Zoological Studies **39**(2): 144-150 (2000)

Zoological Studies

Antagonizing Effects of Type I Antifreeze Protein on Ca^{++} Uptake in Fish TO-2 Cells

Ching-Feng Weng[1], Hong-Yi Gong[1], Jen-Leih Wu[1], Choy-Leong Hew[2] and Pung-Pung Hwang[1,*]

[1]*Institute of Zoology, Academia Sinica, Taipei 115, Taiwan, R.O.C.*

[2]*Department of Laboratory Medicine and Pathology, University of Toronto, Toronto, Ontario, M5G 1L5, Canada*

(Accepted February 14, 2000)

Ching-Feng Weng, Hong-Yi Gong, Jen-Leih Wu, Choy-Leong Hew and Pung-Pung Hwang (2000) Antagonizing effects of type I antifreeze protein on Ca^{++} uptake in fish TO-2 Cells. *Zoological Studies* **39**(2): 144-150. The antifreeze proteins (AFPs) or glycoproteins in several species of polar fish, which prevent the fish from being frozen in icy seawater, have been suggested for use as cryoprotective agents. The present study examines the effect of AFPs on Ca^{++} flux in a fish TO-2 cell line. The results indicate that type I AFP inhibits the uptake of Ca^{++} in a dose-dependent manner, but it does not antagonize the release of Ca^{++}. By contrast, type III AFP shows no effect on Ca^{++} flux. The addition of calcium antagonists, La^{+++} ($LaCl_3$), Co^{++} ($CoCl_2$), or verapamil (L-type Ca^{++} channel blocker), to the uptake medium did not further enhance the blocking effect of AFP on Ca^{++} uptake, indicating that type I AFP is a non-selective Ca^{++} blocker.

Key words: Type I antifreeze protein, Ca^{++} blocker, Ca^{++} uptake, Ca^{++} release, Fish cell.

One type of antifreeze glycoprotein (AFGP) and 4 types of antifreeze proteins (AFPs) have been found and characterized in several species of polar fish (Davies and Sykes 1997). AFGP, a repeat of the glycotripeptide, Ala-Ala-Thr with an O-linked disaccharide linked to each threonine residue (DeVries et al. 1970), was originally isolated from the blood of Antarctic nototheniid fish (DeVries and Wohlschlag 1969). Among them, type I AFP, isolated from various species of flounder and sculpin, is comprised of alanine-rich amphipathic α-helices (Davies and Hew 1990). Type II AFP, a cysteine-rich and larger protein with a folded structure, was isolated from sea raven (Ewart et al. 1992, Ng and Hew 1992, Ewart and Fletcher 1993). Type III AFP, without cysteine and not alanine-rich, but with a β-sheet sandwich structure, was isolated from Newfoundland ocean pout (Hew et al. 1988, Sonnichsen et al. 1993); and type IV AFP, a helix bundle structure, was isolated from the longhorn sculpin (Deng et al. 1997).

The type I AFP gene from winter flounder (*Pleuronectes americanus* Walbaum) has been transfected into Atlantic salmon (*Salmo salar* L.), but the expression of the AFP transgene was insufficient to provide any significant improvement in freeze-tolerance (Shears et al. 1991). Recently, a complete type III AFP gene of Newfoundland ocean pout (*Macrozoarces americanus* Schneider) was introduced into a goldfish (*Carassius auratus* Burgeri) via oocyte microinjection. A subsequent test indicated that 33% of the transgenic goldfish survived while none of the control fish survived at 0 °C for 12 h (Wang et al. 1995). Type I AFP was demonstrated to be capable of enhancing cold tolerance in tilapia and milkfish (Wu et al. 1997). AFPs have been considered to function exclusively by conferring freeze resistance by binding to ice crystals and thereby depressing blood plasma freezing points non-colligatively (Raymond and DeVries 1977, DeVries 1988). Furthermore, previous studies have indicated that AFGP or AFPs facilitate the retention of normal physiological activities in mammalian oocytes during hypothermic storage (Rubinsky et al. 1990 1991a,b) and protect rat livers from the effects of hypothermic

*To whom correspondence and reprint requests should be addressed. Tel: 886-2-27899521. Fax: 886-2-27858059. E-mail: zophwang@ccvax.sinica.edu.tw

exposure (Lee et al. 1992). Rubinsky and colleagues suggested that cold-sensitive oocyte and liver cells become cold tolerant probably because AFPs reduce the leakiness of cell membranes (Negulescu et al. 1992, Rubinsky et al. 1992).

AFPs have been reported to prevent mammalian oocytes from freezing at hypothermic (4 °C) (Rubinsky et al. 1990 1991b) and at cryogenic (-130 °C) temperatures (Rubinsky et al. 1991a, Lee et al. 1992). The addition of AFPs or AFGP to the cryogenic medium may cause an increase in solution viscosity (Eto and Rubinsky 1993), and subsequently to increase the motility of ram spermatozoa (Payne et al. 1994) as well as the survival of red blood cells (Chao et al. 1996a). These proteins also prevent injury to pig oocyte oolemmae (Arav et al. 1993) and attenuate the recovery of cells (Hansen et al. 1993) following cryopreservation. In addition, type II AFP requires Ca^{++} for its activity (Ewart et al. 1992, Ewart and Fletcher 1993) and shows ice-binding activity after specifically binding to Ca^{++} (Ewart et al. 1996). AFGP may protect membrane integrity during cooling by blocking ion fluxes across membranes (Negulescu et al. 1992, Rubinsky et al. 1992). Nevertheless, direct interaction of AFGPs with ion channels is still controversial (Hincha et al. 1993, Payne et al. 1994). Recently, a study has shown that the stabilizing effects of AFGPs on intact cells may be due to a nonspecific effect on the lipid components of native membranes (Hays et al. 1996). However, there is no direct evidence to show the effect of AFP on ionic flux *in vivo* or *in vitro* after administration. In order to study the role of AFPs in Ca^{++} flux, a fish cell line was used to examine the release and uptake of Ca^{++} in the presence of type I AFP by using $^{45}Ca^{++}$ as a tracer. The results show that type I AFP can inhibit the uptake of Ca^{++} as a non-selective Ca^{++} blocker, but it does not antagonize the release of Ca^{++}.

MATERIALS AND METHODS

AFP and fish cells

Type I AFP (from winter flounder) and type III AFP (from Newfoundland ocean pout) were purified from winter flounder serum as described by Fourney et al. (1984) using Sephadex-G75 (Amersham Pharmacia Biotech., Taipei, Taiwan) gel filtration chromatography. AFP was dissolved in saline. The TO-2 cell line derived from tilapia ovary was generously provided by Dr. S.N. Chen (Chen et al. 1983). The cells were kept in 75-cm^2 Falcon flasks containing Leibovitz's growth medium (L-15) supplemented with 10% fetal calf serum, 250 U/ml penicillin, 250 μg/ml streptomycin and 2.5 μg/ml Fungizone. The cells were maintained at 28 °C in a water-saturated incubator and subcultured to confluence by using 0.05% trypsin and EDTA. Approximately 1 x 10^6 cells/well was plated into multiwell dishes (24 wells, Nalgene, Hereford, UK). Ca^{++} uptake and release experiments were performed until the cells reached confluence.

Ca^{++} uptake

Confluent cells (TO-2) were washed twice with Dulbecco's phosphate buffered saline (DPBS). Two hundred μl of uptake medium (L-15) containing 1 μCi $^{45}Ca^{++}$ (specific activity 50 mCi/mg, Amersham, Buckinghamshire UK) plus tested substances were added to the cultured cells at room temperature (26 °C) for various periods of time depending on the experimental design. Ca^{++} uptake was stopped by rapidly washing the cells 3 times with ice-cold DPBS. Cells were then solubilized in 1 N NaOH. Aliquots of cell extracts were taken into vials for β-counting.

Ca^{++} release

Confluent cells (TO-2) were washed twice with DPBS. The cells were preincubated with 0.2 ml uptake medium containing 1 μCi $^{45}Ca^{++}$ for 30 min at room temperature (26 °C). The medium was then removed, and the cells were washed rapidly twice with DPBS and refilled with uptake medium plus tested substances. After 15 min, the media were collected for counting, and the cells were refilled with uptake medium containing 10 μM A23187, a Ca^{++} ionophore, and further incubated for 10 min. The supernatant was then taken into vials for β-counting, and the cells were solubilized and counted.

Statistical analyses

Data are expressed as the mean ± SEM. Statistically significant differences ($p < 0.05$) between treatments were assessed using Student *t*-test after one-way analysis of variance (ANOVA).

RESULTS

Cells were incubated with 1 μCi $^{45}Ca^{++}$ for various periods of time to examine the optimal incubation time. Ca^{++} uptake by TO-2 cells was linear for the first 30 min and reached a plateau after 30 min

(Fig. 1). Type I AFP, ranging from 100 to 1000 μg/ml, dose-dependently inhibited Ca^{++} uptake. Bovine serum albumin (BSA) had no effect on Ca^{++} uptake, while AFP plus BSA blocked Ca^{++} uptake dose-dependently (Fig. 2). By contrast, type III AFP showed no effect on Ca^{++} uptake. Clearly, type I AFP possesses an antagonizing effect on Ca^{++} uptake. Furthermore, the effects of various calcium antagonists alone or with AFP on Ca^{++} uptake are shown in figure 3. La^{+++} ($LaCl_3$; 10^{-8}-10^{-6} M), verapamil (L-type Ca^{++} channel blocker; 10^{-6}-10^{-4} M), or Co^{++} ($CoCl_2$, 10^{-8}-10^{-6} M) inhibited Ca^{++} uptake to the same extent irrespective of the presence or absence of AFP. Furthermore, A23187 (a Ca^{++} ionophore) as a Ca^{++} releaser, type I AFP, and/or BSA did not affect Ca^{++} release in TO-2 cells (Fig. 4).

DISCUSSION

Type I AFP inhibits the uptake of Ca^{++} in a dose-dependent manner in TO-2 cells, but it does not affect the release of Ca^{++}. In contrast, type III AFP shows no effect on Ca^{++} flux. Recently, a study reported that the stabilizing effects of AFGPs on intact cells may be due to a nonspecific effect of BSA on the lipid components of native membranes (Hays et al. 1996). The effect of BSA on Ca^{++} uptake was further examined, and BSA showed no interference with Ca^{++} uptake, nor with inhibition of Ca^{++} uptake by type I AFP in our experiments. Moreover, we thought that AFPs may also interact with cell membrane to prevent Ca^{++} release, but type I AFP and/or BSA did not affect Ca^{++} release in TO-2 cells after

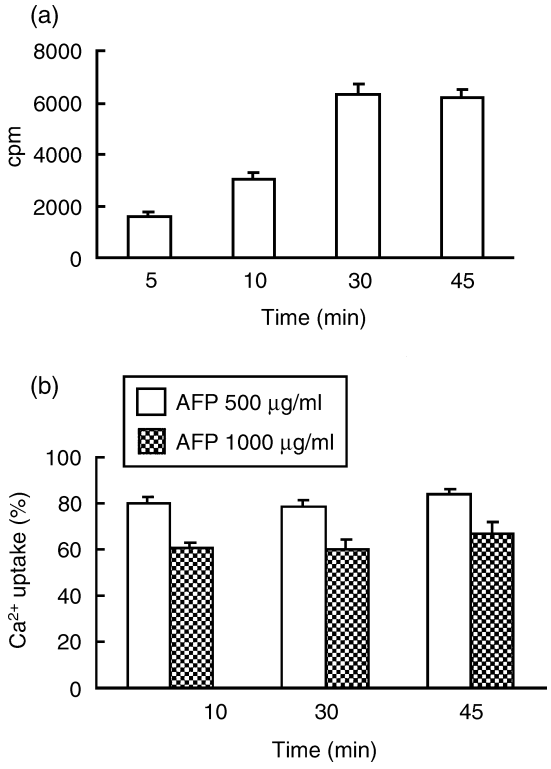

Fig. 1. Ca^{++} uptake in TO-2 cells. (a) incubation time (b) chemical addition time of type I AFP. Confluent cells (TO-2) were washed twice with Dulbecco's phosphate buffered saline (DPBS). Two hundred microliters of uptake medium (L-15) containing 1 μCi $^{45}Ca^{++}$ alone or with tested substances was added to the cultured cells for various times in the experiments. Uptake was stopped by rapidly washing the cells 3 times with ice-cold DPBS. The cells were then solubilized in 1 N NaOH. Aliquots of cell extracts were placed into vials for β-counting. Each value represents triplicate wells from 2 independent experiments. The Ca^{++} uptake of the control group is 100% in panel b.

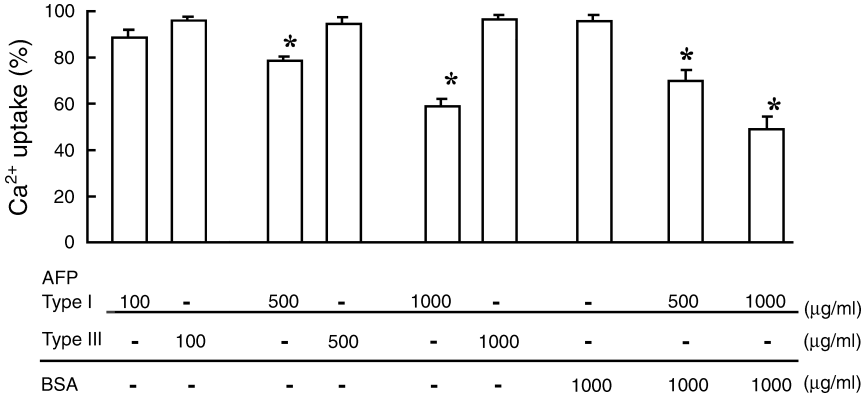

Fig. 2. Effects of type I AFP, type III AFP, or BSA on Ca^{++} uptake in TO-2 cells. Confluent cells (TO-2) were washed twice with Dulbecco's phosphate buffered saline (DPBS). Two hundred μl of uptake medium (L-15) containing 1 μCi $^{45}Ca^{++}$ alone or with tested substances was added to the cultured cells for 30 min. Uptake was stopped by rapidly washing the cells 3 times with ice-cold DPBS. The cells were then solubilized in 1 N NaOH. Aliquots of the cell extracts were placed into vials for β-counting. Each value represents the mean of triplicate wells from 2 different experiments. The Ca^{++} uptake of the control group is 100%. An asterisk (*) represents significant differences between the treatment and control groups ($p < 0.05$).

A23187 (a Ca^{++} ionophore) treatment. This reveals that AFP cannot change the ability of A23187 to form stable complexes with divalent cations. Most current studies have focused on the protection by AFP against the freezing effect of polar environments by its binding to ice surfaces and inhibiting the growth of ice crystals, ice-binding structures, or crystal morphology. The antifreeze effect of AFPs mainly occurs through the binding to ice surfaces and the inhibition of growth of ice crystals (Carpenter and Hansen 1992, Chou 1992, Wen and Laursen 1992 1993, Knight et al. 1993, Sicheri and Yang 1995). In addition, Chao et al. (1996b) found that type I AFP contains four 11-amino acid repeats which produce more active ice-binding than that of the 3-amino acid-repeat winter flounder AFP. Type II AFP specifically binds Ca^{++}, consequently adopting a conformation that is essential for its ice-binding activity (Ewart et al. 1996). For the action of AFPs or AFGPs on ionic flux, however, the effect of AFGPs on ion channels remains controversial (Hincha et al. 1993, Payne et al. 1994). The present study supports previous findings in which AFPs have been shown to be a current blocker for calcium and potassium channels in rabbit parietal cells and porcine granulosa cells (Negulescu et al. 1992, Rubinsky et al. 1992).

On the other hand, type I AFP and $^{45}Ca^{++}$ coexisted in the medium during the experiment; the decrease in Ca^{++} concentration was associated with an increase of AFP dose for cell uptake because AFP may bind most of the Ca^{++}. Thus, the present data could be due to different concentrations of free Ca^{++} in the medium and might not represent an inhibiting effect of type I AFP on Ca^{++} uptake in TO-2 cells. It seems unlikely that most of the Ca^{++} was bound to AFP, resulting in an unavailability of Ca^{++} in the uptake medium during the short-term (30 min) incubation. Furthermore, the AFP gene was transfected into TO-2 cells and then tested for effect on Ca^{++} uptake in a preliminary experiment. TO-2 cells were cotransfected with the AFP gene and green fluorescence protein (GFP) gene to check the efficiency of the transfection. The data revealed no consistent inhibition of Ca^{++} uptake in the transient state of AFP

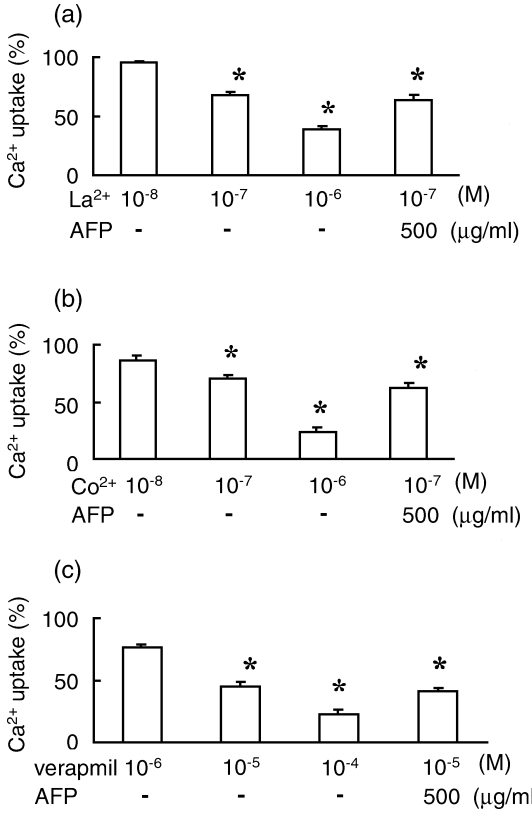

Fig. 3. Effects of calcium antagonists, (a) La^{+++}, (b) Co^{++}, and (c) verapamil on Ca^{++} uptake in TO-2 cells. Confluent cells (TO-2) were washed twice with Dulbecco's phosphate buffered saline (DPBS). Two hundred μl of uptake medium (L-15) containing 1 μCi $^{45}Ca^{++}$ plus tested substances was added to the cultured cells for 30 min. Uptake was stopped by rapidly washing the cells 3 times with ice-cold DPBS. The cells were then solubilized in 1 N NaOH. Aliquots of the cell extracts were placed into vials for β-counting. Each value represents the mean of triplicate wells from 2 different experiments. The Ca^{++} uptake of the control group is 100%. An asterisk (*) represents significant differences between the treatment and control groups ($p < 0.05$).

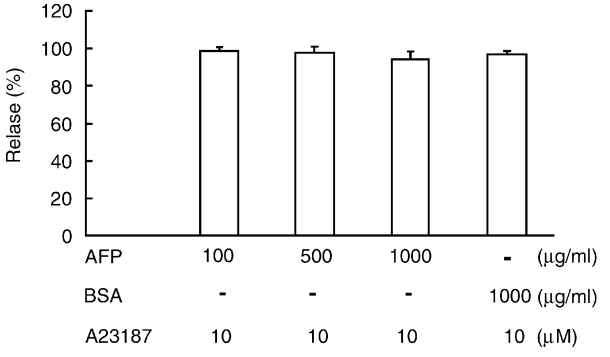

Fig. 4. Effects of type I AFP or BSA on Ca^{++} release in TO-2 cells in the presence of 10 μM A23187. Confluent cells (TO-2) were washed twice with DPBS. Cells were preincubated with 0.2 ml uptake medium containing 1 μCi $^{45}Ca^{++}$ for 30 min. The medium was then removed, and cells were washed rapidly twice with DPBS and refilled with uptake medium plus tested substances. After 15 min, the media were collected for counting, and the cells were refilled with uptake medium containing 10 μM A23187, a Ca^{++} ionophore, and further incubated for 10 min. Then, the supernatant was placed into vials for β-counting, and the cells were solubilized and counted. Each value represents the mean of 4 wells from 1 representative experiment. The Ca^{++} release of the control group is 100%. The statistical test revealed no significant differences between the treatment and control groups ($p > 0.05$).

transfection (data not shown). AFP tranfection may be too low to show the blocking effect on Ca^{++} uptake. This result is similar to those of previous reports in which, even though the AFP gene was successfully incorporated into both Atlantic salmon and goldfish, the expression of the AFP was insufficient to significantly prevent cold shock (Shears et al. 1991, Wang et al. 1995). AFP, a small hydrophobic molecule of 33 to 64 amino acids (including a high content of aspartic acid), is highly α-helical and rod-like in structure (Fourney et al. 1984). This molecule is thought to stabilize the membrane bilayer structure to prevent an irreversible transition to the gel state when the plasma membrane is compressed during cell volume reduction. On the other hand, AFGP or AFP may affect the lipid transition temperature of the membrane, i.e., preventing liposomal leakage induced by chilling the liposome through the transition temperature (Hays et al. 1996). Hochachka (1986) argued that in cold-tolerant animals, cell membranes are less leaky than those of cold-sensitive animals. Temperature differentially affects the transport mechanisms involved in ion balance; and changes in body temperature of poikilothermic animals, such as fish, may disturb the normal cellular steady state (Cossins et al. 1995). In addition, by using different uptake temperatures, no further inhibition of type I AFP on Ca^{++} uptake was observed (data not shown), suggesting that alteration of temperature does facilitate the inhibitory effect of AFP on Ca^{++} uptake. Interestingly, rectal administration of type I AFP can increase the cold tolerance in tilapia and milkfish (Wu et al. 1997). However, recent reports indicate that AFGPs failed to protect rat heart during freezing (Wang et al. 1994), a result of AFGP-induced changes in ice crystal structure and damaging cardiomyocytes of the rat (Mugnano et al. 1995). This difference could be due to a reduction of the extracellular Ca^{++} pool, resulting in increased cold tolerance. This discrepancy still needs to be clarified.

Addition of the calcium antagonists, La^{+++} ($LaCl_3$), Co^{++} ($CoCl_2$), or verapamil (L-type Ca^{++} channel blocker), to the uptake medium caused no further enhancement of the blocking effect of AFP on Ca^{++} uptake, indicating that AFP is a nonselective Ca^{++} blocker. Similar evidence has led to the suggestion that Ca^{++} competes with heavy metals for binding sites in gill epithelial cells (Verbost et al. 1988). Moreover, several studies have reported that Cd^{++} can block calcium channels in several cell types (Nelson 1986, Hinkle et al. 1987). Cobalt is known to inhibit calcium channels and to permeate some of these channels in non-teleost tissues (Simkiss et al. 1996). La^{+++} and Co^{++} inhibit Ca^{++} uptake by competitive-binding to the active site of the Ca^{++} transport system. These chemical reagents are the non-selective Ca^{++} blockers because they inhibit both voltage-dependent and voltage-independent calcium channels. With the presence of non-voltage-gated Ca^{++} channels in ionocytes, blockers of L-type channels (Perry and Flik 1988) have no effect on branchial Ca^{++} influx, whereas La^{+++} or Co^{++} inhibits the transcellular influx of Ca^{++} without entering the ionocyte (Perry and Flik 1988, Verbost et al. 1989). The present study also confirms that calcium antagonists, including non-selective and voltage-dependent ones, inhibit Ca^{++} uptake in TO-2 cells.

Acknowledgments: This study was partially supported by the Council of Agriculture, Executive Yuan, Republic of China.

REFERENCES

Arav A, B Rubinsky, G Fletcher, E Seren. 1993. Cryogenic protection of oocytes with antifreeze proteins. Mol. Reprod. Dev. **36:** 488-493.

Carpenter JE, TN Hansen. 1992. Antifreeze protein modulates cell survival during cryopreservation: mediation through influence on ice crystal growth. Proc. Natl. Acad. Sci. USA **19:** 8953-8957.

Chao H, PL Davies, JE Carpenter. 1996a. Effects of antifreeze proteins on red blood cell survival during cryopreservation. J. Exp. Biol. **199:** 2071-2076.

Chao H, RS Hodges, CM Kay, SY Gauthier, PL Davies. 1996b. A natural variant of type I antifreeze protein with four ice-binding repeats is a particularly potent antifreeze. Protein Sci. **5:** 1150-1156.

Chen SN, SC Chi-Ueno, GH Kou. 1983. A cell line derived from tilapia ovary. Fish Pathol. **18:** 3-18.

Chou KC. 1992. Energy optimized structure of antifreeze protein and its binding mechanism. J. Mol. Biol. **223:** 509-517.

Cossins AR, PJ Schwarzbaum, W Wieser. 1995. Effects of temperature on cellular ion regulation and membrane transport systems. *In* PW Hochachka, TP Mommsen, eds. Environment and biological biochemistry. Amsterdam: Elsevier, pp. 101-126.

Davies PL, CL Hew. 1990. Biochemistry of fish antifreeze proteins. FASEB J. **4:** 2460-2468.

Davies PL, BD Sykes. 1997. Antifreeze proteins. Curr. Opin. Struct. Biol. **7:** 828-834.

Deng G, DW Andrews, RA Laursen. 1997. Amino acid sequence of a new type of antifreeze protein from the longhorn sculpin, *Myoxocephalus octodecimspinosis*. FEBS Lett. **402:** 17-20.

DeVries AL. 1988. The role of antifreeze glycopeptides and peptides in the freezing avoidance of Antarctic fishes. Comp. Biochem. Physiol. **90B:** 611-621.

DeVries AL, SK Komatsu, RE Feeney. 1970. Chemical and physical properties of freezing point-depressing glycoproteins from Antarctic fishes. J. Biol. Chem. **245:** 2901-2908.

DeVries AL, D Wohlschlag. 1969. Freezing resistance in some

Antarctic fishes. Science **163:** 1073-1075.

Eto TK, B Rubinsky. 1993. Antifreeze glycoproteins increase solution viscosity. Biochem. Biophys. Res. Commun. **197:** 927-931.

Ewart KV, GL Fletcher. 1993. Herring antifreeze protein: primary structure and evidence for a C-type lectin evolutionary origin. Mol. Mar. Biol. Biotechnol. **2:** 20-27.

Ewart KV, B Rubinsky, GL Fletcher. 1992. Structural and functional similarity between fish antifreeze proteins and calcium-dependent lectins. Biochem. Biophys. Res. Commun. **185:** 335-340.

Ewart KV, DS Yang, VS Ananthanarayanan, GL Fletcher, CL Hew. 1996. Ca^{2+}-dependent antifreeze proteins. Modulation of conformation and activity by divalent metal ions. J. Biol. Chem. **271:** 16627-16632.

Fourney RM, GL Fletcher, CL Hew. 1984. Heterogeniety of antifreeze polypeptides from the Newfoundland winter flounder *Pseudopleuronectes americanus*. Can. J. Zool. **62:** 28-33.

Hansen TN, KM Smith, KG Brockbank. 1993. Type I antifreeze protein attenuates cell recoveries following cryopreservation. Transplant Proc. 25: 3182-3184.

Hays LM, RE Feeney, LM Crowe, JH Crowe, AE Oliver. 1996. Antifreeze glycoproteins inhibit leakage from liposomes during thermotropic phase transitions. Proc. Natl. Acad. Sci. USA **93:** 6835-6840.

Hew CL, NC Wang, SB Joshi, GL Fletcher, GK Scott, PH Hayes, B Buettner, PL Davies. 1988. Multiple genes provide the basis for antifreeze protein diversity and dosage in the ocean pout, Macrozoarces americanus. J. Biol. Chem. **263:** 12049-12055.

Hincha DK, AL DeVries, JM Schmitt. 1993. Cryotoxicity of antifreeze proteins and glycoproteins to spinach thylakoid membranes–comparison with cryotoxic sugar acids. Biochem. Biophys. Acta **114:** 258-264.

Hinkle PM, PA Kinsella, KC Osterhoudt. 1987. Cadmium uptake and toxicity via voltage-sensitive calcium channels. J. Biol. Chem. **262:** 16333-16337.

Hochachka PW. 1986. Defense strategies against hypoxia and hypothermia. Science **221:** 234-241.

Knight CA, E Driggers, AL DeVries. 1993. Adsorption to ice of fish antifreeze glycopeptides 7 and 8. Biophys. J. **64:** 252-259.

Lee CY, B Rubinsky, GL Fletcher. 1992. Hypothermic protection of whole mammalian organs with antifreeze proteins. Cryoletters **13:** 59-66.

Mugnano JA, T Wang, JR Layne, AL DeVries, RE Lee Jr. 1995. Antifreeze glycoproteins promote intracellular freezing of rat cardiomyocytes at high subzero temperatures. Am. J. Physiol. **269:** R474-R479.

Negulescu PA, B Rubinsky, GL Fletcher, TE Machen. 1992. Fish antifreeze proteins block Ca^{2+} entry into rabbit parietal cells. Am. J. Physiol. **263:** C1310-C1313.

Nelson MT. 1986. Interactions of divalent cations with single calcium channels from rat brain synaptosomes. J. Gen. Physiol. **87:** 201-222.

Ng NFL, CL Hew. 1992. Structure of an antifreeze polypeptide from the sea raven. Disulfide bonds and similarity to lectin-binding proteins. J. Biol. Chem. **267:** 16069-16075.

Payne SR, JE Oliver, GC Upreti. 1994. Effect of antifreeze proteins on the motility of ram spermatozoa. Cryobiology **31:** 180-184.

Perry SF, G Flik. 1988. Characterization of branchial transepithelial calcium fluxes in freshwater trout, *Salmo gairdneri*. Am. J. Physiol. **126:** 277-286.

Raymond JA, AL DeVries. 1977. Adsorption inhibition as a mechanism of freezing resistance in polar fishes. Proc. Natl. Acad. Sci. USA **74:** 2589-2593.

Rubinsky B, A Arav, AL DeVries. 1991a. Cryopreservation of oocytes using directional cooling and antifreeze glycoproteins. Cryoletters **12:** 93-106.

Rubinsky B, A Arav, GL Fletcher. 1991b. Hypothermic protection – a fundamental property of "antifreeze" proteins. Biochem. Biophys. Res. Commun. **180:** 566-571.

Rubinsky B, A Arav, M Mattioli, AL DeVries. 1990. The effect of antifreeze glycopeptides on membrane potential changes at hypothermic temperatures. Biochem. Biophys. Res. Commun. **173:** 1369-1374.

Rubinsky B, M Mattioli, A Arav, B Barboni, GL Fletcher. 1992. Inhibition of Ca^{2+} and K^{+} currents by antifreeze proteins. Am. J. Physiol. **262:** R542-R545.

Shears MA, GL Fletcher, CL Hew, S Gauthier, PL Davies. 1991. Transfer expression and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). Mol. Mar. Biol. Biotechnol. **1:** 58-63.

Sicheri F, DS Yang. 1995. Ice-binding structure and mechanism of an antifreeze protein from winter flounder. Nature **375:** 427-431.

Simkiss K. 1996. Ecotoxicants at the cell-membrane barrier. In MC Newman, CH Jagoe, eds. Ecotoxicology: a hierarchical treatment. New York: Lewis Publ., pp. 59-83.

Sonnichsen FD, BD Sykes, H Chao, PL Davies. 1993. The nonhelical structure of antifreeze protein type III. Science **259:** 1154-1157.

Verbost PM, G Flik, RAC Lock, SE Wendelaar Bonga. 1988. Cadmium inhibits plasma membrane calcium transport. J. Membr. Biol. **102:** 97-104.

Verbost PM, J van Rooij, G Flik, RAC Lock, SE Wendelaar Bonga. 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. J. Expt. Biol. **145:** 185-197.

Wang R, P Zhang, Z Gong, CL Hew. 1995. Expression of the antifreeze protein gene in transgenic goldfish (*Carassius auratus*) and its implication in cold adaptation. Mol. Mar. Biol. Biotechnol. **4:** 20-26.

Wang T, Q Zhu, X Yang, JR Layne, AL DeVries, 1994. Antifreeze glycoproteins from Antarctic notothenioid fishes fail to protect the rat cardiac explant during hypothermic and freezing preservation. Cryobiology **31:** 185-192.

Wen D, RA Laursen. 1992. A model for binding of an antifreeze polypeptide to ice. Biophys. J. **63:** 1659-1662.

Wen D, RA Laursen. 1993. Structure-function relationships in an antifreeze polypeptide. The effect of added bulky groups on activity. J. Biol. Chem. **268:** 16401-16405.

Wu SM, PP Hwang, CL Hew, JL Wu. 1997. Effect of antifreeze protein on cold tolerance in juvenile tilapia (*Oreochromis mossambicus* Peters) and milkfish (*Chanos chanos* Forsskål). Zool. Stud. **37:** 39-44.

Zoological Studies **39**(2): 144-150 (2000)

第一型抗凍蛋白對魚類 TO-2 細胞鈣離子吸收之拮抗作用

翁慶豐[1]　龔紘毅[1]　吳金洌[1]　邱才良[2]　黃鵬鵬[1]

在許多極地魚類身上所發現的抗凍蛋白或醣蛋白，具有防止魚類在冰凍的海水中結凍的能力，所以被視為拮抗劑。本報告是研究抗凍蛋白對魚類 TO-2 細胞鈣離子流動的作用。結果發現，第一型抗凍蛋白能抑制鈣離子之吸收，且其抑制作用隨施用之濃度升高而增加，但它卻不能拮抗鈣離子的釋放。相反的，第三型抗凍蛋白則對鈣離子流動沒有任何影響。另外，我們也發現在 TO-2 細胞的培養液中添加一些鈣離子拮抗物，如 La^{+++} ($LaCl_3$)、Co^{++} ($CoCl_2$) 、或 verapamil (L-型鈣離子通道抑制劑)，並不會加強第一型抗凍蛋白對鈣離子吸收的抑制，此結果顯示第一型抗凍蛋白應是一個非專一性的鈣離子吸收抑制劑。

關鍵詞：第一型抗凍蛋白，鈣離子通道抑制劑，鈣離子吸收，鈣離子釋放，魚類細胞。

[1] 中央研究院動物研究所
[2] 加拿大多倫多大學醫學及病理學系