Cellular distributions of creatine kinase in branchia of euryhaline tilapia (*Oreochromis mossambicus*)

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Lin, Li-Yih, Chia-Chang Chiang, Hong-Yi Gong, Ching-Yi Cheng, Pung-Pung Hwang, and Ching-Feng Weng. Cellular distributions of creatine kinase in branchia of euryhaline tilapia (*Oreochromis mossambicus*). *Am J Physiol Cell Physiol* 284: C233–C241, 2003. First published September 25, 2002; 10.1152/ajpcell.00087.2002.—Although euryhaline teleosts can adapt to environmental fluctuation of salinity, their energy source for responding to changes in salinity and osmolarity remains unclear. This study examines the cellular localization of creatine kinase (CK) expression in branchia of tilapia (*Oreochromis mossambicus*). Western blot analysis of muscle-type CK (MM form) revealed a high association with salinity changes, but BB and MB forms of CK in the gills of fish adapted to seawater did not change. With the use of immunocytochemistry, three CK isoforms (MM, MB, and BB) were localized in mitochondria-rich (MR) cells and other epithelial cells of tilapia gills. In addition, staining intensity of MM-form CK in MR cells increased after seawater transfer, whereas BB and MB forms did not significantly change. To our knowledge, this work presents the first evidence of CK expression in MR cells of tilapia gills, highlighting the potential role of CK in providing energy for ion transport.

CREATINE KINASE (CK; EC 2.7.3.2) catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, regenerating ATP. CK participates in an ubiquitous role to meet the energy demand for homeostasis during environmental changes. The phosphocreatine/creatine kinase is present in some excitable tissues, such as *Narcine brasiliensis* electric organ (2), and in nonexcitable tissues, such as *Squalus acanthias* rectal gland (10), *Gillichthys mirabilis* gills (15), and *Oreochromis mossambicus* gills (37), with high and fluctuating energy demand. Plasma CK exhibits the physiological stress responses in big game fish after capture, perhaps because of muscle damage and subsequent release of cytosolic soluble CK in the plasma (36). Total CK activity significantly declined 20% in the fish (*O. mossambicus*) brain after exposure to hypergravity for 7 days (30). Additionally, some evidence has demonstrated seasonal fluctuations of CK in rainbow trout, *Oncorhynchus mykiss* (3), variability of CK isoenzymes in various tissues of trout (22), and genetic variability in tissue CK among fish species including rainbow trout and salmon (22, 26). Furthermore, CK provides energy for ion transport (Na\(^{+}\)-K\(^{-}\)-ATPase) in the gill of *G. mirabilis* when the CK inhibitor iodoacetamide is used (15). It seems that the phosphocreatine/creatine kinase shuttle in cytosol might coordinate with that in mitochondria to provide more ATP for the extra energy demand of Na\(^{+}\)-K\(^{-}\)-ATPase to pump out excess ions under hypertonic conditions. The results imply that CK may be a good candidate for converting the energy after seawater transfer.

Many CK isoforms are identified by their electrophoretic mobility, tissue and subcellular distribution, and primary sequence (24, 34). Three cytosolic CK isozymes (BB-CK (brain), MM-CK (muscle), and MB-CK (heart, lungs, stomach) (5, 8)) and two mitochondrial forms (sarcomeric MiCK (expressed mainly in heart and skeletal muscle and probably in some brain cells such as Purkinje neurons) and ubiquitous MiCK (expressed in many tissues)) are kinetically very similar but differ in their capacity to associate with subcellular organelles or protein structures (35, 41). Three types (brain, muscle, and mitochondria) of CK have been demonstrated to exist in teleosts (4, 26). The presence of MM-form, BB-form, and mitochondrial CK has been demonstrated, and MM-form CK found to predominate, in the gill of *G. mirabilis* (15). Recently, three different carp (*Cyprinus carpio*) muscle CK isoforms have been found, and their expression may be related to environmental acclimation (31). Multiple isoforms of CK are present in the muscle of channel catfish, *Ictalurus punctatus* (17). Various CK isoforms retain a phosphocreatine energy shuttle by which ATP generated by oxidative phosphorylation in mitochondria CK produces creatine phosphate, which is then transported to the cytosol and used by the cytosolic CK (MM, MB, or BB) to regenerate ATP at sites of high energy demand.

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energy consumption. After the fish is transferred from a hypotonic (freshwater) to a hypertonic (seawater) medium, CK, especially the muscle-form of CK in the tilapia gill, can be affected by salinity, directly or indirectly (37). Interestingly, the altered MM-type CK after seawater transfer seems to be a newly synthesized protein that is involved in osmoregulation. When a euryhaline teleost goes from seawater to freshwater medium, it tends to lose ions and gain water, and vice versa (when transferred from freshwater to seawater). The physiological response maintains a stable internal milieu, ionic regulation, and water balance. The organs involved in osmoregulation in teleosts include the gill, gut, and kidney: the gill is the most prominent organ. Moreover, the gill filaments contain pavement cells, mitochondria-rich (MR) cells, mucous cells, and undifferentiated cells. The MR cells are thought to participate importantly in the ionic regulation of teleostean fishes. This study attempts to elucidate the different forms of CK in the specific cells of gills and to investigate the incremental MM-CK association with the increase of bronchial sodium pump of tilapia after seawater transfer. The results reveal the existence of MM-, MB-, and BB-forms of CK in the MR cells of the tilapia gill. There is a coordinated upregulation of Na⁺-K⁺-ATPase and MM-CK in the MR cells of tilapia gills following transition from freshwater to seawater. Furthermore, three CK isoforms are also expressed in the pavement and other epithelial cells of tilapia gills, which showed no increase in CK level during seawater adaptation.

MATERIALS AND METHODS

Animals. Tilapia (O. mossambicus) were originally obtained from the Tainan Fish Culture Station of the Taiwan Fisheries Research Institute. Euryhaline tilapia can live in both seawater (SW) and freshwater (FW). All fish were maintained in a FW recirculating tank at 25–28°C in a photoperiodic environment (12:12-h light-dark) at the Institute of Zoology, Academia Sinica (Taipei, Taiwan). SW was prepared by adding artificial sea salt to FW. The sampled fish were 5–7 cm long and had a body mass of 2.5–4.0 g. Tilapia were directly transferred from FW to 25 parts per thousand (ppt) SW for various periods after being captured in a nylon net. During the period of the experiment (2 h or 2 wk, SW adaptation), fish were reared in a 25-ppt SW tank without feeding. Fish were anesthetized with ice and killed immediately. The gills were removed and weighed. The tissue was then homogenized in a homogenization solution (100 mM imidazole-HCl buffer, pH 7.0, 5 mM Na₂EDTA, 200 mM sucrose, and 0.1% sodium deoxycholate) with a motorized Teflon pestle that rotated at 600 rpm for 20 strokes on ice. After centrifugation (12,000 rpm, 30 min at 4°C), the supernatant was kept at −70°C until the assay was performed. The total protein content was determined using a protein assay kit (Bio-Rad, Hercules, CA) and calculated by using bovine serum albumin (BSA; Sigma, St. Louis, MO) as a standard.

Western blotting. The homogenate of a gill (total protein 100 µg) was mixed with an equal volume of 2× electrophoresis sample buffer that contained 250 mM Tris-base, 2 mM Na₂EDTA, 2% SDS, and 5% dithiothreitol. The proteins were separated by electrophoresis on a 4–12% gradient polyacrylamide slab gel (NuPAGE, Novex, CA) and electrophoretically transferred to polyvinylidene difluoride (Amersham Life Science, Amersham, UK). The blots were incubated overnight in 3% NET buffer (0.25% gelatin, 50 mM NaCl, 50 mM Tris·HCl, 5 mM EDTA, pH 7.5, and 0.05% Tween 20) and washed three times in PBST buffer (0.01 M phosphate, 0.09% NaCl, pH 7.5, and 0.05% Tween 20). Filters were incubated for 1 h with primary antibody: rabbit anti-human MM-CK polyclonal antibody (Biogenesis) at 1:2,000 dilution and mouse anti-human MB-CK monoclonal antibody (Biogenesis) and rabbit anti-human BB-CK (Biogenesis) at 1:1,000 dilution. Mouse muscle or brain tissues were applied as a positive control to check molecular weight and immunoreactions. After being washed three times with PBST buffer, immunoreactive proteins were visualized with an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL) according to the manufacturer’s instructions. The differences between the band intensity of FW and SW were compared using densitometry (Personal Densitometry SI; Molecular Dynamics, Sunnyvale, CA).

In situ fluorescence staining of gill filaments. For whole mounted branchial staining, tilapia gills were excised and immediately fixed with 4% paraformaldehyde for 2–3 min at 4°C. The fixed gills were then washed three times with PBS (0.1 M phosphate buffer plus 0.09% NaCl) for 5 min each time. The tissues were then stained with 1 mg/ml concanavalin A conjugated with Texas red (Con A; Molecular Probes, Eugene, OR) for 30 min at room temperature (RT). After being washed three times, the tissues were further fixed and permeabilized with 70% ethanol for 10 min at −20°C. After being washed with PBS for 10 min, the Con A-stained gill filaments were incubated with 10% normal goat serum (NGS) for 30 min to block nonspecific binding. The tissues were then incubated for 2 h with mouse anti-chicken Na⁺-K⁺-ATPase α-subunit monoclonal antibody (Developmental Studies Hybridoma Bank), rabbit anti-human MM-CK polyclonal antibody (Biogenesis), rabbit anti-human MB-CK polyclonal antibody (Biogenesis), or mouse anti-human BB-CK monoclonal antibody (Biogenesis) at 1:100 dilution (3% BSA in PBS) at RT. The stained gill filaments were washed three times with PBS for 5 min each time. The stained gill filaments were then incubated with FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:200 dilution, 3% BSA in PBS) for 60 min at RT.

Fluorescence staining in frozen sections. Excised gills from anesthetized tilapia were fixed and permeabilized as previously described. After being washed with PBS, the gills were immersed in 30% sucrose for 2 h and then embedded in a cryomatrix embedding medium (Shandon) at −20°C. Frozen cross sections (15 µm)
were then cut using a cryostat (Bright) and attached to slides coated with poly-L-lysine (Sigma). To stain creatine kinase, the fixed gill sections were rinsed with PBS, blocked with 10% NGS, and then incubated at RT for 2 h with polyclonal antibodies against MM- or BB-CK or with monoclonal antibodies against MB-CK diluted at 1:100 with PBS that contained 3% BSA. After being washed with PBS, the sections were further incubated with goat anti-mouse IgG or goat anti-rabbit IgG conjugated with FITC (Jackson ImmunoResearch Laboratories; 1:200 dilution) at RT for 1 h. The sections was double-stained by an additional incubation with monoclonal α5 antisemur or polyclonal antibody TG3 antiserum against Na+-K+-ATPase (13) diluted at 1:100 for 2 h, followed by incubation with goat anti-mouse or anti-rabbit IgG conjugated with Texas red (Jackson ImmunoResearch Laboratories; 1:200 dilution) at RT for 1 h. After washing, specimens were observed and their positive images were acquisitioned by using a Leica TCS-NT confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with ×10/0.3, ×20/0.4, ×40/1.2 oil, and ×100/1.35 oil lenses and appropriate filter sets for simultaneous monitoring of FITC and Texas red. Branchial sections from FW or SW tilapia were attached side by side on the same slide to compare staining intensities. Therefore, the sections from various groups could be stained under the same conditions to reduce artificial staining bias.

Isolation of epithelial cells. Fish were quickly anesthetized on ice and killed by transection of the spinal cord. The gill filaments were separated from gill arcs and chopped into small segments in PBS. Gill filaments from four tilapia (2.5–4.0 g) were pooled together. The chopped filaments were gently agitated in PBS for 30 min to remove blood cells. The filament segments were incubated in trypsin solution (0.1% trypsin in PBS) for 1 h at RT. After incubation, cells were isolated from digested tissues by gently and repeatedly passing the tissue suspension through a wide-bore pipette. The suspension was then passed through a nylon mesh (mesh size 100 μm) to remove larger tissue fragments. The resulting cell suspension was centrifuged at low speed (200 rpm, 10 min, 4°C), and the isolation solution was replaced with 4% paraformaldehyde for fixation over 10 min. The suspension of fixed cell was washed with PBS and centrifuged again. This cell suspension was then dropped on poly-L-lysine-coated slides and stored at −20°C for further staining. Isolated cells from FW or SW tilapia were dropped next to each other on the same slide to compare staining intensities. Accordingly, the isolated cells treated differently could be stained under the same conditions to reduce artificial staining bias. The staining procedure used was the same described previously for the staining of frozen sections.

Acquiring and analyzing images. The period of time over which the image was captured, the photomultiplier tube gain, and the scanning rules of confocal microscope were optimized before each experiment and maintained throughout each experiment to standard-

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**Fig. 1.** Western blot of 3 creatine kinase (CK) isoforms (MM-, MB-, and BB-CK) in freshwater (FW) tilapia, tilapia that were transferred to seawater (SW) for 2 h, and SW-adapted tilapia. Each lane contained 100 μg of protein.

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<td>MM</td>
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<td>BB</td>
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<td>MB</td>
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**Fig. 2.** In situ fluorescence staining of gill filaments: a, double labeling of Con A (red) and sodium pump (green); b, double labeling of Con A (red) and MB-CK (green) in FW-adapted tilapia gills. Magnification, ×400.
ize the intensity of fluorescence among experiments. Images were taken with ×40/1.2 oil lenses and quantified with MetaMorph software (Universal Imaging, Philadelphia, PA). With respect to the stained bronchial sections, the fluorescence intensities of the sodium pump and CK double-labeled regions of the FW gill section were measured and compared with those of the SW gill section on the same slide. The staining intensities of sodium pump and CK were determined separately from the averaged pixel depth of the double-labeled regions. The average calculated intensity of one branchial section referred to a single sample. Ten samples of FW or SW gill sections were measured and compared. In the cell isolation experiment, CK and sodium pump double-labeled cells were identified as MR cells (38), and other cells that expressed only CK were identified as non-MR cells. MM-CK and sodium pump staining intensities obtained from 500 non-MR cells and 300 MR cells were measured in both FW and SW specimens.

Statistics. Values are given as means ± SD. The level of significance is P < 0.05 in a two-tailed test. Student’s t-test was used to compare the staining intensities of FW sections or cells with those of SW sections or cells.

RESULTS

After tilapia were transferred to SW within 2 h and SW adapted, Western blot showed that MM-form CK in the gill of tilapia was highly associated with salinity change (Fig. 1). However, no significant elevations in the prevalence of BB and MB forms were observed in 25-ppt SW after transfer and SW adaptation (Fig. 1).

Con A-conjugated Texas red used to stain the gills yielded positive staining, confirming that Con A was located at the apical surface of MR cells in whole mount gill filaments. Con A staining was used to identify MR cells and to analyze distribution of sodium pumps and CK isoforms in MR cells and other cell types in the gill filaments. Thereafter, the Con A-positive gill filaments were stained with mouse anti-chicken Na⁺-K⁺-ATPase α-subunit monoclonal antibody, with polyclonal antibodies against MM- or BB-form CK or with monoclonal antibodies against MB-form CK. The images revealed that Na⁺-K⁺-ATPase or MB-CK was labeled concomitantly with Con A in the MR cells of the FW tilapia gill (Fig. 2, a and b). Results demonstrate that three CK antibodies labeled on the gill filaments exhibited a similar pattern; that is, MR cells and other epithelial...
cells are labeled with three CK isoforms (Fig. 2b; images of MM- and BB-CK not shown).

Frozen sections were then used for Na\(^+\)-K\(^+\)-ATPase and CK isoform double staining to further establish the expression pattern of the three CK isoforms in the various cell types of the branchial epithelium. The confocal images of FW branchial sections verified the expression of three CK isoforms in MR cells, which extensively expressed Na\(^+\)-K\(^+\)-ATPase on their tubular systems (Figs. 3–6). Moreover, CK was also expressed in other epithelial cells, including pavement cells and undifferentiated cells of gill filaments. The elevation of MM-CK expression in MR cells of SW tilapia suggests that MM-form CK provides an energy source to regulate ion transport during hyperosmoregulation. Under normal conditions, the activity of CK is already many times higher than that of the Na\(^+\)-K\(^+\)-ATPase in the gills of euryhaline teleost G. mirabilis (15). The CK

Furthermore, the expression of MM-CK in isolated epithelial cells from FW individuals was compared with that in such cells from SW individuals. Figure 8 depicts the double staining of MM-CK and sodium pump in isolated epithelial cells from FW or SW tilapia. The results revealed that MM-CK was expressed in MR cells of SW and FW, and the expression in MR cells of SW was more intense than in MR cells of FW (Fig. 9). However, other sodium pump-negative cells (non-MR cells) did not significantly differ between FW and SW.

**DISCUSSION**

This work is the first evidence of CK expressions localized in MR cells of tilapia gills. MM-form CK in MR cells is increased after SW transfer, whereas BB and MB forms did not show a significant change. Three forms of CK (MM, MB and BB) are also present in other epithelial cells, including pavement cells and undifferentiated cells of gill filaments. The elevation of MM-CK expression in MR cells of SW tilapia suggests that MM-form CK provides an energy source to regulate ion transport during hyperosmoregulation. Under normal conditions, the activity of CK is already many times higher than that of the Na\(^+\)-K\(^+\)-ATPase in the gills of euryhaline teleost G. mirabilis (15). The CK

![Fig. 5. Fluorescence staining of frozen sections: a, double labeling of sodium pump (red) and MB-CK (green) in branchial frozen sections of FW tilapia; b, signal from MB-CK staining is isolated by double labeling. Original magnification, ×1,000. Arrowheads, MR cells.](image)

![Fig. 6. Fluorescence staining of frozen sections: a, double labeling of sodium pump (red) and BB-CK (green) in branchial frozen sections of FW tilapia; b, signal from BB-CK staining is isolated by double labeling. Magnification, ×1,000. Arrowheads, MR cells.](image)

![Fig. 7. Fluorescence staining of frozen sections: a, double labeling of sodium pump (red) and MM-CK (green) in branchial frozen sections of FW tilapia; b, signal from MM-CK staining is isolated by double labeling. Magnification, ×1,000. Arrowheads, MR cells.](image)
system (likely MM-CK) is directly functionally coupled with Na\(^{+}\)-K\(^{-}\)-ATPase, which allows ATP generated by membrane-bound CK to be directly channeled to the ATPase without equilibration with the cytosolic bulk phase in G. mirabilis gills. CK activity is \(80\) times higher than that of Na\(^{+}\)-K\(^{-}\)-ATPase in tilapia gills (37). The results obtained elsewhere (37) and in the present study appear to support the hypothesis that a coordinated upregulation of Na\(^{+}\)-K\(^{-}\)-ATPase and MM-CK occurs in tilapia MR cells. Furthermore, Kultz and Somero (15) also demonstrated the existence of all the other CK isoenzymes, including mitochondria CK, in G. mirabilis gills, suggesting the existence of a complete phosphocreatine shuttle. Recently, mitochondria CK of tilapia gills have been cloned, and the expressions of RNA are consistent with changes in salinity (C. F. Weng, C. J. Wu, M. J. Lo, and J. L. Wu, unpublished data). These results suggest that the phosphocreatine shuttle could be an important system in energetic cross talk between the sites of ATP production and those of its utilization in MR cells, as previously suggested (15). The fact that MM-CK is upregulated during transition from FW to SW also reinforces the plasticity of this system. After SW transfer or SW adaptation, the number and size of MR cells and the activity and amount of Na\(^{+}\)-K\(^{-}\)-ATPase in tilapia gills are increased (14, 38). In the context of this shuttle, which acts as a spatial rather than a temporal energy buffer, an increase in the number of Na\(^{+}\)-K\(^{-}\)-ATPase units will be accompanied by an almost stoichiometric increase in the expression of MM-CK. This coordinated upregulation of MM-CK preserves the function of the CK shuttle as a network for transferring intracellular energy and feedback signals among the sites of ATP production and utilization. However, the mechanisms

Table 1. Comparisons of relative staining intensities of FW and SW branchial sections

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<td>Na pump</td>
<td>78.7 ± 12.7</td>
<td>114.2 ± 20.5*</td>
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<tr>
<td>MM-CK</td>
<td>84.9 ± 15.4</td>
<td>127.0 ± 16.2*</td>
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<tr>
<td>BB-CK</td>
<td>60.2 ± 12.1</td>
<td>67.5 ± 7.5</td>
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<tr>
<td>MB-CK</td>
<td>77.2 ± 11.3</td>
<td>74 ± 13.0</td>
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Values are means ± SD \((n = 10)\). Student’s \(t\)-test was used to analyze the difference between freshwater (FW) and seawater (SW) groups. MM-CK, BB-CK, and MB-CK are cytosolic creatine kinase isozymes in muscle (MM), brain (BB), and heart, lungs, and stomach (MB). *Significant difference \((P < 0.05)\).
by which an increase in MM-CK activity promotes the acclimation of SW must be elucidated further.

Previous studies reported that fish experience two phases after transfer to SW. The first is a crisis phase, in which fish critically respond to the removal of water from the gill and the gut epithelia (dehydration), and the second phase is stabilization, during which the fish remains alive or dies, according to the extent of dehydration (1, 12, 14). Tilapia died within 4 h after being directly transferred to SW (35 ppt); however, they survived after transfer from FW to 25-ppt SW (14, 37). Fish must expend much energy to react to and compensate for a salinity challenge, particularly in the gill, intestine, kidney, and brain. Most previous studies have focused on physiological responses, including those involving hormones (growth hormone, prolactin, cortisol), plasma ions (Na\(^+\), Cl\(^-\), K\(^+\)) and osmolarity, glucose and oxygen consumption rate (19), and water-drinking activity (16) during SW transfer. The exposure of common carp to salt stress reduces their intake of food and depletes both muscle and liver glycogen stores to meet the requirement for extra energy (6). Few reports have addressed the energy source of the osmoregulation in fish that faced a change in salinity. The energy source for the responding organ remains an interesting issue in understanding osmoregulation after fish are transferred to SW. Normal demands placed on the Na\(^+\)-K\(^+\)-ATPase during the generation of electrical currents require large and rapid changes in activity of CK in the electric organ of N. brasiliensis (2). By using the CK inhibitor iodoacetamide and measuring the CK activity, recent studies have demonstrated that CK is an energy source for ion transporter (Na\(^+\)-K\(^+\)-ATPase) in the gills of G. mirabilis and tilapia (O. mossambicus) after transfer from FW to SW (15, 37). This study is consistent with previous studies and further demonstrates the existence of three forms of CK (MM, MB, and BB) in MR cells and other epithelial cells in tilapia gills.

The serum levels of CK showed significant variation in the presence of environmental stressors (acute handling and transport stress) in channel catfish (7). Plasma CK exhibited physiological stress responses in big game fish (36). Brain CK activity responded to hypergravity (30) or hypertonic conditions (37) in tilapia. The rising CK levels were associated with pathology in Atlantic salmon (Salmo salar) (9, 25) and in sea bass (Dicentrarchus labrax) (20). Marine fish, red seabream (Pagrus major), and Pacific mackerel (Scomber japonicus) have less thermostable muscle CK than carp (C. carpio) (21). The expression of three different muscle CK isoforms in carp may be related to thermoacclimation (31). Recently, we determined that CK activity and content were elevated within 2 h after transfer from FW to SW, and only MM-form CK was affected by changes in salinity (37). In the present study, the MR cells of SW-adapted tilapia expressed MM-form CK more strongly than did than FW individuals, confirming that the elevations of MM-form CK are associated with changes in salinity. Furthermore, two different muscle-type isoforms (CKM1 and CKM2) were cloned in our laboratory. The expression of the two isoforms shows opposite responses, and CKM1 expresses an increasing pattern, suggesting that different CKMs are influenced by an acute salinity challenge (11).

In the past decade, some enzymes [Na\(^+\)-K\(^+\)-ATPase (38), H\(^+\)-ATPase and Na\(^+\)/NH\(_4\)\(^+\)-ATPase (41), carbonic
anhydride (29), ion exchangers and cotransporters [Cl-/HCO₃ and Na⁺/H⁺ (39), Na⁺/NH₄⁺ (40), Na⁺/K⁺-2Cl⁻ (23)], ion channels [Na⁺ channel (39), K⁺ channel (32), CFTR-like Cl⁻ channel (40), and receptors [prolactin receptor (38), glucocorticoid receptor (33), angiotensin II receptor (18)] were found to be localized in MR cells of fish gills. This study reports the first evidence of the localization of CK expression in MR cells of tilapia gills. The function of BB-CK protein in neurons may involve ATP regeneration to maintain ATP availability to, for example, the Na⁺-K⁺-ATPase. Accordingly, in vivo studies have suggested a direct coupling of CK-ATPase (2). An increase in CKB activity and brain energy metabolism constitutes a mechanism for coupling energy production (ATP) and energy storage (creatinine phosphate) to meet rapidly increasing cellular energy demands (35). Muscle-type CK is functionally coupled to Na⁺-K⁺-ATPase activity, providing ATP for the ATPase reaction (27, 28). Taking all these facts into account, the increase in CK is associated with increased Na⁺-K⁺-ATPase activity and more MR cells in fish gills after SW transfer. The CK localized in MR cells may couple with the Na⁺-K⁺-ATPase to maintain ion balance under hypertonic conditions.

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