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Calcium in Infectious Hematopoietic Necrosis Virus (IHNV) Infected Fish Cell Lines

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Infection of fish cells with IHNV resulted in gradual increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in CHSE, gradual decrease in $[Ca^{2+}]_i$ in FHM, and no significant change in RTG cells. The degree of $[Ca^{2+}]_i$ increase or decrease was dependent on the amount of infectious virus, and these $[Ca^{2+}]_i$ variations were maximal at 16 hours after virus infection (p.i.) in both cell lines. When the fish cells were infected with inactivated IHNV, evident variation in $[Ca^{2+}]_i$ was not observed. Thus, infectivity of IHNV appears to correlate with changes in $[Ca^{2+}]_i$ in virus-infected cells. These IHNV-induced $[Ca^{2+}]_i$ changes were partially blocked by cycloheximide, but not affected by cordycepin. It seems to be that virus-induced Ca^{2+} variations were more related with protein synthesis than RNA synthesis. Various Ca^{2+} related drugs were used in search for the mechanisms of the $[Ca^{2+}]_i$ changes following IHNV infection of CHSE cells. Decreasing extracellular Ca^{2+} concentration or blocking Ca^{2+} influx from extracellular media inhibited the IHNV-induced increase in $[Ca^{2+}]_i$ in CHSE cells. Similar results were obtained with intracellular Ca^{2+} blockers. Thus, it is suggested that both the extracellular and the intracellular Ca^{2+} sources are important in IHNV-induced $[Ca^{2+}]_i$ increase in CHSE cells.

Key words: Infectious Hematopoietic Necrosis Virus (IHNV), calcium, fish cell lines

Infectious hematopoietic necrosis (IHN) is an acute, systemic, and usually virulent rhabdoviral disease that can occur in the wild salmonids, but it is more typically seen in epizootic proportion among young trouts and certain Pacific salmons under husbandry in coastal North America from California to Alaska (2, 3, 26) and rainbow trout in Korea. An epizootic of IHN usually begins with a sudden rise in mortality. Moribund fish shows dark coloring, loss of appetite, anemia, exophthalmia, distension of the abdomen with ascites, general viremia and fecal casts. Although detected on cell culture and characterized as infectious hematopoietic necrosis virus (IHNV) initially from sockeye salmon (30), this infectious agent seems to be more specific for chinook salmon (4). After the first detection in North America's Pacific Northwest (12, 15, 33), IHNV was detected in Taiwan (10), Canada (31), Italy (7), France (14), and recently in Korea (24).

IHNV is a bullet shape enveloped (84 nm×194 nm) rhabdovirus. The genomic RNA is nonsegmented, linear, single-stranded and negative sense. It has been shown to be composed of a polymerase (L), a surface glycoprotein (G), a nucleocapsid (N), and two matrix proteins (M1 and M2), and the gene order in the genome is 5'-L-G-M2-M1-N-3' (11, 22). IHNV is transmitted through water, by feeding on infected carcasses or by exposure to eggs or fry from infected fish. Gills and gastrointestinal tract are the most probable route of entry. Recently, IHNV has been detected in an invertebrate (*Callibaetis* sp.) (26) as a carrier of IHNV. Three conditions are essential for occurrence of IHN; low temperature (10-15°C) (4), susceptible young fish, and a probable source of virus. Hematopoietic tissue is the prime target of this virus. Thus, epizootic transmission of IHNV to fingerlings of salmonid fish could follow seasonal changes (3, 8). Serious epizootic diseases were reported in the world, and there are little chemotherapeutic agents (13, 17) or vaccines available for prevention or control of the diseases. Presently, the development of a vaccine against IHNV would be important in practical and economical value (28). However, serological discrimination is not clear

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among geographic strains (6, 27). Also, isolates from Korea are somewhat different in neutralizing epitopes from other serotypes of IHNV (16, 21, 24).

To control or prevent the diseases caused by IHNV, a highly sophisticated and productive investigation of the molecular aspects of this virus and physiological aspects of infected cells are needed. Virus infection may have some effects on the cellular biochemical and physiological machineries. The cellular responses caused by some viruses appear to involve cellular signal transduction system which could be observed in cells stimulated with hormones or growth factors (1). Recently, attention has been focused on changes of signal transduction system following virus infection. However, there has not been a report on changes of intracellular levels of signal transduction elements following IHNV infections. In this study, changes in cytosolic free calcium concentration in three fish cell lines infected with IHNV were examined.

Materials and Methods

Virus and cell culture.

IHNV strain PRT and the cell lines used in this study were supplied by Dr. J. W. Park (Ulsan University). IHNV-PRT is a Korean strain of IHNV isolated from fish tissue samples collected during epizootics at several hatcheries in Kangwon Province (24). To support the multiplication of IHNV permissive cell lines including CHSE (chinook salmon embryo)-214, FHM (fathead minnow) and RTG (rainbow trout gonad) cells were used. Cells were grown in EMEM (Eagle's minimum essential medium) supplemented with 10% newborn calf serum (NCS) for CHSE-214 cells, or with 10% fetal bovine serum (FBS) for FHM and RTG cells. All cells were grown at 17.5°C without exogenous supply of CO_2. To produce IHNV-PRT stock, confluent monolayer of CHSE cells was infected with virus at m.o.i. (multiplicity of infection) of 0.1 PFU (plaque forming unit)/cell. The infected cells were incubated until extensive cytopathic effect appeared, and cells were frozen at -70°C. IHNV was harvested by rapid thawing of the infected cells and sonicating for 1 min 30 sec. The virus stock was stored at -70°C.

Drugs

EGTA (ethylene glycol tetraacetic acid), dantrolene, diltiazem, cordycepin, cycloheximide and nifedipine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EGTA, diltiazem and cycloheximide were dissolved in H_2O at 100 mM, 100 mM and 1 mg/ml, respectively. Nifedipine was dissolved in DMSO (dimethyl sulfoxide) at 1 mM immediately before use. Dantrolene and cordycepin were also dissolved in DMSO at 2 mM and 1 mg/ml, respectively. Ca^{2+} ionophore bromo-A23187 and intracellular Ca^{2+} sequester BAPTA/AM were purchased from Molecular Probes (Eugene, OR, U.S.A.) and dissolved in DMSO at concentration of 2 M and 1 mM, respectively. Verapamil was obtained from LymphoMed (Melrose Park, IL, U.S.A.) and dissolved in H_2O at 100 mM. Cytotoxicity of drugs was performed by determining the number of viable cells with trypan blue exclusion method.

Plaque assay

To determine the infectious titer of virus, serially diluted virus sample was inoculated (0.2 ml per 35 mm dish) to confluent monolayer of CHSE-214 cells and incubated for 1 hr with gentle rocking at every 15 minutes. Then, cells were washed with PBS (phosphate buffered saline: KCl 0.2 g, KH_2PO_4 0.24 g, NaCl 8 g, $Na_2HPO_4 7H_2O$ 2.16 g, distilled water upto 1 liter) and overlay medium (EMEM with 2% NCS, 0.075% sodium bicarbonate, 0.25% agarose) was added. Cells were fixed with 10% formalin in 0.85% saline about 4 days after virus infection, and the overlay medium was removed on the following day. Then, cell monolayer was stained with 0.03% methylen blue and the number of plaques was counted under stereo microscope.

Fluorescence measurement of cytosolic free Ca^{2+} concentration

To examine the possible Ca^{2+} response and the role of Ca^{2+} in IHNV-PRT infection, Fura-2/AM (Molecular Probes) was used as a fluorescent Ca^{2+} indicator. Confluent monolayer of cells grown in tissue culture dishes was infected with IHNV at m.o.i. of 1-3 PFU/cell or mock-infected. After virus infection, cells were incubated, washed twice with PBS and loaded with Fura-2/AM (5-7.5 μM in loading buffer: 10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM sodium pyruvate, 0.02% pluronic-F127) for 1 hr at 17.5°C. Thereafter, cells were washed with PBS, harvested by trypsinization and centrifugation, and washed twice with loading buffer. Fluorescence was measured by using fluorescence spectrophotometer (Hitachi, model F-3000). Excitation wavelength was 340 nm, and emission wavelength was 500 nm. Most experiments were performed more than three times in duplicate.

Results

One step growth curve of IHNV in fish cell lines

Confluent monolayers of each fish cell lines were in-

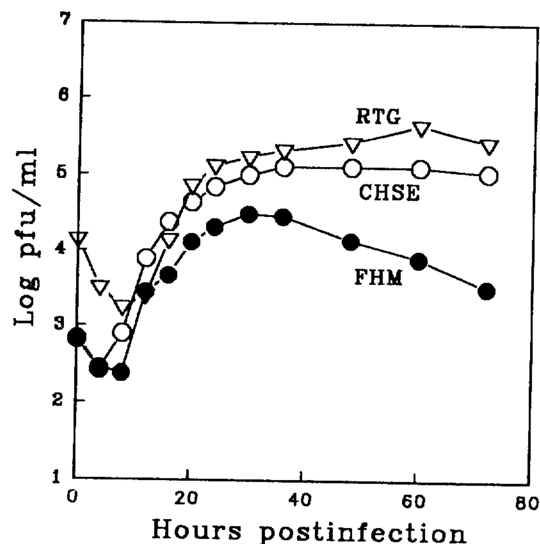

Fig. 1. Growth curve of IHNV-PRT in three fish cell lines. Confluent monolayer of each cells in 35 mm tissue culture dishes was infected with IHNV-PRT at 3 pfu/cell. Virus samples were prepared by freezing and thawing the entire dish. The infectivity of virus was determined at the time indicated by plaque assay in CHSE cells.

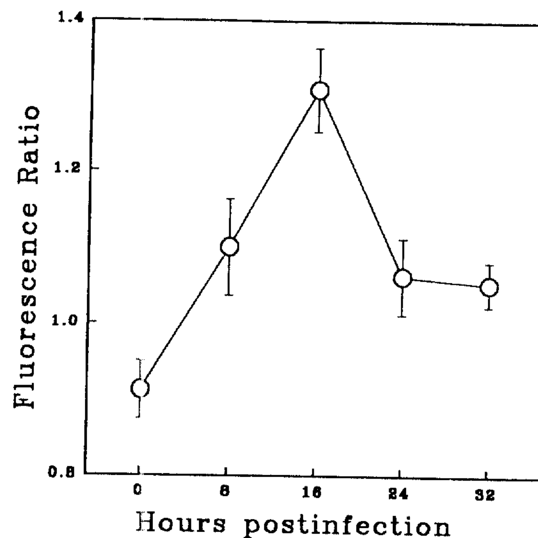

Fig. 2. The increase in Fura-2 fluorescence in CHSE cells following IHNV-PRT infection. Confluent monolayer of CHSE cells grown in 35 mm tissue culture dishes was mock-infected or infected with IHNV-PRT at 3 pfu/cell. At selected times after virus infection, cells were loaded with Fura-2/AM at 7.5 μM and harvested. The fluorescence was measured, and data were presented as the average ratio of fluorescence in IHNV-infected cells compared with that in mock-infected cells.

fected with IHNV at 3 pfu/cell, and the virus samples were prepared by freezing at -70℃ at indicated time points until 72 hr p.i. The amount of infectious virus produced was determined by plaque assay. Overall growth pattern of IHNV indicates that after a latent period of 8 hrs, infectious viruses were produced and they were rapidly released after 24 hr p.i. Although each cell lines show some difference in supporting the multiplication of IHNV, IHNV seemed to replicate at similar efficiency in the three cell lines (Fig. 1). Maximum production of the viruses in each cell lines was observed after 30 hr p.i.

Changes of $[Ca^{2+}]_i$ in IHNV infected fish cells

Confluent monolayer of fish cell lines was infected with IHNV. At selected time points after virus infection, cells were loaded with a fluorescent Ca^{2+} indicator, Fura-2/AM, and the fluorescence intensities were measured. The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) data were presented as the ratio of fluorescence in IHNV-infected cells compared with that in mock-infected cells. Infection of CHSE cells with IHNV resulted in gradual increase in $[Ca^{2+}]_i$ with the maximal increase observed at 16 hr p.i., and followed by gradual recovery to basal level until 32 hr p.i. (Fig. 2). On the contrary, as shown in Fig. 3, infection of FHM cells with IHNV resulted in gradual decrease in $[Ca^{2+}]_i$, maximal decrease at 16 hr p.i. and gradual recovery to basal level after that. Significant change in $[Ca^{2+}]_i$ was not noticed in IHNV-infected RTG cells (data not shown). Thus, it is concluded that IHNV infection resulted in elevation of $[Ca^{2+}]_i$ in CHSE cell and decrease in FHM cell, and these $[Ca^{2+}]_i$ variations were maximal at 16 hr p.i.

Relationship between IHNV infectivity and $[Ca^{2+}]_i$ variation

To examine whether these changes of $[Ca^{2+}]_i$ following IHNV infection of fish cell lines were direct consequence of viral infectivity, cells were infected with various amount of virus (m.o.i.=0-5 PFU/cell) and the Fura-2 fluorescence was measured at 16 hr p.i. The fluorescence in IHNV-infected CHSE cells was found to increase linearly and decrease in FHM cells as m.o.i. increased (Fig. 4). These data suggest that the increase or decrease in $[Ca^{2+}]_i$ observed in IHNV-infected cells may be a consequence of viral activity. When the infectivity of IHNV was abolished by UV irradiation for 30 minutes on 366 nm or heating for 1 hr at 50℃, $[Ca^{2+}]_i$ variations were not as evident as when infectious virus was used (Fig. 5). Therefore the variations in $[Ca^{2+}]_i$ in IHNV-infected fish cells appear to require virus infectivity. In order to examine whether viral or cellular gene expression is involved in IHNV-induced changes of $[Ca^{2+}]_i$, specific inhibitors of metabolism were used. The result shown in Fig. 6 indicates that the IHNV-induced increase in $[Ca^{2+}]_i$ was partially blocked by cycloheximide,

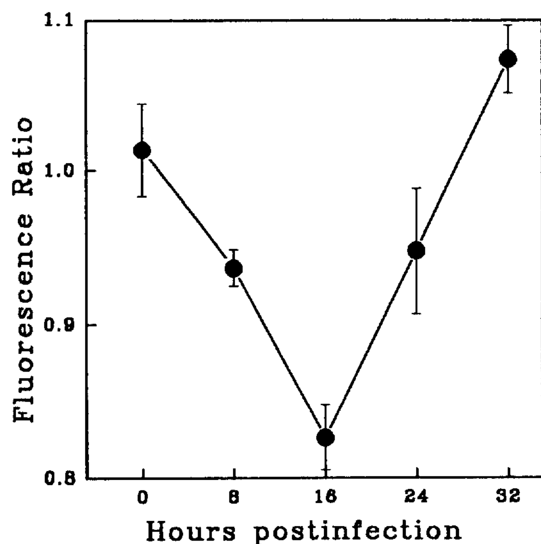

Fig. 3. The decrease in Fura-2 fluorescence in FHM cells infected with IHNV-PRT. Confluent monolayer of FHM cells grown in 35 mm tissue culture dishes was mock-infected or infected with IHNV-PRT at 3 pfu/cell. At selected times after virus infection, cells were loaded with Fura-2/AM at 5 μM, and the fluorescence was measured. Data are average ratios of fluorescence from separate experiments.

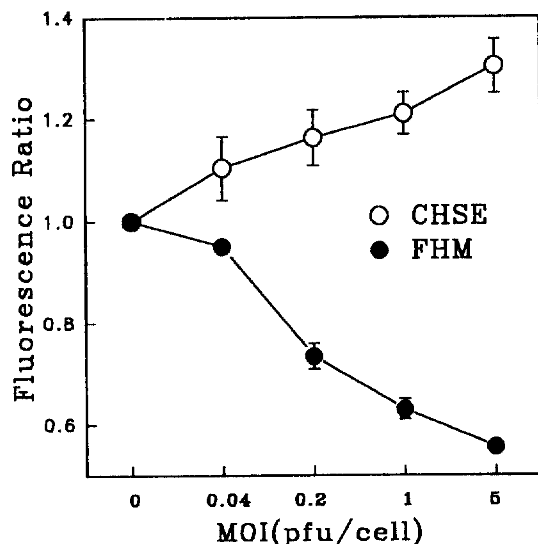

Fig. 4. The m.o.i. (multiplicity of infection) dependent changes in $[Ca^{2+}]_i$ in CHSE and FHM cells following infection with IHNV. Confluent monolayer of each cells was grown in 35 mm tissue culture dishes and infected with IHNV-PRT at various m.o.i. Cells were loaded with Fura-2/AM, and the Fura-2 fluorescence was measured at 16 hr p.i.

an inhibitor of protein synthesis. But cordycepin, RNA synthesis inhibitor, exerted little effect on $[Ca^{2+}]_i$. Thus, it appears that IHNV-induced increase in $[Ca^{2+}]_i$ in CHSE cells is more related to protein synthesis than cellular RNA synthesis.

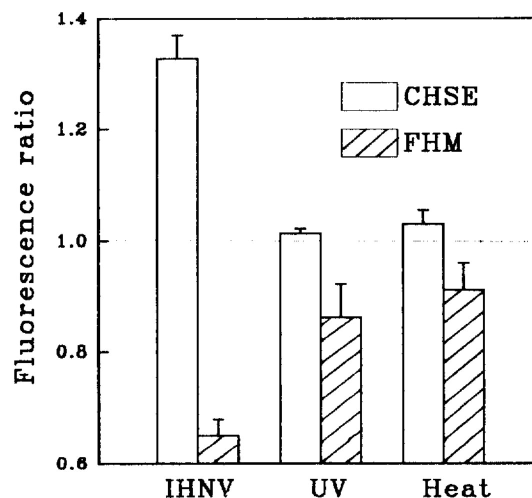

Fig. 5. Effect of virus inactivation on IHNV-PRT induced variations in $[Ca^{2+}]_i$. Fura-2 fluorescence was measured at 16 hr p.i. Virus was inactivated by UV irradiation for 30 minutes at 366 nm or by heating for 1 hour at 50℃. Mock; mock-infected cells, IHNV; infectious IHNV, UV; UV-inactivated IHNV, Heat; heat-inactivated IHNV.

Table 1. Effect of calcium modulators on IHNV-induced increase of $[Ca^{2+}]_i$ in CHSE cells.

Drugs	Fluorescence ratio[1] (±SD)	Significance[2]
None	1.34±0.06	
EGTA, 100 mM	1.09±0.02	*
Verapamil, 10 μM	1.04±0.04	*
Nifedipine, 10 μM	1.13±0.09	*
Diltiazem, 10 μM	1.11±0.06	*
BAPTA, 100 μM	1.05±0.02	*
Dantrolene, 100 μM	0.99±0.06	*

Confluent monolayer of FHM cells grown in 35 mm tissue culture dishes was mock-infected or infected with IHNV-PRT at 3 pfu/cell. At selected times after virus infection, cells were loaded with Fura-2/AM at 5 M, and the fluorescence was measured

[1]Fluorescence in IHNV-infected cells compared with that in mock-infected cells.

[2]Asterisks signify the difference of fluorescence ratio in drug-treated cells from that in untreated cells at the level of 95%.

Effect of Ca^{2+} modulators on $[Ca^{2+}]_i$

In order to understand the mechanism for the increase in $[Ca^{2+}]_i$, the effects of Ca^{2+} modulators on IHNV-induced $[Ca^{2+}]_i$ increase in CHSE cells were investigated (Table 1). Decreasing extracellular Ca^{2+} concentration by treating IHNV-infected CHSE cells with EGTA resulted in a diminution of the IHNV-induced $[Ca^{2+}]_i$ increase. Treatment with Ca^{2+} influx blockers such as nifedipine, diltiazem or verapamil also recovered $[Ca^{2+}]_i$ in IHNV-infected CHSE cells. As shown in Table 1, reduction of IHNV-induced increase in $[Ca^{2+}]_i$ by BAPTA/AM, an in-

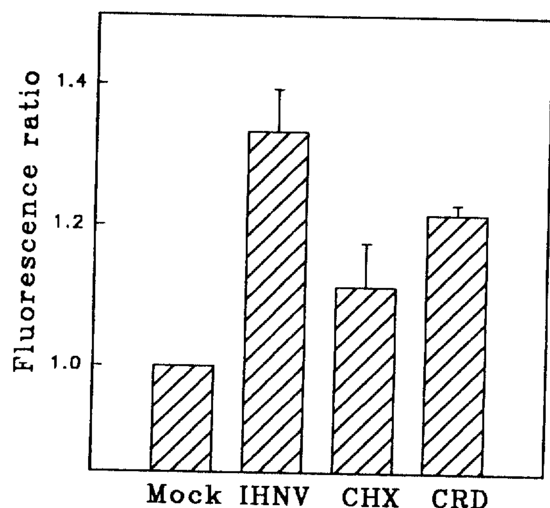

Fig. 6. Effect of metabolic inhibitors on IHNV-induced increase in $[Ca^{2+}]_i$ in CHSE cells. Cycloheximide is a protein synthesis inhibitor, and cordycepin is an RNA synthesis inhibitor. Both were treated at concentration of 10 μg/ml. CHX; cycloheximide, CRD; cordycepin.

tracellular Ca^{2+} chelator, or by dantrolene, a blocker of Ca^{2+} release from the intracellular Ca^{2+} stores, suggests the contribution of intracellular Ca^{2+}. Thus, both extracellular and intracellular Ca^{2+} sources appear to be important in IHNV-induced increase in $[Ca^{2+}]_i$ in CHSE cells.

Discussion

In this report, we have demonstrated for the first time that infection of fish cells with IHNV induces elevated or decreased levels of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Elevation of $[Ca^{2+}]_i$ was observed in CHSE cell while decrease in $[Ca^{2+}]$ was observed in FHM cell and there was no significant change in RTG cell following IHNV infection. The $[Ca^{2+}]_i$ increase or decrease seems to require infectious virus.

Previous reports have demonstrated a decrease in $[Ca^{2+}]_i$ in CHSE cells infected by IPNV (infectious pancreatic necrosis virus) (18), which infects salmonid fishes and causes necrosis of pancreas. Not all viruses, however, have been found to elevate or reduce $[Ca^{2+}]_i$ levels; human cytomegalovirus (HCMV) promotes the increase in $[Ca^{2+}]_i$ in fibroblast (19, 23). The increase in $[Ca^{2+}]_i$ in HCMV-infected human fibroblasts requires infectious virus and active gene expression, suggesting a crucial role of Ca^{2+} in the successful multiplication of HCMV. Other evidences support close relationships between Ca^{2+} response and viral infection (23). Why the viruses bring to changes in $[Ca^{2+}]_i$? In cell death, altered regulation of ions, especially Ca^{2+}, plays a key role in the processes leading to irreversible injury of cell. Elevated Ca^{2+} may initiate a cascade of signals leading to activation of phospholipase A2 (PLA2) and C (PLC), endonucleases, or proteases, and the expression of several immediate-early genes including *c-fos*, *c-jun*, *c-myc*, and *egr-1* (5). Virus may need to alter intracellular biochemical and physiological environment to accomodate its better status for replication. These alteration caused by some viruses are somewhat similar to signal transduction cascade induced by hormones or growth factors (1). Different cells provide different intracellular environment. Thus, biochemical and physiological changes induced by a certain virus might be different in different cells (see below).

There are several possibilities for the source of increased $[Ca^{2+}]_i$ in IHNV-infected CHSE cells. First possible way is to stimulate the Ca^{2+} entry from the extracellular medium. This possibility was examined with Ca^{2+} influx blockers (nifedipine, verapamil or diltiazem) and EGTA in this study. The data suggest that extracellular Ca^{2+} source may be associated with the increase in $[Ca^{2+}]_i$ following IHNV-infection of CHSE cells. The second possibility is the release of Ca^{2+} from intracellular Ca^{2+} compartments such as endoplasmic reticulum, mitochondria and Golgi complex (32). Data showing that the treatment with BAPTA/AM or dantrolene led to the reduction of increased $[Ca^{2+}]_i$ in IHNV-infected CHSE cell support the possible involvement of the intracellular Ca^{2+} stores. Other possibilities include the release of Ca^{2+} from intracellular Ca^{2+}-binding proteins, or the block of Ca^{2+} efflux to extracellular medium or intracellular Ca^{2+} compartment by specific Ca^{2+} pump (9, 29). In short, present data suggest that both the extracellular and intracellular Ca^{2+} sources may be responsible for the variations in $[Ca^{2+}]_i$ following IHNV infection.

Alhough the role of $[Ca^{2+}]_i$ in IHNV infected fish cells was not fully understood at this time, including different $[Ca^{2+}]_i$ patterns in different cells, any consideration of the underlying mechanisms responsible for the different responses in different fish cells needs to be elucidated. They may include species and tissue origin differences in potential for functionally important differences in tissue-specific and inducible expression attributable to IHNV-induced $[Ca^{2+}]_i$ changes, even if they were already transformed cell lines (20). Also they may include differences in unknown cellular factors for cell's metabolic activity, differentiation, signal cascade system, membrane affinity binding with virus, expression of Ca^{2+} binding protein calmodulin and regulators of maintenance or activation of cellular enzyme system for viral gene expression. No matter what factors are involved, however,

different responses in $[Ca^{2+}]_i$ of each cell types to IHNV infection may have a same goal, alteration of the cellular environment for viral multiplication.

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