Intercellular Ice Propagation: Experimental Evidence for Ice Growth through Membrane Pores

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ABSTRACT Propagation of intracellular ice between cells significantly increases the prevalence of intracellular ice in confluent monolayers and tissues. It has been proposed that gap junctions facilitate ice propagation between cells. This study develops an equation for capillary freezing-point depression to determine the effect of temperature on the equilibrium radius of an ice crystal sufficiently small to grow through gap junctions. Convection cryomicroscopy and video image analysis were used to examine the incidence and pattern of intracellular ice formation (IIF) in the confluent monolayers of cell lines that do (MDCK) and do not (V-79W) form gap junctions. The effect of gap junctions on intracellular ice propagation was strongly temperature-dependent. For cells with gap junctions, IIF occurred in a directed wave-like pattern in 100% of the cells below −3°C. At temperatures above −3°C, there was a marked drop in the incidence of IIF, with isolated individual cells initially freezing randomly throughout the sample. This random pattern of IIF was also observed in the V-79W monolayers and in MDCK monolayers treated to prevent gap junction formation. The significant change in the low temperature behavior of confluent MDCK monolayers at −3°C is likely the result of the inhibition of gap junction-facilitated ice propagation, and supports the theory that gap junctions facilitate ice nucleation between cells.

INTRODUCTION

Tissues are complex arrangements of multiple cells and cell types that interact together to perform specific functions. During freezing, cell-cell and cell-matrix connections influence tissue responses at low temperatures. One of the most dramatic effects of cell adhesion is the propagation of intracellular ice that occurs between adjacent cells in tissues and tissue models. This phenomenon was first documented in plant tissues (Molich, 1897; Chambers and Hale, 1932; Luyet and Gibbs, 1937; Stuckey and Curtis, 1938; Asahina, 1956; Levitt, 1966; McLeester et al., 1969; Brown and Reuter, 1974; Brown, 1980; Tsuruta et al., 1998) where researchers noted a strong relationship between cell-cell contact and intracellular ice formation (IIF; Molisch, 1897; Chambers and Hale, 1932; Stuckey and Curtis, 1938). Cell-cell propagation of intracellular ice has also been reported in muscle fibers (Chambers and Hale, 1932), insect salivary glands (Berger and Uhrik, 1996), cultured cell monolayers (Larese et al., 1992; Acker and McGann, 1998; Acker et al., 1999) and intact Antarctic nematodes (Wharton and Ferns, 1995). The increasing requirement for cryopreservation of natural and biosynthetic tissues has renewed interest in understanding the role of cell interactions and tissue architecture on IIF (Karlsson and Toner, 1996; Acker, 1997; Acker and McGann, 2000).

A number of studies have demonstrated nonrandom initiation of IIF in tissue systems (Stuckey and Curtis, 1938; Brown and Reuter, 1974; Brown 1980; Tsuruta et al., 1997; Tsuruta et al., 1998). IIF in the constituent cells of cucumber (Brown and Reuter, 1974) and onion tissue (Brown, 1980) was shown to be influenced by the presence of ice in adjacent cells. Mapping the sequence of IIF pointed to two types of intracellular ice nucleation: a random process without interference from adjacent cells and a nonrandom process where ice in one cell triggers nucleation in adjacent cells (Brown, 1980). Using high-speed video microscopy to examine the propagation of intracellular ice in onion epidermal tissue, Tsuruta et al. (1998) confirmed nonrandom intracellular ice nucleation. Acker and McGann (1998) used a statistical approach to demonstrate that the probability of intracellular ice nucleation increases when an adjacent cell in a confluent monolayer is already frozen.

There are two current theories describing the mechanism for propagation of intracellular ice between adjacent cells. Surface-catalyzed nucleation (SCN) is based on the premise that the cell plasma membrane, interacting with extracellular ice, acts as an intracellular nucleation site (Toner et al., 1990, Tsuruta et al., 1997, 1998). Although the SCN theory was developed to explain intracellular nucleation by extracellular ice, it does not preclude the possibility that intracellular ice in an adjacent cell functions in a similar manner to extracellular ice, resulting in propagation of ice between cells. This mechanism was suggested for ice propagation between adjacent fibroblasts in a confluent monolayer (Acker, 1997; Acker and McGann, 1998).

Another theory (Berger and Uhrik, 1996) proposes that the propagation of ice between cells occurs via gap junctions in the plasma membrane. Gap junctions are intercellular pores that permit the passage of small molecules between adjacent cells. Using single strands of salivary tissue, Berger and Uhrik demonstrated the induction of ice between cells and the inhibition of this propagation by the

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addition of chemical agents that uncoupled adjacent cells. The rate, pattern, and cumulative incidence of IIF were compared in confluent monolayers of cultured cells with and without gap junctions (Acker, 1997; Acker and McGann, 1997), and concluded that the presence of gap junctions increased the induction of intracellular ice between adjacent cells. Critical to this theory is the ability of ice to grow through small-diameter pores the size of gap junctions.

The concept of intracellular ice nucleation by ice growth through aqueous pores was proposed by Mazur (1960, 1965, 1966), using the following equation (roughly analogous to the Kelvin equation) to model the change in equilibrium ice crystal radius with temperature:

$$\Delta T = \frac{2v_1^{\mathrm{L}} T_{\mathrm{fp}}^{\mathrm{o}} \sigma_{\mathrm{SL}} \cos\,\theta}{aL_{\mathrm{f}}} \tag{1}$$

where Mazur defined ΔT as the difference between the freezing point of water in the pore of radius a and the freezing point of a planar ice-water interface ($T_{\mathrm{fp}}^{\mathrm{o}}$); σ_{SL} as the interfacial tension between the ice and the liquid water; v_1^{L} as the molar volume of pure liquid water; θ as the contact angle between the ice-water interface and the pore wall measured through the liquid water; and L_{f} as the molar heat of fusion (Mazur, 1965, 1966). Mazur calculated that the equilibrium radius of an ice crystal below -10°C was small enough to pass through pores of 8 Å if the contact angle of the ice-water interface with the pore wall was $\sim$75°.

Until recently, it was not possible to prove or disprove Mazur's pore theory because of a lack of accurate data on the physical interaction of ice and pores. However, the work by Berger and Uhrik (1996) on the propagation of ice via gap junctions demonstrated a strong temperature dependence of the propagation of ice between adjacent cells in salivary gland tissue, providing strong evidence to support the pore theory. They defined an upper temperature above which intercellular ice induction was not observed. Although the nature of the experiment did not allow determination of the exact temperature at which intracellular ice propagation ceased, the observations strongly support the idea that there is a critical radius, and hence, a temperature at which ice cannot propagate to adjacent cells through intercellular pores. Accurate determination of this temperature and its correlation with known physical properties of gap junctions, would permit validation of the assumptions made by Mazur using his equation and, hence, Mazur's pore theory.

This study is a theoretical and experimental investigation of the temperature-dependence of ice propagation in confluent monolayers to test the hypothesis that ice growth through gap junctions is a plausible mechanism for the propagation of intracellular ice. Resolution of this question is an important element in the development of a model to describe the formation of intracellular ice in tissues.

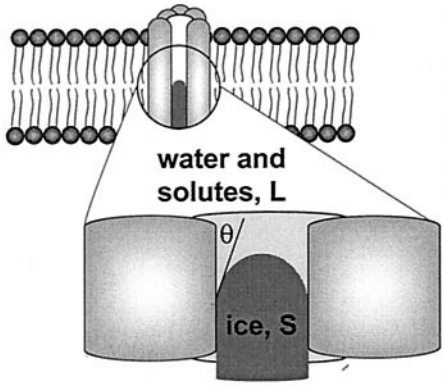

FIGURE 1 Schematic representation of ice propagation through a transmembrane pore.

Theoretical derivation

As dendritic ice grows in the extracellular solution, the concentration of solutes increases in the residual liquid, and this concentrated solution becomes trapped between dendrites as the freezing proceeds. Consider the equilibrium of ice with an aqueous solution in a capillary as shown in Fig. 1. When ice forms in a sufficiently small capillary, interfacial forces will cause the interface to assume the shape of a spherical cap. In this case, one can assume that the ice formed contains no solutes. For a spherical interface, one can show that the conditions for the interface to be in equilibrium are:

$$T^{\mathrm{S}} = T^{\mathrm{L}} = T_{\mathrm{fp}} \tag{2}$$

$$\mu_1^{\mathrm{S}} = \mu_1^{\mathrm{L}} \tag{3}$$

$$P^{\mathrm{S}} - P^{\mathrm{L}} = \frac{2\sigma_{\mathrm{SL}}}{a}\cos\theta \tag{4}$$

where T^{S} is the temperature of the ice; T^{L} is the temperature of the liquid; T_{fp} is the new freezing point; μ_1^{S} is the chemical potential of the ice; μ_1^{L} is the chemical potential of the water in the liquid phase; P^{S} is the pressure of the ice, and P^{L} is the pressure of the liquid.

Assuming that the liquid is incompressible (specific volume is independent of pressure) and that over the temperature range of interest the specific entropy is independent of temperature, then the chemical potential of the water in the liquid phase can be written:

$$\mu_1^{\mathrm{L}}(T^{\mathrm{L}}, P^{\mathrm{L}}, x_2) = \mu_1^{\mathrm{o}}(T_{\mathrm{fp}}^{\mathrm{o}}, P_{\mathrm{atm}}) + v_1^{\mathrm{L}}(P^{\mathrm{L}} - P_{\mathrm{atm}}) + s_1^{\mathrm{L}}(T_{\mathrm{fp}}^{\mathrm{o}} - T^{\mathrm{L}}) - v_1^{\mathrm{L}}\pi^{\mathrm{L}}(T^{\mathrm{L}}, x_2) \tag{5}$$

where μ_1^{o} is the chemical potential of pure water at the planar freezing point ($T_{\mathrm{fp}}^{\mathrm{o}}$) and atmospheric pressure (P_{atm}); S_1^{L} is the specific entropy of pure liquid water at standard

pressure and temperature; π^L is the osmotic pressure of the solution and x_2 is the solute mole fraction in the liquid. It is assumed that the osmotic pressure of the solution is only a function of temperature and mole fraction of the solute.

If one assumes that there are no nonisotropic stresses on the ice, that the ice is incompressible, and that over the temperature range of interest, the specific entropy of the ice is independent of temperature, then the chemical potential of ice can be written:

$$\mu_1^S(T^S, P^S) = \mu_1^o(T_{fp}^o, P_{atm}) + v_1^S(P^S - P_{atm}) + s_1^S(T_{fp}^o - T^S) \quad (6)$$

where v_1^S is the molar volume of ice and s_1^S is the specific entropy of ice at standard conditions.

According to Eq. 3 (making use of Eq. 2), at equilibrium, Eqs. 5 and 6 may be equated to give:

$$v_1^L(P^L - P_{atm}) + s_1^L(T_{fp}^o - T_{fp}) - v_1^L\pi^L(T_{fp}, x_2) = v_1^S(P^S - P_{atm}) + s_1^S(T_{fp}^o - T_{fp}) \quad (7)$$

To make use of all the information contained in the conditions for equilibrium, Eqs. 2–4, the Laplace equation, Eq. 4, must be substituted into Eq. 7. This can not be done in a way that eliminates a variable unless a further assumption is made. At this point, some authors (Everett, 1961; Mazur, 1965; Christenson, 1997; Morishige and Kawano, 1999) have made the assumption that the specific volumes of the solid and pure liquid phases are the same, which introduces a ~10% error in the case of water. There is no need to make this assumption if one assumes that either the pressure in the liquid or the pressure of the ice is controlled to be at atmospheric pressure. Assuming that it is the liquid phase pressure that is controlled, Eqs. 4 and 7 may be combined to give:

$$T_{fp}^o - T_{fp} = \frac{2v_1^S\sigma_{SL}\cos\phi}{(s_1^L - s_1^S)a} + \frac{v_1^L\pi^L}{(s_1^L - s_1^S)} \quad (8)$$

From the Clapeyron equation:

$$\frac{1}{s_1^L - s_1^S} = \frac{T_{fp}^o}{L_f} \quad (9)$$

where L_f is the molar latent heat of fusion, substituting Eq. 9 into 8 gives the following equation that describes capillary freezing-point depression:

$$\Delta T = \frac{2\sigma_{SL}v_1^S T_{fp}^o\cos\phi}{aL_f} + \frac{v_1^L\pi^L T_{fp}^o}{L_f} \quad (10)$$

The equation derived by Mazur (Eq. 1), is an approximation for the effect of temperature on the radius of an ice crystal. In his derivation, Mazur did not consider the effect of solutes, and assumed that there was pure water on both sides of the pore. Clearly, this is not the condition that exists in biological systems. The effect of solutes on the freezing point of water in a pore is represented by the second term of the righthand side of Eq. 10. An additional difference between Eqs. 1 and 10 is the appearance of the molar volume of liquid water in Eq. 1 rather than the molar volume of ice as appears in Eq. 10. Eq. 10 is the correct form for the capillary freezing-point depression if it is assumed that: 1) the liquid is maintained at atmospheric pressure; 2) the liquid and solid water are incompressible; and 3) the molar entropies are independent of temperature.

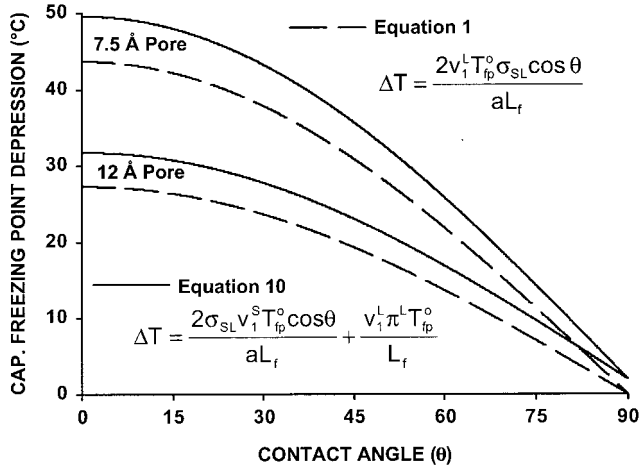

FIGURE 2 Comparison of Eqs. 1 and 10. Capillary freezing-point depression is calculated for Eq. 1 (*dashed line*) and Eq. 10 (*solid line*) as a function of contact angle for pores of radii 7.5 and 12 Å.

Without considering solute effects, Eqs. 1 and 10 differ by ~10%. The difference between the calculated freezing-point depression using Eqs. 1 and 10 is shown in Fig. 2. We used Eq. 10 for all calculations presented in this paper; as it more precisely accounts for the conditions encountered during intercellular ice propagation and does not make the specific volume assumption. However, because of errors inherent in the experimental system, the difference in the capillary freezing-point depression predicted by Eqs. 1 and 10 is not significant for analysis of the data.

Application of the capillary freezing-point depression equation

Consider the propagation of ice through a pore that connects two cells with intracellular ice on one side of the pore and supercooled cytoplasm on the other (Fig. 1). We wish to determine the minimum temperature difference from the planar freezing point that will permit the propagation of an ice crystal through the pore. Stated differently, what is the temperature that will result in an equilibrium ice crystal of small enough size to pass through the pore? To answer this, we will use the capillary freezing-point depression equation (Eq. 10).

Of the parameters in Eq. 10, only the interfacial tension (σ_{SL}) and the contact angle (θ) are unknown. From the work

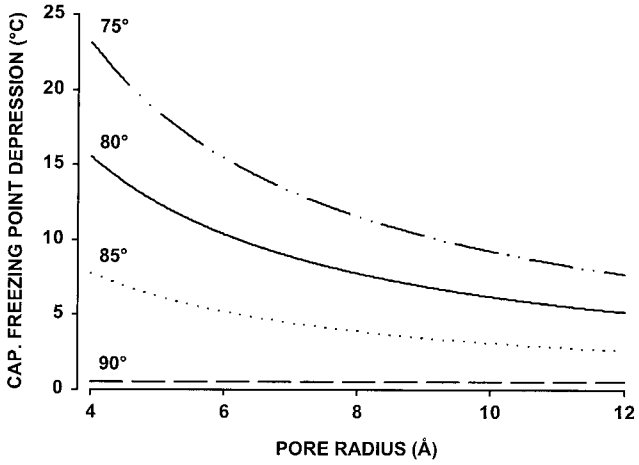

FIGURE 3 Capillary freezing-point depression as a function of pore radius for various contact angles.

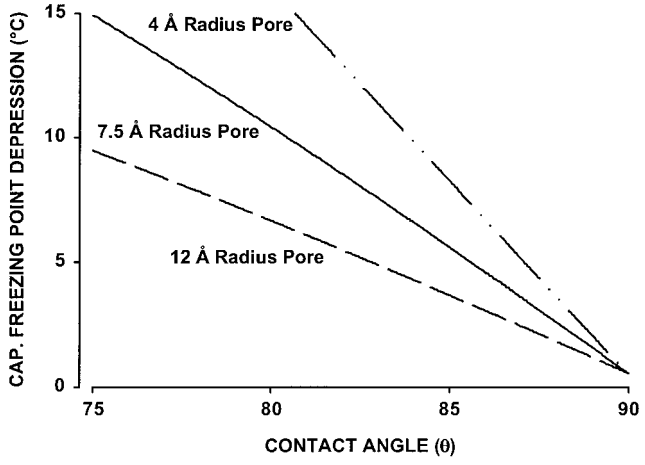

FIGURE 4 Capillary freezing-point depression as a function of contact angle for three pores with defined radii.

of Fletcher (1962), Mazur proposed that the interfacial tension for ice-pure water interfaces is in the range of 1.7×10^{-2} J/m^2 at -40°C to 2.0×10^{-2} J/m^2 at 0°C (Mazur, 1965, 1966). We will assume that the interfacial tension is linear with temperature. The only other unknown is the contact angle of the ice-water interface. In his work, Mazur concluded that the contact angle is much greater than 0° and may well approach 90° as a result of the unique structure of water in transmembrane pores (Mazur, 1965, 1966). Recent studies have confirmed that water in transmembrane pores (Verkman et al., 1995; Breed et al., 1996) is highly structured.

With values for all parameters other than the contact angle, we are able to model the behavior of intracellular ice propagation via gap junctions. Using Eq. 10 we can plot the capillary freezing-point depression as a function of pore radius for various contact angles (Fig. 3). For a given pore radius, as the contact angle increases, the capillary freezing-point depression decreases and approaches the freezing point of the solution at 90°. Similarly, as the pore radius increases, the freezing-point depression will decrease for any given contact angle. This means that the larger the pore, or the greater the contact angle, the higher is the temperature that is sufficient for intercellular ice propagation to occur.

A critical piece of information that is known is the radius of gap junctions. Current literature assumes that the diameter of mammalian gap junctions is approximately 1.5 nm (15 Å; Loewenstein, 1981). Pore diameter is a function of the structure of the gap junction and is highly dependent on the connexin subcomponents. As a result, the determination of a suitable pore diameter for gap junctions will be highly dependent on the cell type and species being investigated. From channel conductance experiments, gap junction pore diameters range from a minimum of 8 Å for connexin 45 to 24 Å for the human connexin 37 (Veenstra, 1996). For this study we will assume an average pore diameter of 15 Å (7.5 Å radii) with the understanding that this may be too small or too large for the cell model systems being studied herein.

With this information, we can calculate a temperature range, and hence ice crystal size, where the propagation of intracellular ice through pores will be plausible. Fig. 4 shows the capillary freezing-point depression as a function of contact angle for three different pore radii. For a pore radius of 7.5 Å, and a contact angle of >80°, Eq. 10 predicts that ice will be able to propagate between cells if the temperature is approximately 10°C or more below the planar freezing point. For a pore radius of 12 Å, or a contact angle >85°, the required temperature is approximately 5°C below the planar freezing point. These results are in approximate agreement with those proposed by Mazur (1960, 1965, 1966). Note, however, that the equation used herein is more precisely defined and relevant for this experimental system than that proposed by Mazur.

Care must be taken when using the capillary freezing-point depression equation, Eq. 10, for pores of too small a radius. To our knowledge, there has been no direct experimental verification of the capillary freezing-point equation, attributable to the difficulty in determining the required interfacial tension and contact angle independently. However, the liquid-vapor Kelvin equation has been verified down to 4 nm (40 Å; Fisher and Israelachvili, 1979). With decreasing pore diameter, there may be significant deviations in the microscopic interactions of water and the pore as one approaches molecular dimensions. For gap junctions with a radius of 7.5 Å, fewer than 5 water molecules will fit across the entire pore. Clearly, the use of Eq. 10 to model the effect of temperature on ice crystal size for small-diameter pores may not be accurate. Further studies are required to validate the capillary freezing equation down to 1 nm. Nevertheless, we can show qualitatively that the phenomenon described by Eq. 10 can explain the experi-

mentally observed temperature-dependent ice propagation between cells in a monolayer.

Ice growth and gap junctions: experimental evidence

Eq. 10 shows that there must be a defined upper temperature limit where the equilibrium curvature of an ice crystal is too large to pass through a pore. Using directional solidification, Berger and Uhrik (1996) demonstrated the existence of such a temperature, but did not provide sufficient information to calculate the temperature.

In this study, three different experimental model systems were used to determine the temperature at which ice propagation through gap junctions occurs. Hamster fibroblast (V-79W) and Madin-Darby canine kidney (MDCK; CCL 34, ATCC, Rockville, MD) cells were cultured to confluency on glass coverslips over 3 days at 37°C in supplemented media. The first model system was the V-79W confluent monolayer, where under standard culture conditions, gap junctions are not present (Yang et al., 1996; Acker and McGann, 1998; Acker et al., 1999). The second model system was the MDCK monolayer cultured to confluency in standard culture media, resulting in the expression of gap junctions (Yang et al., 1996). The formation of intercellular junctions, including gap junctions, can be inhibited by reducing the concentration of calcium in the culture media to 5 μmol/L (Peracchia and Peracchia, 1980; Armitage and Juss, 2000). Thus, MDCK cells with gap junctions (standard media) and without gap junctions (low Ca^{2+} media) represent the second and third experimental model systems. The confluent monolayers were stained with 12.5 μM SYTO 13 (Molecular Probes, Eugene, OR) to assist in the detection of intracellular ice (Acker, 1997; Acker and McGann, 2000). Cell monolayers on glass coverslips were transferred to a convection cryomicroscope and cooled at 25°C/min to a predetermined subzero experimental temperature where ice was nucleated in the extracellular solution with a cold copper probe. The cooling power of the cryomicroscope was sufficient to remove the latent heat of fusion within 0.5 s after extracellular ice nucleation so that the cells remained approximately isothermal during freezing. The occurrence of intracellular ice was indicated by a sudden darkening of the cytoplasm (Chambers and Hale, 1932; Luyet and Gibbs, 1937) and the presence of a honeycomb pattern in the fluorescent staining (Acker, 1997; Acker and McGann, 2000). The entire freezing procedure was videotaped for later analysis.

The incidence of intracellular ice in V-79W and MDCK confluent monolayers as a function of temperature is shown in Fig. 5. For V-79W cells, there is an increase in the incidence of intracellular ice between −5 and −11°C. The temperature in which 50% of the cells in the monolayer have formed intracellular ice (T_{50}^{IIF}) was −7.3°C. These data are consistent with those previously reported for V-79W

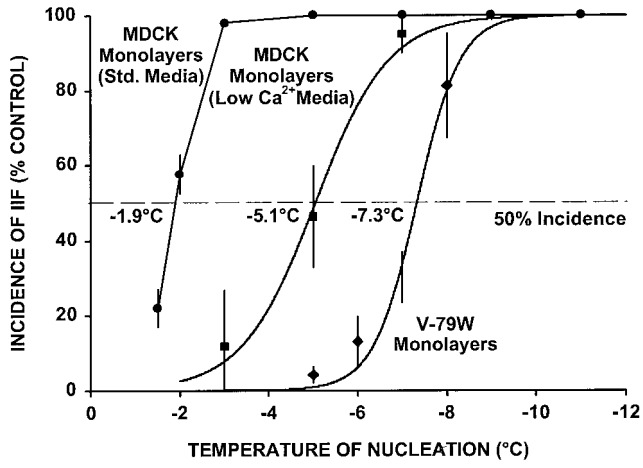

FIGURE 5 Incidence of IIF in three experimental model systems. Mean ± SEM (n = 6).

monolayers (Larese et al., 1992; Acker, 1997; Acker et al., 1999). The incidence of intracellular ice was different for the MDCK monolayers cultured in standard media, where 50% of the cells formed intracellular ice at −1.9°C. At temperatures below −3°C, there was complete formation of intracellular ice in all cells of the confluent MDCK monolayer when gap junctions were present, and the incidence of intracellular ice decreased sharply at temperatures above −3°C. In the absence of gap junctions (low Ca^{2+} media), the incidence of intracellular ice in the confluent MDCK monolayer increased as the temperature decreased between −3° and −7°C, with a T_{50}^{IIF} of −5.1°C. Fig. 5 provides a static picture of intracellular ice nucleation.

The patterns of IIF and intercellular ice propagation were determined from analysis of sequential video frames. Figs. 6–8 show schematic representations of the propagation of intracellular ice. In confluent V-79W monolayers (Fig. 6), there was initially a random dispersion of spontaneous IIF, followed by a nonrandom propagation of ice to adjacent cells as previously reported (Acker, 1997; Acker and McGann, 1998; Acker et al., 1999). This pattern was the same at all temperatures examined. An examination of the isothermal kinetics of intracellular freezing have been presented elsewhere (Acker, 1997; Acker and McGann, 1998; Acker et al., 1999).

For confluent monolayers of MDCK cells with gap junctions (Fig. 7), the freezing pattern depended on the temperature of nucleation. Below −3°C, IIF occurred in a well defined, wave-like manner from the initial site of IIF (Fig. 7). A wave of intracellular ice nucleation in 100% of the cells followed the extracellular ice front across the field of view. This is similar to the pattern of IIF reported for a number of tissues (Stuckey and Curtis, 1938; Wharton and Ferns, 1995; Berger and Uhrik, 1996; Tsuruta et al., 1997; Tsuruta et al., 1998), including MDCK monolayers (Acker, 1997; Acker and McGann, 1997). At temperatures above

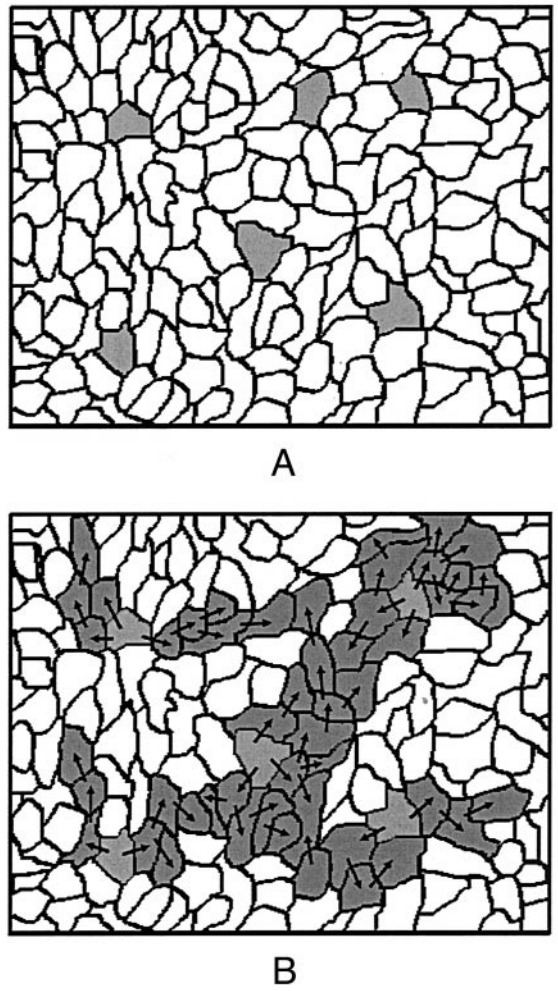

FIGURE 6 A schematic representation of the pattern of IIF in V-79W confluent cell monolayers. Ice forms initially in a random number of cells that are dispersed throughout the monolayer (*A*). This is followed by the nonrandom propagation of intracellular ice to adjacent cells (*B*).

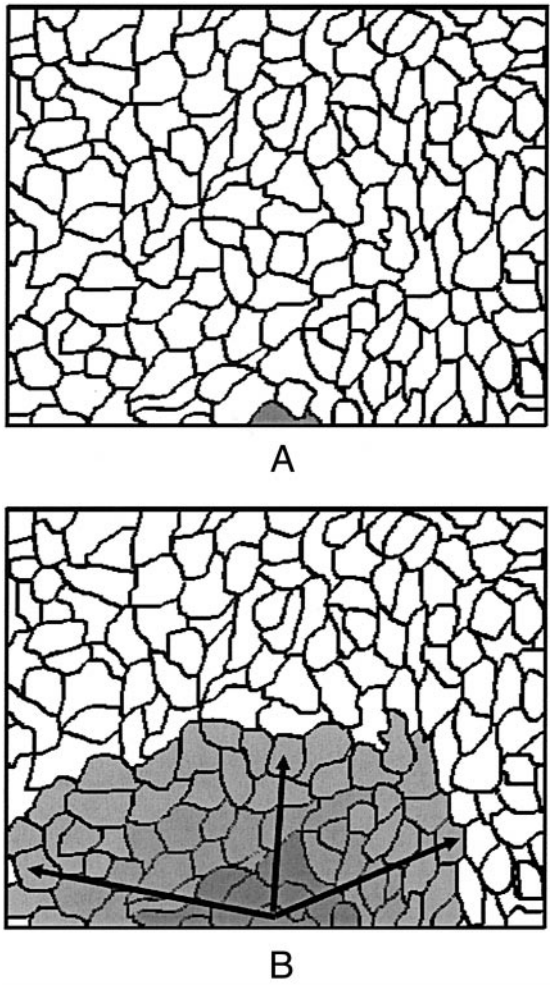

FIGURE 7 A schematic representation of the pattern of IIF in MDCK confluent cell monolayers (standard media) frozen below −3°C. As the extracellular ice front passes through the field of view, intracellular ice will form in a cell (*A*). This is followed by the propagation of intracellular ice to adjacent cells in a wave-like pattern (*B*).

−3°C, ice formed initially in a number of single cells in the sample and then propagated to a portion of the adjacent cells. This pattern of IIF in confluent MDCK monolayers (Fig. 8) above −3°C was quite different from the pattern in MDCK cells below −3°C (Fig. 7), but similar to the pattern for V-79W (Fig. 6).

Upon suppression of gap junction formation in MDCK confluent monolayers using a low concentration of extracellular calcium, the pattern of IIF was also different from that observed in the MDCK monolayer with gap junctions. In the absence of gap junctions, intracellular ice formed initially in a number of single cells randomly dispersed throughout the sample, and then intracellular ice propagated from these cells to a portion of the adjacent cells. This pattern of IIF was as shown for the V-79W monolayers (Fig. 6) and for MDCK monolayers with gap junctions that were nucleated above −3°C (Fig. 8). For the MDCK monolayers with gap junctions below −3°C, nucleation of ice in a cell will induce intracellular nucleation in all adjacent cells. However, for MDCK monolayers without gap junctions, V-79W monolayers, and MDCK monolayers with gap junctions above −3°C, cells may remain unfrozen even with an adjacent cell containing intracellular ice. These observations indicate that ice grows more readily between cells when gap junctions are present, and the temperature is below −3°C.

To quantify IIF in V-79W and MDCK confluent monolayers, we determined the number of independent nucleation sites from the cryomicroscope video images. An independent nucleation site was defined as a cell that froze intracellularly with no adjacent cells already frozen. The number of independent nucleation sites as a function of temperature is expressed as a percentage of the total number of cells in the sample that formed intracellular ice at that temperature (Fig. 9). A high percentage of nucleation sites

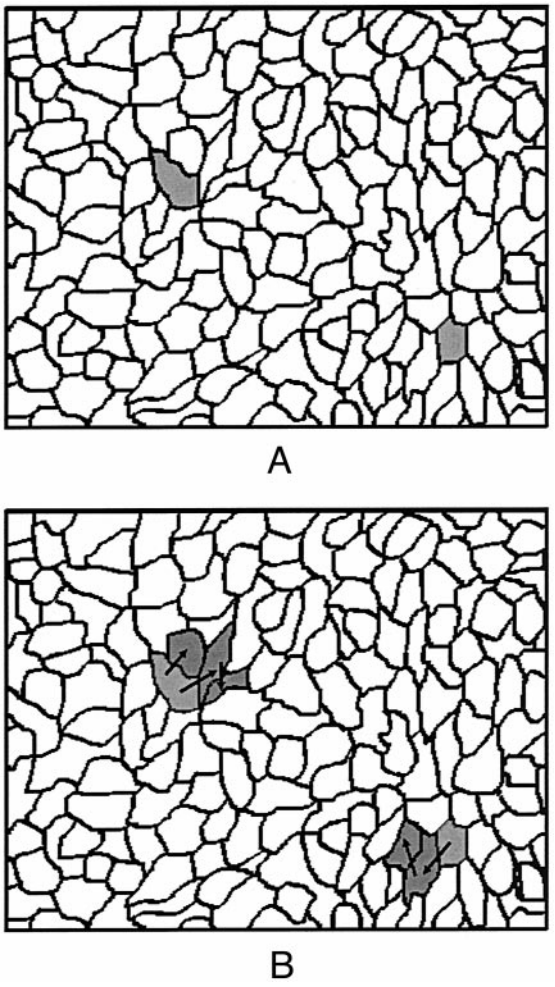

FIGURE 8 A schematic representation of the pattern of IIF in MDCK confluent cell monolayers (standard media) frozen above −3°C. Ice forms initially in a random number of cells that are dispersed throughout the monolayer (*A*). This is followed by the propagation of intracellular ice to adjacent cells (*B*). This pattern is identical to that observed in V-79W confluent monolayers.

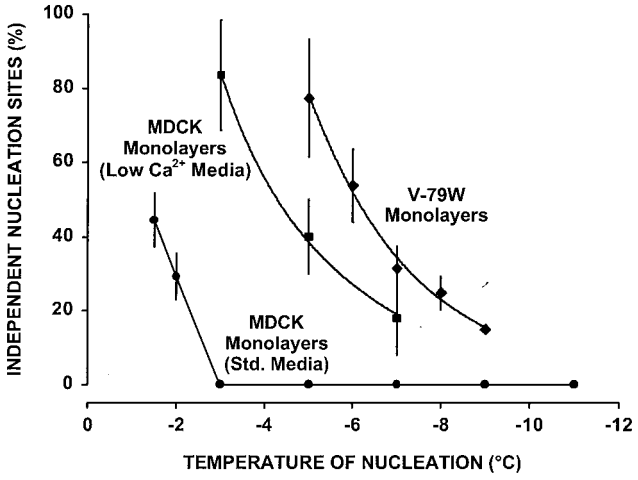

FIGURE 9 Percentage of independent nucleation sites as a function of the temperature of nucleation for the three experimental model systems. An independent nucleation site is defined as a cell that forms intracellular ice without any contact with an already frozen cell. The percentage of independent nucleation sites is calculated as the total number of independent sites divided by the total number of cells that form intracellular ice. Mean ± SEM ($n = 6$).

indicate that a large proportion of cells formed intracellular ice independent of freezing in an adjacent cell. A low value indicates a high degree of interaction between adjacent cells resulting in intercellular ice nucleation. Fig. 9 shows differences in the percentage of independent nucleation sites for the three experimental model systems. For V-79W monolayers and MDCK monolayers without gap junctions (low Ca^{2+} media), the percentage of independent sites increased with increasing temperature. In the MDCK monolayer with gap junctions (standard media), there were no independent nucleation sites (in the cryomicroscope field of view) at or below −3°C. Above −3°C, the percentage of independent nucleation sites increased with increasing temperature.

In confluent MDCK monolayers with gap junctions, there is a transition at −3°C in the incidence of IIF, the pattern of propagation and in the percentage of independent nucleation sites. This is different from observations for confluent MDCK monolayers without gap junctions and for confluent V-79W monolayers.

DISCUSSION

It has been proposed that during freezing gap junctions in the plasma membrane facilitate the passage of ice between adjacent cells. Using the capillary freezing-point depression equation (Eq. 10), we have shown that it is plausible for there to exist a relatively high subzero temperature where an ice crystal of equilibrium curvature is of sufficient size to propagate between adjacent cells through gap junctions. Experimentally, we observed a significant change in the formation and propagation of intracellular ice in confluent monolayers with and without gap junctions that were nucleated at −3°C. As this temperature is within the range predicted by the capillary freezing equation with intermediate contact angles, we believe the observed behavior at this transition represents the inhibition of gap junction-facilitated ice propagation. In this study we have qualitatively coupled the predictive value of the capillary freezing-point equation with experimental evidence to support the proposed theory that gap junctions can facilitate the intercellular propagation of intracellular ice. Note, however, that not all the parameters are known well enough to make a quantitative comparison, with the least well known parameter being the contact angle.

Although the data presented herein strongly support the involvement of gap junctions in the propagation of intracellular ice, the complete mechanism of such action is still

unresolved. We provide evidence to support the theory that intercellular ice growth occurs when intracellular ice physically propagates through gap junctions. However, alternative mechanisms may account for the observed changes in IIF in MDCK confluent monolayers. Mazur (1966) proposed a model for the response of two membrane-bound compartments connected by a narrow pore where the formation of ice in one compartment could result in expansion of the pore and/or membrane rupture. Although the disruption of adjacent plasma membranes and/or an increase in the radii of gap junctions could result in the propagation of intracellular ice between neighboring cells, recent evidence on the postthaw integrity of the cell plasma membrane suggests that intercellular ice propagation occurs without significant damage to these structures. Using a dual fluorescent labeling technique, the formation of intracellular ice does not necessarily result in a disruption in the integrity of the constituent cells in MDCK and V-79W confluent monolayers (Acker, 1997; Acker and McGann, 2000). If mechanical disruption of adjacent cell membranes or even transient enlargement of gap junctions did occur, it would be expected that the fluorescent probes used in their study would have leaked into adjacent cells, giving the impression that there was a complete loss of membrane integrity. As this did not occur, we can propose that the integrity of adjacent cells after intercellular ice propagation is not appreciable affected by the process.

SCN (Toner et al., 1990) has been proposed as an alternative mechanism for the intercellular propagation of intracellular ice (Acker, 1997; Tsuruta et al., 1997, 1998). It has been suggested that the close cell-cell contact that exists in confluent monolayers results in the nucleation of intracellular ice on the intracellular plasma membrane surface of adjacent cells (Acker, 1997; Acker and McGann, 1998). Although none of the data as presented herein preclude the possibility that gap junctions function solely to enhance the propagation of intracellular ice by SCN, there is evidence in the literature that suggests intercellular ice propagation requires more that just adjoining extracellular protein complexes. Using dinitrophenol and heptanal to uncouple gap junctions in cell strands from insect salivary gland, Berger and Uhrik (1996) showed that intercellular ice propagation could be inhibited. As heptanal functions to close (uncouple) gap junctions without removing them from the plasma membrane (Bernardini et al., 1984), intercellular ice propagation required functioning pores and not just the presence of connexin-connexin complexes (gap junctions).

Although this study addresses ice propagation in systems with gap junctions, the fact that intercellular ice propagation occurs to some degree in tissue systems that do not form gap junctions (V-79W confluent monolayers for example) suggests that this can occur by multiple mechanisms. Understanding the mechanisms by which intracellular ice can pass from one cell to another will be critical to developing mechanistic and mathematical models to predict the occurrence of IIF in tissue systems. Clearly, further work is required to understand these mechanisms and to integrate them into our understanding of the complex low-temperature response of tissue systems.

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