). Cytochalasin D treatment resulted in a significant increase in $^{86}Rb^{+}$ transport (at all concentrations; Student–Neuman–Kuels test, $P < 0.05$) over control embryos. No significant differences were observed among cytochalasin treatments (5, 10, 20, or 30 μg/ml cytochalasin D). Each data point (mean ± SE) represents the mean $^{86}Rb^{+}$ uptake (five embryos per cytochalasin concentration) obtained in five independent experiments. Data are presented as net ouabain-sensitive uptake (difference between + and − ouabain treatments for each group). Bars with the different letters are significantly different from one another.

uptake over all cleavage stage groups and untreated morulae, respectively (Fig. 7). Ouabain-sensitive $^{86}Rb^{+}$ uptake was significantly enhanced ($P < 0.05$) in untreated blastocysts compared to untreated cleavage stage embryos and morulae (approximately 6.5- and 8.6-fold increases in uptake over one-cell embryos and morulae, respectively). When tight junction integrity was disrupted by cytochalasin D treatment, blastocysts displayed significant ($P < 0.05$) 4.5- and 3-fold increases in $^{86}Rb^{+}$ uptake over similarly treated morulae and untreated blastocysts, respectively. In contrast, $^{86}Rb^{+}$ uptake underwent a significant ($P < 0.05$) 18.1-fold increase between cytochalasin D-treated blastocysts and treated and untreated six- to eight-cell embryos (Fig. 7).

Na/K-ATPase α1-Subunit Immunofluorescence in Cytochalasin D-Treated Blastocysts

Blastocysts (20–25) from control, cytochalasin D-treated, and cytochalasin D-recovered treatment groups were examined and the same immunofluorescence procedures were applied as described. Blastocysts incubated in TCM-199 medium alone (controls) for 1.5 h displayed an identical Na/K-ATPase α1-subunit immunofluorescent distribution pattern as described above. The signal was restricted to the basolateral membrane domains of the polar and mural trophectoderm and also encircling the cells of the ICM (Figs. 8a and 8b). Blastocysts cultured in cytochalasin D (5 μg/ml) were composed of rounded trophectoderm cells and displayed an apolar α1-subunit immunofluorescent signal encircling the entire periphery of both trophectoderm and ICM cells (Figs. 8c and 8d). Recovered cytochalasin D-treated blastocysts primarily displayed a restored polarized basolateral trophectoderm Na/K-ATPase α1-subunit immunofluorescence pattern, identical to nontreated control blastocysts (Figs. 8e and 8f). However, some immunofluorescence was observed in the apical and lateral domains between a few trophectoderm cells, indicating that not all blastomeres recover from cytochalasin D treatment in a 2-h interval (Fig. 8f).

DISCUSSION

Localization of Na/K-ATPase α1- and α3-Subunit Polypeptides in Bovine Preattachment Embryos

The timing of appearance and distribution of the Na/K-ATPase α1-subunit throughout preimplantation development, reported in the present study, varies from our earlier report. Na/K-ATPase was first detected by immunofluorescence in the late morula of preimplantation mouse embryos

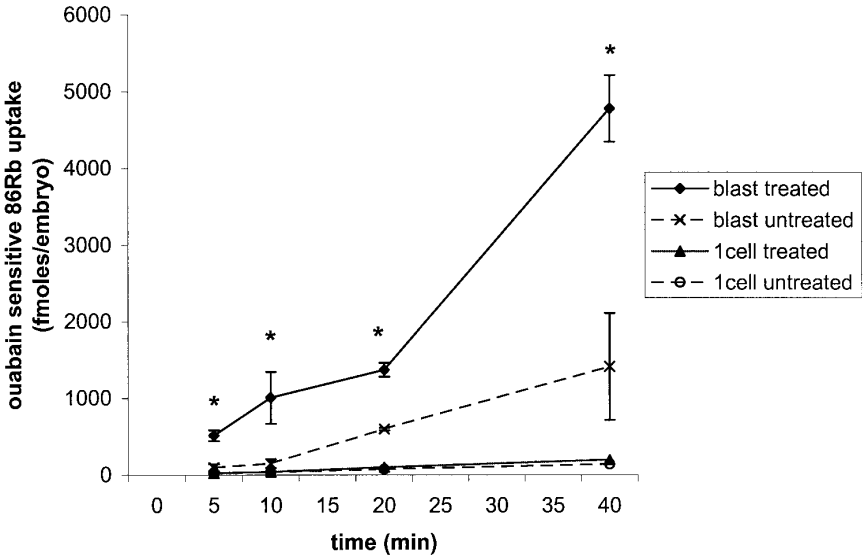

FIG. 6. Uptake time course for ouabain-sensitive $^{86}Rb^{+}$ transport by bovine blastocysts. Blastocysts (day 8 p.i.) and one-cell embryos (day 1 p.i.) were labeled with 0.4 mM $^{86}Rb^{+}$ for intervals of 5, 10, 20, and 40 min to assess the linear range of $^{86}Rb^{+}$ uptake. $^{86}Rb^{+}$ uptake was linear between 5 and 40 min for treated one-cell ($y = 5.14x - 4.57$, $P < 0.05$) and untreated blastocysts ($y = 44.69x - 793.2$, $P < 0.05$). In contrast, uptake in treated blastocysts was linear only over the 5- to 20-min labeling interval ($y = 54.1x + 333.6$, $P < 0.05$). Uptake was significantly ($P < 0.05$) higher in treated versus untreated blastocysts over this time course; there was no significant difference in uptake between treated and untreated one-cell embryos. Each data point (mean ± SE) represents the mean $^{86}Rb^{+}$ uptake (five embryos per time point) obtained in three independent experiments. Data are presented as net ouabain-sensitive uptake (difference between + and − ouabain treatments for each group and time point).

(Watson and Kidder, 1988). The dramatic transition from an apolar morula pattern to a polarized basolateral trophectoderm cell distribution was observed for murine blastocysts; however, no immunofluorescence was observed within the inner cell mass or polar trophectoderm (Watson and Kidder, 1988). The results from this earlier murine study were at variance with other evidence reporting Na/K-ATPase activity in mouse oocytes and early cleavage stage embryos derived from ion flux measurements (Powers and Tupper, 1975, 1977). Additionally, Gardiner *et al.* (1990a) detected Na/K-ATPase α- and β-subunit protein by Western blot analysis throughout mouse preimplantation development. Immunohistochemical methods also detected Na/K-ATPase in the closely apposed membranes of both trophectoderm and ICM cells of murine blastocysts (Vorbrodt *et al.*, 1977). The results reported in our present study clearly support the presence of active Na/K-ATPase throughout bovine early development. These findings are further supported by Baltz *et al.* (1997) who also detected Na/K-ATPase activity throughout murine preimplantation development by measuring effects of ouabain on embryonic intracellular Na^{+} and K^{+} levels. Our earlier mouse embryo results (Watson and Kidder, 1988) were obtained from 1-μm sections and our failure to detect the protein in early cleavage stages may simply have stemmed from a low abundance of the protein in these stages. The whole-mount approach applied in the present study clearly has overcome this problem and documents the distribution of the immunodetectable Na/K-ATPase α1-subunit throughout the first week of mammalian development.

The present study has also demonstrated that multiple α-isoform polypeptides are expressed and distributed in preimplantation embryos. To date, α3 polypeptides have been primarily localized to neural tissue membranes (Shyjan and Levenson, 1989). The most unexpected result in our study was that the α3-subunit distribution in blastocysts was distinct from the pattern observed for the α1 polypeptide.

Based on substrate–saturation kinetic analyses (stemming from ouabain-sensitive $^{86}Rb^{+}$ transport studies), Van Winkle and Campione (1991) predicted that different forms of Na/K-ATPase may be present in preimplantation mouse conceptuses. The presence of more than one isoform of Na/K-ATPase during preimplantation development was also predicted from Western blot results revealing three protein bands comigrating for the Na/K-ATPase α-subunit in mouse preimplantation embryos (Gardiner *et al.*, 1990a). It was suggested that the three bands could represent the three different α-subunit isozymes (α1, α2, and α3). Betts *et al.* (1997) recently reported the detection of transcripts encoding α1, α2, and α3 isoforms throughout bovine preattachment development. Taken together, these results provide definitive evidence supporting the expression of multiple Na/K-ATPase isoforms throughout the first week of mammalian development.

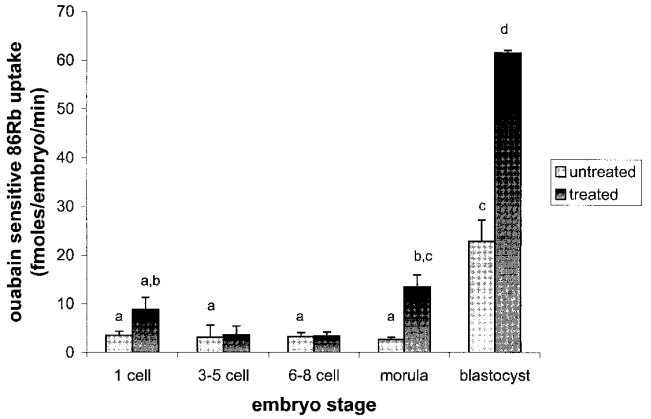

FIG. 7. Developmental profile of ouabain-sensitive $^{86}Rb^+$ uptake by bovine preattachment embryos *in vitro*. Pools of staged embryos (five embryos/determination; $n = 5$) were treated with (treated) or without (untreated) 5 μg/ml cytochalasin D for 1.5 h prior to measuring ouabain-sensitive $^{86}Rb^+$ uptake (0.4 mM $^{86}Rb^+$ in K^+-depleted KSOMaa for 20 min). $^{86}Rb^+$ uptake did not vary significantly from the inseminated oocyte to the six- to eight-cell stage in either intact or cytochalasin D-treated groups. Untreated morulae (day 6 p.i.) did not vary significantly from one-, three- to five-, or six- to eight-cell embryo groups. Cytochalasin D-treated morulae displayed a significant ($P < 0.05$) increase in $^{86}Rb^+$ uptake over all cleavage stage groups and untreated morulae. $^{86}Rb^+$ uptake was significantly enhanced ($P < 0.05$) in untreated blastocysts compared to untreated cleavage stage embryos and morulae (approximately 6.5- and 8.6-fold increase in uptake over one-cell embryos and morulae, respectively). Treated blastocysts displayed a significant ($P < 0.05$) 4.5- and 3-fold increases in $^{86}Rb^+$ uptake over similarly treated morulae and untreated blastocysts, respectively. $^{86}Rb^+$ uptake underwent a significant ($P < 0.05$) 18.1-fold increase between cytochalasin D-treated blastocysts and treated and untreated six- to eight-cell embryos. Data represent mean values for fmol $^{86}Rb^+$/embryo/min ± SE. Bars with different letters (a–d) are significantly different ($P < 0.05$).

Ouabain-Sensitive $^{86}Rb^+$ Uptake in Bovine Preattachment Embryos

Our results clearly support the presence of ouabain-sensitive cation-transporting activity throughout bovine preattachment development. $^{86}Rb^+$ uptake was largely maintained at a constant level from the one-cell zygote to the six- to eight-cell stage, but then increased dramatically to the blastocyst stage. This increase in ouabain-sensitive $^{86}Rb^+$ uptake is coordinately timed with blastocyst formation and parallels the increased abundance of Na/K-ATPase subunit mRNAs (Watson *et al.*, 1990b; MacPhee *et al.*, 1994; Betts *et al.*, 1997), subunit proteins (Watson and Kidder, 1988; Gardiner *et al.*, 1990b), and enzyme activity (Vorbrodt *et al.*, 1977; Van Winkle and Campione, 1991; Baltz *et al.*, 1997) observed during murine and bovine blastocyst formation. In contrast to the 2-fold increase in ouabain-sensitive $^{86}Rb^+$ uptake reported for murine embryos throughout the first week of development (Van Winkle and Campione, 1991), we observed more than an 18-fold increase in uptake from the six- to eight-cell to bovine blastocyst stages (cytochalasin-treated groups).

Considerable ouabain-sensitive $^{86}Rb^+$ uptake was measured in untreated bovine blastocysts. Van Winkle and Campione (1991) also reported $^{86}Rb^+$ transport within intact murine blastocysts raising the possibility that a component of this Na/K-ATPase transport activity may be directed from the apical trophectoderm membrane of mammalian blastocysts. Alternatively, the junctions between trophectoderm cells might be leaky and allow the paracellular movement of $^{86}Rb^+$ and ouabain into the blastocyst cavity.

Although vectorial transport of Na/K-ATPase to the basolateral surface of membranes is conventional (Caplan *et al.*, 1986; Gottardi and Caplan, 1993a; Zurzolo and Rodriguez-Boulan, 1993), apical localization of the sodium pump has been reported in the choroid plexus (Marrs *et al.*, 1993; Villalobos *et al.*, 1997), cockroach salivary glands (Just and Walz, 1994), and retinal pigment epithelium (Sugasawa *et al.*, 1994). Experiments performed on one clone of MDCK cells (Siemers *et al.*, 1993) and A6 epithelia (Coupaye-Gerard *et al.*, 1997) indicate that initially the Na/K-ATPase is randomly delivered to both cell surface domains. The polarized distribution of Na/K-ATPase occurs through the selective stabilization of the enzyme at the basolateral plasma membrane through interactions with the cytoskeleton (Hammerton *et al.*, 1991; Marrs *et al.*, 1993). Na/K-ATPase binds with high affinity to a complex of membrane–cytoskeletal proteins containing ankyrin and fodrin (Morrow *et al.*, 1989; Nelson and Veshnock, 1987; Nelson *et al.*, 1990; Marrs *et al.*, 1993). Although ankyrin and fodrin are restricted to the basolateral surfaces of polarized MDCK cells (Morrow *et al.*, 1989), they have been associated with the apical membrane domains in other epithelia (Gundersen *et al.*, 1991), and isoforms of these proteins are found in both membrane domains of some polarized cells (Kordeli and Bennett, 1991; Kunimoto *et al.*, 1991). Assembly of the membrane–cytoskeletal complexes in selective regions may provide a flexible mechanism for generating different distributions of Na/K-ATPase isoforms in other polarized epithelial cells in which the subunits are localized to the apical membrane (Bok, 1982; Byers and Graham, 1990; Gosh *et al.*, 1990; Gundersen *et al.*, 1991) or to the apical and lateral membranes (Avner *et al.*, 1992; Hammerton *et al.*, 1991). Whether this selective inclusion occurs for the Na/K-ATPase α1 polypeptide in the basolateral and the α3 polypeptide in the apical cell domains of the mural and polar trophectoderm in bovine blastocysts must be determined by future experiments.

The immunofluorescence localization of the α3-subunit to the apical trophectoderm membrane domains in bovine blastocysts may, in part, explain their greater sensitivity (over that displayed by their murine counterparts) to the specific inhibitor ouabain (Betts *et al.*, 1997). The rodent Na/K-ATPase α3-subunit displays a greater ouabain sensitivity and a lower affinity for Na^+ than the α1-subunit (Sweadner, 1989). In addition, bovine blastocysts can be col-

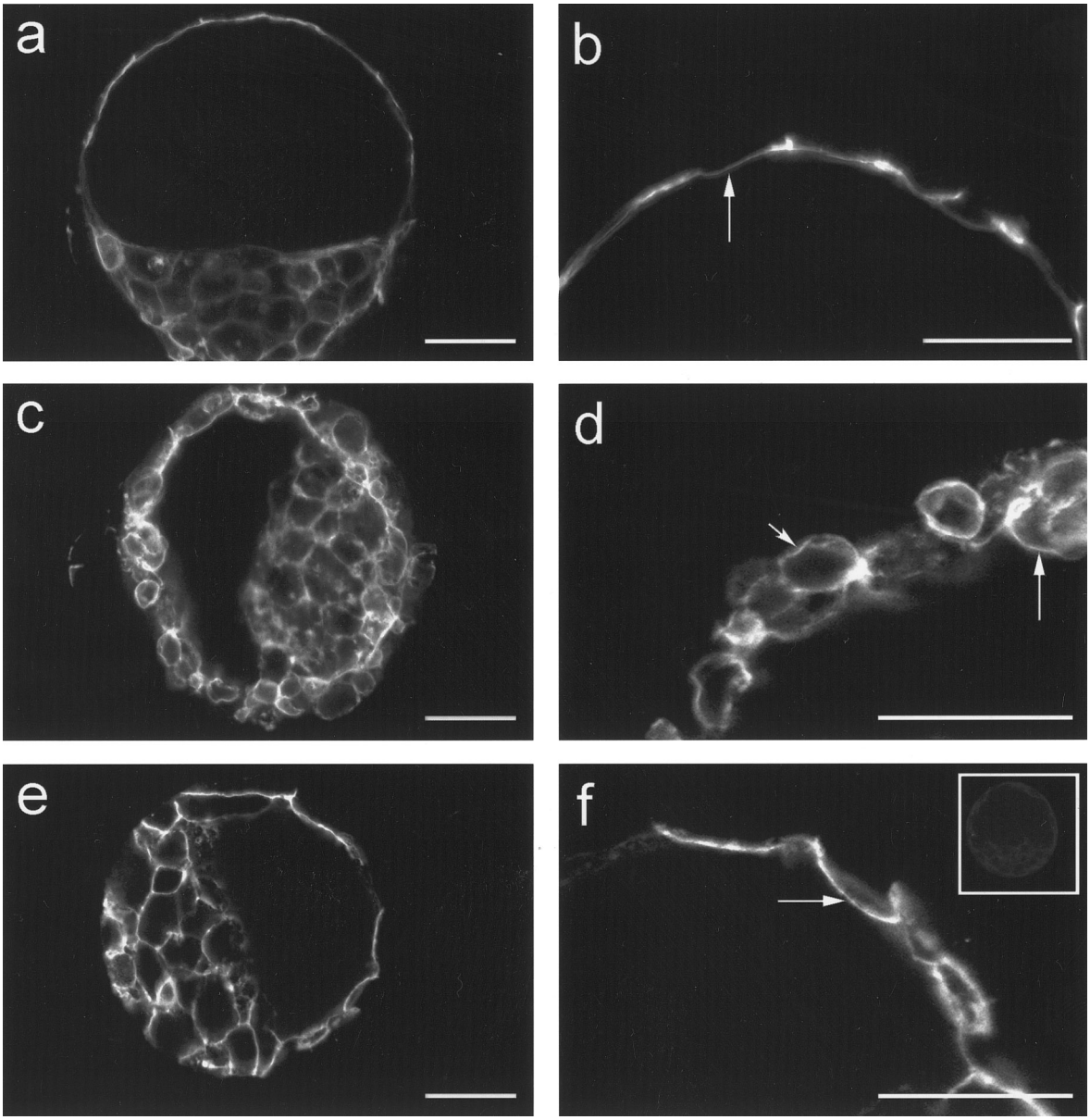

FIG. 8. Na/K-ATPase α1-subunit immunofluorescent distribution in cytochalasin D-treated blastocysts. α1 immunofluorescence staining in control blastocysts (a) revealed the expected α1-subunit distribution restricted to the basolateral cell margins of the mural and polar trophectoderm. Enlarged image of control trophectoderm highlighting this basolateral distribution (b, arrow). After a 1.5-h treatment in 5 μg/ml cytochalasin D all trophectoderm cells rounded up and the α1 immunofluorescence changed to an apolar pattern encircling the entire cell periphery of each outer cell (c). Enlarged image of the cytochalasin D-treated trophectoderm highlighting the variation in α1 immunofluorescence displayed in these embryos (d, arrows). The Na/K-ATPase α1-subunit immunofluorescence signal was observed to return to the basolateral trophectoderm cell margins after a 2-h recovery period in fresh cytochalasin D-free medium (e). Enlarged immunofluorescent image of the recovered trophectoderm highlighting the return to a polarized basolateral distribution (f, arrow). Secondary control (no primary antibody) is shown (f, inset). Scale bars = 50 μm.

lapsed simply by treatment with high (10^{-6} to 10^{-3} M) ouabain, without the initial treatment with cytochalasin D (data not shown). Mouse blastocysts require cytochalasin-induced collapse presumably in order for ouabain to gain access to their extracellular binding sites located along the basolateral membrane domains facing the cavity (Dizio and Tasca, 1977). Wiley and Obasaju (1989) demonstrated that with lower concentrations of ouabain (10^{-5} M), blastocyst

fluid accumulation was accelerated and at higher concentrations (10^{-4} M), fluid accumulation was delayed. These data support the possibility that the apical α3-subunit plays an active role in blastocyst formation in the bovine embryo.

Immunofluorescent localization patterns do not shed any light on the functional significance of α1 and α3 polypeptides to the overall mechanism of cavitation, although treatment of collapsed bovine blastocysts with 10^{-9} M ouabain causing reduced blastocyst reexpansion is highly suggestive of the α3 isoform contributing to blastocyst formation (Betts *et al.*, 1997). We propose that an apical trophectoderm α3 isoform could participate in regulating the steepness of the transtrophectoderm Na^+ gradient which is required to drive the osmotic accumulation of water across this epithelium to form the blastocyst cavity. Support for this role comes from studies investigating the kinetics of transfected α isoforms in Hela cells (Zahler *et al.*, 1997). This study reports that the α3 isoform may regulate high intracellular Na^+ loads in order to restore physiological Na^+ levels. We are uncertain as to how high trophectoderm cell intracellular Na^+ levels rise during blastocyst formation. Baltz *et al.* (1997) measured intracellular Na^+ and K^+ levels up to the murine morulae stage. We propose that the hypothesized increase in intracellular Na^+, required to drive blastocyst formation, must be regulated to ensure that intracellular Na^+ levels do not reach excessive levels. An apical α3 Na/K-ATPase could perform this role and maintain the trophectoderm cell Na^+ gradient within physiological limits. The α3-subunit has a fourfold lower affinity for cytoplasmic Na^+ than the α1-subunit (Munzer *et al.*, 1994), so localization of the α3-subunit to the apical membrane domains would not necessarily disrupt a transtrophectoderm Na^+ gradient (driven by the basolateral Na/K-ATPase; Wiley, 1988; Manejwala *et al.*, 1986, 1989), but instead could serve to modulate the gradient by moving Na^+ from inside the cell to the extraembryonic environment. Functional studies applied to isoform-specific null mutants will be required to determine the precise ion transport properties of each Na/K-ATPase isoform during blastocyst formation.

To conclude, our results demonstrate that multiple Na/K-ATPase α-subunit isoforms are expressed throughout the first week of mammalian embryonic development. α1 and α3 polypeptides are distributed asymmetrically to basolateral and apical (respectively) cell margins in bovine blastocysts. Ouabain-sensitive Na/K-ATPase-mediated $^{86}Rb^+$ uptake undergoes a dramatic increase coincident with bovine cavitation. These results have advanced our earlier murine studies and clearly indicate that the role of the Na/K-ATPase in blastocyst formation is more complex than first thought by raising the possibility of multiple isozymes of the Na/K-ATPase contributing to blastocyst formation.

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