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Hideki Ushio

Shugo Watabe

Muneaki Iwamoto

Kanehisa Hashimoto

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ULTRASTRUCTURAL EVIDENCE FOR TEMPERATURE-DEPENDENT Ca^{2+} RELEASE FROM FISH SARCOPLASMIC RETICULUM DURING RIGOR MORTIS

Hideki Ushio[1], Shugo Watabe[1], Muneaki Iwamoto[2], and Kanehisa Hashimoto[3]

[1]Laboratory of Marine Biochemistry, Faculty of Agriculture, University of Tokyo, Bunkyo, Tokyo 113, Japan
[2]Shimane Prefectural Fisheries Experimental Station, Setogashima, Hamada, Shimane 697, Japan
[3]Food Science Laboratory, Faculty of Education, Ibaraki University, Mito, Ibaraki 310, Japan

Abstract

The release or leakage of Ca^{2+} from the sarcoplasmic reticulum (SR) during rigor mortis of fish muscle was investigated by transmission electron microscopy using pyroantimonate and related biochemical changes.

Ca^{2+}-pyroantimonate deposits were observed in the SR immediately after spiking the fish. At the onset of rigor for fish stored at 0°C, no deposits were found in the SR; however, fish stored for the same period at 10°C which were still in the pre-rigor state, clearly showed Ca^{2+} deposits in the SR.

In association with the Ca^{2+} translocation, ATP degraded faster at 0°C than at 10°C, probably due to enhancement of myofibrillar ATPase activity by the increasing Ca^{2+} concentrations.

Therefore, rapid Ca^{2+} release from the SR at 0°C seemed to trigger the acceleration of fish rigor mortis at this temperature, analogous to the phenomenon called "cold shortening."

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Direct inquiries to S. Watabe
Telephone number: 81 3 3812 2111 ext. 5297
Fax number: 81 3 5684 0622

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Introduction

"Cold shortening" was first observed for avian and bovine muscles which show a long delay of rigor mortis when stored at 15 - 20°C, compared with those muscles stored at lower temperatures including 0°C (De Fremery and Pool, 1960; Locker and Hagyard, 1963; Cassens and Newbold, 1967). Subsequently, a similar acceleration of rigor mortis at 0°C was reported for several temperate and tropical fish species such as red sea bream, plaice and tilapia (Poulter *et al.*, 1981; Iwamoto *et al.*, 1985, 1987, 1990; Curran *et al.*, 1986) and this can be regarded as a cold shortening phenomenon also. However, fish living in the cold waters do not undergo cold shortening (Amlacher, 1961). Cold shortening has been of practical significance in meat industry, since almost all quality attributes of livestock meat are established during the first 24 to 48 hours postmortem when muscle is transformed from its normal, living state into meat (Goll *et al.*, 1971). A related situation is also applicable to the fisheries industry. If commercially valuable bottom fish such as red sea bream and plaice can be supplied to the market in a pre-rigor state, they have the same commercial value as do live specimens. This is because live fish are considered to be highest in freshness and best to be consumed raw. Therefore, it would be desirable for the fishing and meat industries to elucidate the mechanisms underlying cold shortening and to suggest possible treatments to prevent it.

The fact that the sarcoplasmic reticulum (SR) plays an important role in muscle contraction and relaxation and in controlling myofibrillar ATPase activity (Hasselbach, 1964; Ebashi and Endo, 1968) suggests that the maintenance or loss of its functional integrity is related to postmortem changes in muscle (Greaser *et al.*, 1967, 1969a, 1969b). Greaser *et al.* (1969b) reported that low muscle pH and high temperature are responsible for the inactivation of Ca^{2+} accumulation ability of the SR that occurs *in situ*.

Whiting (1980) also examined the effects of pH and temperature on Ca^{2+} uptake ability of the SR and mitochondria, and claimed that the latter organelles could

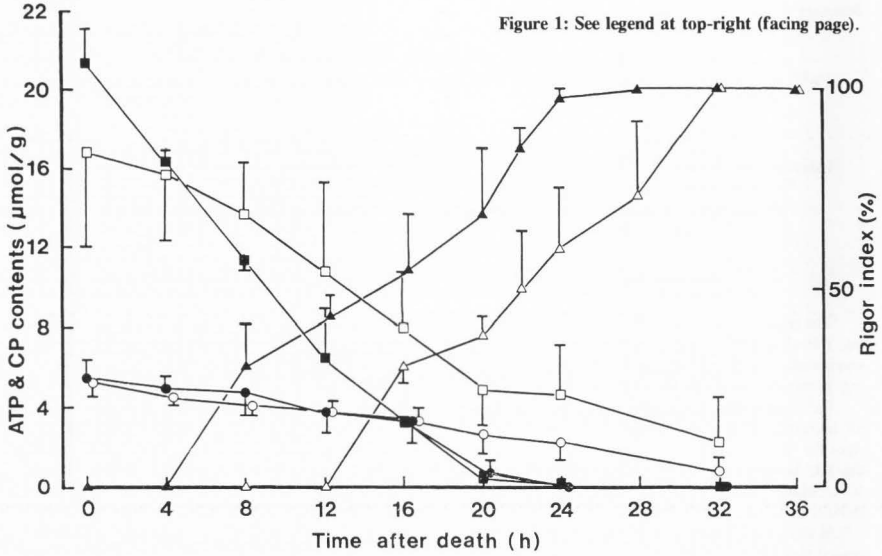

Figure 1: See legend at top-right (facing page).

account for the Ca^{2+} release since mitochondria lost function more rapidly than the SR. This idea was supported by the fact that cold shortening occurs more frequently in muscles which are red-colored and rich in mitochondria (Davey and Gilbert, 1974; Buege and Marsh, 1975).

Most fish musculature is composed of two homogeneous populations of fiber type, white ordinary muscle and smaller amounts of dark muscle under the lateral line. For example, flatfish, like flounder have significantly less dark muscle than tuna (Love, 1988). The proportion of dark to white muscle varies for different species. These two muscles correspond to mammalian fast and slow skeletal muscles, respectively. When rigor mortis progress was examined on sardine using the sag of fish tail as a parameter, it was more closely related with biochemical changes of fast muscle than with those of slow muscle (Watabe *et al.*, 1991). Since fast muscle contains much less mitochondria than slow muscle (Patterson and Goldspink, 1973), the SR may play the primary role in rigor mortis acceleration of fish. The SR from fish fast muscle indeed decreases its Ca^{2+} uptake ability at 0°C, possibly accelerating rigor mortis at this temperature (Ushio *et al.*, 1989; Watabe *et al.*, 1989a). In addition, the SR from carp fast muscle has been demonstrated to be responsible for acclimation temperature-dependent changes in rigor mortis progress (Watabe *et al.*, 1989b, 1990a).

This paper deals with ultrastructural studies in association with related biochemical changes of plaice fast muscle which demonstrated direct evidence for temperature-dependent Ca^{2+} release or leakage from the SR during rigor mortis progress.

Materials and Methods

Materials

Eight live specimens of plaice *Paralichthys olivaceus* (0.9 - 1.1 kg in body weight) were sacrificed by cranial spiking and used for storage experiments (two groups of 4 fish each at 0°C and 10°C storage temperatures).

Analytical methods

"Rigor index" of 4 fishes each for the two storage groups (0°C and 10°C), used as a parameter of rigor tension, was measured by the sag of the tail when half the body length was supported on a horizontal table with the tail end allowed to overhang the edge (Iwamoto *et al.*, 1987).

ATP and creatine phosphate were determined for 4 fishes each for the two groups by high performance liquid chromatography as described previously (Watabe *et al.*, 1990b).

The muscle pH was measured using a NPN - 10 S

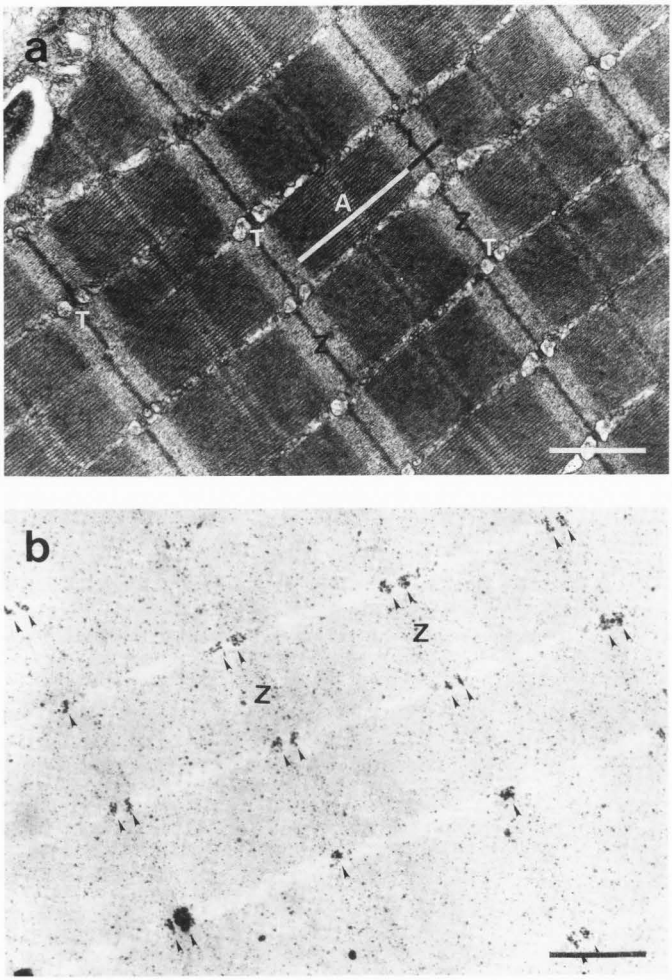

Figure 1 (on page 268). Rigor mortis progress of spiked plaice and degradation of muscle ATP and creatine phosphate (CP) during storage at 0°C and 10°C. Rigor mortis development was measured as the increase of rigor index (RI). Storage at 0°C (● ATP, ■ CP, ▲ RI) and 10°C (○ ATP, □ CP, △ RI). Data are given with mean ± S.E. (n = 8).

Figure 2 (at right). Electron micrographs of longitudinal views of plaice muscle showing ultrastructure (**a**) and distribution of Ca^{2+} (**b**) immediately after death. Excised muscles were fixed with glutaraldehyde and OsO_4 (**a**) and OsO_4 and pyroantimonate (**b**). Bars represent 1 μm. Arrowheads indicate the sarcoplasmic reticulum (SR). Note that pyroantimonate precipitates are localized in the SR where triad (T) structures are constructed. The A and I bands and Z disks indicated in (a) are applicable to (b).

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pH meter (Nissin - Rika, Tokyo) with a surface electrode directly on the muscle tissue.

Electron microscopy

Samples for electron microscopy were taken from 3 fishes each for the two groups which used for the determination of rigor mortis progress and related biochemical changes. Since muscle tissues were taken from the dorsal part of the ordinary muscle within one-third distance of the body length from the head, it was assumed that there would be no effect on the subsequent measurements of rigor index.

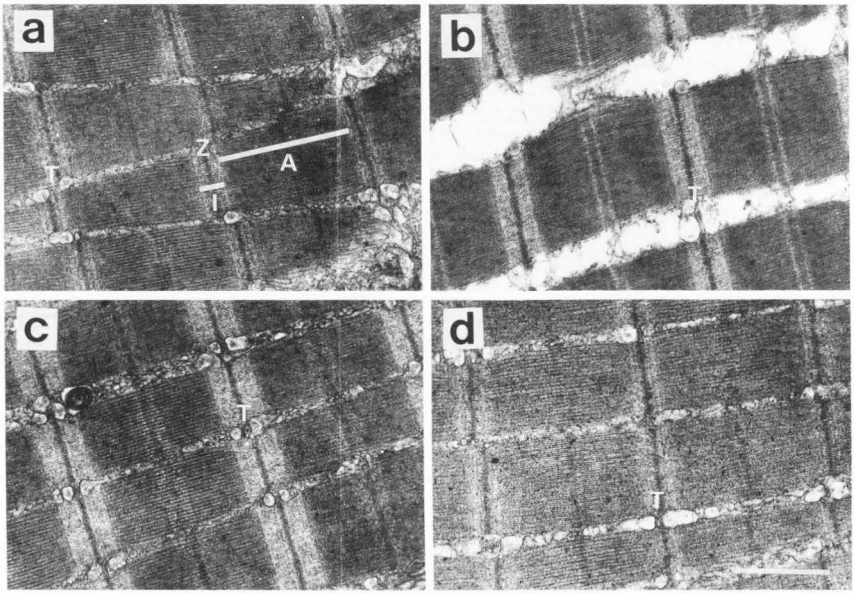

Figure 3. Electron micrographs showing longitudinal ultrastructure of plaice muscle. Spiked fish were stored at 0°C (**a, b**) or 10°C (**c, d**) for 8 hours (**a, c**) or 24 hours (**b, d**). Excised muscles were fixed with glutaraldehyde and OsO_4. Bars represent 1 μm. The A and I bands are indicated together with Z disks and triad structures (T).

White ordinary muscle was exclusively used in the present study for biochemical and histological analysis. Ordinary muscle strips of 3 x 3 x 20 mm were excised at various stages during rigor mortis progress from the dorsal part about 10 mm deep from the skin. They were set in plastic supports where both ends of muscle strips were tied with strings to prevent them from shortening during fixation. They were prefixed *in situ* for 30 minutes in a solution containing 0.1 M cacodylate-KOH (pH 7.5) and 2% glutaraldehyde for conventional electron microscopy at either 0°C or 10°C which corresponded to the storage temperature of fish. Subsequently, inner parts of the strips were cut into smaller pieces (1 x 1 x 5 mm) and fixed again at 4°C for 1 hour. After washing with 0.1 M cacodylate buffer, samples were postfixed in a solution containing 2% osmium tetroxide (OsO_4) at 0°C for 2 hours, dehydrated in a series of increasing ethanol concentrations from 50 to 100% and 100% acetone, embedded in a resin (Poly/Bed 812, Polyscience), and polymerized at 60°C for 3 days. For visualizing intracellular Ca^{2+} localization, muscles were fixed for 30 minutes in a solution containing 1% OsO_4 and 2% potassium pyroantimonate (adjusted to pH 7.5 by 0.01 M acetic acid) at the same temperatures as for storage according to the method of Legato and Langer (1969) after Komnick and Komnick (1963), and fixed again at 0°C for 1 hour.

Fixed tissues were cut into ultrathin sections by an ultramicrotome model MT - 5000 (DuPont Instruments - Sorvall). Longitudinal sections were prepared from the portions about 0.3 mm deep from the surface of the fixed pieces. Sections from conventionally fixed samples were stained sequentially with uranyl acetate and lead citrate, while those fixed with pyroantimonate were directly subjected to electron microscopic observation. Micrographs were taken with a JEM - 1200 EX or JEM - 2000 EX transmission electron microscope (JOEL, Tokyo) operating at an accelerating voltage of 80 kV.

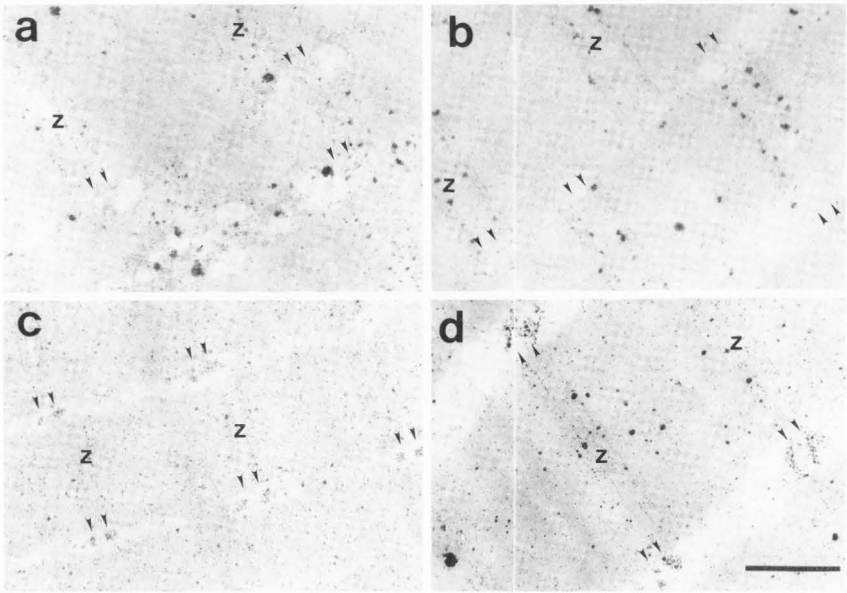

Figure 4. Electron micrographs of longitudinal views of plaice muscle showing location of Ca^{2+}. Spiked fishes were stored at 0°C (**a, b**) or 10°C (**c, d**) for 8 hours (**a, c**) or 24 hours (**b, d**). Excised muscles were fixed with OsO_4 and pyroantimonate. Bars represent 1 μm. Arrowheads indicate terminal cisterna of the sarcoplasmic reticulum.

Results

Rigor mortis progress and related biochemical changes

The onset time of rigor mortis of the plaice (0.9 - 1.1 kg) was observed after 8 hours of storage at 0°C (Fig. 1). When stored at 10°C, the fish showed a longer pre-rigor period and reached rigor mortis onset after 16 hours. The trend of faster rigor mortis progress in the fish stored at 0°C continued until it attained the full-rigor state after 24 - 28 hours. The fish stored at 10°C reached the full-rigor state after 32 hours.

Muscle ATP concentration in plaice fast muscle was 4 - 7 μmol/g at the start of storage. In association with rigor mortis progress, ATP degraded faster at 0°C than at 10°C (Fig. 1). However, ATP concentration was less than 1 μmol/g at the full- rigor state irrespective of storage temperature. Creatine phosphate breakdown was also slower during storage at 10°C where about half of the initial level remained even after 16 hours (Fig. 1). The muscle pH was 7.2 - 7.4 immediately after death and not less than 6.5 even after 36 - 48 hours.

Ultrastructural changes

Figs. 2 - 4 deal with electron micrographs from one representative pair of fish stored for the same length of time. Fig. 2a shows an electron micrograph of a longitudinal section of plaice fast muscle. Immediately after spiking, essentially no contraction was observed in each muscle, where the longest length of the sarcomere was about 2.20 μm and Z disks well arranged. The typical triad structure was maintained in the SR of these muscles. Electron micrographs of fish muscle stored at 0°C before rigor were principally similar to those immediately after death (data not shown).

After 8 hour-storage at 0°C where the fish was at the onset of rigor, the sarcomeres shortened slightly, whereas the SR structure was still well maintained (Fig. 3a). When the fish was stored at 0°C for 24 hours, disordered structures were observed in myofilaments and SR, suggesting that muscle structure underwent severe changes probably by an unusually strong force (Fig. 3b). The fish stored at 10°C for 8 hours (Fig. 3c) and 24 hours (Fig. 3d) showed structures less affected than those stored at 0°C. The 10°C stored fish has after 24 hours, however, reached over 60% in rigor index.

Identification of pyroantimonate precipitation

There is some inaccuracy in the pyroantimonate method to localize Ca^{2+} in muscle tissues, since pyroantimonate precipitates not only with Ca^{2+} but also with other cations such as Na^{+} and Mg^{2+} (see "Discussion"). Nevertheless, we adopted this method to examine Ca^{2+} release or leakage from the SR during rigor mortis progress, and obtained reasonable results as follows.

Fig. 2b shows an electron micrograph of the longitudinal view of pyroantimonate-fixed muscle immediately after death. The micrograph demonstrated that precipitation of pyroantimonate possibly with Ca^{2+} was mainly in the SR. After 8 hour-storage at 0°C where the onset of rigor was recorded, no deposits appeared in the SR (Fig. 4a). It should be emphasized that the SR structure in the fish stored at 0°C remained still intact (Fig. 3a). Therefore, Ca^{2+} concentration in the SR was not decreased by the destruction of the SR structure. Deposits were clearly observed in the fish which were stored at 10°C for the same period and in pre-rigor state (Fig. 4c). When the fish stored at 0°C reached 100% in rigor index after 24 hours, no deposits were observed in the SR (Fig. 4b). At 10°C, deposits still remained in the SR after 24 hours when the fish reached over 60% in rigor index (Fig. 4d). Other deposits observed around myofibrils, especially at I bands (Figs. 2b and 4), could be also pyroantimonate complexes with cations, which will be interpreted in "Discussion".

The differences in the cross-sectional view after pyroantimonate staining between fishes stored at 0 and 10°C were consistent with those observed in the longitudinal view (data not shown).

Discussion

Degradation rates of muscle ATP and creatine phosphate were clearly higher at 0°C than at 10°C. These results were consistent with our earlier findings on the same fish species (Iwamoto *et al.*, 1987). It was also reported that lactate accumulation in the spiked plaice muscle was faster at 0°C than at 10°C and accompanying the rigor mortis (Iwamoto *et al.*, 1987, 1988).

It was demonstrated in our previous paper (Watabe *et al.*, 1989a) that Ca^{2+} uptake rate of plaice SR was higher at 10°C than at 0°C. We claimed that cytosolic Ca^{2+} concentration of 0°C-stored plaice could rapidly be increased, resulting in activation of myofibrillar Mg^{2+}-ATPase and subsequent acceleration of ATP degradation.

Somlyo *et al.* (1981) measured *in situ* Ca^{2+} movement across the SR of frog skeletal muscle fibers by electron probe microanalysis. Pyroantimonate precipitate is associated with Ca^{2+} (Klein *et al.*, 1972; Wick and Hepler, 1982; Kashiwa and Thiersch, 1984). Unfortunately, this compound is also known to form precipitates with other metal ions such as K^{+}, Mg^{2+} and Na^{+} (Shiina *et al.*, 1970; Torack and LaValle, 1970; Klein *et al.*, 1972; Simson and Spicer, 1975). However, the pyroantimonate method for measuring Ca^{2+} distribution in chicken epiphyseal cartilage (Kashiwa and Thiersch, 1984), mouse skeletal muscle (Mentre and Halpern, 1988) and *Mytilus* muscle cell (Suzuki and Sugi, 1989) was successfully evaluated by electron probe microanalysis. It is likely to be also the case in the plaice muscle of the present study. In addition, precipitate observed after OsO_4-pyroantimonate fixation cannot demonstrate the initially free Ca^{2+} concentration which is lower than 10^{-6} M (Klein *et al.*, 1972) but rather reflects free Ca^{2+} plus the Ca^{2+} liberated from complexes by OsO_4 plus the contaminating Ca^{2+} in the OsO_4 solutions (Nicaise *et al.*, 1989). Ca^{2+} -pyroantimonate precipitates were sparsely observed in the cytosol area even when rigor mortis occurred (see Fig. 4). It is probably because free Ca^{2+} concentration was around 10^{-6} M (Ebashi and Endo, 1968). It has been known that Ca^{2+} ions are stored as complexes with Ca^{2+}- binding proteins such as calsequestrin in the SR when the muscle is relaxed (MacLennan *et al.*, 1983). Therefore, it can be assumed that Ca^{2+} ions released from Ca^{2+}- binding proteins by OsO_4 precipitate with pyroantimonate as was probably the case in the present study. The precipitate observed in the I bands could be produced by the reaction of pyroantimonate with Ca^{2+} bound to troponin (Legato and Langer, 1969).

Despite such uncertainty, the pyroantimonate method was very effective in the present study to follow postmortem changes in Ca^{2+} location in fish muscle during storage at 0°C and 10°C. With the above assumptions and interpretations, it was evident that Ca^{2+} release or leakage from the SR during storage of plaice more rapidly occurred at 0°C than at 10°C. Therefore, it is not in doubt that cytosolic Ca^{2+} concentration increased faster during storage at 0°C than at 10°C. In association with a rapid decrease in Ca^{2+} concentration in the SR at 0°C, ATP and creatine phosphate both degraded

quickly, compared with those at 10°C. Izumi *et al.* (1978) showed with glycerinated rabbit psoas muscle fiber that the onset and extent of rigor was faster at alkaline than at acidic pH value (9.0 compared with 6.2), and it increased with Ca^{2+} (10^{-9} - 10^{-5} M). It was, however, demonstrated that rigor contraction occurred in the absence of both ATP and Ca^{2+} at pH 7.0 (Izumi *et al.*, 1978).

A high ATP concentration in fish muscle was often experienced even at the onset of rigor. This phenomenon, which was always observed to precede rigor mortis very rapidly (Watabe *et al.*, 1989a; Iwamoto *et al.*, 1990), is difficult to explain. There might have been some problem in sampling muscle tissues for ATP determination.

The rigor index was the parameter which expressed the stiffness of the fish body and probably correlated with the rigor stage of muscles located near the vertebrae. The sample for ATP determination was taken from the dorsal surface of the muscle. When rigor mortis proceeded rapidly, there might have been some differences in the stages of rigor between different areas of the same muscle. Nevertheless, Ca^{2+} concentration in the SR, even in the muscle surface, decreased more rapidly when the fish was stored at 0°C (see Fig. 4). The SR plays an important role in modulating Ca^{2+} concentration in intact skeletal muscle (Hasselbach, 1964; Ebashi and Endo, 1968). We recently demonstrated that rigor mortis progress of 10°C- acclimated carp was faster than that of 30°C- acclimated carp and the change of SR Ca^{2+} uptake activity by thermal acclimation would be responsible for this phenomena (Watabe *et al.*, 1989b; Hwang *et al.*, 1990). The participation of mitochondria in Ca^{2+} release has been claimed as a consequence of postmortem anoxia to explain the acceleration of rigor mortis development at low temperatures ("cold shortening") of mammalian red muscle (Davey and Gilbert, 1974; Buege and Marsh, 1975). However, Mickelson (1983) demonstrated that Ca^{2+} was not released during anoxia if ATP was available to the mitochondrial ATPase, suggesting that a theory involving the SR in cold shortening is indeed correct. The present study gave direct, electron microscopic evidence that the SR also plays an important role in cold shortening of fish muscle.

Acknowledgements

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References

Amlacher E (1961). Rigor mortis in fish. In: Fish as Food, Borgstrom G (ed.), Academic Press, New York, pp. 385-409.

Buege DR, Marsh BB (1975). Mitochondrial calcium and postmortem muscle shortening. Biochem. Biophys. Res. Commun. **65**, 478-482.

Cassens RG, Newbold RP (1967). Effect of temperature on the time course of rigor mortis in ox muscle. J. Food Sci. **32**, 269-272.

Curran CA, Poulter RG, Brueton A, Jones NR (1986). Cold shock reactions in iced tropical fish. J. Food Technol. **21**, 289-299.

Davey CL, Gilbert KV (1974). The mechanism of cold-induced shortening in beef muscle. J. Food Technol. **9**, 51-58.

De Fremery D, Pool MF (1960). Biochemistry of chicken muscle as related to rigor mortis and tenderization. Food Res. **25**, 73-87.

Ebashi S, Endo M (1968). Calcium ion and muscle contraction. Prog. Biophys. Mol. Biol. **18**, 123-183.

Goll DE, Stromer MH, Robson RM, Temple J, Eason BA, Busch WA (1971). Tryptic digestion of muscle components simulates many of the changes caused by postmortem storage. J. Animal Sci. **33**, 963-982.

Greaser ML, Cassens RG, Hoekstra WG (1967) Changes in oxalate-stimulated calcium accumulation in particulate fractions from post-mortem muscle. J. Agric. Food Chem. **15**, 1112-1117.

Greaser ML, Cassens RG, Briskey EJ, Hoekstra WG (1969a) Post-mortem changes in subcellular fractions from normal and pale, soft, exudative porcine muscle. 1. Calcium accumulation and adenosine triphosphatase activities. J. Food Sci. **34**, 120-124.

Greaser ML, Cassens RG, Briskey EJ, Hoekstra WG (1969b) Post-mortem changes in subcellular fractions from normal and pale, soft, exudative porcine muscle. 2. Electron microscopy. J. Food Sci. **34**, 125-132.

Hasselbach W (1964). Relaxing factor and the relaxation of muscle. Prog. Biophys. Mol. Biol. **14**, 167-222.

Hwang GC, Ushio H, Watabe S, Iwamoto M, Hashimoto K (1991) The effect of thermal acclimation on rigor mortis progress of carp stored different temperatures. Nippon Suisan Gakkaishi, **57**, 541-548.

Iwamoto M, Ioka H, Saito M, Yamanaka M (1985). Relation between rigor mortis of sea bream and storage temperature. Nippon Suisan Gakkaishi **51**, 443-446.

Iwamoto M, Yamanaka H, Watabe S, Hashimoto K (1987). Effect of storage temperature on rigor-mortis and ATP degradation in plaice *Paralichthys olivaceus* muscle. J. Food Sci. **52**, 1514-1517.

Iwamoto M, Yamanaka H, Abe H, Ushio H, Watabe S, Hashimoto K (1988). ATP and creatine

phosphate breakdown in spiked plaice muscle during storage, and activities of some enzymes involved. J. Food Sci. **53**, 1662-1665.

Iwamoto M, Yamanaka H, Watabe S, Hashimoto K (1990). Rigor-mortis progress and its temperature-dependency in several marine fishes. Nippon Suisan Gakkaishi **56**, 93-99.

Izumi K, Ito T, Fukuzawa T (1978). Effect of pH, calcium and ATP on rigor contraction in glycerinated rabbit psoas muscle fiber. J. Food Sci. **43**, 1188-1190.

Kashiwa HK, Thiersch NJ (1984). Evaluation of potassium pyroantimonate/sucrose/glutaraldehyde concentration and incubation time as essential variables for localizing calcium bound to organic compounds in epiphyseal chondrocytes. J. Histochem. Cytochem. **32**, 1055-1065.

Klein RL, Yen S-S, Thureson-Klein A (1972). Critique on the K-pyroantimonate method for semiquantitative estimation of cations in conjugation with electron microscopy. J. Histochem. Cytochem. **20**, 65-78.

Komnick H, Komnick U (1963). Electronenmikroskopische Untersuchungen zur funktionellen Morphologie des Ionentransportes in der Salzdruse von *Larus argentatus*. Z. Zellforsch. **60**, 163-203.

Legato MJ, Langer GA (1969). The subcellular localization of calcium ion in mammalian myocardium. J. Cell Biol. **41**, 401-423.

Locker RH, Hagyard CL (1963). A cold shortening effect in beef muscle. J. Sci. Food Agric. **14**, 787-793.

Love RM (1988). The physical structure of fish muscle and its chemistry. In: The Food Fishes, Farrand Press, London, pp. 3-23.

MacLennan DH, Campbell KP, Reithmeier RAF (1983). Calsequestrin. In: Calcium and Cell Function, Cheung WY (ed.), Academic Press, New York, pp. 151-173.

Mentre P, Halpern S (1988). Localization of cations by pyroantimonate. II. Electron probe microanalysis of calcium and sodium in skeletal muscle of mouse. J. Histochem. Cytochem. **36**, 55-64.

Mickelson JR (1983). Calcium transport by bovine skeletal-muscle mitochondria and its relationship to post-mortem muscle. Meat Sci. **9**, 205-229.

Nicaise G, Gillot I, Julliard AK, Keicher E, Blaineau S, Amsellem J, Meyran JC, Hernandez-Nicaise ML, Ciapa B, Gleyzal C (1989). X-ray microanalysis of calcium containing organelles in resin embedded tissue. Scanning Microscopy **3**, 199-220.

Patterson S, Goldspink G (1973). The effect of starvation on the ultrastructure of the red and white myotomal muscle of the crucian carp (*Carassius carassius*). Z. Zellforsch. **146**, 375-384.

Poulter RG, Curran CA, Disney JG (1981). Chill storage of tropical and temperate water fish - differences and similarities. In: Advances in Technology in the Chilling, Freezing, Processing, Storage and Transport of Fish, Especially Underutilized Species, International Institute of Refrigeration, Paris, p. 111-123.

Shiina S-I, Mizuhira V, Amakawa T, Futaesaku Y (1970). An analysis of the histochemical procedure for sodium ion detection. J. Histochem. Cytochem. **18**, 644-649.

Simson JAV, Spicer SS (1975). Selective subcellular localization of cations with variants of the potassium (pyro)antimonate technique. J. Histochem. Cytochem. **23**, 575-598.

Somlyo AV, Gonzalez-Serratos H, Shuman H, McClellan G, Somlyo AP (1981). Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: An electron-probe study. J. Cell Biol. **90**, 577-594.

Suzuki S, Sugi H (1989). Evaluation of the pyroantimonate method for detecting intracellular calcium localization in smooth muscle fibers by X-ray microanalysis of cryosections. Histochemistry. **92**, 95-101.

Torack RM, LaValle M (1970). The specificity of the pyroantimonate technique to demonstrate sodium. J. Histochem. Cytochem. **18**, 635-643.

Ushio H, Watabe S, Hashimoto K (1989). Purification and characterization of carp sarcoplasmic reticulum. Nippon Suisan Gakkaishi **55**, 1641-1648.

Watabe S, Ushio H, Iwamoto M, Yamanaka H, Hashimoto K (1989a). Temperature-dependency of rigor-mortis of fish muscle: Myofibrillar Mg^{2+}-ATPase activity and Ca^{2+} uptake by sarcoplasmic reticulum. J. Food Sci. **54**, 1107-1110, 1115.

Watabe S, Hwang GC, Ushio H, Hashimoto K (1989b). Biochemical responses to thermal acclimation in muscle proteins of carp. In: Current Topics in Marine Biotechnology, Miyachi S, Karube I, Ishida Y (eds.), The Japan Society for Marine Biotechnology, Tokyo, pp. 323-326.

Watabe S, Hwang GC, Ushio H, Hashimoto K (1990a). Changes in rigor-mortis progress of carp induced by temperature acclimation. Agric. Biol. Chem. **54**, 219-221.

Watabe S, Hwang GC, Ushio H, Hatae K, Yamanaka H, Hashimoto K (1990b). Acceleration of physicochemical change in carp muscle by washing in either chilled or heated water. J. Food Sci. **55**, 674-677.

Watabe S, Kamal M, Hashimoto K (1991). Post-mortem changes in ATP, creatine phosphate, and lactate in sardine muscle. J. Food Sci. **56**, 151-153.

Whiting RC (1980). Calcium uptake by bovine muscle mitochondria and sarcoplasmic reticulum. J. Food Sci. **45**, 288-292.

Wick SM, Hepler PK (1982). Selective localization of intracellular Ca^{2+} with potassium antimonate. J.

Histochem. Cytochem. **30**, 1190-1204.

Discussion with Reviewers

Reviewer V: Is there a relationship between the onset of rigor at 0°C and the rapid or slow decrease of ATP in small and large fish? This could raise the question of temperature control during handling of these fish and/or while the sag tests were being done.
Authors: We often observed rigor onset of fish which had considerable amounts of ATP. This is the case when fish rigor mortis proceeds rapidly. Also, size might have less to do with rapidity of ATP decrease than proportion of fast and slow muscle.

Reviewer I: The faster rate of ATP and creatine phosphate decline at 0°C is puzzling. I would expect the ATPase and the kinase to be more active at 10°C than at 0°C. Please comment.
Authors: The ATPase and the kinase are without doubt more active at 10°C as reported previously (Iwamoto *et al.*, 1988, text reference). We demonstrated that Ca^{2+} uptake ability of the SR decreases when the fish is stored at 0°C. We then assumed that this decrease will result in an increase in Ca^{2+} concentration, activating myofibrillar Mg^{2+}- ATPase. Enhancement of the ATPase will accelerate ATP and creatine phosphate degradations (Watabe *et al.*, 1989a, text reference).

Reviewer II: There are several inconsistencies in the data in Figure 1. Why is the rigor index not at 100 when the ATP levels drop to 0 at 20 hours in fish held at 10°C? How can the rigor be complete at 24 hours at 10°C while the ATP levels are still greater than 2μmole/g? This suggests to me that either the sag test is not a reliable measure of rigor or there are errors in the analytical determination of ATP.
Authors: As suggested by the reviewer, the sag test is not so reliable measure of rigor, but is a very convenient one for following rigor mortis progress and related biochemical changes together with changes in ultrastructure of fish muscle. We agree that small differences will be produced by our methods, but we obtained many reproducible data which were published elsewhere.

Reviewer II: The inconsistent quality of the staining between the two prints in Figs. 3c and 3d suggests that processing artifacts may be responsible for some of the differences reported. Please comment.
Authors: We tried to adopt the same staining and printing conditions, but the inconsistent quality of photographs was reluctantly introduced.

Reviewer II: White muscles do not cold shorten; the electron micrographs in the paper are from white muscle. Thus conclusions about SR calcium release causing cold shortening are not warranted.
Authors: Cold shortening occurs more frequently in mammalian muscles which are red-colored and rich in mitochondria (Davey and Gilbert, 1974; Buege and Marsh, 1975; text references). This phenomenon is also observed with chicken fast muscles (De Fremery and Pool, 1960, text reference). In addition, cold shortening was demonstrated for sardine stored at 0°C [Watabe *et al.*, Nippon Suisan Gakkaishi, 55: 1833 (1989)]. As described in "Introduction", rigor mortis progress of sardine was more clearly related with biochemical changes of fast muscle than with those of dark slow muscle (Watabe *et al.*, 1991). Therefore, faster biochemical changes in fish white fast muscle at 0°C than at 10°C suggest that cold shortening really occurs in this muscle.

Reviewer III: In relation to Figure 4 caption, why large spots are not Ca^{2+} deposits with pyroantimonate? Did the authors analyze them by microanalysis?
Authors: The large spots after pyroantimonate staining in Figs. 2 and 4 were difficult to explain as Ca^{2+} localization in the muscle. Since we did not perform any microanalysis, we could not positively identify these spots.

Reviewer IV: How were the sarcomere length measurements carried out?
Authors: The sarcomere length was approximated from the photograph which showed the largest size according to suggestions from Dr. P. Mentre.