JSHC
Japan Society of
Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 48 (2): 47–52, 2015
doi: 10.1267/ahc.14049

Localization of Rod Bipolar Cells in the Mammalian Retina Using an Antibody Against the α_1c L-type Ca^{2+} Channel

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Received October 8, 2014; accepted March 16, 2015; published online April 24, 2015

Bipolar cells transmit stimuli via graded changes in membrane potential and neurotransmitter release is modulated by Ca^{2+} influx through L-type Ca^{2+} channels. The purpose of this study was to determine whether the α_1c subunit of L-type voltage-gated Ca^{2+} channel (α_1c L-type Ca^{2+} channel) colocalizes with protein kinase C alpha (PKC-α), which labels rod bipolar cells. Retinal whole mounts and vertical sections from mouse, hamster, rabbit, and dog were immunolabeled with antibodies against PKC-α and α_1c L-type Ca^{2+} channel, using fluorescein isothiocyanate (FITC) and Cy5 as visualizing agents. PKC-α-immunoreactive cells were morphologically identical to rod bipolar cells as previously reported. Their cell bodies were located within the inner nuclear layer, dendritic processes branched into the outer plexiform layer, and axons extended into the inner plexiform layer. Immunostaining showed that α_1c L-type Ca^{2+} channel colocalized with PKC-α in rod bipolar cells. The identical expression of PKC-α and α_1c L-type Ca^{2+} channel indicates that the α_1c L-type Ca^{2+} channel has a specific role in rod bipolar cells, and the antibody against the α_1c L-type Ca^{2+} channel may be a useful marker for studying the distribution of rod bipolar cells in mouse, hamster, rabbit, and dog retinas.

Key words: immunocytochemistry, rod bipolar cell, L-type Ca^{2+} channel, protein kinase C, mammalian retina

I. Introduction

Bipolar cells are glutamatergic second-order neurons that transmit signals from photoreceptors to amacrine and ganglion cells in the inner plexiform layer (IPL) of the retina. According to the source of presynaptic input, bipolar cells are classified into two types: rod and cone bipolar cells. According to the current concept, rod photoreceptors connect only to rod bipolar cells, and cone photoreceptors connect only to cone bipolar cells. Multiple subtypes of cone bipolar cells have been reported, but there is only one type of rod bipolar cell [6].

Various cellular processes are controlled by Ca^{2+} concentration, and neurotransmitter release is related to the influx of Ca^{2+} at presynaptic terminals [12, 17, 26]. Thus, the voltage-gated Ca^{2+} channels play an important role in bipolar cell synaptic transmission. Two types of Ca^{2+} currents have been measured in mouse bipolar cells using patch-clamp recordings: transient (T-) and long lasting (L-) types [5, 21]. The L-type Ca^{2+} channel is composed of four subunits (α_1, $\alpha_2\delta$, β, and γ), shows pharmacological sensitivity to dihydropyridines (DHP), and requires relatively strong depolarization for activation. The α_1 subunit is a transmembrane protein that forms the ion channel containing the DHP-binding site [19]. Among the four subunits of the L-type Ca^{2+} channels, α_1 is the key subunit that modulates voltage-gated Ca^{2+} channel activity. There are several α_1 subunits [8], and an antibody against the α_1c subunit of L-type voltage-gated Ca^{2+} channel (α_1c L-type Ca^{2+} channel) was used in the present study.

Protein kinase C (PKC) is an important secondary messenger in the nervous system. It plays a fundamental

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role in the control of cell proliferation, differentiation, gene expression, and modulation of the sensitivity of channels and receptors. Enzymatic and cloning studies have revealed that PKC exists as a family of isoforms that are closely related in structure, size, and mechanism of activation. Eleven PKC isoforms have been identified, and these can be subdivided into three major groups based on their activation conditions. PKC isoforms are classified as conventional (α, β_I, β_{II}, γ), novel (δ, ε, η, θ), and atypical (ζ, λ) types [13]. Immunocytochemical studies indicate that conventional PKC isoforms are found in the central nervous system [9, 22]. Negishi and coworkers published the study of PKC alpha (PKC-α) localization in the retina and demonstrated intense PKC-α-immunoreactivity in rod bipolar cells [18]. PKC-α is now a commonly used marker to identify rod bipolar cells in the vertebrate retina [3, 4, 15, 16, 27].

The purpose of this study was to investigate whether the α_1c L-type Ca^{2+} channel localizes to a specific cell type in the mammalian retina. Our results show that cells labeled with an antibody against α_1c L-type Ca^{2+} channel exhibited the morphology of rod bipolar cells with PKC-α immunoreactivity. This study indicates that α_1c L-type Ca^{2+} channel may be another marker for rod bipolar cells in the mammalian retina.

II. Materials and Methods

Animals and tissue preparation

Four different mammalian species were used in the present study: adult C57BL/6 mice (20–25 g), golden hamsters (25–30 g), New Zealand white rabbits (2.5–3.0 kg), and Korean mixed-breed dogs (4.0–5.0 kg). Animals were anesthetized with a mixture of ketamine hydrochloride (30–40 mg/kg) and xylazine (3–6 mg/kg). Proparacaine HCl (200–300 μl) was applied to the cornea to suppress blink reflexes. Immediately after eye enucleation, the retinas were isolated from the eyecup. The isolated retinas were fixed for 1–2 hr in a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) with 0.002% $CaCl_2$ added. The experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) guidelines for the use of animals with the approval of the committee of Kyungpook National University.

Immunocytochemical procedure

The retinal tissues were processed as whole mounts or 50 μm vertical sections, which were cut using a Vibratome 3000 Plus Sectioning System (St. Louis, Missouri, USA). The immunocytochemical methods have been described in detail previously [14]. The primary antibodies used were monoclonal anti-PKC (1:100, Amersham, Arlington Heights, Illinois, USA) and polyclonal anti-α_1c L-type voltage-gated Ca^{2+} channel (1:200–1000, Chemicon, Temecula, California, USA). Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG (1:50–250, Vector Laboratories, Burlingame, California, USA) and Cy5-conjugated goat anti-rabbit IgG (1:50, Jackson ImmunoResearch, West Grove, Pennsylvania, USA). Following the immunocytochemical procedures, the tissues were mounted in Vectashield mounting medium (Vector Laboratories).

Characterization of antibodies

(1) A monoclonal antibody against PKC has been found to react with the α and β forms, but not the γ form. We checked its cross-reactivity using an antibody against PKC-α (1:100, #MA1-157, Thermo Fisher Scientific, Rockford, Illinois, USA), which recognizes only the α form. We found no difference between the immunostaining patterns using antibodies against PKC or PKC-α. Besides, many previous studies proved that the antibody used in this study is a specific marker of rod bipolar cells [6, 9, 18].

(2) The information provided with the polyclonal antibody against the α_1c L-type Ca^{2+} channel states that the antibody does not cross-react with any other Ca^{2+} channel antigens tested. To demonstrate the specificity of the antibody against the α_1c L-type Ca^{2+} channel, we performed a preabsorption control test in the retina of four species. To inactivate the antibody, blocking peptide of the anti-α_1c L-type Ca^{2+} channel (#sc-16229-P, Santa Cruz Biotechnology, Santa Cruz, California, USA) was mixed with the primary antibody at 10:1 ratio and the mixture was pre-incubated for 1 hr at room temperature. Tissue sections were incubated in the preabsorbed antibody in place of the primary antibody. We confirmed that no immunoreactivity was visible in retinal cells (Fig. 1).

Data analysis

A differential interference contrast (DIC) microscopy was used to clear the immunostaining area. All labeled tissues were imaged using a laser scanning confocal microscope (LSM 700, Carl Zeiss Meditec Incorporation, Jena, Germany). Images were adjusted for brightness and contrast using Adobe Photoshop CS (Adobe Systems, Mountain View, California, USA).

III. Results

Figure 2 shows PKC-α- and α_1c L-type Ca^{2+} channel-immunoreactive cells in vertical sections of the retina from mouse (Fig. 2A–D), hamster (Fig. 2E–H), rabbit (Fig. 2I–L), and dog (Fig. 2M–P). Antibodies against PKC-α and α_1c L-type Ca^{2+} channel labeled processes in the retina, which is consistent with the morphology of rod bipolar cells. In Figures 2A, 2E, 2I, and 2M, the DIC images were shown to clarify the layers of retina. Immunoreactive cell bodies were visible in the outermost region of the inner nuclear layer (INL), with branched dendritic processes in the outer plexiform layer (OPL). The cells also had long and thick axonal processes in the innermost region of the

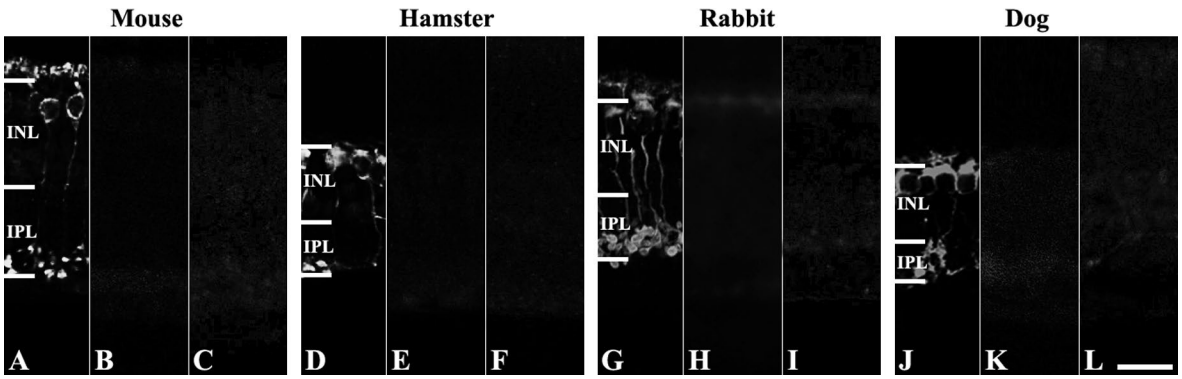

Fig. 1. Photomicrographs of the distribution and control of α_1c L-type Ca^{2+} channel-immunoreactive cells in the retina. (**A, D, G, J**) Anti-α_1c L-type Ca^{2+} channel-immunoreactive cells. (**B, E, H, K**) Sections before preabsorption. (**C, F, I, L**) The α_1c L-type Ca^{2+} channel antibody was preabsorbed with antigen prior to tissue incubation. Preabsorption control test of antibody against the α_1c L-type Ca^{2+} channel showed no immunoreactive cells in four different mammalian retinas. INL, inner nuclear layer; IPL, inner plexiform layer. Bar=20 μm.

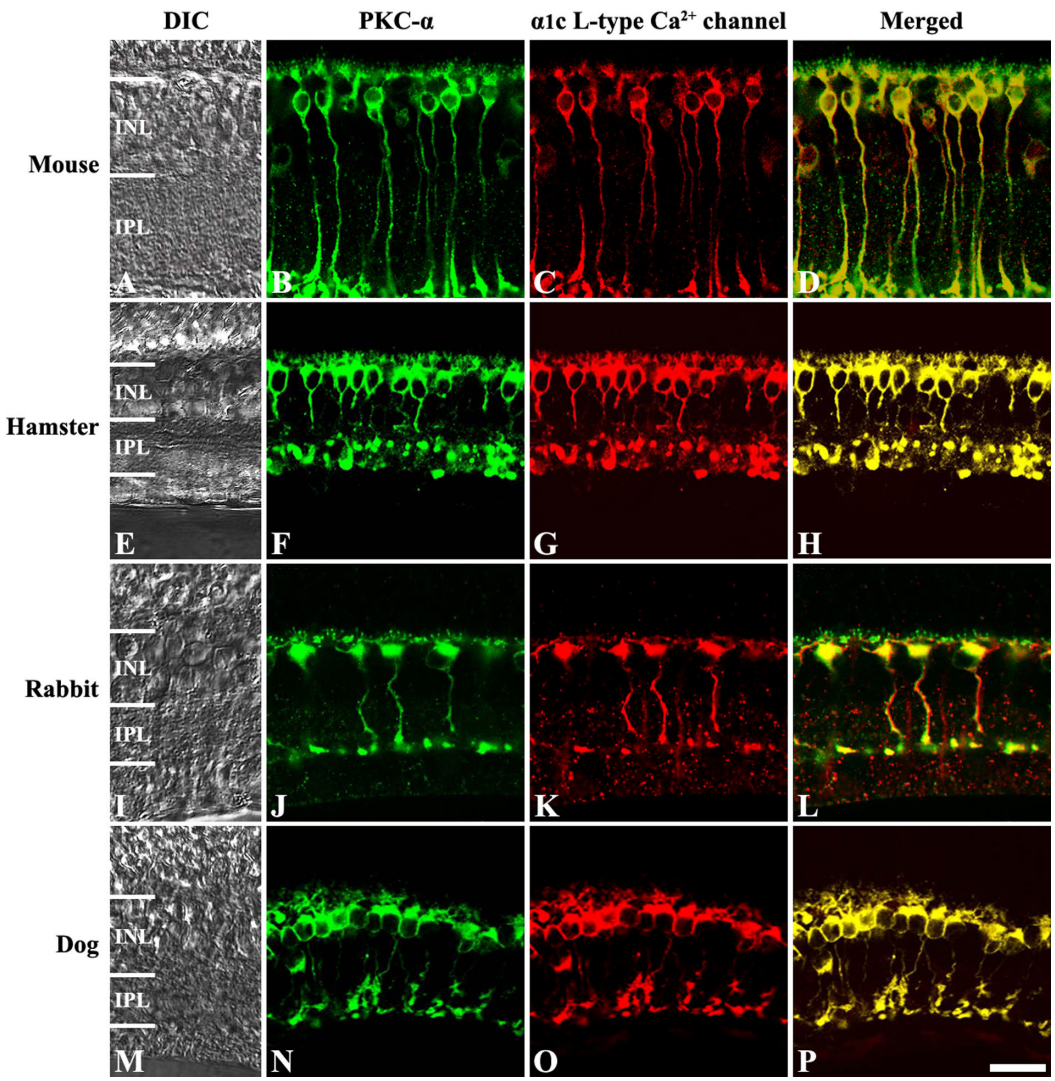

Fig. 2. Confocal micrographs of vertical sections from the mouse, hamster, rabbit, and dog retinas. (**A, E, I, M**) DIC images were used to reveal the layers of retina. (**B, F, J, N**) Retinal sections were immunostained for PKC-α. (**C, G, K, O**) Retinal sections were immunostained for α_1c L-type Ca^{2+} channel. In both labelings, the immunoreactive cells displayed the morphology of rod bipolar cells as their cell bodies in the INL extended long axonal process throughout the IPL. (**D, H, L, P**) Superimposed images of PKC-α and α_1c L-type Ca^{2+} channel immunolabeling revealed an almost complete of colocalization. DIC, differential interference contrast; INL, inner nuclear layer; IPL, inner plexiform layer. Bar=20 μm.

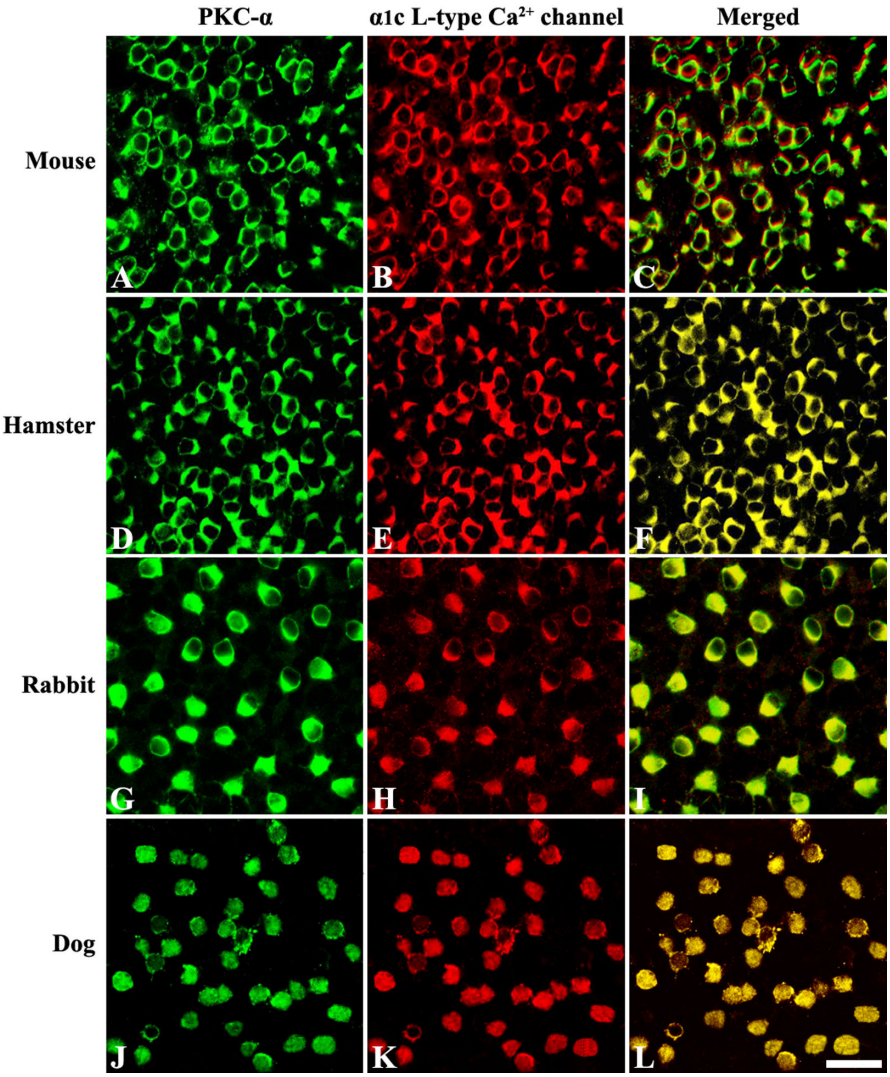

Fig. 3. Confocal micrographs of whole mounts from the mouse, hamster, rabbit, and dog retinas. (**A, D, G, J**) Whole mounts were immunostained for PKC-α. (**B, E, H, K**) Whole mounts were immunostained for α_1c L-type Ca^{2+} channel. Immunolabeling was visible within the cytoplasm of rod bipolar cells. The immunoreactive cells in the mouse and hamster formed a tight mosaic compared to those in the rabbit and dog. (**C, F, I, L**) Superimposed images of PKC-α and α_1c L-type Ca^{2+} channel immunostaining revealed that these proteins were colocalized. Bar=20 μm.

IPL, close to the ganglion cell layer (GCL). In Figures 2D, 2H, 2L, and 2P, the superimposed images of PKC-α- and α_1c L-type Ca^{2+} channel-immunoreactive cells demonstrate the extent of colocalization. All the rod bipolar cells stained with PKC-α were also labeled for α_1c L-type Ca^{2+} channel.

Figure 3 shows the immunoreactivity of PKC-α and α_1c L-type Ca^{2+} channel on the retinal whole mounts. The labeled cells were imaged focusing on the cell bodies of rod bipolar cells in the INL, and the images show the rod bipolar cell body mosaic in the retina of each species. Figures 3C, 3F, 3I, and 3L show that PKC-α-immunoreactive cells colocalize with α_1c L-type Ca^{2+} channel-labeling in mouse, hamster, rabbit, and dog. These results confirm that the rod bipolar cells labeled for PKC-α are also immunoreactive for α_1c L-type Ca^{2+} channel.

IV. Discussion

The present study has revealed one important feature of the expression of α_1c L-type Ca^{2+} channel in mouse, hamster, rabbit, and dog retinas: the expression of PKC-α and α_1c L-type Ca^{2+} channel is identical and localized exclusively to rod bipolar cells in mouse, hamster, rabbit, and dog. These results show that an antibody against the α_1c L-type Ca^{2+} channel can be used as a novel marker for rod bipolar cells.

In early experimental studies investigating the expression of PKC in the retina, PKC-α was found to be expressed in cells with a morphology corresponding to rod bipolar cells [9, 18]. Since then, PKC-α has been used as a specific marker for rod bipolar cells in the retina. Our results for PKC-α expression in mouse, hamster, rabbit, and dog retinas are consistent with earlier results in other verte-

brates, including cat, chicken, goldfish, guinea pig, human, rabbit, rat, and zebrafish [3, 9, 15, 16, 18, 27]. In the human retina, the antibody against PKC-α revealed the morphology of rod bipolar cells. However, besides rod bipolar cells, another cell type was also weakly immunoreactive in the retina. These were the cone bipolar cells, which stratify in the IPL [10, 15]. In the rat retina, PKC-α was not exclusively expressed in rod bipolar cells, but in some amacrine cells as well [18]. In addition, a class of amacrine cells were PKC-α-immunoreactive in the retina of catfish, turtle, and frog. However, rod bipolar cells were not labeled for PKC-α. Although many previous studies had been reported that PKC-α was expressed in rod bipolar cells in vertebrates, the reasons for the specific localization in rod bipolar cells and the different expression pattern of PKC-α among species have not been investigated yet. It is only possible to presume that these might be related to species variation. However, PKC-α is still used as a specific marker of rod bipolar cells.

The L-type Ca^{2+} channel is a type of voltage-dependent Ca^{2+} channels whose activation result in the excitation of neurons or release of neurotransmitters [1, 11, 12, 17]. In the synaptic transmission of retinal cells, Ca^{2+} influx through L-type Ca^{2+} channel is particularly important. A previous study reported an attempt to localize L-type Ca^{2+} channels in the chicken retina. In that study, antibodies against several L-type Ca^{2+} channel α_1-subunits (α_1c, α_1d, and α_1f) were used, and each L-type Ca^{2+} channel α_1-subunit showed a specific localization pattern in the retinal cells. The α_1c-subunit was expressed in Müller cells, and the α_1d-subunit labeled the cell bodies of most retinal cells. The α_1f-subunit was present in the photoreceptor terminals [7].

Our results clearly show that α_1c L-type Ca^{2+} channel-immunoreactive cells are rod bipolar cells: their cell bodies were located in the INL, dendritic processes branched into the OPL, and axons extended into the innermost region of the IPL. Although our results differ from the results shown previously in the chicken retina, we clearly show that an antibody against the α_1c L-type Ca^{2+} channel labels rod bipolar cells in mouse, hamster, rabbit, and dog. The differences between our results and previously reported data might be due to unidentified variations between species.

It has been reported that L-type Ca^{2+} channels mediate glutamate release [11, 23, 25]. It is intriguing that only rod bipolar cells express α_1c L-type Ca^{2+} channel, as these are one of several types of glutamate-releasing cells in the retina, including cone bipolar and ganglion cells. The reason for the selective expression of α_1c L-type Ca^{2+} channel is currently unclear. In previous study, PKC-α was shown to function in modulatory mechanism at rod–rod bipolar cell synapse [20]. PKC is activated by an increase in the concentration of diacylglycerol (DAG) or Ca^{2+}. The antibody used in our study recognizes PKC isoforms that require Ca^{2+} for activation [13]. We propose that these related mechanisms may explain colocalization of PKC-α and α_1c L-type Ca^{2+} channel in rod bipolar cells. It has been reported that the α_1f L-type Ca^{2+} channel is responsible for visual transduction and Ca^{2+} entry into photoreceptors [8]. In addition, a mutation in the α_1f L-type Ca^{2+} channel is associated with incomplete X-linked congenital stationary night blindness [2, 24]. Although the physiological function of the α_1c L-type Ca^{2+} channel in the visual system is unknown, previous results suggest the possible important role for α_1c L-type Ca^{2+} channel; this needs further investigation in the future. Our results also imply the possibility of a close functional relationship between the α_1c L-type Ca^{2+} channel and PKC-α in rod bipolar cells.

In conclusion, our results demonstrate that α_1c L-type Ca^{2+} channel is expressed in rod bipolar cells in the retina of mouse, hamster, rabbit, and dog. The colocalization of PKC-α and α_1c L-type Ca^{2+} channel suggests that the α_1c L-type Ca^{2+} channel is a novel specific marker for rod bipolar cells in retina.

V. Acknowledgments

We thank Cactus Communications for proofreading the manuscript. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by Ministry of Education (NRF-2013R1A1A2059568).

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