Expression of Beta Subunit 2 of Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase I in the Developing Rat Retina

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Expression of beta 2 subunit of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase 1 (CaMKI\textsubscript{β}2) of the rat retina during the developmental period and in the adulthood was studied immunohistochemically. The immunoreactivity of CaMKI\textsubscript{β}2 was detected in the earliest development of the primordial retina at embryological day (E) 12. The inner neuroblastic layer from which the presumptive ganglion cells are generated showed the ubiquitous CaMKI\textsubscript{β}2 immunoreactivity at E15 and persistently expressed at the same level until postnatal day (P) 0 when the inner neuroblastic layer divides into the ganglionic cell layer and the inner plexiform layer. The strong immunoreactivity was detected in the ganglion cell layer and the moderate one in the internal plexiform layer. CaMKI\textsubscript{β}2 immunoreactivities were persistently expressed throughout the postnatal development at the same level. The low level of intensity was first found in the inner nuclear layer at P7, followed by the outer plexiform, outer nuclear and rod-cone cell layers at the age of P12, respectively. The intensities of CaMKI\textsubscript{β}2 immunoreactivities in the inner nuclear and rod-cone cell layers were gradually increased to the strong level by P18 and persisted until adulthood. The present study revealed that the expression of CaMKI\textsubscript{β}2 in the retina was detected from the earliest development until adulthood, indicating that CaMKI\textsubscript{β}2 may be required in both proliferation and differentiation of the retinal precursor cells and subsequent formation of the functional layers. In addition, CaMKI\textsubscript{β}2 immunoreactivity in the rod-cone cell layer implies that this protein may be involved in the visual signaling process.

INTRODUCTION

The intracellular Ca\textsuperscript{2+} is known to regulate a broad spectrum of cellular functions including neurotransmitter release, excitation-contraction coupling in muscle, ion channel permeability and the active state of a variety of intracellular enzyme (Bading, 2013). The effects of Ca\textsuperscript{2+} ion on cellular functions may be mediated by the ubiquitously distributed Ca\textsuperscript{2+} receptor, calmodulin (CaM) (Burgoyne, 2007). The complex of Ca\textsuperscript{2+} ion and CaM activates a variety of enzymes which include Ca\textsuperscript{2+}/CaM-dependent protein kinases (CaMKs) (Wayman et al., 2011; Cohen et al., 2015). CaMKs (i.e., CaMKI-IV) constitute a family of structurally related protein kinases, including phosphorylase kinase, myosin light chain kinase, death-associated protein kinase (Sakakgami and Kondo, 1996; Song et al., 2004; Sakagami et al., 2005; Takemoto-Kimura et al., 2007; Ciani and Salinas, 2008; Ageta-Ishihara et al., 2009).

CaMKI was identified originally in rat brain on the basis of its ability to phosphorylate synaptic vesicle-associated protein, synapsin I (Kennedy and Greengard, 1981) and has been purified from bovine and rat brains (DeRemer et al., 1992a, b). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the purified enzyme revealed multiple 37-43 kDa polypeptides, suggesting the existence of isoforms of this enzyme. Four isoforms of CaMKI, i.e., α, β, γ and δ, have already been cloned from embryonic rat brain cDNA libraries (Yokokura et al., 1997) or from Hela cells (Ishikawa et al., 2003). It has been known that β isoform of CaMKI consists of two forms, CaMKI\textsubscript{β}1 and CaMKI\textsubscript{β}2, as a result of alternative splicing on the rat CaMKI\textsubscript{β} transcript (Loseth et al., 2000). Furthermore, the amino acid sequences of CaMKI\textsubscript{β}2 between mouse and rat are very similar at more than 99% (Ueda et al., 1999), suggesting the high homology of this protein in both species.
CaMKIβ2 is expressed mainly in the central nervous system (CNS) of adult rat whereas CaMKIβ1 is distributed rather ubiquitously in the rat tissue (Rina-Susilowati et al., 2001; Jusuf et al., 2002; Nishimura et al., 2003), suggesting some important roles of CaMKIβ2 in the CNS. During embryogenesis, the murine CaMKIβ2 transcripts are detected mainly in the nervous system, including brain, spinal cord, trigeminal ganglion and retina (Ueda et al., 1999). In adult brain, CaMKIβ2 transcripts are detected at high level in the anterior olfactory nuclei, piriform cortex, septal nuclei, granule cells in the dentate gyrus, amygdala, hypothalamic nuclei, parabrachial nuclei, and nucleus of solitary tract (Ueda et al., 1999).

Retina embiologically arises from an evaginated portion of the brain and expresses a variety of neurotransmitters and protein kinases in a similar manner as seen in the CNS (Yang, 2004; Masland, 2012). Little is known about the expression of CaMKIβ2 in the rat retina during developmental periods and in the adulthood, although expression patterns of other CaMKs in the retina have been reported (Terashima et al., 1994; Xue et al., 2002). In the present study, we immunohistochemically investigated expression of CaMKIβ2 in the rat retina from midgestational ages until the adulthood.

## MATERIALS AND METHODS

### 1. Animals

Rat pups or fetuses were obtained from time-pregnant Wistar rats purchased from a local supplier (Clea Co; Osaka, Japan). For fetuses, the embryological (E) ages were determined by day of insemination (E0). For

### Table I. Numbers of Animals

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### Figure 1.

Localization of CaMKIβ2 immunoreactivity in the rat eye during prenatal development at embryological day (E) 12 (A, E, I, M), E13 (B, F, J, N), E15 (C, G, K, O), E18 (D, H, L, P). A-D: Weak (A) and strong (B-D) immunoreactivities of CaMKIβ2 in the rat retina are detected during prenatal developmental periods. Arrowheads and asterisks indicate cells of lens and optic fiber layer, respectively. E-H: High magnification of neural retinal cells (E and F) and inner neuroblastic layer cells (G and H). Arrows indicate that CaMKIβ2 protein is expressed in the cellular nuclei. I-L: Unmyelinated nerve fibers in the optic stalk (I, J) and the optic nerve (K and L) are immunonegative. Arrows in J indicate the obliterating lumen of optic stalk. M-P: Control stainings. Strong staining in M is an endogeneous peroxidase activity of red blood cells. **Abbreviations in Figures 1-3 and Table II:** 2n, optic nerve; GCL, ganglion cell layer; IBL, inner neuroblastic layer; INL, inner nuclear layer; IPL, inner plexiform layer; Lns, lens; NRL, neural retinal layer; OBL, outer neuroblastic layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OpV, optic vesicle; PL, pigmented epithelial layer; RCL, photoreceptor or rod-cone cell layer; Ret, retina. Scale bars : 100 µm (A-D, M-P), 50 µm (E-H), 200 µm (I-L).
Development of CaMKIβ2 Expression in the Retina

Postnatal animals, the time of birth was closely monitored. Pups were born on E22. The first 24h of life was designated as postnatal day (P) 0. Rats from E10 until the adulthood were examined for this study (Table I). All animals were housed in a temperature-controlled (22°C±1°C) colony room with a 12h/12h L/D cycle and in groups in acrylic cages with woodchip bedding and unlimited access to normal laboratory chow and water. All procedures were standarized by the Institutional Animal Care Ethics Committee of Kobe University School of Medicine.

2. Production and purification of the antibody against CaMKIβ2
The production, purification and specificity of the present polyclonal antibodies raised against CaMKIβ2 were previously described in detail (Rina-Susilowati et al., 2001).

3. Immunohistochemistry
Rat pups at the age of P0, 2, 7, 12, 18 and adult rats were deeply anesthesized by an intraperitoneal injection of chloral hydrate (3.5mg/100g body weight). Perfusion was started transcardially by a brief wash with 0.1M phosphate buffer (PB; pH 7.4) containing 0.9% sodium chloride (PBS) at room temperature (RT), followed by a mixture of fixative agents containing 4% paraformaldehyde and 7% saturated picric acid in PB at 4°C. Immediately after perfusion, the eyeballs were enucleated from the skull. The retinas were removed from the eyeballs and then immersed in the same fixative at 4°C for additional 2 hours. For retinal preparations of rat fetuses, the pregnant rats were anesthesized with the injection of chloral hydrate (3.5 mg/100 g body weight) and the rat embryos at E10, 12, 13, 15 and 18 were obtained from the uterus of pregnant rats. The crania were then cut immediately and immersed with the same fixative agent for 4 hours. After fixation, all of these tissues were washed and cryoprotected in 0.1 M PB containing 15% sucrose for overnight at 4°C. After embedding in the OCT compound, the tissues were cut on a cryostat at a thickness of 10 µm and mounted on the gelatin-coated slides. The tissue sections were immunostained using the avidin-biotin-peroxidase complex (ABC) method. Briefly, after washing with PBS, tissue sections were incubated in the blocking solution containing 0.5% bovine serum albumin (BSA), followed by incubating with purified rabbit polyclonal antibody against murine CaMKIβ2 at a dilution of 1:200 (1.5 µg/ml) overnight at 4°C. After washing with PBS (3 times), sections were incubated with biotinylated goat anti-rabbit IgG (dilution 1:200 in 0.1 M PBS; Vectastain ABC kit) for 1 h at RT and avidin-biotin-peroxidase complex solution (dilution 1:100 in 0.1 M PBS; Vectastatin ABC kit) for 30 min at RT. After each step, the tissue sections were washed thoroughly with PBS (3 times). Immunoreactive products

Figure 2. Localization of CaMKIβ2 immunoreactivity in the rat retina during postnatal development at P0 (A, E, I), P7 (B, F, J), P12 (C, G, K), and P18 (D, H, L). A-D: CaMKIβ2 immunoreactivities are detected in the retina during the postnatal period. At P0 the immunoreactivity is detected strongly in the nuclei of ganglion cells and moderately in the neuropile of the immature IPL (A). At P7 the newly generated INL is immunostained with CaMKIβ2 antibody (B). At P12 the RCL, ONL and OPL are weakly immunostained (C). At P18 the strong CaMKIβ2 immunoreactivities are detected in the GCL, INL and RCL. E-H: Arrows in these high-power photographs show the nuclear localization of CaMKIβ2 protein in ganglion cells at P0 (E), P7 (F), P12 (G) and P18 (H). The squares in A and C are enlarged into E and G, respectively. I-L: Control staining for CaMKIβ2 in the retina at defined ages. Scale bars: 25 µm (A-D and I-L), 75 µm (E-H).
were visualized by a 15 min incubation with a chromogen, 3.3% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxidase dissolved in 0.1 M PB (pH 7.4), until an optimal immunoreaction was obtained. The slides were dehydrated in a series of ethyl alcohol, cleared with xylene and then coverslipped by a cover glass. Tissue slides were examined under the light microscope (Olympus AX80 Microscope).

RESULTS

1. CaMKIβ2 expression in the retina during prenatal period

During embryonic period, the immunoreactivity of CaMKIβ2 first appeared in the earliest development of eyes when the optic vesicles were generated by an evagination process occurring in the prosencephalon at E10. CaMKIβ2 immunoreactivity was detected in the optic vesicle and in the three brain vesicles (prosencephalon, mesencephalon and rhombencephalon) with low level of the intensity (data not shown). The optic vesicle developed to become optic cup from which the primordial retina was generated at E12. At this age the expression of CaMKIβ2 in the retina was detected with a low level (Fig. 1A) and gradually increased to the strong level of intensity at E13 (Fig. 1B). The same level of intensity was also identified in other parts of eye such as optic stalk (Fig. 1I), lens (Fig. 1A, arrowheads) and cornea (data not shown).

At E13 the intensity of CaMKIβ2 immunoreactivity in the neural layer of the retina was almost the same as that in the pigmented layer of the retina (Fig. 1B), both of which are known to originate from the optic vesicle. The strong immunoreactivity was also detected in the lens cells (Fig. 1B, arrowheads) which are derived from the surface ectoderm contacting to the underlying optic vesicle. Strong immunoreactivity was also found in the optic stalk (Fig. 1J) but absent in the unmyelinated nerve fibers (optic nerve layer) (Fig. 1B, asterisks). These fibers, which originated from the primitive ganglion cells of the neural retina, proceeded toward the optic fissure and entered into the optic stalk. At this stage the lumen of optic stalk was almost obliterated (arrows in Fig. 1J). At E15 the neural retina was divided into the inner and outer neuroblastic layers. The outer neuroblastic layer consists of undifferentiated neuroepithelial cells that produce horizontal cells and photoreceptor cells during the further developmental stages. Meanwhile the inner neuroblastic layer develops to become ganglion cells (Braekevelt and Hollenberg, 1970). The intensity of CaMKIβ2 immunoreactivity was stronger for the cells located in the inner neuroblastic layer than that in the outer neuroblastic layer and continually persisted until E18 (Fig. 1C and D). However, the intensity in the pigment layer was not changed during E15-18. Since presumptive ganglion cells arise at E15 and E18 from the inner neuroblastic layer (Braekevelt and Hollenberg, 1970), CaMKIβ2 may be expressed by postmitotic, presumptive ganglion cells. Optic fibers were not immunostained during retina development (Fig. 1C and D, asterisks).
The expression of CaMKIβ2 protein in the lens (arrowheads in Fig. 1A-D), uvea and cornea was also detected during embryonic days (data not shown). CaMKIβ2 protein was clearly located in the nuclei of developing retinal cells (arrows in Fig. 1E-H), suggesting the involvement of that protein in some processes within the nucleus (Cohen et al., 2015). Control slides without the primary antibody showed no immunopositive products (Fig. 1M-P).

2. CaMKIβ2 expression in the retina during the postnatal (P0-P18) periods

At P0, CaMKIβ2 immunoreactivities were strongly detected in cells in the ganglionic cell layer. The newly generated inner plexiform layer showed moderate immunoreactivity as well as cells in the outer neuroblast layer (Fig. 2A). At P2, the distribution and intensity of CaMKIβ2 immunoreactivities in retina were similar to those at P0 (data not shown). At P7, CaMKIβ2 was strongly expressed in the ganglionic cell layer, and the newly generated inner nuclear layers started to express CaMKIβ2 immunoreactivity (Fig. 2B). The immunostaining in the inner nuclear layer was weak in its early appearance at P7 and increased in intensity during the future course of the development, meanwhile the outer neuroblastic neurons were immunostained with less intensity in comparison with those in the early stage. At P12, the weak immunoreactivity of CaMKIβ2 was first detected in the outer nuclear layer, the outer plexiform layer and the rod-cone cell layer (Fig. 2C), while CaMKIβ2-immunoreactivity was persistently strong in the ganglionic cell layer and moderate in the inner plexiform and nuclear layers. At P18, all of the layers of the rat retina showed CaMKIβ2 immunoreactivity with the pattern and intensity similar to the adult rat (Fig. 2D). The strong immunoreactivity was detected in the ganglionic cell layer, inner plexiform layer and rod-cone layer, and moderate intensity was detected in the inner nuclear layer. The neuropile in the outer nuclear layer and outer plexiform layer were immunostained weakly. Expression of CaMKIβ2 protein in the nuclei of ganglion cells was identified during postnatal developmental periods (arrows in Fig. 2E-H). Negative reactivity was detected in the control slides of P0, P7, P12 and P18 retinas (Fig. 2I-L).

During developmental stage, CaMKIβ2 was expressed in the retina in an inside-out sequence. This phenomenon suggests that the chronological pattern of CaMKIβ2 expression is a maturation-dependent increasing pattern (Sidman, 1961). The similar pattern of CaMKIβ2 expression has been also identified for CaMKIV in the developing retina (Sakagami and Kondoh, 1996) and cerebelum (Sakagami et al., 1992).

3. Expression of CaMKIβ2 protein in the adult retina

In the adult rat retina, CaMKIβ2 was strongly expressed in the ganglionic cell layer, inner nuclear layer, and rod-cone cell layer, moderately in the inner plexiform layer, and weakly in the outer plexiform layer and outer nuclear layer (Fig. 3B). In the cellular components of ganglionic cell layer, strong immunoreactivity was recognized in the nucleus, whereas very faint immunoreactivity was found in the cytoplasm (Fig. 3D).

Table II. Summary of the Expression Pattern of CaMKIβ2 in the rat retina during developmental period and adulthood1)

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1) The immunoreactivity level: (-) not detected; (+), weak; (++), moderate; (+++), strong.
2) At this stage, a thin moderately immunostained band is detected in the OPL. Abbreviations are described in the legend for Figure 1.
ganglionic cell layer consists of two neuronal components: ganglion cells and displaced amacrine cells. Ganglion cells have a large soma of more than 14 μm in width with several primary dendrites (Masland, 2012) while displaced amacrine cells have a smaller soma with a single dendritic shaft extending innerwards and terminating within the inner plexiform layer (Lee et al., 2015). Since CaMKIIβ-immunopositive neurons in the ganglionic cell layer have a wide spectrum of cell somata in width (Fig. 3B and D), suggesting that CaMKIIβ2 is expressed by both ganglion cells and displaced amacrine cells. Further characterization using a specific marker or retrograde labeling of the ganglion cells (Terashima et al., 1994) will be required to determine the types of these cells. The neuropile in the inner plexiform layer where axons of bipolar cells and dendrites of ganglion cells and amacrine cells make synaptic connections was moderately immunostained by CaMKIIβ2 antibody (Fig. 3B). In the inner nuclear layer, CaMKIIβ2 immunoreactivity was detected in all depths of this layer (Fig. 3B), indicating that all cellular components in this layer (i.e., horizontal, amacrine and bipolar cells) expressed CaMKIIβ2. The nuclei of these cells were strongly immunostained but their cytoplasm was poorly immunostained (Fig. 3E). In the outer plexiform layer, a thin, weakly immunostained band was detected (Fig. 3B, arrowheads). In contrast to the inner nuclear layer, weak immunoreactivity of CaMKIIβ2 was found in the outer nuclear layer (Fig. 3B). Strong CaMKIIβ2 expression was detected in the cytoplasm of photoreceptor cells in the rod-cone cell layer (Fig. 3F).

The onset time and intensity levels of CaMKIIβ2 immunoreactivity in the rat retina from the mid-gestational days until the adulthood are summarized in Table II.

**DISCUSSION**

In the present study, we have revealed distribution pattern of CaMKIIβ2 in the rat retina from the earliest developmental stage until the adulthood by the immunohistochemical method using the polyclonal antibody specific for CaMKIIβ2.

The previous immunohistochemical studies have reported expression patterns of two other members of CaMKs family (i.e., CaMKII and CaMKIV) in the retina (Bronstein et al., 1988b; Terashima et al., 1994; Sakagami and Kondo, 1996; Calkins et al., 2005). CaMKII expression is distributed in both ganglion cells and displaced amacrine cells in the ganglionic cell layer and amacrine cells in the inner nuclear layer (Bronstein et al., 1988a; Terashima et al., 1994; Liu et al., 2000). CaMKIV is expressed strongly in bipolar cells of inner nuclear layer and weakly in the ganglionic cell layer (Sakagami and Kondo, 1996). The present study has shown that CaMKIIβ2 is strongly expressed in the ganglionic cell layer, inner nuclear layer, and rod-cone cell layer, moderately in the inner plexiform layer, and weakly in the outer nuclear layer. In addition, a thin, weakly immunostained line in the outer plexiform layer was detected. CaMKIIβ2 is more widely expressed in the retina during the developmental period and in the adulthood in comparison with CaMKII and CaMKIV, indicating that CaMKIIβ2 plays a crucial role in the retina during the developmental periods and in the adulthood.

Tsumura et al. (1999) reported that CaMKI immunoreactivities are detected in the cell bodies of ganglion cells and a subset of cells in the inner nuclear layer, and the neuropile of the inner and outer plexiform layers. The present study has demonstrated that CaMKIβ2 immunoreactivities are localized not only in these layers but also in the outer nuclear layer with weak intensity of immunostaining and outer segments of photoreceptor cells in the rod-cone cell layer with strong intensity. These differences may be attributed to the antibodies used. We used the antibody specific for CaMKIβ2 whereas Tsumura et al. (1999) used the antibody that detects broad spectrum of CaMKI isoforms, which may result in differences of expression pattern of CaMKI between the present and previous studies. Since CaMKIβ2 is expressed in the retina from the earliest developmental stage, CaMKIβ2 may be involved in the proliferation and differentiation processes of the precursor cells to differentiate into specific cells in the retina and the formation of the functional layers. Although little is known about the role of CaMKI in cell cycle regulation, CaMKI is known to be regulate G1 progression through the activation of cdc4 in human diploid fibroblasts (Kahl and Means, 2004) and that CMKB, a CaMKI homologue in Aspergillus nidulans, plays an essential role in S phase entry (Joseph and Means, 2000). CaMKI induces Rb phosphorylation and cyclin D1 upregulation and CDK4 activation for overall G1 to S phase transition (Skelding et al., 2011). The expression of CaMKIβ2 in the primordial retina may suggest a possible role of this kinase in the proliferation and early differentiation of neuronal precursors (Kamata et al., 2007).

The present study has demonstrated that CaMKIβ2 is very strongly expressed by the nuclei in the ganglionic cell layer and the inner nuclear layer of the adult retina, suggesting that CaMKIβ2 may phosphorylate certain nuclear proteins such as transcription factors and is involved in the modulation of gene transcription. The cAMP-responsive binding protein (CREB) is a transcription factor that is regulated by phosphorylation. CREB is a substrate for CaMKIβ2 and CaMKII, and the amino acid residue (Ser133) of this protein is known to be the major site of phosphorylation by CaM-kinases (Sheng et al., 1991; Fukuchi et al., 2014). Light stimulation
induces the expression of c-fos and somatostatin genes in the ganglionic cell layer and the inner nuclear layer, both of which are regulated by CREB (Yoshida et al., 1996). Since the CaMKIβ2 expression in the nuclei of neurons in these two layers, CaMKIβ2 may regulate the phosphorylation level of CREB and subsequent CREB-dependent genes. Waymen et al. (2006) clarified that activity-dependent dendritic outgrowth and branching of cultured hippocampal neurons required sequential activation of the NMDA receptors, CaMK kinase (CaMKK), CaMKI and MEK/ERK to enhance CREB-mediated transcription of Wnt-2. Wnt is a secreted glycoprotein that binds to the Frizzled family receptors to activate the scaffold protein Dishevelled (Jiang et al., 2014). This event in turn activates signalling pathways essential for multiple aspects of neuronal development (Ciani and Salinas, 2005). However, such an activity-dependent dendritic arborization of cultured hippocampal neurons is mediated by CaMKIγ, but not by CaMKIβ2, and therefore, further studies must be awaited to examine whether the similar mechanism of enhanced CREB-dependent transcription of Wnt-2 occurs in the retinal cells or not.

CaMKIβ2 expression is firstly detected in the rod-cone cell layer around on P12. This immunoreactivity is increased until P18 and permanently preserved during the further development and in the adulthood. The expression of other two members of CaMKs (i.e., CaMKII and IV) are not found in this layer (Bronstein et al., 1988b; Sakagami and Kondo, 1996; Terashima et al., 1994). Since light alters CaMKs activity in crude synaptic membranes of rat retina (Bronstein et al., 1988b) and the first electroretinographic responses are recorded around on P13 (Weidman and Kuwabara, 1968), this enzyme may play an important role in regulating of visual signaling processes during the postnatal period.

ACKNOWLEDGEMENTS

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