

Acidification of the synaptic cleft of cone photoreceptor terminal controls the amount of transmitter release, thereby forming the receptive field surround in the vertebrate retina

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Abstract In the vertebrate retina, feedback from horizontal cells (HCs) to cone photoreceptors plays a key role in the formation of the center-surround receptive field of retinal cells, which induces contrast enhancement of visual images. The mechanism underlying surround inhibition is not fully understood. In this review, we discuss this issue, focusing on our recent hypothesis that acidification of the synaptic cleft of the cone photoreceptor terminal causes this inhibition by modulating the Ca channel of the terminals. We present evidence that the acidification is caused by proton excretion from HCs by a vacuolar type H⁺ pump. Recent publications supporting or opposing our hypothesis are discussed.

Keywords Proton · pH · Feedback · Horizontal cell · Calcium channel · V-ATPase

Abbreviations

CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
DNQX	6,7-Dinitroquinoxaline-2,3(1H,4H)-dione
NBQX	1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
Tris	Tris(hydroxymethyl)aminomethane
MOPS	3-(<i>N</i> -morpholino) propanesulfonic acid

PIPES	Piperazine- <i>N,N'</i> -bis(2-ethanesulfonic acid)
MES	2-(<i>N</i> -morpholino) ethanesulfonic acid
<i>I</i> _{Ca}	Voltage-dependent calcium current
HC	Horizontal cell
BC	Bipolar cell
GC	Ganglion cell
GABA	γ-aminobutyric acid
V-ATPase	Vacuolar type adenosine triphosphatase, proton pump, H ⁺ -ATPase

Introduction

In the retina, the photoreceptors are arranged two dimensionally and serve as pixels. They convert the incoming light signal into the electrical activity. The strength of the light stimulus is first converted into the amplitude of receptor potentials, which is coded finally to the train of action potentials of output neurons, i.e., retinal ganglion cells (GCs). The retinal photoreceptors not only convert the image faithfully pixel by pixel, but also enhance the contrast of the image by lateral inhibition. This lateral inhibition contributes to the center-surround receptive field organization of GCs. Since its discovery by Kuffler [1], many investigators have presented hypotheses that account for the underlying neural mechanisms. In lower vertebrates (fish, turtle and amphibian), it has been well documented that cones receive a negative feedback message from horizontal cells (HCs) that serves to provide a receptive field surround for the individual cones. The most classical demonstration is found in the turtle retina [2].

In 1971, Baylor et al. [2] showed that the cone photoreceptors of the turtle respond by hyperpolarization to direct illumination of the recorded cone, but by delayed

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depolarization to surround illumination of an area comparable to the receptive field size of the HCs. In a more direct experiment, they showed that hyperpolarization of the HCs by extrinsic current injection depolarizes the nearby cones. Thus, it has been widely accepted that negative feedback from the HCs plays a key role in the center-surround antagonism of retinal neurons. In fish retinas there is morphological evidence that HC lateral elements at the triad synapse in the cone terminal produce spinules at which feedback signaling could occur to the cone [3]. Furthermore, chromaticity-type HCs that can depolarize in their spectral response have been reported in the lower vertebrates, which is consistent with the evidence of feedback to cones. In mammalian retina, however, physiological evidence for the feedback synapses has been clearly found only in the macaque [4, 5], but not in other mammals such as rabbit (but see [6]), rat and mouse. This means that the horizontal cell feedback model is applicable to mammals, including the human retina, but needs further examination to apply to all mammals.

There is considerable evidence to suggest that the feedback from the HCs to cones is mediated by GABA. In many non-mammalian animal species, the HCs are shown to be GABAergic; they have glutamic acid decarboxylase (GAD, [7]), a GABA-synthesizing enzyme. They accumulate GABA via a selective uptake mechanism [8, 9] and release it when they are depolarized [10]. It has also been shown that cone photoreceptors in turtles are highly sensitive to GABA at their terminals where they connect with HCs [11, 12]. The presence of GABA receptors has also been shown in mammalian cones [13, 14]. These observations have presented evidence that suggests GABA-mediated feedback from HCs to cone photoreceptors. On the other hand, there are several reports that argue against the GABA feedback hypothesis. The most critical opposition is that the surround response of the cone photoreceptors remains even after application of GABA antagonists [15–17] or agonists [15, 16].

Modulation of the voltage-dependent calcium current (I_{Ca}), hence the amount of transmitter release, in cones has also been proposed as a mechanism for feedback. Verweij et al. [17] reported that surround illumination shifts the activation voltage of the cone I_{Ca} to negative direction in the goldfish retina. Feedback by modulation of the cone I_{Ca} is also consistent with the observation in the turtle retina that surround illumination causes calcium-dependent spikes in the cones [18, 19]. Furthermore, Verweij et al. [17] also proposed that GABA is not likely the main mediator of this feedback mechanism, since this modulation of the cone I_{Ca} induced by surround light was resistant to GABA_{A&C} receptor antagonists and GABA transporter antagonists.

Byzov and Shura-Bura [20] proposed an electric field feedback mechanism by which a local current in the cone-HC synapse causes a voltage drop in the cone synaptic clefts, and results in modulation of the transfer function between the cones and bipolar cells (BCs). More recently, Kamermans et al. [21] suggested that an ephaptic effect¹ (field effect) of the current flowing through the hemi-gap junctional channel at the dendritic tips of the HCs shifts the activation voltage of the cone I_{Ca} . However, the validity of the hemichannel-mediated feedback hypothesis is still under debate, since the pharmacological specificity of carbenoxolone is uncertain [24–26], and the hemi-channels are not likely working since they are closed in physiological condition at around 1 mM $[Ca^{2+}]_o$ [27].

Provoked by the ephaptic hypothesis, we tried to follow the experiments of Kamermans et al. [21]. We confirmed that the surround illumination shifts the activation voltage of the cone I_{Ca} toward negative voltages, as reported by Verweij et al. [17], but arrived at a different conclusion [28]. We found that the cone I_{Ca} was enhanced along all the voltages we examined (not merely voltage shift of $I-V$ relation of I_{Ca}). This enhancement of I_{Ca} could not be explained by an ephaptic effect, but it was very similar to that caused by alkalization of the extracellular space. Hyperpolarization of the HCs induced by CNQX, an antagonist of postsynaptic glutamate receptors of HCs, increased cone I_{Ca} , whereas depolarization of the HCs induced by kainate decreased cone I_{Ca} . Enrichment of the pH-buffering capacity of the extracellular solution increased cone I_{Ca} and suppressed the light-induced surround effect. These data thus strongly suggest that feedback from HCs to cone photoreceptors is mediated by pH changes in the synaptic cleft. In later studies we suggested that HCs have a voltage-sensitive proton pump (vacuolar type adenosine triphosphatase, V-ATPase), which is thought to be responsible for the proton release from HCs [29, 30]. The proton feedback mechanism seems to be conserved throughout vertebrate retinas, which is shown by many reports demonstrating similar results in lower vertebrate as well as in mammalian retinas.

¹ Usage of the terminologies “ephaptic effect” and “field effect” may vary according to the context. The word ephapse (Greek: meaning to touch) coined by Arvanitaki [22] appeared to describe electrical interaction between touching *Sepia* axons. Afterwards Jefferys [23] described the term “ephaptic interaction” as highly localized communications such as axo-axonal interactions and the term “field effect interaction” as electrical communication between neurons that depend on large voltage fields generated by synchronous activity of many neurons. On the analogy of a word “Field Effect Transistor” in use frequently, the term “(electrical) field effect” seems intuitive to describe the changes in electrical field. Thus, in the authors’ view, the term “local effect of electrical field” or just simply “field effect” could be an optional term.

In this article, we will review the past papers regarding the receptive field surround formation and examine the present knowledge concerning the proton hypothesis.

Protons and retinal function

The importance of protons to neuronal functions is widely recognized because neuronal activity in the nervous tissue can result in pH shifts that may be large enough to influence functions of a number of voltage/ligand-gated ion channels [31]. The extracellular pH changes may be generated by the activation of ion channels (e.g., [32]) or ion transporters (e.g., [33]). A far faster pH change may also occur in the context of synaptic vesicle fusion [34]. As a target of the pH changes, protonation of ionizable residues is emerging as a central feature of voltage-gated channel function [35] and ligand-gated channel function [36].

In the retina, light stimulation evokes alkalization of up to 0.2 pH in the intraretinal extracellular space [37], probably because of the change in proton release caused by the energy metabolism in retinal cells. The activity-dependent changes in the external pH can play an active role in the activity of the retina, as well as in the basal metabolism of retinal systems. For example, a transient inhibition of a presynaptic I_{Ca} by proton release from fused synaptic vesicles is described in retinal photoreceptors [34, 38] and BCs [39]. These observations of proton release are consistent with a preceding study of hippocampal slices by Krishtal et al. [40] who noted that synaptic transmission was accompanied by a short latency transient change in a brief (few ms) extracellular acidification.

The mechanisms that organize the center-surround antagonism of the receptive field in the retinal synapses have not yet been fully explained. Currently, intersynaptic pH changes at the photoreceptor terminals appear to be responsible for the generation of receptive field surround in the retina [28]. This theory proposed that hyperpolarization of HCs, caused by surround illumination, elevates pH in the HC-cone synaptic cleft, leading to an increase in calcium current in cones and a higher rate of glutamate release that depolarizes OFF-type BCs and hyperpolarizes ON-type BCs. Main evidence for the proton hypothesis is that the enrichment of pH-buffering capacity² of superfusate prevents the generation of surround response in

² pH-Buffering capacity is a quantitative measure of pH-buffering power, calculated as

$$\frac{2.3K_a[H^+][B]}{(K_a + [H^+])^2}$$

where B is the buffer concentration, K_a is the buffer dissociation constant, and $[H^+]$ is the proton concentration at pH used for experiments [41].

photoreceptors and other retinal neurons. The proton hypothesis should serve as the simplest description, so far, for the mechanism of feedback in the outer retina.

Ca²⁺ channels in photoreceptors and their modulation by protons

In photoreceptors, a great deal is known about the pharmacology and electrophysiological properties of the L-type Ca²⁺ channels in their synaptic terminals [42]. Functionally, these channels are responsible for the sustained release of the neurotransmitter, glutamate. It has been demonstrated that extracellular acidification regulates I_{Ca} by (a) charge-screening effects of protons on voltage-gated calcium channels [43–45] or (b) proton-binding effects on the Ca²⁺ channels [46, 47]. On the other hand, extracellular alkalization increases the peak of I_{Ca} with shifting of its activation voltage toward negative potential [35]. The consequence of the alkalization for synaptic transmission from photoreceptors to HCs is that the synaptic efficacy is increased as extracellular pH is increased [48, 49].

In fact, Ca²⁺ channels appear much more pH-sensitive than any other types of voltage/ligand-gated channels [35]. The amount by which external pH shifts Ca²⁺ channel gating is ~ 1 mV per 0.1 pH unit [50]. Notably, this pH shift can occur within physiological pH values of 7.2–7.4. Thus, even modest pH shifts (0.1 pH unit) located near Ca²⁺ channels could modulate synaptic transmission by acting presynaptically [49].

Surround illumination augments photoreceptor I_{Ca} by hyperpolarizing HCs

Fundamentally, surround illumination augments cone I_{Ca} by shifting its activation voltage toward negative potentials [17, 28]. In the case of cones in newt retinal slices, a voltage-dependent I_{Ca} was found to be activated by depolarization to voltages more positive than -30 mV (Fig. 1) and was augmented by surround illumination in the presence of spot illumination at all holding voltages (Fig. 1A, Ba) [28]. This augmentation was voltage dependent; greater augmentation was seen at voltages more negative than -15 mV, at which the standing inward current was maximal, whereas little augmentation was seen at voltages between 0 and $+10$ mV. This effect of surround illumination on cone I_{Ca} was observed even in the presence of blockers for GABA receptors, bicuculline or picrotoxin [17, 28], which indicates that GABAergic systems are not involved in the augmentation of cone I_{Ca} . The augmentation of I_{Ca} by surround illumination was also observed in rod photoreceptors [51, 52].

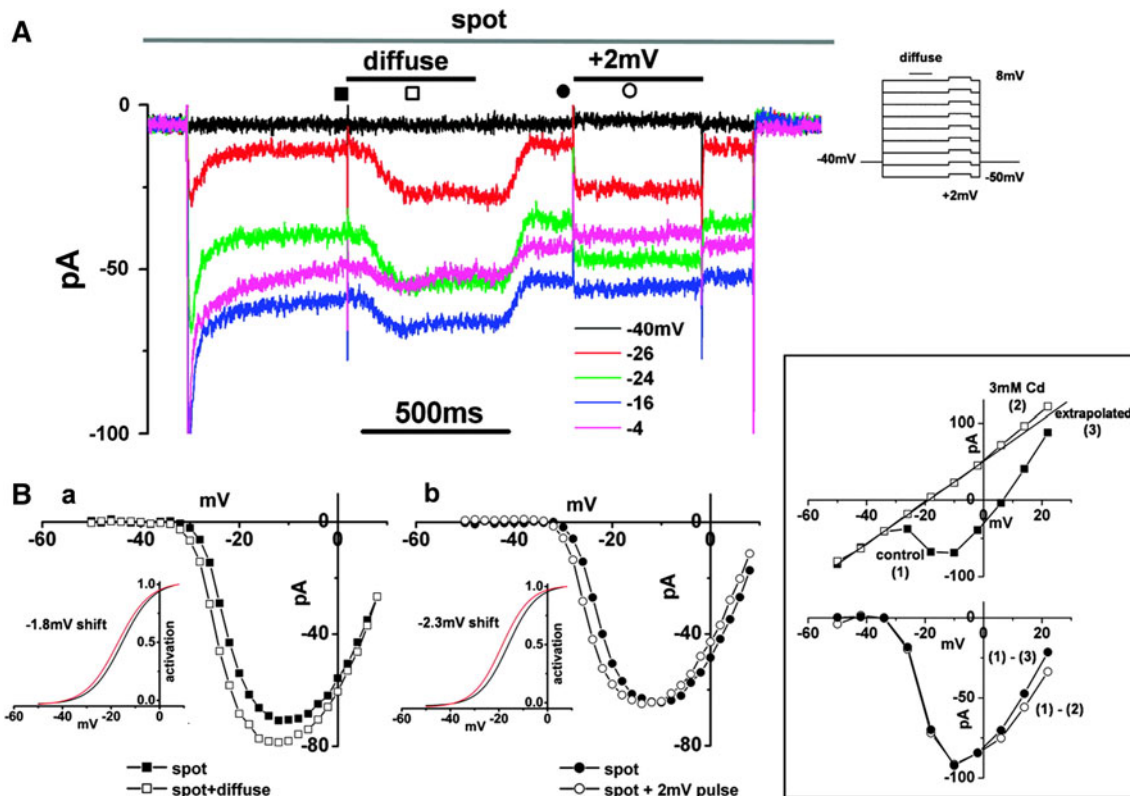


Fig. 1 Surround illumination augments cone I_{Ca} . **A** I_{Ca} of a cone in newt retinal slice was recorded under the whole-cell voltage clamp condition at a holding potential of -40 mV. The Cs^+ -based pipette solution contained 20 mM BAPTA to minimize any Ca^{2+} -activated currents. Depolarizing voltage steps from -50 mV to $+8$ mV (2 mV step) induced inward currents. I_{Ca} of a cone in newt retinal slice was recorded under the whole-cell voltage clamp condition at a holding potential of -40 mV. The I_{Ca} was abolished by extracellular cadmium, a blocker of Ca channels (plots with open squares shown in the right panel). Five representative traces, voltage-clamped at -40 , -26 , -24 , -16 and -4 mV, are shown. Diffuse illumination (4,000 μm in diameter used for ‘surround illumination’: shorter bar) was applied every 4 s, while the spot illumination (30 μm in diameter: top bar) was maintained. An additional 2-mV depolarization was applied to mimic an ephaptic (field) effect (external voltage drop) after withdrawing the diffuse illumination. Note that at -4 mV (pink trace), diffuse illumination evoked an inward current, while a $+2$ -mV pulse evoked an outward current. The current amplitude was

sampled at the time indicated by the symbols to construct the $I-V$ curves shown in **Ba** and **Bb**. **Ba** Leak-subtracted $I-V$ curve of cone I_{Ca} in the presence of the spot (filled squares) and during diffuse illumination (open squares). Inset shows activation curves fitted to the Boltzmann function derived from the $I-V$ curves. **Bb** Leak-subtracted $I-V$ curve of cone I_{Ca} in the presence of the spot light (filled circles) and during a $+2$ -mV depolarizing pulse (open circles). Inset shows activation curves fitted to the Boltzmann function derived from the $I-V$ curves. Boxed inset isolation method of $I-V$ curves of cone I_{Ca} obtained from a different cone in **A**. Top The $I-V$ curves were obtained in the control solution [filled squares (1)] and in a 3 mM Cd containing solution [open squares (2)]. Bottom (open circles) $I-V$ curve of the cone I_{Ca} obtained by subtracting the $I-V$ curve in a 3 mM Cd containing solution from that in the control solution [(1)–(2)]. Filled circles $I-V$ curve obtained by subtracting the $I-V$ curve from the extrapolated leakage current from that in the control solution [(1)–(3)] (from Hirasawa and Kaneko [28])

Three pieces of evidence support the conclusion that the membrane potential of HCs controls cone I_{Ca} . First, the enhancement of cone I_{Ca} caused by a small (0.25 mm) diameter illumination was less than that by a large (4 mm) diffuse light [28], which is consistent with evidence that the response amplitude of HCs depends on the illuminated area of the retina [2, 53]. Second, glutamatergic drugs to manipulate HC membrane potential can significantly influence the amplitude and activation voltage of cone/rod I_{Ca} . Kainate, which depolarizes HCs by acting as an agonist of the postsynaptic glutamate receptors of HCs, suppressed cone/rod I_{Ca} recorded in sliced retinas [28]. CNQX/DNQX,

blockers for AMPA/kainate type glutamate receptors which hyperpolarize HCs by acting as an antagonist of the postsynaptic glutamate receptors of HCs, augmented cone/rod I_{Ca} recorded in sliced/whole-mounted retinas [17, 28, 52]. In retinal slices, Ca^{2+} signals in cone/rod terminals (enhanced by depolarization with elevated extracellular K^+) were suppressed by kainate and increased by CNQX/NBQX [52, 54], which is consistent with the results suggesting that polarization of HCs alters photoreceptor I_{Ca} . Third, by using dual whole-cell recordings, direct manipulation of HC membrane potential was found to significantly influence the voltage dependence and amplitude of

I_{Ca} in synaptically connected cones/rods [51, 55]. As surround illumination does, hyperpolarizing a single HC increased the peak amplitude of cone I_{Ca} and shifted its activation voltage (Fig. 4A) [51].

Surround illumination augments photoreceptor I_{Ca} : a pH effect

Kamermans et al. [21] proposed hemichannel-mediated electric field feedback in the goldfish retina, in which model they proposed that a current flowing into hemichannels at the HC dendritic tips creates a voltage drop in the dead-end of the synaptic cleft at the cone terminal. In this model, the cone terminals are relatively depolarized, and the cone I_{Ca} is enhanced [21, 56]. They call this electric field effect an ‘ephaptic effect’. This ephaptic model is based on following evidence: (1) the presence of hemichannel proteins in the dendritic tips of the HCs and (2) the blocking effect of carbenoxolone, a blocker of hemichannels, on the surround responses of the cones. This model has recently been modified by incorporating an additional ephaptic effect mediated by ionotropic type glutamate receptors at HC dendritic tips [57]. In Hirawasa and Kaneko’s experiment, the holding potential of cones was depolarized by 2 mV after switching off the surround illumination to mimic the presumable ephaptic effect (Fig. 1A) [28]. During the 2-mV depolarization, the I - V curve of the cone I_{Ca} was shifted by 2 mV toward the negative direction (Fig. 1Bb). The change was clearly different from that induced by surround illumination. A difference was especially apparent at membrane voltages between -10 and $+8$ mV, where surround illumination increased the current amplitude, whereas 2-mV depolarization decreased it (Fig. 1B): surround illumination did not simply shift the voltage dependence in cone I_{Ca} along the voltage axis, the change that the ephaptic feedback hypothesis has suggested. The failure of a parallel shift of the I - V relationship was a strong reason for the authors to consider that an alternative effect other than the ephaptic effect might be working. Moreover, the validity of the blocking effect of carbenoxolone on feedback response can be called into question. For example, carbenoxolone appears also to act as an inhibitor for Ca channels [25].

The effect of surround illumination on cone I_{Ca} , increasing peak current and shifting its activation voltage toward negative potentials, is strongly reminiscent of the effect of extracellular alkalization on photoreceptor I_{Ca} [34, 49, 50]. Indeed, pressure ejection of high pH Ringer’s solution (pH 9.0) onto the outer plexiform layer enhanced cone I_{Ca} recorded in newt retinal slices (Fig. 2a) [28]. The high pH-induced inward current was voltage dependent: the inward current was more pronounced at voltages

between -40 and $+5$ mV. Thus, the external alkalization augments the peak of cone I_{Ca} and shifts its activation voltage toward negative potential (Fig. 2b), the effect similar to that of the surround illumination (Fig. 1Ba). Ca^{2+} channels are localized in the terminal regions of the photoreceptors [58, 59] and are thus presumably localized near the center of the invaginating synapses. This localization suggests that surround illumination alkalizes the synaptic clefts of the invaginating synapses, resulting in the augmentation of the cone I_{Ca} locally.

pH clamp suppresses surround response in retinal neurons

Enrichment of the buffering capacity for extracellular protons, “extracellular pH clamp”, is a powerful approach to examine the involvement of intersynaptic protons in the synaptic functions. The elegant work examining the effect of the pH clamp on the synaptic transmission performed by DeVries [34] demonstrated that the protons released from presynaptic vesicles together with glutamate provide a fast and potent autoinhibition of cone transmitter release and that 20 mM HEPES suppresses it. Considerable proton-dependent modulation of Ca^{2+} channels is feasible in the extracellular fluid [34]. If pH changes in the synaptic clefts mediate the surround response of cones by modulating Ca^{2+} channels, clamping extracellular pH by enrichment of the pH-buffering capacity of the external solution would be expected to prevent the generation of surround response in photoreceptors and their postsynaptic retinal neurons. In fact, as described in the following section, evidence that the pH clamp suppresses surround responses in retinal neurons has been accumulated.

pH clamp suppresses surround response in photoreceptors

First of all, the pH clamp suppresses surround response in photoreceptors. In newt retinal slices, changing the superfusate from control Ringer’s solution to one supplemented with 10 mM HEPES (having an identical pH value) increased cone I_{Ca} and prevented any additional inward currents caused by surround illumination (Fig. 3A) [28]. Such changes in I_{Ca} were seen throughout the entire voltage range at which I_{Ca} is activated (Fig. 3Ba, b). As a result, the use of HEPES shifted the Boltzmann-fit activation curve of cone I_{Ca} toward the negative direction to the similar extent as that induced by surround illumination in control solution, and prevented any additional shift caused by surround illumination (Fig. 3Bc). HEPES did not block the light response of the newt HCs to diffuse light illumination (Fig. 3A: inset). The effects of HEPES on

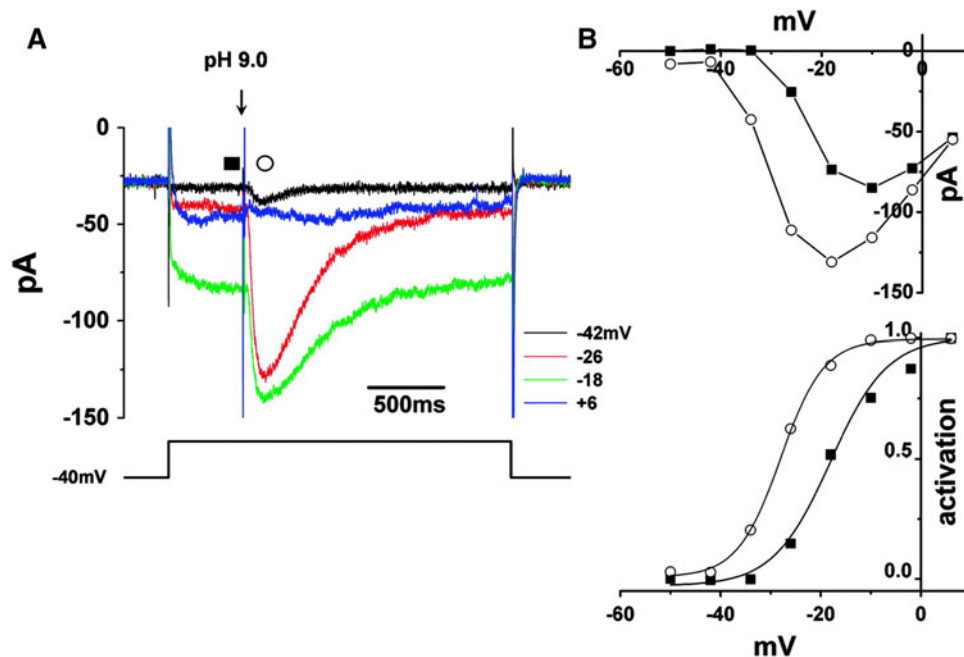


Fig. 2 Modulation of cone I_{Ca} by a high-pH solution applied to the cone terminal layer. **A** Alkalinized Ringer's solution (pH 9.0) was focally ejected to cone synaptic terminal layer in newt retinal slice. The cone was voltage-clamped at various voltages in the range of -50 to $+6$ mV in 8-mV steps. The representative four traces, voltage clamped at -42 , -26 , -18 and $+6$ mV, are shown. The current was sampled at the points marked by a symbol to construct the I - V curves

shown in **B**. Small spot illumination ($30\ \mu\text{m}$ in diameter) was maintained throughout. **B** *Top* leak-subtracted I - V curve of cone I_{Ca} in normal Ringer's solution (pH 7.4, filled squares) and in response to a high-pH solution (pH 9.0, open circles). *Bottom* activation curves derived from the I - V curves fitted to the Boltzmann function (from Hirasawa and Kaneko [28])

cone I_{Ca} suggest that the external pH clamp increases cone I_{Ca} and blocks any additional increase in cone I_{Ca} caused by surround illumination.

HEPES, MES and TAPS, commonly used "Good buffers", have an aminosulfonate moiety that has been reported in the protonated form to decrease the permeability of connexin-26 hemichannels [60]. One concern is that HEPES might close the connexin-26 hemichannels on HCs ([21] but see [61–63]), which results in blockade of the proposed ephaptic effect [21]. However, the effect of pH buffering is not unique to HEPES because the superfusate supplemented with Tris, which does not possess an aminosulfonate moiety, also enhanced cone I_{Ca} and suppressed additional increment of I_{Ca} caused by surround illumination [28]. pH buffers other than HEPES were also effective in suppressing the feedback response in HCs (see the following section [54, 64]).

In retinal slices, high K^+ -induced Ca^{2+} signals in photoreceptors detected by Ca^{2+} indicators were suppressed by kainate and increased by CNQX/DNQX in zebrafish [54] and mouse [52]. The effects produced by the glutamatergic drugs were significantly attenuated by increasing HEPES concentration: HEPES at 20 mM eliminated the CNQX/DNQX-mediated increase in Ca^{2+} signal and significantly suppressed the kainate-mediated decrease in the Ca^{2+}

signal [52, 54], which is consistent with the effect of HEPES on photoreceptor I_{Ca} [28, 52].

Consistent with studies using light or glutamatergic drugs to manipulate HC membrane potential [17, 28], directly manipulating the membrane potential of a voltage-clamped HC can significantly influence the voltage dependence and amplitude of I_{Ca} in synaptically connected cones/rods [51, 55]. Hyperpolarizing a single HC, similar to the membrane hyperpolarization evoked by a bright light flash, increased the peak amplitude of photoreceptor I_{Ca} and produced a negative shift in its activation voltage (Fig. 4A). Application of HEPES almost suppressed the changes in I_{Ca} produced by membrane potential changes in voltage-clamped HCs (Fig. 4B) [51, 55].

In Hirasawa and Kaneko [28], cone I_{Ca} under no surround illumination was increased by pH buffers, which prevented additional increment in inward I_{Ca} caused by surround illumination (Fig. 3Bb). Cadetti and Thoreson [55] demonstrated in tiger salamander retinal slices with the dual voltage-clamp method that pH buffers prevent cone I_{Ca} increments caused by hyperpolarization of HCs (Fig. 4). The former finding suggests that "depolarized HCs release a proton/acidic substance" to the intersynaptic space, and the latter finding that "hyperpolarized HCs release an alkalinizing substance." The possible mechanism by which

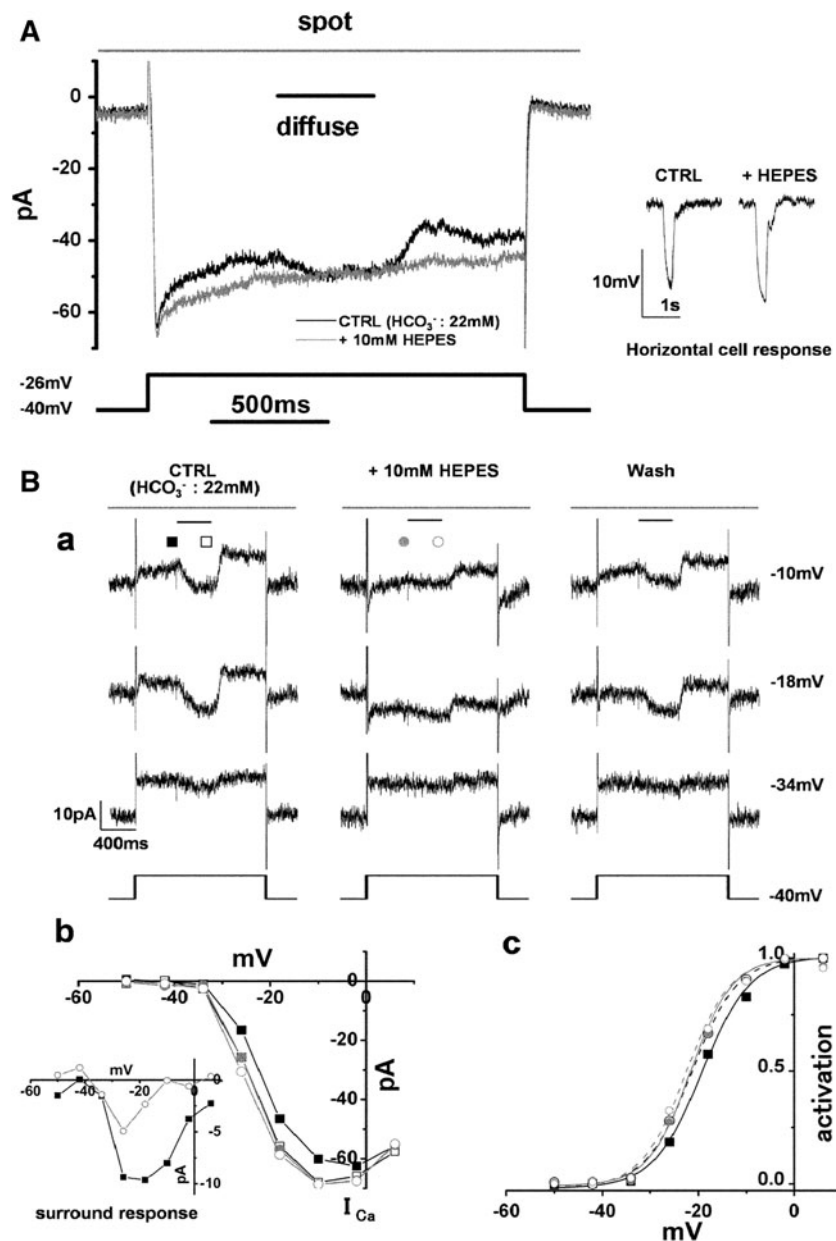


Fig. 3 Cone I_{Ca} and its surround response recorded in a superfusate enriched with HEPES. **A** Effects of 10 mM HEPES-enriched buffer on cone I_{Ca} and surround response. The cone in newt retinal slice was depolarized from the holding voltage of -40 to -26 mV. Diffuse light illumination ($4,000 \mu\text{m}$ in diameter used for surround illumination: *shorter bar*) was given during the step depolarization in the presence of a small spot light ($30 \mu\text{m}$ in diameter: *top bar*). Changing the superfusate from control solution (*black trace*) to one supplemented with 10 mM HEPES increased cone I_{Ca} and prevented any additional inward current upon surround illumination (*gray trace*). *Inset* shows horizontal cell (HC) responses to a large light spot ($4,000 \mu\text{m}$ in diameter) in the control solution and in the solution enriched with HEPES. **Ba** Reversible effects of 10 mM HEPES-enriched buffer on cone I_{Ca} and surround response. The small spot light ($30 \mu\text{m}$ in diameter: *top bars*) was kept on throughout. Diffuse light illumination ($4,000 \mu\text{m}$ in diameter: *shorter bar*) was given during the step depolarization in the presence of the small spot. The cone was held at -40 mV and polarized to voltages ranging from -50 to $+6$ mV in 8 mV steps. The current traces before, during and after application of

10 mM HEPES-enriched buffer. The recording sequence was *left column* (-34 , -18 and -10 mV) followed by the *middle column* and finally the *right column*. The leak conductance of 2.26 nS did not change either in the HEPES-containing solution or during the washout (current traces at -34 mV). In the HEPES-containing solution, inward I_{Ca} in darkness was reversibly increased, and the surround response was reversibly suppressed (current traces at -18 and -10 mV). *Symbols* denote the sampling points for calculation of the $I-V$ curves of I_{Ca} in **Bb**. In the control solution, the inward current produced by I_{Ca} was counterbalanced by the outward leak current (at -18 and -10 mV in the control and washout solutions). **Bb** $I-V$ curves of cone I_{Ca} recorded in **Ba**. The leak conductance was subtracted. *Filled and open squares* in the control solution without and with surround illumination, respectively; *filled and open gray circles* in the HEPES-containing solution without and with surround illumination, respectively. *Inset* shows the voltage dependence of the surround response in the control solution (*filled squares*) and in the HEPES-containing solution (*open circles*). **Bc** Activation curves fitted to the Boltzmann function derived from the data in **Bb** (from Hirasawa and Kaneko [28])

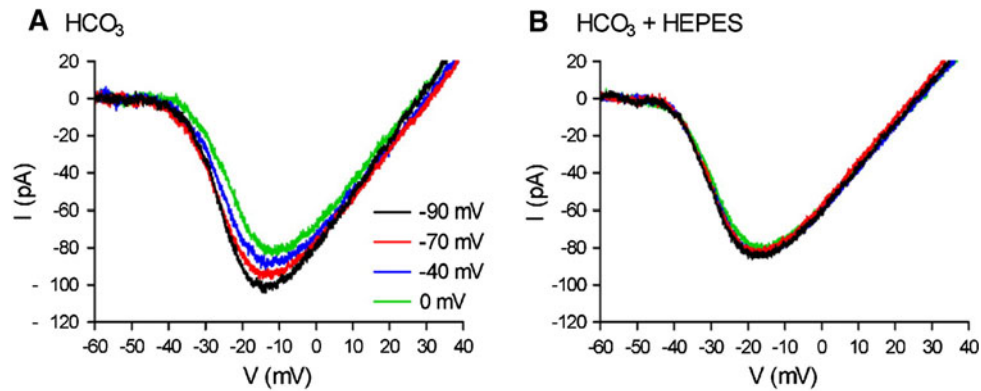


Fig. 4 Changes in horizontal cell (HC) membrane potential alter the amplitude and voltage dependence of I_{Ca} in an adjacent cone simultaneously recorded: these effects are blocked by application of HEPES. **A** I_{Ca} of cone in tiger salamander retinal slice was recorded under the whole-cell voltage clamp condition with a ramp-function from -90 to $+60$ mV (0.5 mV/ms) applied from a steady holding potential of -70 mV. By step-wise depolarization of HC

simultaneously voltage-clamping at -90 , -70 , -40 and 0 mV, the cone I_{Ca} was progressively decreased in amplitude and positively shifted in the activation voltage to more positive potentials. **B** Addition of HEPES (10 mM) abolished the voltage shift and amplitude changes of cone I_{Ca} curves produced by the HC polarization (from Cadetti and Thoreson [55])

HC depolarization controls extracellular pH will be discussed in the following section.

The efficacy of HEPES to block the feedback response in photoreceptors supports the hypothesis that HC hyperpolarization alkalizes the extracellular space in the synaptic cleft, leading to increase in photoreceptor I_{Ca} . Since the center-surround antagonism formed at the photoreceptor synapse is suppressed by pH buffer, it is expected that the pH clamp will also suppress the surround/feedback response in some 2nd and 3rd order retinal neurons, as described below.

pH clamp suppresses feedback response in HCs

In the cone pathway of lower vertebrates, the roll-back response in luminosity-type (H1) HCs, a slow depolarization after the rapid hyperpolarization at light onset, is commonly attributed to HC feedback to cones. In goldfish retina, pH buffers (HEPES and Tris) were found to reduce the roll-back response in H1 HCs [54, 57, 65]. pH buffers also reduced the depolarizing response in chromaticity-type HCs [54, 57, 66], which is also believed to result from negative feedback from HCs to cones in the cyprinid fish retina [67]. The proton-mediated negative feedback can be suggested to the formation of opponent color signaling in HCs [54, 66]. The effect of pH buffers on HC light responses is consistent with the efficacy of HEPES to block the feedback response in cones.

Also in the macaque retina, HEPES reduces the roll-back response in HCs, which is attributed to feedback from HCs to cones: HEPES significantly increased the hyperpolarization amplitude and decreased the slow depolarizing component in a concentration-dependent manner (Fig. 5) [64]. The effects of pH buffering are not unique to HEPES

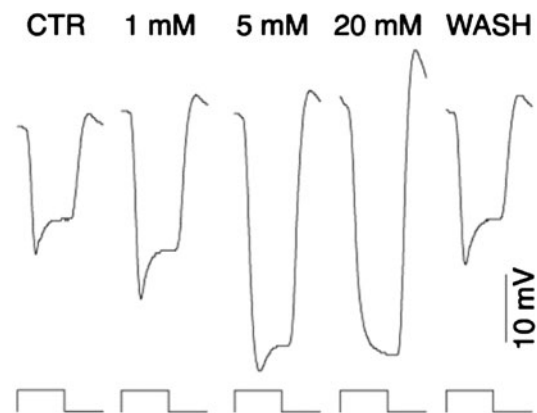


Fig. 5 Concentration-dependent effects of HEPES on monkey HC light response. Light responses of an HC in macaque monkey retina illuminated to a $2,000$ - μ m diameter spot light were obtained by intracellular recording. HEPES at 1 , 5 and 20 mM increased the hyperpolarization amplitude and decreased the slow depolarization in a dose-dependent manner (from Davenport et al. [64])

(pK_a 7.4) because other pH buffers, such as MOPS (pK_a 7.06), PIPES (pK_a 6.7), MES (pK_a 6.03) and Tris (pK_a 7.8), diminished the slow depolarization of HCs in a concentration-dependent manner. In addition, buffers with pK_a values farther away from the pH used for their experiments, such as MES, were less effective in reducing the feedback effect [64]. Thus, the effect of exogenous buffers is likely attributable to their pH buffering capacity rather than nonspecific effects of pH buffers.

pH clamp suppresses surround response in BCs

It is widely considered that feedback from HCs to photoreceptors is involved in the center-surround organization of

the receptive field of BCs. In OFF-type BCs in newt retinal slices, spot illumination evoked a sustained outward current and surround illumination evokes an inward current (Fig. 6A) [28], which is consistent with the result that OFF-type BCs are hyperpolarized by spot illumination and depolarized by annulus illumination [68]. Changing the superfusate to HEPES-enriched buffer solution suppressed the inward surround response (Fig. 6B). Consistent with the evidence that pH clamp suppresses feedback response in photoreceptors and HCs, external HEPES-enriched buffer can suppress the surround response in BCs. This effect of surround illumination on OFF-type BCs was observed even in the presence of picrotoxin and bicuculline, blockers for GABA receptors, which indicates that GABAergic systems are not involved in the generation of the surround response. Besides the suppression of the surround response, a marked observation in the OFF-type BCs is that the transient inward current at the offset of the spot illumination was prominently increased by HEPES-enriched buffer (Fig. 6B). This augmentation of the offset response in the OFF-type BCs may reflect the increase of transmitter release from photoreceptors, which is consistent with the fact that HEPES augments cone I_{Ca} in newt retinal slices (Fig. 3).

Additional evidence that HEPES-enriched buffer suppresses the surround response of OFF-type BCs is also reported in the primate retina. In macaque retina, the small bistratified, “blue-yellow” color-opponent ganglion cell receives ON-depolarizing and OFF-hyperpolarizing inputs from short (S)-wavelength sensitive and combined long (L)- and middle (M)-wavelength sensitive cones, respectively. The LM component, isolated from S component pharmacologically, shows a center-surround receptive field structure consistent with an input from OFF-center, ON-surround “diffuse” cone BCs. Increasing the buffering capacity of superfusate with HEPES suppresses the LM-ON surround component, indicating that the surround component of LM-OFF BCs is suppressed by the pH-buffer [69].

pH clamp attenuates surround response in GCs

In primate retina, the receptive field surrounds of GCs in light-adapted retinas appear to be primarily mediated by a non-GABAergic mechanism because the GABA receptor antagonist picrotoxin only slightly reduces the basic center-surround structure of parasol GC receptive fields [70]. Instead, pH buffers reduced their inhibitory surround [64]. A large 2,000- μm diameter spot, stimulating both the center and the surround of the ON-center parasol GC receptive field, evoked relatively small transient depolarizing responses at light onset and transient hyperpolarizations at light offset (Fig. 7B left), indicative of cancellation

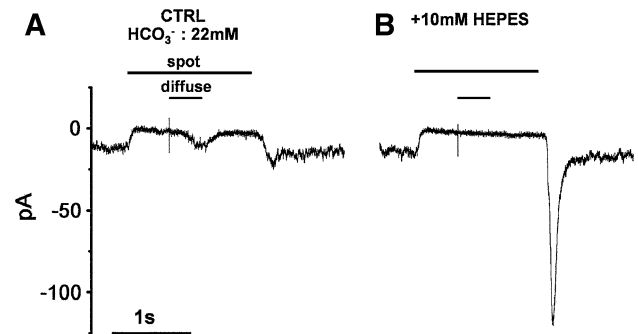


Fig. 6 Center and surround responses of an OFF-type bipolar cell (BC) recorded in a solution enriched with HEPES. Light responses of an OFF-type BC in newt retinal slice were recorded under the voltage-clamp conditions (holding voltage, -40 mV). **A** In control Ringer's solution, spot illumination (50 μm in diameter) evoked an outward current as a center response and surround illumination (diffuse light superimposed on the spot light: $4,000$ μm in diameter) evoked an inward current as a surround response. **B** Changing the superfusate to the HEPES-enriched solution (10 mM) abolished the surround response. The transient inward current at the time of switch-off of spot illumination was prominently increased in HEPES buffer (from Hirasawa and Kaneko [28])

due to center-surround antagonism. HEPES buffering at 20 mM strongly reduced the surround strength by $\sim 65\%$: the responses at light onset became enhanced and more sustained, and those at the light offset were attenuated (Fig. 7B right) [64].

At mesopic (relatively low light) level, surround inhibitory response in most GCs is partially attenuated by picrotoxin [71, 72] and the sodium channel blocker tetrodotoxin [71–73], which indicates that the contribution of inner retinal amacrine cell circuitry to the surround antagonism may be substantial at mesopic condition. Under the photopic condition, however, picrotoxin and tetrodotoxin only slightly reduce the surround response in parasol GCs, although HEPES attenuates it [64, 70]. Thus, the effect of pH buffer on the receptive field surround of GCs may vary with adaptive condition.

An additional factor that may affect the role of amacrine cells in surround formation is the diversity of GC types. Even under conditions in which GABAergic surround inhibition has been found, its contribution may vary with GC types. Taylor [73] found that the surrounds of a number of ON-center GCs are attenuated by TTX, although it produces little effect on OFF-center cells. Kirby and Enroth-Cugell [74] reported that, under scotopic conditions, picrotoxin substantially reduces the surrounds of cat Y cells, but has no effect on X cells. McMahon et al. [70] and Davenport et al. [64], who found the inhibitory surround sensitive to pH buffers but insensitive to GABA receptor blockers, collected data only from ON- and OFF-center parasol GCs.

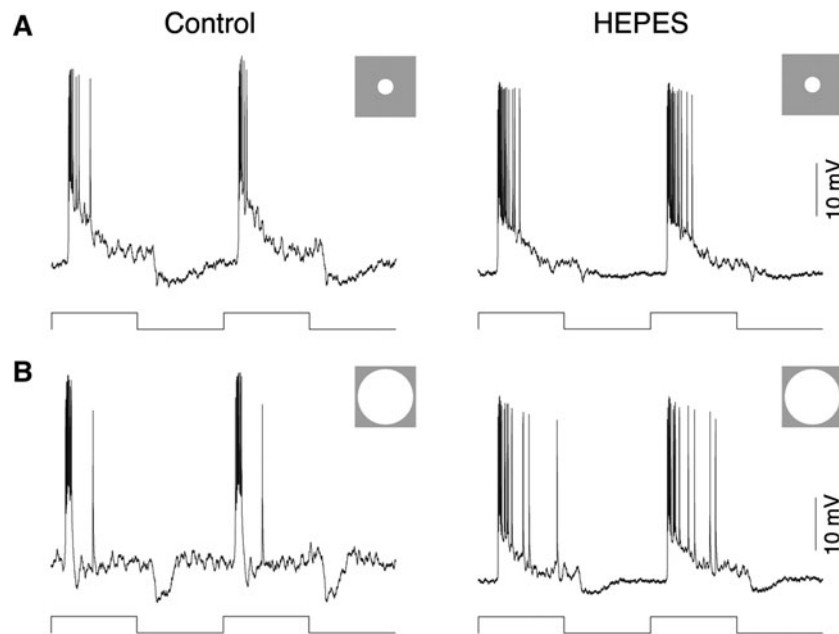


Fig. 7 HEPES attenuates parasol ganglion cell (GC) surround. **A** An ON parasol GC in macaque monkey retina was illuminated to a 300- μm -diameter spot light (stimulus trace below) and its ON responses in the absence (*left*) and presence (*right*) of 20 mM HEPES were obtained by intracellular recording. The responses were similar in both conditions. **B** Responses of a parasol GC to a larger 2,000- μm -

diameter spot (stimulus trace below) were obtained in the absence (*left*) and presence (*right*) of HEPES. The control response was small and transient because of surround antagonism. The response in HEPES was larger and more sustained, indicating diminished surround antagonism (from Davenport et al. [64])

A hypothesis emerged: do protons released by HCs mediate the surround signal?

As described above, the external pH clamp reliably suppresses surround/feedback response in photoreceptors and other retinal neurons [28, 51, 52, 54, 55, 64]. The most plausible interpretation of the effects of an external pH clamp is that the surround response is modulated by pH changes in the invaginating synaptic clefts at cone terminals, and thus the following sequence has been proposed. In a normal bicarbonate-buffer solution, pH in the synaptic cleft is slightly more acidic (by about pH 0.2–0.7: see below) than the pH in the large extracellular pool (\sim pH 7.4). Thus, cone I_{Ca} is relatively suppressed. The HC hyperpolarization caused by surround illumination neutralizes this acidic condition, thus restoring cone I_{Ca} and the surround response (Fig. 10A). On the other hand, in an external solution with high buffering capacity, pH in the synaptic cleft is already fixed to the same pH as that of the extracellular pool even in darkness, and thus the enhancement of cone I_{Ca} by surround illumination does not occur. Surround illumination shifts the activation curve of cone I_{Ca} to negative voltage by 2.5–7.5 mV (e.g. [17, 28]), which corresponds to the alkalization-mediated shift of pH by 0.2–0.7 (estimated from [49]) in a pH 7.4 external

solution. The pH change in the invaginating synapse may be prominent because the microenvironment in the invaginating synaptic cleft is relatively isolated from these systemic changes. Furthermore, a tiny proton extrusion interrelated with HC membrane potentials could induce a large change in pH enough to decrease because of the narrow space of the invaginating synapses. In fact, the volume of the invaginating synaptic cleft is roughly estimated in the range of 3×10^{-18} l [75], a volume in which approximately two protons give rise to pH 6 [54].

Modulation of cone I_{Ca} by the intersynaptic pH changes may be strategic for direct control of the amount of transmitter release. That is, the modulatory effect of protons on Ca channels at the cone synaptic terminal has the dual roles of contributing to the formation of the receptive field surround in the cones and of controlling transmitter release from the cones to HCs/BCs (Fig. 10). External protons also inhibit the glutamate response of AMPA type receptors by increasing steady-state desensitization [76]. However, the IC_{50} values for proton inhibition exceed the physiological range (pH 5.7–6.3) [77]. Thus, during the generation of the cone surround response, pH changes in the cone synaptic clefts may affect the presynaptic cone Ca channels selectively, but not the postsynaptic glutamate (AMPA/kainate type) receptors on HCs and OFF-type BCs.

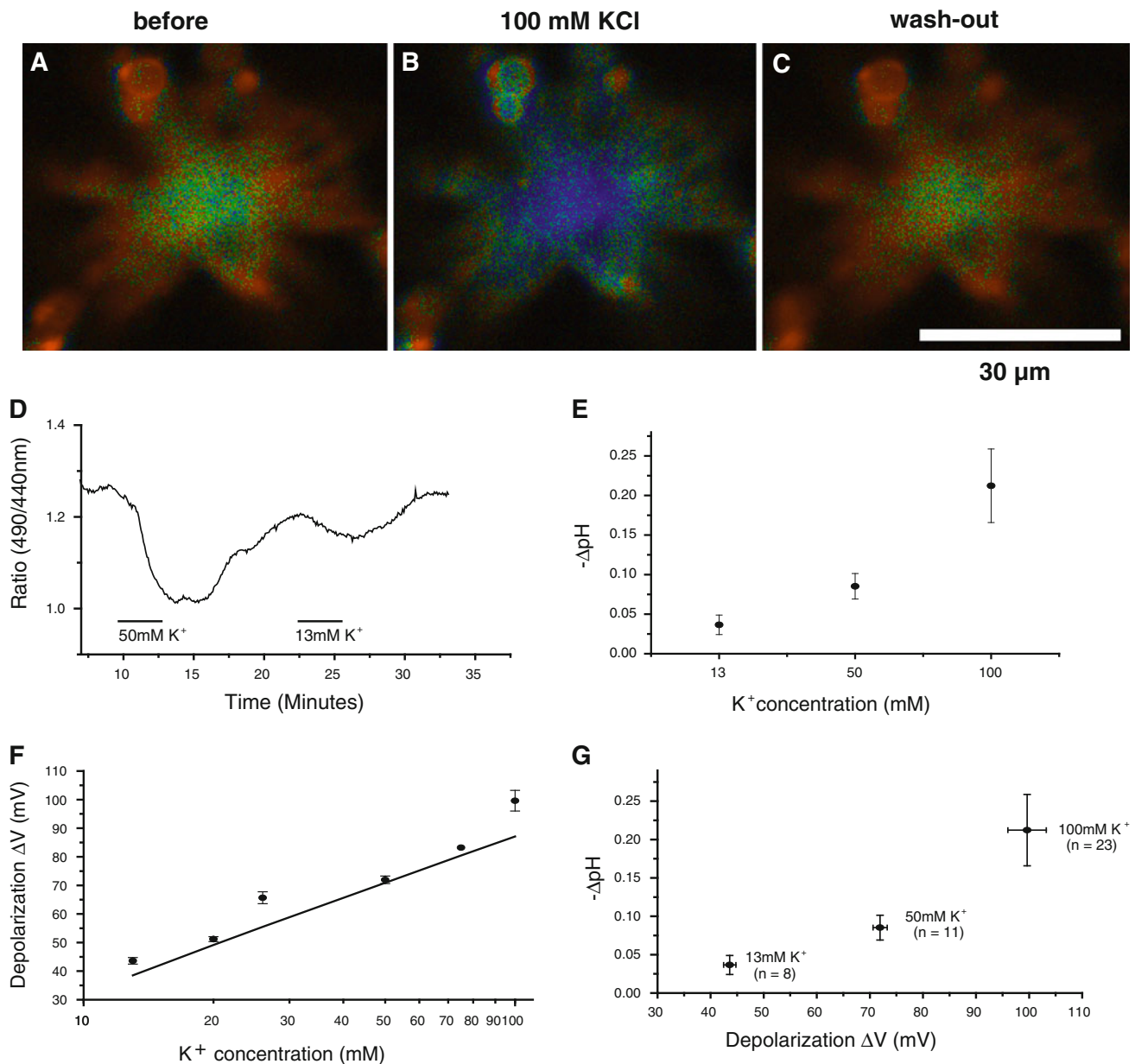


Fig. 8 Acidification of extracellular surface of cone-driven HCs dissociated from goldfish retinas by high-K⁺-induced membrane depolarization. **A–C** A series of time-dependent surface pH (pH_s) changes of an HC (pH_s reduction: 0.10 pH units from 7.60 during 100 mM K⁺ application for 3 min). The pH_s change is measured by the ratio imaging method (*bluish and yellowish colour* corresponds to the lower and higher pH_s, respectively). **D** Acidification responses of the HC extracellular surface serially induced by 2 min bath applications of 50 and 13 mM KCl containing Ringers to depolarize the membrane potential. **E** Acidification of the measured HC extracellular

surface is dependent on the KCl concentration. **F** HC membrane depolarization is dependent on the KCl concentration measured by patch-clamp study in current-clamp mode in comparison with the control Ringer having 2.6 mM KCl. *Straight line* Nernst equation for K⁺ shown by $V = \frac{RT}{F} \log \frac{[K^+]_o}{2.6}$. **G** HC acidification induced by high-K⁺ is dependent on the membrane depolarization. This graph was composed by combining *E* and *F* at 13, 50 and 100 mM KCl (from Jouhou et al. [29])

Extracellular acidification driven by depolarization of HCs

This proton hypothesis is supported by the existence of V-ATPase on the plasma membrane of HCs as a proton-releasing mechanism [29, 30]. In order to investigate

whether the membrane polarization of HCs really modulates their extracellular surface pH, the pH-sensitive fluorescent dye 5-*N*-hexadecanoyl-aminofluorescein (HAF), which sticks to the cell membrane surface [78] like a detergent molecule, was used to measure pH at the immediate external surface (pH_s) of dissociated goldfish or

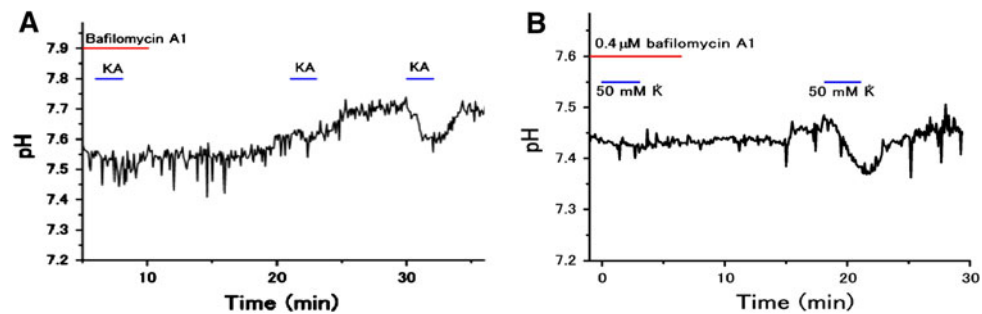


Fig. 9 Bafilomycin A1, a specific inhibitor of V-ATPase inhibitor, blocks extracellular surface acidification of goldfish HCs induced by 20 μM kainate or 50 mM K^+ . **A** Kainate-induced acidification is blocked by 0.4 μM bafilomycin A1, and partially recovered after

10 min wash-out and then fully recovered after 20 min wash-out; **B** 0.4 μM bafilomycin A1 suppressed 50 mM K^+ -induced HC acidification, which was recovered after 13 min wash-out (from Jouhou et al. [29])

A H^+ negative feedback onto cone terminals induced by HC polarization

Surround light	HC	V-ATPase at HC	Synaptic cleft $[\text{H}^+]$	Cone I_{Ca}	Transmitter release	OFF-BC	ON-BC
OFF	depolarize	\uparrow	\uparrow	\downarrow	\downarrow	hyperpolarize	depolarize
ON	hyperpolarize	\downarrow	\downarrow	\uparrow	\uparrow	depolarize	hyperpolarize

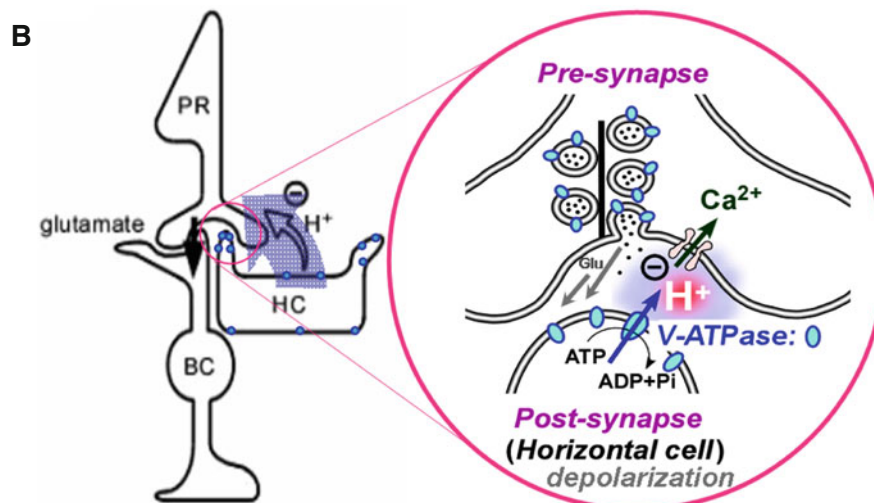


Fig. 10 Schematics of H^+ negative feedback onto BCs via H^+ concentration change at synaptic clefts of cone terminals induced by HC polarization. **A** H^+ negative feedback onto OFF- and ON-type BCs via the series of effects of depolarization or hyperpolarization of HCs. The depolarization or hyperpolarization of HCs is induced either by OFF or ON of light illumination, respectively. \uparrow denotes an activation or increase, whereas \downarrow denotes an inactivation or decrease. In the presence of HEPES, synaptic cleft pH changes will be suppressed and bring about a change to the opposite direction to the control effect of HC polarization. These effects of HEPES imply the

suppression of negative feedback ordinarily present in the control condition. **B** A schematic drawing of H^+ negative feedback onto BCs via the concentration increase of H^+ released from depolarized HCs into the synaptic cleft of cone terminals, illustrating OFF case in **A**. H^+ release is due to V-ATPase (a proton pump located on plasma membrane shown by blue circles in an expanded figure) on depolarized HCs, resulting in suppression of glutamate release from cones. V-ATPase is also localized at the membrane of synaptic vesicles at the cone terminals

carp HCs [29, 65]. Jouhou et al. [29] found by a ratio imaging method that depolarization of HC (by 72 or 100 mV) induced by high K^+ Ringer (50 or 100 mM, respectively) caused acidification of the HC extracellular surface (by 0.09 and 0.21 pH units, respectively). It is thus

evident that the degree of surface acidification is dependent on HC membrane depolarization (Fig. 8). Depolarization stimulated by glutamate or kainate also induced extracellular acidification of HCs. These observations of surface acidification suggest an existence of voltage-dependent

proton efflux from the cell [29]. The increase of proton concentration at the membrane surface occurred within 2.5 s, a limit of time resolution of their measurements [29]. Much faster pH change could be revealed if a faster photo-detector was available for the pH imaging.

Jacoby et al. [79] measured the pH of dissociated cone-driven HCs of catfish stained with HAF and claimed that depolarization of the cell induced an intracellular acidification and extracellular alkalization of HCs. They claimed that HAF molecules resided inside the cell. If their logic is correct, hyperpolarization of HC will induce acidification of the synaptic cleft. This is opposite to what Jouhou et al. [29] detected and cannot account for I_{Ca} increase after HC hyperpolarization.

Involvement of vacuolar-type proton pump in HC surface pH change

Extracellular surface acidification of dissociated fish HCs depolarized by the application of kainate or high- K^+ containing Ringer was suppressed by 0.4 μ M bafilomycin A1, a highly specific and potent inhibitors of V-ATPase (proton pump, H^+ -ATPase) in a dose-dependent manner (Fig. 9) [29]. Bafilomycin A1 is known to be effective on V-ATPase at nM concentration [80, 81]. This finding suggests that the proton extrusion mechanism is primarily due to V-ATPase driven by membrane depolarization. Contribution of either a Na^+/HCO_3^- co-transporter or a H^+ channel, both voltage-dependent mechanisms, was not likely the major source of the depolarization-induced acidification of the HC surface, since high K^+ Ringer-induced extracellular acidification was blocked neither by Na-free Ringer, bicarbonate-free Ringer nor 0.2 mM $ZnCl_2$ (an H^+ channel blocker)-containing Ringer [29].

V-ATPase is known to be present on the plasma membrane as well as subcellular organelles where it was originally found [82]. In fact, isolated HCs of goldfish retina are immunoreactive for V-ATPase [29]. Furthermore, plasma-membrane V-ATPase was detected on HC dendritic tips in the invaginating synapses of cone terminals as well as on HC somata in fish and monkey retinas [30]. Intracellular Ca^{2+} imaging of photoreceptor cells revealed that activation of V-ATPase by hydrogen sulfide, whose synthase is localized in the retina, enhanced proton release, resulting in the suppression of Ca^{2+} channels serving to prevent cell death brought about by excess irradiation [83].

Based on these results, we conclude that the suppression of cone I_{Ca} by kainate-induced HC depolarization [28] would be due to acidification of the synaptic cleft of cone terminals brought about by proton release via V-ATPase from the HC surface (Fig. 10) [29]. Whereas the enhancement of cone I_{Ca} by HC hyperpolarization induced

by the application of CNQX or by surround illumination [28] is due to alkalization of the synaptic cleft of cone terminals; this alkalization is ascribed to suppression of proton release from HCs, or moving protons into the HCs through ion channels or proton transporters on the HC plasma membrane. In the latter case, V-ATPase would be a reversible coupling device that can also utilize the proton gradient energy for ATP synthesis, dependent on the thermodynamics of the proton pump (e.g., [84, 85]). However, other possibilities, such as a release of alkalizing molecules from HC surface, cannot be excluded.

The Ca^{2+} pump (Ca^{2+}/H^+ -ATPase) has been proposed to work in rod- and cone-driven HCs in skate and catfish retinas, respectively [86, 87]. This Ca^{2+} pump in the plasma membrane of HCs would induce extracellular alkalization at the photoreceptor synaptic clefts in darkness and potentiate transmitter release from the photoreceptors via enhancement of I_{Ca} . Such an extracellular alkalization mechanism proposed by Malchow and his colleagues [79, 86, 87] would facilitate positive feedback upon photoreceptors and, thus, could not explain the formation of center-surround antagonistic receptive field in BCs. Moreover, the Ca^{2+} pump is a type of electro-neutral (non-electrogenic) pump, and thus it is not dependent on the membrane potential of HCs. Therefore, this mechanism does not seem to play a major role in the electric signal processing between HCs and cones.

Criticisms: what are the problems of pH-clamp experiments and proton hypothesis?

As pointed out by Kamermans and Fahrenfort [56] and Fahrenfort et al. [57], the external pH clamp with a solution containing high buffer capacity might also somehow influence metabolic activity controlling intracellular pH. These possible changes in intracellular pH might affect pH-sensitive components including several types of voltage-dependent ion channels, ligand-gated ion channels and transporters, all of which might induce secondary effects. One concern is that a nonspecific effect of an external pH clamp might result in closure of connexin 26 (carp, [21]) or 55.5 (zebrafish, [88]) in HC dendritic tips induced by intracellular acidification and the blockade of the ephaptic effect. However, in macaque retina, Davenport et al. [64] found that external HEPES increases monkey H1 receptive field size rather than decreasing it. In a study on the carp HCs, Yamamoto et al. [89] reported that HEPES increases the receptive field size and the dye coupling. These observations concerning HEPES seem to be inconsistent with the idea that HEPES induced intracellular acidification and closure of hemichannels/connexins in the HCs.

According to the proton hypothesis, it is expected that intrinsic systems for pH buffering, the bicarbonate/carbonic anhydrase system, may regulate the amplitude of the surround response in cones. Fahrenfort et al. [57] thus studied the effect of interfering with the endogenous pH buffer and reported that benzolamide, an inhibitor for carbonic anhydrase, failed to enhance the cone feedback responses in goldfish retina. On the basis of intrinsic pH-buffering of the bicarbonate/carbonic anhydrase system, however, removal of bicarbonate from the perfusate would have been expected to result in intracellular acidification and, therefore, blockade of connexin as well as Ca^{2+} channels. However, the feedback signals in zebrafish cone terminals, measured by Ca imaging, were observed even in the absence of external bicarbonate [54]. This result indicates that the bicarbonate/carbonic anhydrase system does not seem to contribute to the proton feedback mechanism significantly.

HCs and GABA

In the lower vertebrate retina, synaptic vesicle-like structures containing GABA have not been found in HC dendritic tips. Thus, it has been a matter of debate whether non-vesicular release of GABA from HCs, mediated by plasma membrane transporters [90], would also be involved in the formation of the receptive field surround in cones. In lower vertebrates, however, there is a component of evidence that does not require GABA because GABA antagonists fail to suppress surround response in cones [17, 28]. Tiny GABA-mediated synaptic inputs from HCs to cones were detected in turtles only when the GABAergic inputs to cones were pharmacologically potentiated by pentobarbital [91]. Thus, under physiological conditions, GABA may not play a major role as the feedback messenger in the HC-cone synapses. GABA can, however, change membrane voltages of the HCs by modulating GABA-gated chloride currents and GABA transporter currents in the HCs [92–94]. Thus, it is assumed that GABA may play some role in the information processing in the outer retina by modulating activities of HCs as an autocrine messenger.

In the mammalian retina, GABA in HCs is more controversial. In fact, the vesicular GABA transporter, which mediates the uptake and storage of GABA in synaptic vesicles [95], is reliably expressed in the mammalian HCs [96–99], but GABA plasma membrane transporter has not yet been identified [3, 100, 101]. Furthermore, SNARE-proteins, release machinery for exocytosis, are also present in the mammalian HCs [102–104], suggesting that mammalian HCs may have the capacity to utilize a regulated exocytosis of GABA. However, as reported in lower

vertebrates, GABA antagonists fail to suppress surround response in macaque cones [105]. Thus, mammalian HCs might utilize an alternative mechanism of transmitter release although the physiological role of the presumable exocytosis of GABA is unknown.

What remains to be elucidated?

Although accumulating evidence that an external pH clamp reliably suppresses the surround/feedback response in photoreceptors and other retinal neurons [28, 51, 52, 54, 55, 64] provides strong support for the proton hypothesis of surround inhibition, it is insufficient to prove the hypothesis. Problems are the lack of (1) experimental evidence for the proton concentration change in the invaginating synapse at photoreceptor terminals, and that the pH change is large enough to control Ca^{2+} channels, and (2) evidence that the speed of the pH change following HC depolarization or hyperpolarization is sufficiently rapid to account for the surround response of BCs and other retinal neurons.

To date, enriching pH buffers in the external solution is the most popular method to clamp extracellular pH. However, it is hard to exclude the possibility that this extracellular pH clamp may induce secondary effects on the still unknown feedback mechanisms by affecting any other buffer-sensitive components. This means that a variety of approaches to clamp extracellular pH should be necessary to ascertain the proton hypothesis.

We do not know whether proton feedback is involved in the receptive field surround in all types of BCs and GCs. In all mammalian retinas, the classes of BCs and GCs are each further subdivided; there are 3 to 5 distinct types of ON-type BCs, 3 to 5 types of OFF-type BCs and more than 10 to 15 morphologically different types of GCs [106]. Thus, it is likely that heterogeneous synaptic circuitry, in addition to feedback circuit from HCs to photoreceptors, may participate in the formation of receptive field surround in some BCs [107] and GCs. Much remains to be clarified about the mechanisms underlying the receptive field properties of BCs and GCs—despite a very basic feature of visual functions.

In summary, we would like to propose, at this moment, a hypothesis that intersynaptic H^+ concentration changes in the invaginating synaptic clefts at photoreceptor terminals contribute to the generation of the receptive field surround in the outer retina. Although a major breakthrough in research methodology is necessary to prove the proton feedback hypothesis beyond a doubt, protons appear to be the most likely candidate as the messenger in one of the most fundamental steps in visual processing. We would like to assume that there are other proton-mediated mechanisms in the nervous system, since protons have

been shown to be a novel intercellular messenger, e.g., in muscle contraction in *C. elegans* [108].

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