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Aquaporin proteins in murine trophectoderm mediate transepithelial water movements during cavitation

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Abstract

Mammalian blastocyst formation is dependent on establishment of trophectoderm (TE) ion and fluid transport mechanisms. We have examined the expression and function of aquaporin (AQP) water channels during murine preimplantation development. AQP 3, 8, and 9 proteins demonstrated cell margin-associated staining starting at the 8-cell (AQP 9) or compacted morula (AQP 3 and 8) stages. In blastocysts, AQP 3 and 8 were detected in the basolateral membrane domains of the trophectoderm, while AQP3 was also observed in cell margins of all inner cell mass (ICM) cells. In contrast, AQP 9 was predominantly observed within the apical membrane domains of the TE. Murine blastocysts exposed to hyperosmotic culture media (1800 mOsm; 10% glycerol) demonstrated a rapid volume decrease followed by recovery to approximately 80% of initial volume over 5 min. Treatment of blastocysts with p-chloromercuriphenylsulfonic acid (pCMPS, ≥ 100 μM) for 5 min significantly impaired ($P < 0.05$) volume recovery, indicating the involvement of AQPs in fluid transport across the TE. Blastocysts exposure to an 1800-mOsm sucrose/KSOMaa solution did not demonstrate volume recovery as observed following treatment with glycerol containing medium, indicating glycerol permeability via AQPs 3 and 9. These findings support the hypothesis that aquaporins mediate $trans$-trophectodermal water movements during cavitation.
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Introduction

Mammalian preimplantation development culminates in the formation of the fluid-filled blastocyst, composed of a group of apolar cells (the inner cell mass; ICM) and a fluid cavity whose boundary is formed by the trophectoderm epithelium (TE). Differentiation of the trophectoderm occurs at the morula stage of development, when molecular and cellular events leading to the maturation of the epithelial junctional complexes (adherens and tight junctions) are initiated (reviewed by Collins and Fleming, 1995; Fleming et al., 2001). E-cadherin-mediated cell–cell adhesion coordinates cellular polarity and maturation of the tight junction, resulting in the formation of distinct apical and basolateral membrane domains within the TE cell layer. The resulting

polarity of ion/solute transporters mediates the establishment of ionic gradients across the TE, leading to accumulation of water within the nascent blastocoel cavity in response to the newly established $trans$-trophectoderm osmotic gradient (reviewed by Biggers et al., 1988; Watson, 1992; Watson and Barcroft, 2001).

Unidirectional Na^+ movements occur predominantly in a trophectoderm-to-blastocoel direction, similar to that observed in other transporting epithelia (Neilsen et al., 1987). Borland et al. (1976) demonstrated active Na^+ and Cl^- transport into the blastocoel cavity in response to hyperosmotic conditions induced by sucrose supplementation of blastocyst culture media in vitro. Addition of 2 mM sucrose to culture media results in increased accumulation of Na^+ and Cl^- (1 mM each) across the trophectoderm in a stoichiometry indicative of active Na^+ transport (Borland et al., 1976). Establishment of the $trans$-trophectoderm ionic gradients appears to be energized by activity of the

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Na/K-ATPase within the basolateral membrane domains of the TE (Betts et al., 1997; Biggers et al., 1978; Watson et al., 1988; Wiley, 1984) as Na$^+$-pump inhibition blocks blastocyst formation in vitro (Biggers et al., 1978; Wiley, 1984) and following experimental collapse of blastocysts with cytochalasins (Betts et al., 1997; DiZio and Tasca, 1977). Expression of the NHE-3 Na$^+$/H$^+$-exchanger has been demonstrated in the apical membranes of mouse trophectoderm cells (Barr et al., 1998). Apical Na$^+$/glucose and Na$^+$/amino acid cotransporter expression has also been identified within the apical margins of the trophectoderm (DiZio and Tasca, 1977; Manejwala et al., 1989; Miller and Schultz, 1985). An amiloride-sensitive Na$^+$ conductance also occurs within the trophectoderm (Manejwala et al., 1989; Notarianni and Hirst, 1999; Robinson et al., 1991) where a high-amiloride affinity Na$^+$-channel has been localized within the apical membranes (Robinson et al., 1991). In a porcine trophectoderm cell line, Notarianni and Hirst (1999) demonstrated that amiloride treatment resulted in loss of the Na$^+$ and Cl$^-$ gradient across these cells, suggesting that amiloride-sensitive Na$^+$ channels play an important role in the apical entry of Na$^+$ in trophectoderm cells.

Although fluid accumulation during cavitation has been historically attributed to the process of simple diffusion, it is evident that blastocyst formation occurs in the absence of a steep osmotic gradient across the trophectoderm of rabbit blastocysts, where the blastocoel fluid has been demonstrated to be hyperosmotic to culture media by approximately 8 mOsm/kg (Biggers et al., 1988; Borland et al., 1977). Recent evidence also suggests that, in some species, such as the horse, cavitation occurs in the presence of blastocoel fluid that is hypoosmotic to the surrounding environment of the embryo (Waelchli and Betteridge, 1996). Despite the presence of shallow osmotic gradients across the TE cell layer, fluid accumulation occurs rapidly, increasing in the rabbit blastocyst from 6.8 nL/h on embryonic day 5 to 18 μL/h by day 8 (Borland et al., 1976).

Aquaporins (AQPs) are transmembrane channel proteins within animal and plant cells, that function as molecular water channels allowing water to flow rapidly across the membrane in the direction of osmotic gradients, mostly in epithelial tissues (Shiels and Griffin, 1993). To date, 11 mammalian homologues belonging to this family of water channel proteins have been identified (reviewed by Badaut et al., 2001). This protein family can be further subdivided into 2 groups based on selectivity of the water pore for water and small solutes. The "true" aquaporins (AQPs 0, 1, 2, 4, 5, 6, 8, and 10) have a pore structure that is highly selective for water molecules, while the "aquaglyceroporins" (AQPs 3, 7, and 9) have less selectivity, allowing passage of small solutes, such as glycerol, urea, purines, pyrimidines, carbamides, and polyols (Borgnia et al., 1999; Ko et al., 1999; Kuriyama et al., 1997; Sansom and Law, 2001; Tsukaguchi et al., 1998; van Os et al., 2002). In addition to differences in solute permeability, aquaporins

also differ in their global sensitivity to mercuric inhibition, with AQPs 4 and 7 representing the mercury-insensitive water channels (Jung et al., 1994; Ishibashi et al., 1997a).

Rapid fluid transport during cavitation in response to a relatively small osmotic gradient suggests that a channel-mediated process for *trans*-trophectodermal fluid accumulation exists. In various epithelial systems, aquaporins are involved in mediating rapid near-isosmotic fluid transport across cell layers (Verkman et al., 1996; Verkman, 1999, 2000). Data from our lab and others have demonstrated that murine preimplantation embryos express mRNAs for multiple AQPs throughout preimplantation development (Edashige et al., 2000; Offenberg et al., 2000). We have demonstrated that murine preimplantation embryos express mRNAs for AQP1, 3, 5, 6, 7, 8, and 9 (Offenberg et al., 2000). AQP3 mRNA expression increased at the morula–blastocyst transition, and AQP 8 mRNA expression was first detected at the morula stage (Offenberg et al. 2000). Expression of aquaporin family member mRNAs by the preimplantation embryo (Edashige et al. 2000; Offenberg et al. 2000) predicts that AQPs could be involved in mediating fluid transport during mammalian cavitation. Although there may be mouse strain-specific differences in the AQP mRNA subtypes present during preimplantation development, expression of mRNA for AQPs 3, 7, 8, and 9 are all observed at the morula/blastocyst stage in both ICR (Edashige et al., 2000) and CD-1 × CB6F1/J (Offenberg et al., 2000) mice. In the present study, we have examined expression of the mercury-sensitive AQPs, AQP3, 8, and 9, in order to determine their role in mediating fluid accumulation during blastocyst formation. We have characterized the expression of AQP 3, 8, and 9 proteins during murine preimplantation development and have established a role for AQP-mediated fluid transport across the trophectoderm of the murine blastocyst.

Materials and methods

Superovulation and mouse embryo collection

Female CD-1 mice (Charles River, Canada; 3–5 weeks old) were injected with 5 IU PMSG (Intervet Canada Ltd, Whitby ON), followed by 5 IU hCG (Intervet) 47 h later and mated with CB6F1/J males. Successful mating was determined the following morning (day 1) by detection of a vaginal plug. Time post-hCG was used to measure the developmental age of the embryos. Preimplantation mouse embryos were collected at 18 (unfertilized oocytes), 48 (2-cell), 60 (4-cell), 65–68 (8-cell and compacting 8-cell), 80–85 (morulae), and 90 h (blastocysts) post-hCG. Zygote through compacted 8-cell-stage embryos were flushed from the reproductive tract by using the method of Spindle (1980) employing Flushing Medium I (1.71 mM calcium lactate, 0.25 mM sodium pyruvate, and 3 mg/ml bovine serum albumin added to 10× Leibovitz-modified Hank's Balanced

Salt Solution (HBSS) and diluted to 1× with water), while morulae and blastocysts were recovered by using Flushing Medium II [1.8 mM $CaCl_2$; amino acids: 0.1 mM L-arginine, 0.5 mM L-cysteine, 1.03 mM L-histidine, 0.2 mM L-isoleucine, 1.0 mM L-leucine, 2.0 mM L-lysine, 0.25 mM L-methionine, 0.5 mM L-phenylalanine, 2.0 mM L-threonine, 0.1 mM L-tryptophan, 0.1 mM L-tyrosine, 1.0 mM L-valine, and 2.0 mM L-glutamine (Sigma, St. Louis, MO); and 1× BME vitamins (GIBCO, Mississauga ON, Canada) in 10× Leibovitz-modified HBSS, diluted to 1× with water]. Embryos were washed four to five times in flushing media and either fixed for immunofluorescence or transferred to 20-μl drops of KSOMaa (Jones et al., 1997) under light paraffin oil and maintained in culture under 5% CO_2 in air atmosphere at 37°C, depending on their experimental destination.

Antibodies

Immunolocalization of AQPs 3, 8, and 9 during murine preimplantation development was determined by using proven commercially available antisera from Alpha Diagnostic Int. (San Antonio, TX) consisting of: (1) rabbit polyclonal anti-rat AQP3, directed against a 15-amino-acid sequence from the C terminus of rat AQP3; (2) rabbit polyclonal anti-rat AQP8, directed against a 16-amino-acid synthetic peptide from the C-terminal domain of rat AQP8; and (3) rabbit polyclonal anti-rat AQP9, raised against a 19-amino-acid synthetic peptide from the rat AQP9 C-terminal domain. All three antisera were employed at a dilution of 1:100 in conjunction with a goat anti-rabbit whole IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA). Each antiserum has been extensively characterized in Western blot and immunocytochemistry studies on mouse and rat tissues and established as specific for their target proteins (Ishibashi et al., 1994, 1997b, 1998; Ecelbarger et al., 1995; Echevarria et al., 1996; Koyama et al., 1997; Ma et al., 1994).

Indirect immunofluorescence detection of AQPs 3, 8, and 9 during murine preimplantation development

Murine embryos were collected from the reproductive tracts of superovulated female CD-1 mice as described above. Embryo pools were washed in 1× PBS (GIBCO) and fixed for indirect immunofluorescence in 2% paraformaldehyde in PBS at room temperature for 30 min. Fixed embryos were washed once in 1× PBS and either used immediately or stored at 4°C in Embryo Storage Buffer (1× PBS + 0.9% sodium azide) for up to 1 week before processing for whole-amount indirect immunofluorescence as previously described (Barcroft et al., 1998, 2002; Betts et al., 1998; Jones et al., 1997; MacPhee et al., 2000). Fixed embryos were permeabilized in Blocking Buffer (0.1 M lysine + 0.01% Triton X-100 + 1% normal Goat Serum in 1× PBS) at room temperature for 30 min followed by two washes in fresh PBS. Embryos were incubated with primary antisera for AQPs 3, 8, or 9 at a 1:100 dilution in Antibody Dilution/Wash Buffer (ADB; 0.1 M lysine + 0.005% Triton X-100 + 1 % normal Goat Serum in 1× PBS) at 4°C overnight. Embryos were then washed 3 times for 20 min in ADB and incubated with rhodamine-conjugated secondary antibody (1:50 in ADB) at room temperature for 2 h, followed by 3 washes for 20 min in ADB. Fully processed embryos were mounted onto glass slides in 20 μl of FluoroGuard Anti-fade Reagent (BioRad, Montreal PQ) under elevated 22 × 22-mm glass coverslips (No. 1 thickness), and slide preparations were sealed with nail polish. Slides were stored for up to 2 days at −20°C in a light tight box prior to immunofluorescence imaging employing a BioRad MRC600 Confocal Laser Scanning Microscope in conjunction with the COMOS Image Capture System (BioRad). In total, 20–30 embryos of each developmental stage were examined for each of the 3 primary antisera.

Western blot analysis of control mouse tissues was performed in order to confirm efficacy of each of the antisera to their target proteins (data not shown). Secondary control preparations for embryos were also generated by treating samples with secondary antiserum alone to control for nonspecific staining contributing to the second antibody.

Analysis of aquaporin mediated water transport in murine blastocysts

In order to determine the mechanism of water transport across the trophectoderm, acute changes in media osmolality were induced to examine the osmotic behavior of mouse blastocysts. Acute osmolality changes were achieved by employing a 10% glycerol solution as previously described in bovine blastocysts by Kaidi et al. (2000). Murine blastocysts collected at 90 h post-hCG were transferred individually to 1 μl KSOMaa (288 mOsm) drops under light paraffin oil in a 5% CO_2 in air incubator at 37°C. All experimental manipulations were performed at room temperature. Initial blastocyst volume was recorded by making a measurement of diameter along two axes of the embryo and assuming that the blastocyst represents a prolate sphere as described previously by Manejwala et al. (1989, 1986). Hyperosmotic conditions were induced by the addition of 1 μl of 20% glycerol in KSOMaa to individual blastocyst culture drops (final glycerol = 10% v/v; approximately 1800 mOsm final), and osmotic response of each embryo was recorded starting at 15 s postglycerol addition at 10-s intervals for a period of 5:30 min on a Nikon TS100 inverted microscope employing the Nikon DXM1200 digital camera in conjunction with the Act-1 Image Capture System (Version 2.0; Nikon). Post hoc measurements of blastocyst diameter were made every 10 s, and data were expressed as % initial blastocyst volume over the treatment interval. Involvement of aquaporins in mediating the osmotic response of murine blastocysts to 10% glycerol solutions was determined by employing the mercuric com-

pound p-chloromercuriphenylsulfonic acid (pCMPS; Sigma), which has been employed at concentrations of 1–2 mM in other studies to inhibit AQP-mediated water transport in experimental systems (Abrami et al., 1996; Cooper and Boron, 1998; Echevarria et al., 1996; Virkki et al., 2001). For murine blastocysts, concentration dependence for pCMPS inhibition of water transport was determined by pretreating embryos for 5 min at 37°C with 10, 100, or 500 μM pCMPS prior to determining initial blastocyst volume and addition of glycerol. Osmotic response was measured as for embryos treated without pCMPS and plotted as % initial blastocyst volume over the 5-min interval of 10% glycerol exposure. Data from the first minute of blastocysts reexpansion for each treatment were converted to pL/s fluid transport and plotted as cumulative rate of reexpansion in relation to experimental treatment.

The involvement of glycerol diffusion into the blastocoel cavity in the osmotic response to murine blastocysts was demonstrated by treating embryos with either 10% glycerol (approximately 1800 mOsm) or 1.4 M sucrose (approximately 1800 mOsm) solutions. Osmotic response of embryos under hyperosmotic conditions generated by these two solutions was determined as described above by measuring embryo response to hyperosmolality as % of initial blastocyst volume over the 5-min experimental interval.

Statistical analysis

Rate of reexpansion (pL/s) of blastocysts treated with 10% glycerol, for control vs 10–500 μM pCMPS, was determined, and inhibition of the rate of fluid accumulation over the first 60 s of blastocyst reexpansion in the 10% glycerol/pCMPS concentration-dependence assay was determined by analyzing the rates of fluid accumulation (pL/s) over the first minute of blastocyst reexpansion by one-way ANOVA with Bonferroni's Test ($P < 0.05$). Differences in mean blastocyst shrinkage (minimum % of initial blastocyst volume for blastocysts in each group) for pCMPS vs control treatments were assessed by one-way ANOVA with Bonferroni's T-test for differences ($P < 0.05$).

Results

Expression of AQP3, 8, and 9 polypeptides during murine preimplantation development

In total, 30–40 embryos from each developmental stage were examined from 3 replicate embryo collections for immunolocalization employing each of the antisera. The described immunolocalization patterns were consistently observed in all embryos examined for each antiserum.

AQP3 immunolocalization was not observed in membrane regions in one-cell, two-cell, and eight-cell embryos (Fig. 1a–c), although levels of fluorescence above those observed for control embryos (Fig. 1f) suggest that AQP3 is present within the cytoplasm of early cleavage-stage embryos. Cytoplasmic localization of AQP3-associated immunofluorescence was particularly evident in the blastomeres of the eight-cell-stage embryo (Fig. 1c), where distinct foci of fluorescence were seen throughout the cytoplasm of all embryonic cells. At the morula stage, AQP3 was localized in an apolar fashion within the membrane domains of all blastomeres of the compacted morula (Fig. 1d). At the blastocyst stage, an apolar distribution of membrane-associated AQP3 was maintained within the inner cell mass, while trophectoderm expression became restricted to the basolateral cell margins (Fig. 1e).

Expression of AQP8 protein was first detected by immunofluorescence at the eight-cell stage of mouse development (Fig. 2a–c) as distinct foci within the cytoplasm of embryonic blastomeres. Within the compacted morula, AQP8 immunostaining was evident within the cell margins of the outer blastomeres at cell–cell contacts (Fig. 2d) and was associated with the basolateral cell margins of the trophectoderm with no detectable expression in the inner cell mass (Fig. 2e).

Murine one-cell embryos immunostained with the AQP9 antisera demonstrated a low level of membrane-associated immunofluorescence (Fig. 3a) that was not evident by the two-cell stage (Fig. 3b). Membrane-associated AQP9 immunofluorescence was again observed at the eight-cell stage at cell–cell contacts (Fig. 3c) and also in the outer blastomeres of the compacted morula (Fig. 3d) with some evidence of perinuclear staining within the cytoplasm of individual blastomeres. At the blastocyst stage, AQP9 immunofluorescence was observed within the cytoplasm of inner cell mass cells and was associated with the apical membrane domains of mural and polar trophectoderm (Fig. 3e).

Mercury-sensitive water transport in murine blastocysts

Exposure of murine blastocysts to a 10% glycerol gradient (media osmolality shift from 290 to ~1800 mOsm) resulted in a rapid decrease in blastocyst volume followed by sustained reexpansion over the 5.5-min data collection interval (Fig. 4A). At the end of this time interval, blastocysts had recovered to 85.9 ± 0.81% of initial blastocyst volume. Aquaporin-mediated water transport during this recovery interval was demonstrated by a concentration-dependent inhibition of blastocyst reexpansion following exposure to a 10% glycerol gradient in the presence of increasing concentrations of the mercuric compound pCMPS (Fig. 4A). Over the 5.5-min data collection interval, embryos treated with 10, 100, and 500 μM pCMPS recovered from initial hyperosmolarity-induced blastocyst collapse to 81.44 ± 1.16%, 49.47 ± 0.96%, and 50.08 ± 1.0% of initial blastocyst volume, respectively (Fig. 4A). Determination of the rate of blastocyst reexpansion for each treatment over the first 60 s in which the blastocyst underwent volume recovery demonstrated that control blastocysts
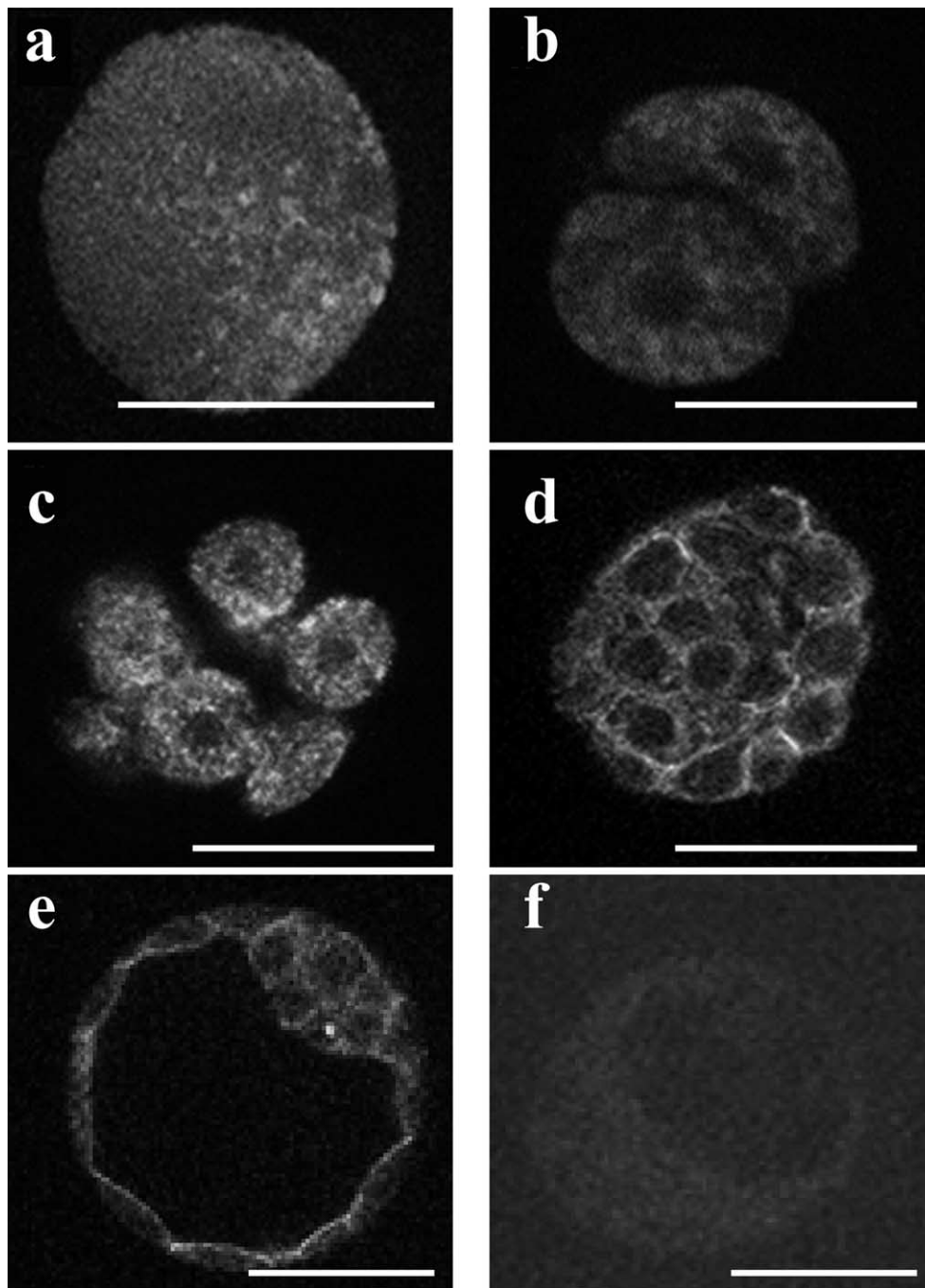
Fig 1. Immunofluorescence localization of AQP3 in murine preimplantation embryos. (a) One-cell; (b) two-cell; (c) eight-cell; (d) morula; (e) blastocyst, and (f) secondary control. AQP3 protein expression is first detected as cytoplasmic foci, followed by association with the cell margins of all blastomeres of the compacted morula (d). Within the blastocyst, AQP3 remains apolar within the inner cell mass (ICM) while becoming confined to the basolateral cell margins of trophectoderm cells (e). Scale bars represent 50 μM.

transported water at a rate of 3.15 $\pm$ 0.55 pL/s (Fig. 4B). Treatment with 10 μM pCMPS resulted in no significant decrease in the rate of blastocyst reexpansion compared with control (10 μM = 2.95 $\pm$ 0.25 pL/s; $P < 0.05$). Exposure of murine blastocysts to 100 and 500 μM pCMPS, however, resulted in a significant ($P < 0.05$) decrease in the rate of reexpansion compared with controls, with rates of expansion of 1.48 $\pm$ 0.23 and 0.9 $\pm$ 0.2 pL/s, respectively (Fig. 4B).

Immunofluorescence localization of AQPs 3 and 9 within murine blastocysts suggests that part of the mechanism of blastocyst volume recovery following exposure to a 10% glycerol gradient involves the movement of glycerol through the "aquaglyceroporins." In order to determine whether the nature of the solute used to generate the osmotic gradient impacts on blastocyst reexpansion behavior, we exposed murine blastocysts to either a 10% glycerol solu-
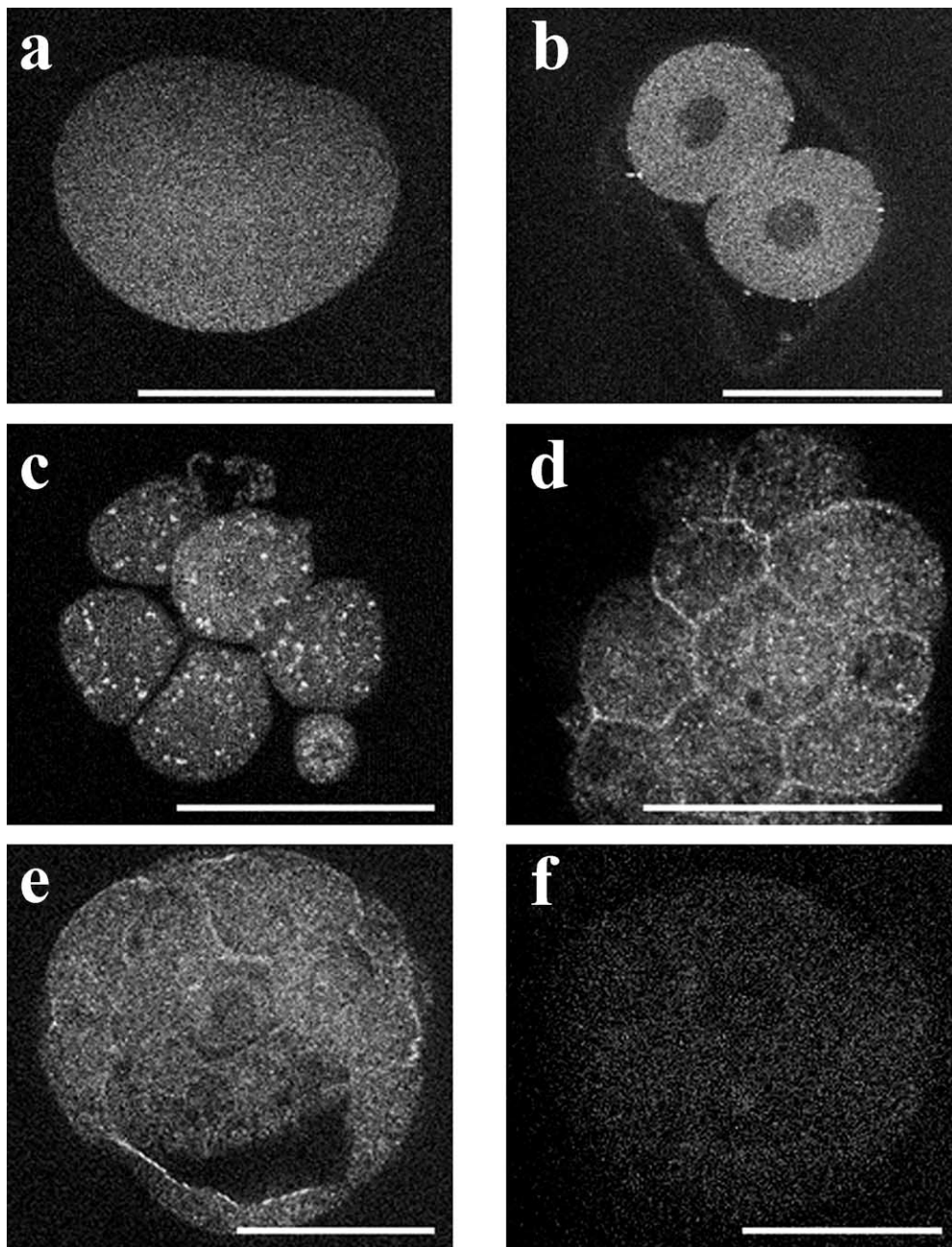
Fig. 2. Expression of AQP8 polypeptides during murine preimplantation development. Immunolocalization of AQP8 was not evident in one-cell (a) and two-cell (b) embryos. Expression of AQP8 was first evident as cytoplasmic foci in the eight-cell embryo (c), becoming localized within the cell margins of the layer of outer cells in the compacted morula (d; tangential section through outer cells of morula). At the blastocyst stage, AQP8 associated immunofluorescence was primarily localized within the basolateral cell margins of the trophectoderm (e). Secondary control (f). Scale bars represent 50 μM.

tion or an equiosmolar sucrose solution (1.4 M sucrose; ~1800 mOsm). Exposure to the 10% glycerol solution resulted in blastocyst shrinkage and reexpansion (Fig. 5) as observed in the pCMPS concentration-dependence assay. Exposure to an equiosmolar sucrose solution, however, resulted in continued blastocyst volume decrease over the 5.5-min recording interval (Fig. 5). Following 45 min of exposure to the 1800-mOsm sucrose solution, blastocyst volume showed no signs of recovery, whereas blastocysts treated with or without 500 μM pCMPS and exposed to the

1800-mOsm glycerol solution all recovered to approximately their initial volume in 45 min (Fig. 6).

Discussion

We have demonstrated for the first time that aquaporin proteins are expressed within the mammalian blastocyst and are involved in the *trans*-trophectoderm water movements occurring during the process of cavitation. The presence of
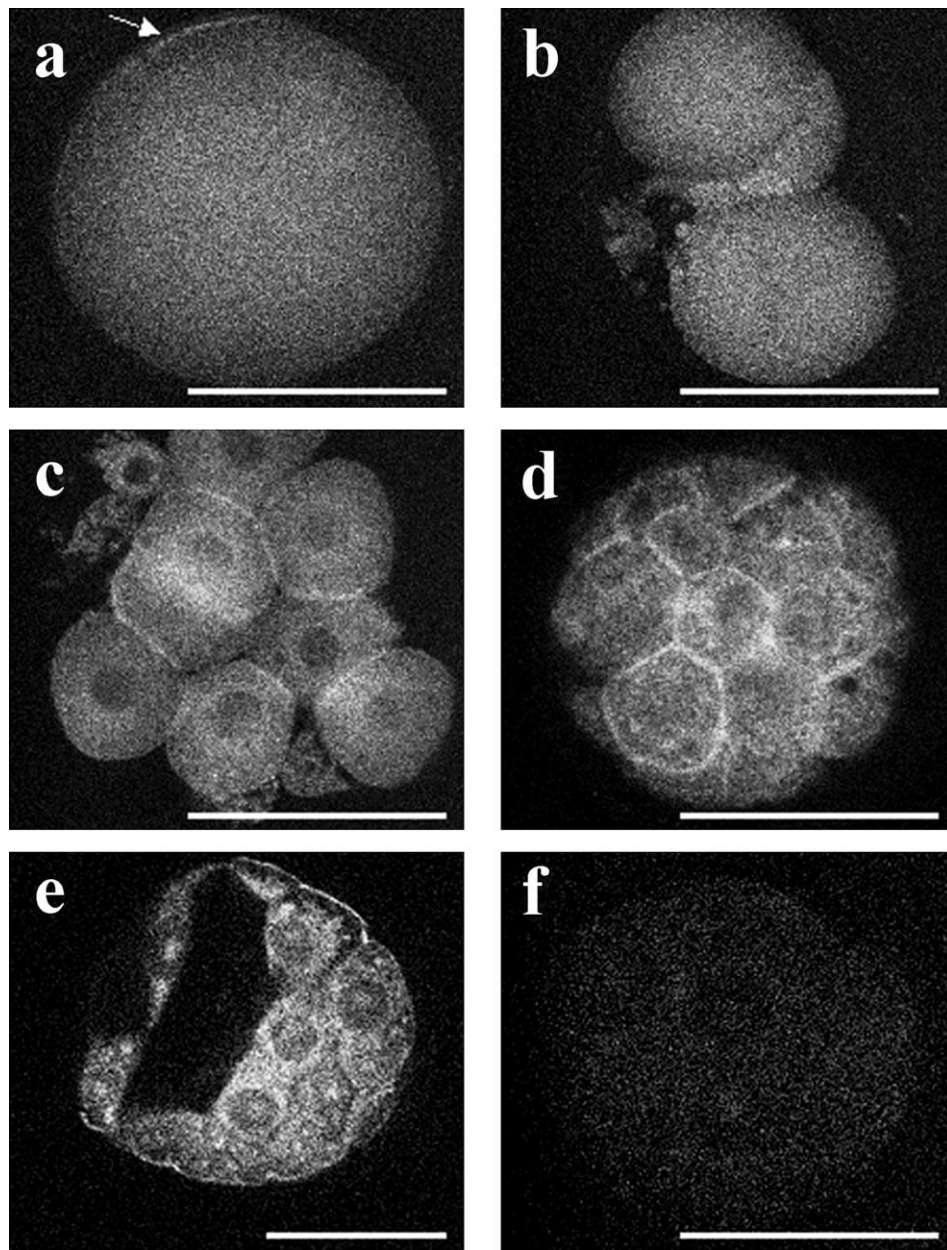
Fig. 3. Immunolocalization of AQP9 polypeptides in the murine preimplantation embryo. Membrane-associated expression in one-cell zygotes (a; arrow) disappeared by the two-cell stage (b) and was again detectable at the cell surface of blastomeres in eight-cell-stage embryos (c). At the compacted morula stage, AQP9 immunofluorescence was detected at cell contacts between the outer most blastomeres (d; tangential section through presumptive TE cells). Blastocyst-stage embryos (e) demonstrated a high level of cytoplasmic immunofluorescence in ICM and trophectoderm cells in conjunction with localization to the apical cell margins of trophectoderm cells. (f) secondary antibody control. Scale bars, 50 μM.

both apical and basolateral AQPs within the murine trophectoderm establishes a mechanism whereby channel-mediated *trans*-cellular water movements can occur during fluid accumulation within the nascent blastocoel cavity. Increasing evidence suggests that localization of distinct AQP family members within opposing membrane domains of transporting epithelia of the renal collecting duct (reviewed by Knepper et al., 1996; Verkman, 1999), secretory glands (Gresz et al., 2001; Moore et al., 2000; Wellner et al., 2000), and airway epithelium (Song and Verkman, 2001)

mediate *trans*-cellular water movements in these tissues. The patterns of immunolocalization observed within the trophectoderm epithelium for AQPs 3, 8, and 9 are consistent with membrane localization patterns in other epithelial tissues. AQP3 has been localized to the basolateral membranes of principle cells of the renal collecting duct (Ecelbarger et al., 1995; Ishibashi et al., 1997b), urinary tract (Spector et al., 2002), and salivary glands (Gresz et al., 2001; Moore et al., 2000). Localization of AQP3 within the trophectoderm provides a major entry/exit point for water
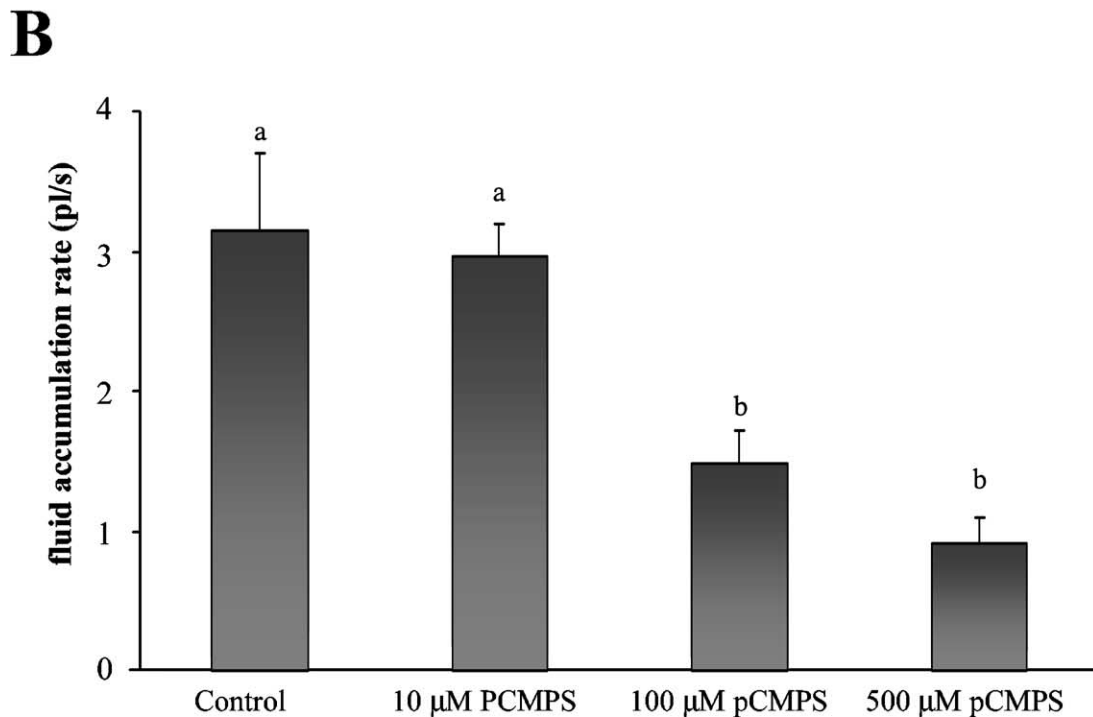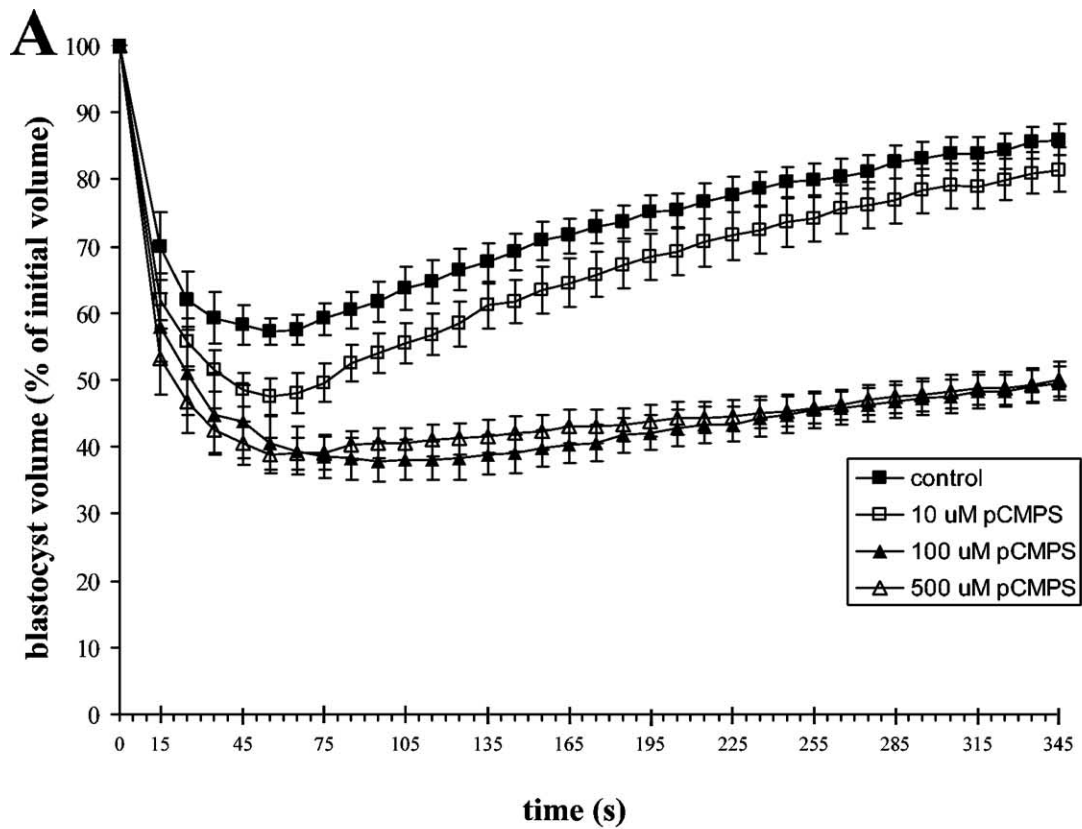
Fig. 4. Concentration-dependent inhibition of water transport by pCMPS following exposure of murine blastocysts to a 10% glycerol (~1800 mOsm) gradient. (A) Effect of pCMPS concentration on the response of murine blastocysts to 10% glycerol. Data are presented as percent of initial blastocyst volume following exposure to 10% glycerol following treatment with or without 50, 100, or 500 μM pCMPS (mean % $\pm$ S.E.; $n = 9$ blastocysts per treatment). (B) Rate of blastocyst expansion (pl/s) over the first 60 s of reexpansion following exposure of murine blastocysts to 10% glycerol (reexpansion data were measured starting at 55 s postosmolarity shift for control, 10 and 500 μM pCMPS and at 85 s for the 100-μM pCMPS treatment). Reexpansion rate was significantly decreased by pretreatment of blastocysts with 100 and 500 μM pCMPS ($P < 0.05$; $n = 9$ embryos per treatment). Bars with different letters represent significant differences in rate of fluid accumulation ($P < 0.05$).
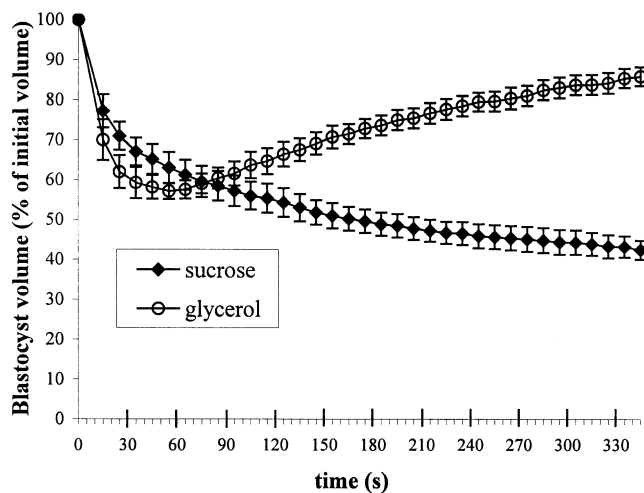
Fig. 5. Effect of solute composition in the response of murine blastocysts to acute exposure to hyperosmolarity (1800 mOsm). Embryos exposed to a 10% glycerol solution underwent blastocyst shrinkage and reexpansion to approximately 80% of initial blastocyst volume over a 5-min recording interval. Substitution of glycerol with sucrose in the 1800-mOsm solution resulted in continued blastocoel shrinkage over a 5-min interval following induction of hyperosmotic conditions. Data presented as mean % of initial blastocyst volume (± S.E.) for each time point ($n = 9$ blastocysts per data point for each treatment).

across the basolateral membranes during blastocyst formation. In contrast, AQP3 has been localized to the apical membrane domains of human syncytiotrophoblast (Damiano et al., 2001) and ovine cytotrophoblast (Johnston et al., 2000) cells, suggesting that this early localization pattern may undergo a marked shift during later trophoblast differentiation. Localization of the aquaglyceroporins AQP3 and 9 within the apical membranes of the human syncytiotrophoblast are suggested to facilitate transport of water and solutes from the mother to fetus due to their broad permeability to neutral solutes in addition to water (Damiano et al., 2001). Similar to the observation of apical AQP9 expression within the syncytiotrophoblast (Damiano et al., 2001), murine blastocysts express AQP9 within the apical membrane domains of the trophectoderm. This localization pattern would provide a mechanism for channel-mediated water movement across the apical domains of the trophectoderm epithelium, and may also allow movement of other solutes such as glycerol, urea, purines, and pyrimidines across the apical membrane. Cytoplasmic localization of all three of the AQP proteins examined prior to the eight-cell/morula stage suggests that a mechanism for near-isosmotic fluid transfer may not be required by the preimplantation embryo prior to the onset of cavitation.

AQP8 immunolocalization within the basolateral membrane domains of the trophectoderm provides and additional route for water passage across this membrane. This pattern of AQP8 localization has been observed in MDCK cells (Wellner and Baum, 2001) and submandibular gland acinar cells (Wellner et al., 2000). At the mouse eight-cell stage, AQP8 immunofluorescence was observed as distinct cyto-

plasmic foci that underwent a shift to a membrane localized pattern by the compacted morula stage within the mouse embryo. Cytoplasmic immunolocalization of AQP8 has been documented in a variety of tissues (Calamita et al., 2001; Elkjaer et al., 2001; Garcia et al., 2001; Hurley et al., 2001), and in hepatocytes, this protein undergoes a shift in distribution from intracellular vesicles to the plasma membrane in response to cAMP stimulation (Garcia et al., 2001). It is possible that a similar mechanism is involved in the subcellular shift observed during murine preimplantation development, as adenylate cyclase levels undergo a marked increase at the morula/blastocyst stage (Manejwala et al., 1986) and intracellular cAMP levels are increased in blastocysts (Dardik and Schultz, 1992). Expression of multiple AQP family members within the trophectoderm may explain the observation that ablation of individual AQPs by gene targeting do not result in preimplantation developmental lethality (reviewed by van Os et al., 2000; Verkman, 2000), although a significant reduction in null offspring is observed at day 5 postpartum in the AQP1 knockout (Ma et al., 1998; Verkman, 1999), suggesting impaired in utero survival. We have demonstrated the expression of seven AQP mRNA subtypes within the preimplantation embryo, suggesting that the three AQPs examined in the present study may not be the only members of this family present at the time of blastocyst formation.

Immunolocalization of AQP polypeptides in apical and basolateral membrane domains of the mammalian trophectoderm suggested that *trans*-trophectodermal water movements occur via a channel-mediated process. This idea has been confirmed by the observation that murine blastocysts exposed to a 10% glycerol gradient undergo volume recovery via a mercury-sensitive mechanism. All three of the AQP family members localized within the trophectoderm in the present study are inhibited by exposure to mercuric compounds, and we have demonstrated mercuric-inhibition of *trans*-trophectodermal water movements at concentrations of pCMPS 10- to 20-fold lower than those routinely employed in previous studies involving *Xenopus* oocyte expression systems (Abrami et al., 1996; Cooper and Boron, 1998; Echevarria et al., 1996; Virkki et al., 2001). We also observed that a 20-min exposure to 100 and 500 μM pCMPS did not prevent subsequent blastocyst hatching following transfer to fresh embryo culture drops free of pCMPS, suggesting that the concentrations utilized in the present study were not detrimental to further embryo development and thus did not compromise embryo viability (data not shown).

The observed response of murine blastocysts to a 10% glycerol gradient is consistent with that observed previously in the bovine blastocyst by Kaidi et al. (2000). Bovine blastocysts transferred to a 10% glycerol solution underwent rapid decrease in blastocyst volume and reexpanded to approximately 80% of initial volume over the 5-min data collection interval (Kaidi et al., 2000). This study suggested that embryo response to 10% glycerol was predictive of
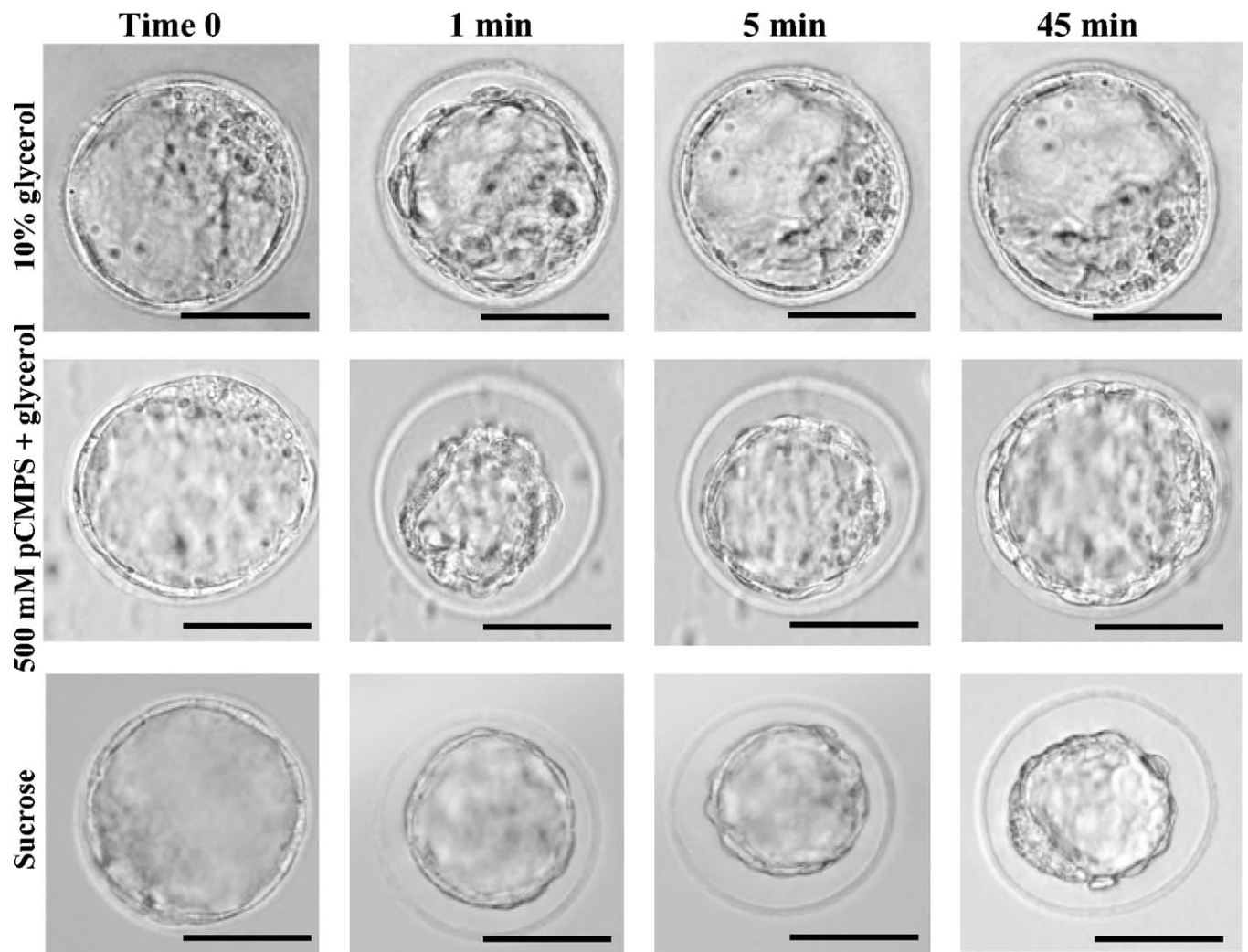
Fig. 6. Phase contrast images of murine blastocysts exposed to 10% glycerol, 500 μM pCMPS + 10% glycerol, or sucrose (1.4 M). At 1 min post-exposure to hyperosmotic conditions, all blastocysts demonstrate marked decreases in volume. By 5 min, glycerol-treated embryos have recovered to approximately initial blastocyst volume, although reexpansion is retarded in embryos treated with 500 uM pCMPS. Embryos exposed to sucrose demonstrate further volume decrease at the 5-min time point. Following 45 min of exposure to hyperosmotic conditions, embryos from treatments consisting of 10% glycerol and 10% glycerol + 500 μM pCMPS have recovered to approximately initial volume. Sucrose exposure completely prevented the reexpansion observed following exposure to glycerol. Scale bar, 25 μm.

postcryopreservation survival. Coupled with our data demonstrating that this response is mediated by aquaporins in the murine embryo, these studies suggest that AQP expression by mammalian blastocysts may have important implications for success of cryopreservation and post-freezing embryo transfer success in assisted reproductive technologies. Kaidi et al. (2000) suggested that the rapid decrease in blastocyst volume was due to two factors. First, the 10% glycerol solution represents a hyperosmotic shift from ~290 to 1800 mOsm, and second, that the higher permeability of plasma membranes to water than to glycerol (2000–3000 times more permeable to water than most permeating cryoprotectants; Jakowski et al., 1980; Saha and Suzuki, 1997) results in rapid efflux of water from the blastocoel cavity. Efflux of water from the blastocoel, how-

ever, was halted when a balance between glycerol influx and water efflux was reached (Kaidi et al., 2000). We have demonstrated in the present study that the sequential events of blastocyst shrinkage and reexpansion characteristic of exposure to 10% glycerol solutions is likely mediated by glycerol movement through apical AQP9 and basolateral AQP3 within the murine trophectoderm. The greater degree shrinkage observed in blastocysts treated with 100 and 500 μM pCMPS is likely due to inhibition of glycerol movement through these water channels, such that it takes longer to reach a balance between water efflux and glycerol influx. Water efflux, however, is not affected due to the scale of the hyperosmotic gradient across the trophectoderm (approximately a 6-fold increase), providing a sufficient driving force for rapid diffusive water loss across this cell layer. We

have further demonstrated that this response is dependent on glycerol movement into the blastocoel cavity, as substitution of sucrose for glycerol in induction of acute hyperosmolarity resulted in continued blastocyst volume decrease with no evidence of reexpansion following 5 or 45 min of exposure to the sucrose solution.

Involvement of the AQP family of proteins in mediating fluid movements across the trophectoderm layer may explain the ability of the preimplantation embryo to exhibit rapid near-isosmotic water transport as previously described (Borland et al., 1977; Biggers et al., 1988; Waelchli and Betteridge, 1996). Diffusion of water across the cell membrane requires a relatively large osmotic gradient, as the activation energy (E_a > 10 kcal/mol) of *trans*-cellular water transport is much higher than that observed in the presence of AQP water channels (Verkman et al., 1996). In general, an osmotic permeability (P_f) higher than 10 μm/s and an Arrhenius activation energy (E_a) lower than 6 kcal/mol is suggestive of water transport through AQPs (Verkman et al. 1996). A role for AQPs in oocytes is debatable as P_f values have been measured as 9.86 $\pm$ 0.67 μm/s (0.44 μm/min/ atm; Leibo, 1980) in mouse and 10.53 $\pm$ 4.7 μm/s (0.47 μm/min/atm; Ruffing et al., 1993) in cow oocytes at 20°C with E_a values between 14.5 (Leibo, 1980) and 7.84 kcal/ mol (Ruffing et al., 1996), respectively. Biggers et al. (1988) reported hydraulic conductivity values (L_p) for rabbit trophectoderm that were 1188 μm/min/atm on day 5 and 1033 μm/min/atm on day 6 of embryonic development. These values are considerably higher than those calculated for oocytes (Leibo, 1980; Ruffing et al., 1993) and may reflect the change in membrane-associated AQP expression observed by immunofluorescence in the present study. Further evidence for the role of membrane association of AQPs (particularly AQPs 3 and 9) in the process of cavitation is provided by the observation that glycerol permeability occurs at very low rates in oocytes (Jackowski et al., 1980; Pedro et al., 1996) but increases dramatically at the morula/ blastocyst stage (Kasai, 1996; Pedro et al., 1996). These findings are consistent with our observation that membrane association of the AQPs examined in the current study is not observed until the late 8-cell to compacted morula stages of murine development in addition to the ability of murine blastocysts to respond rapidly to the introduction of a glycerol gradient.

In summary, we have demonstrated that mammalian blastocysts possess aquaporin water channel-mediated mechanisms for fluid transport across the trophectoderm epithelium. Expression of AQP polypeptides in both apical and basolateral membrane domains provides a route for *trans*-trophectoderm water fluxes during blastocyst formation. These findings greatly advance our understanding of the cellular and physiological mechanisms that underlie fluid accumulation in the mammalian blastocyst.

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