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Protein kinase C modulates synaptic vesicle acidification in a ribbon type nerve terminal in the retina

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Abstract

The driving force for neurotransmitter accumulation into synaptic vesicles is provided by the generation of a transmembrane electrochemical gradient ($\Delta\mu\text{H}^+$) that has two components: a chemical gradient (ΔpH , inside acidic) and an electrical potential across the vesicular membrane ($\Delta\Psi$, inside positive). This gradient is generated in situ by the electrogenic vacuolar H^+ -ATPase, which is responsible for the acidification and positive membrane potential of the vesicle lumen. Here, we investigate the modulation of vesicle acidification by using the acidic-organelle probe LysoTracker and the pH-sensitive probe LysoSensor at goldfish Mb-type bipolar cell terminals. Since phosphorylation can modulate secretory granule acidification in neuroendocrine cells, we investigated if drugs that affect protein kinases modulate LysoTracker staining of bipolar cell terminals. We find that protein kinase C (PKC) activation induces an increase in LysoTracker-fluorescence. By contrast, protein kinase A (PKA) or calcium/calmodulin kinase II (CaMKII) activation or inhibition did not change LysoTracker-fluorescence. Using a pH-dependent fluorescent dye (LysoSensor) we show that the PKC activation with PMA induces an increase in LysoSensor-fluorescence, whereas the inactive analog 4alpha-PMA was unable to cause the same effect. This increase induced by PMA was blocked by PKC inhibitors, calphostin C and staurosporine. These results suggest that phosphorylation by PKC may increase synaptic vesicle acidification in retinal bipolar cells and therefore has the potential to modulate glutamate concentrations inside synaptic vesicles.

Keywords

Retina; Bipolar cell; PKC; Acidification; Confocal microscopy; Synaptic vesicle

1. Introduction

Information exchange at chemical synapses is mediated by messengers released from synaptic vesicles during regulated secretion. When an action potential invades the nerve

terminal, a rapid and local increase of intracellular calcium triggers the fusion of neurotransmitter-loaded vesicles with the plasma membrane (Katz and Miledi, 1969). In order to avoid depletion of synaptic vesicles and interruption of neurotransmission, vesicles must be reinternalized (endocytosis), recycled, acidified and refilled with neurotransmitter in a rapid fashion (Sudhof, 2004). Recycled synaptic vesicles must be refilled with neurotransmitters by special transporters that take up cytosolic neurotransmitter upon the generation of a transmembrane electrochemical gradient ($\Delta\mu\text{H}^+$) generated *in situ* by the electrogenic vacuolar H^+ -ATPase ($\text{V-H}^+\text{ATPase}$; Liu and Edwards, 1997). The $\Delta\mu\text{H}^+$ has two components: a chemical gradient for protons (ΔpH , inside acidic) and an electrical potential across the vesicular membrane ($\Delta\Psi$, inside positive) (Liu and Edwards, 1997; Rudnick, 1998). Although there is still controversy on whether glutamate uptake is driven solely by $\Delta\Psi$ (Maycox et al., 1988; Hartinger and Jahn, 1993) or by both $\Delta\Psi$ and ΔpH of the $\Delta\mu\text{H}^+$ (Naito and Ueda, 1985; Tabb et al., 1992; Wolosker et al., 1996), it appears that the $\Delta\Psi$ has the primary role for glutamate uptake (Cousin and Nicholls, 1997). However, ΔpH may still be essential to antagonize glutamate efflux and retain glutamate inside the vesicles (Wolosker et al., 1996).

It is unknown whether phosphorylation and dephosphorylation can control vesicular acidification in nerve terminals. Considering that neuronal activity can effectively change cycles of phosphorylation and dephosphorylation in nerve endings, it is important to evaluate the potential of such modulation of synaptic vesicles acidification. Here we use the large nerve terminals of the goldfish Mb bipolar retinal neurons, which contain nearly 10^6 synaptic vesicles (von Gersdorff et al., 1996), to investigate the potential of phosphorylation cycles in modulating synaptic vesicle acidification. Therefore, this study provides a new methodology to investigate molecular mechanisms that can modulate the amount of glutamate inside synaptic vesicles.

2. Experimental procedures

2.1. Drugs and chemicals

LysoSensor Green® DND 189 (100 nM) and LysoTracker Red® DND-99 (100 nM) were purchased from Molecular Probes, Eugene, OR; methylamine (10 nM), staurosporine (100 nM) and bafilomycin (500 nM) were purchased from Sigma-Aldrich. SP-cAMP (100 nM), RP-cAMP (100 nM), KN93 (1 μM), PMA (100 nM and 1 μM), alpha-PMA (100 nM) and calphostin C (100 nM) were obtained from Calbiochem (Germany). All other chemicals and reagents were of analytical grade.

2.2. Preparation of retinal bipolar cells

Bipolar cells were acutely dissociated from the retina of goldfish (*Carassius auratus auratus*) according to von Gersdorff and Matthews (1994). Briefly, 10–15 cm standard length goldfishes were decapitated, the eyes were removed and the retinas were dissected and placed in Fish Ringer (120 mM NaCl, 2.5 mM CaCl_2 , 2.5 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 10 mM HEPES, pH 7.3). The retinas were then exposed to hyaluronidase (0.1 mg/ml) for 20 min and after washing in Fish Ringer, the retinas were cut in small pieces and digested in solution containing papain (2.5 mg/ml) and cysteine free-base. After digestion with papain, retina pieces were washed in Fish Ringer, dissociated with glass Pasteur pipette and maintained on ice until use. Three or four pieces of retina were dissociated each time, plated in a coverslip and mounted in a perfusion chamber.

2.3. Fluorescence imaging of synaptic vesicle acidification

The LysoTracker (LT) and LysoSensor (LS) are fluorescent acidotropic probes that accumulate inside acidic secretory vesicles (Cousin and Nicholls, 1997; Giner et al., 2007).

These probes have several important features, including high selectivity for acidic organelles and effective labeling of live cells at nanomolar concentrations.

LT is a fluorophore linked to a weakly basic amine that selectively accumulates in cellular compartments with low internal pH. Retinal bipolar cells were stained with LT (100 nM) for 10 min and mounted in a perfusion chamber. When cells previously stained with LT were perfused with Fish Ringer without the fluorescent dye, a steep decrease of fluorescence was observed within few minutes (data not shown), indicating that the labeling is readily reversible. Therefore, in experiments with kinase activators and inhibitors, bipolar cell terminals were first labeled with LT for 10 min and then the cells were exposed to drugs for 10 min in the continuous presence of the dye. It is important to mention that after 10 min labeling, peak fluorescence levels were reached and further incubation with LT did not alter the levels of fluorescence (data not shown), suggesting that maximum labeling was attained within 10 min exposure to dye.

LysoSensor also accumulates in acidic organelles but exhibits a pH-dependent increase in fluorescence intensity upon acidification, in contrast of LT. The protocol used in our experiments was similar to that described by Cousin and Nicholls (1997). Bipolar cells were exposed to LS (100 nM) for 20 min, were then briefly washed and mounted in a perfusion chamber. In some control experiments, cells were incubated with bafilomycin, methylamine and NH_4Cl before and during LS loading. In experiments using PKC activator and inhibitors, bipolar cells were incubated for 15 min with drugs before and during staining with LS. Fluorescence imaging was achieved with a Zeiss water immersion objective (40 $\times$, 1.2 NA) or Zeiss oil immersion objective (63 $\times$, NA) coupled to a Zeiss LSM510 laser scanning confocal microscope (CEMEL-UFMG) or a Biorad MRC1024 confocal system (Department of Pharmacology, ICB-UFMG). Cells were excited with a He/Ne laser selecting at 568 nm and emitted light was collected using a filter selecting for 598/40 nm for LT and images were captured at 450 nm excitation and emission at 510 nm for LS.

2.4. Imaging analysis

Fluorescence measurements were performed using the software Metamorph Imaging System 4.0 (Universal Imaging Corporation). Briefly, Z-sections of bipolar cells were obtained and a Z-section containing the in focus image of cell terminal was selected. We measured the fluorescence in nerve terminals by drawing a line and saving it as regions of interest (ROI) to be used at the subsequent images of the same cell after various treatments. By comparing ROI images before and after dye and drug treatments, we noticed that there were no significant modifications in nerve terminals diameter. The fluorescence measurements were expressed in grey levels (8 bit).

2.5. Statistics

Data are expressed as means values with standard errors from (*n*) number of cells. Statistical differences were analyzed using paired Student's *t*-test. *P*-values <0.05 were considered to be statistically significant.

3. Results

3.1. Staining of bipolar neurons with LysoTracker

Bipolar cells loaded with LT (100 nM) for 10 min exhibited a pronounced fluorescence in cell terminal (Fig. 1A). Staining at the cell body was much weaker than that of terminals (Fig. 1B) and likely reflects the presence of lysosomes or other acidic organelles. The fluorescence levels after 10 min in LT was not statistically different from that obtained

during 40 min of incubation with medium containing dye (not shown), indicating that the fluorescence signal was stable for this time period.

Since bipolar cell terminals are densely packed with small clear core synaptic vesicles (von Gersdorff et al., 1996), and these vesicles release protons via exocytosis (Palmer et al., 2003), we assume that these organelles are likely to be the major source of dye fluorescence. Indeed, Stenovec et al. (2007) demonstrated that LT staining colocalizes with the vesicular glutamate transporter (VGLUT1) in astrocytes indicating that the major fraction of LT fluorescent signal might be confined to synaptic vesicles. Thus, in the bi-dimensional images shown in this work, we assumed that the fluorescent signal along the cell terminals membrane might correspond to synaptic vesicles that are located near the plasma membrane.

If we consider that LT labels synaptic vesicles in retinal bipolar cells, the membrane permeable weak base NH_4Cl is expected to decrease the fluorescence intensity in cell terminal stained with this dye by dissipation of vesicular pH (Camacho et al., 2006). Bipolar cells pre-incubated with NH_4Cl (30 mM) for 10 min were stained with LT (100 nM) in association with NH_4Cl (30 mM) for 10 min (Fig. 2A second panel). NH_4Cl produced a steep decrease in the fluorescence intensity level of these cells (Fig. 2B). Bafilomycin, a H^+ -V-ATPase blocker that collapses the transmembrane electrochemical gradient (Moriyama and Futai, 1990), and methylamine, a hydrophilic weak that accumulate in acidic compartments eliminating pH gradients without dissipating the $\Delta\psi$ (Cousin and Nicholls, 1997) decreased the fluorescence signal in bipolar cell terminals labeled with LT (Vigh et al., in preparation). These data suggest that the LT fluorescence signal originates mainly from synaptic vesicles in bipolar cell terminals.

3.2. Protein kinase A (PKA) and calcium/calmodulin kinase II (CaMKII) do not alter LysoTracker staining of retinal bipolar neurons

Protein kinase A and calcium/calmodulin kinase II are second messenger-activated protein kinases implicated in mediating short- and long-term changes in synaptic transmission (Mayford et al., 1995; Weisskopf et al., 1994). PKA and CaMKII activation modulates exocytosis and increase neurotransmitter release in presynaptic terminals (reviewed by Leenders and Sheng, 2005). Tamir et al. (1994) have previously reported that PKA inhibitors interfere with vesicle acidification and secretion in parafollicular cells. In order to determine if PKA interferes on synaptic vesicle acidification in bipolar terminals, bipolar cells were pre-incubated for 10 min with SP-cAMP (100 nM), which is a potent membrane permeable activator of PKA, or with RP-cAMP (100 nM), a specific inhibitor of PKA (Wang et al., 1991). The nerve terminals that were treated with SP-cAMP exhibited similar staining to those terminals stained with LT alone (Fig. 2A, third panel). Fig. 2B shows that fluorescence of LT-stained bipolar terminals remained similar after treatment with SP-cAMP comparing to control. In addition, we did not observe any significant changes in fluorescence in bipolar terminals stained with LT in the presence of RP-cAMP (Fig. 2A fourth panel and Fig. 2B). No detectable changes in fluorescence were also observed when KN93 (1 μM), a selective CaMKII inhibitor (Sumi et al., 1991), was added to LT-stained bipolar neurons (Fig. 2A, fourth panel). Fig. 2B shows that KN93 do not cause significant change in fluorescence of LT-stained bipolar terminal.

3.3. PKC activation increases fluorescence in retinal bipolar terminals stained with LysoTracker

We next asked if other neuron enriched proteins kinases, such as protein kinase C (PKC), interferes with LT staining in bipolar cells. To investigate if phosphorylation by PKC modulates synaptic vesicle acidification in ribbon nerve terminals, we tested the effect of the PKC activator PMA (Phorbol 12-myristate 13 acetate) on LT staining of bipolar neurons. A

representative nerve terminal that was marked with LT for 10 min and then exposed to PMA (100 nM) for 10 min is shown in Fig. 3A (second panel). The increased fluorescence induced by PMA remained for at least 30 min (data not shown). The same results were observed with PMA at 1 μ M but did not at 10 nM (data not shown). Calphostin C (100 nM), a highly specific inhibitor of PKC (Kobayashi et al., 1989), did not cause any significant change in fluorescence after 10 min treatment indicating that this PKC-inhibitor alone does not interfere with LT staining (Fig. 3, third panel). However, when bipolar cells were stained with LT in the presence of calphostin C and then exposed to PMA and calphostin C associated, we could not detect the additional increase in signal observed by PKC activation with PMA (Fig. 3, fourth panel). In fact, after 10 min of exposure to PMA and calphostin C, LT stained neurons presented the same levels of fluorescence found in terminals labeled without any drug (Fig. 3B). The results above indicate that PKC activation might modulate synaptic vesicle acidification. However, considering that LT probes accumulate in acidic compartments but their increase in fluorescence is not largely dependent on pH, we had to confirm this result using a pH-sensitive probe. We therefore chose LS, which exhibit a pH-dependent increase in fluorescence, to confirm that PKC phosphorylation increases synaptic vesicle acidification in retinal bipolar neurons.

3.4. PKC activation increases fluorescence in retinal bipolar terminals stained with LysoSensor

Fig. 4A shows a representative bipolar cell stained with LS (100 nM) for 20 min. As observed with LT labeling, LS fluorescence was stronger in cell terminal if compared to cell body (Fig. 4B). The cell terminals fluorescence peak reached a maximum value at 20 min and no major alterations were observed during 40 min experiments (data not shown). The pattern of LS fluorescence staining was quite similar of LT and some spots are visible along the nerve terminal.

We performed a series of control experiments with methylamine (10 nM), NH_4Cl (30 mM) and bafilomycin (500 nM) to confirm that LS fluorescence changes according to synaptic vesicles proton-electrochemical gradient. Cells were pre-incubated with these pharmacological agents for 10 min and exposed to LS (100 nM) for 20 min associated with these drugs (Fig. 5). In the presence of the three pharmacological agents, nerve terminals showed less fluorescence compared to control (Fig. 5A). The reduction in fluorescence staining with LS was statistically significant in the three experimental conditions if compared to their controls (see Fig. 5B). These results are in agreement with that obtained by Cousin and Nicholls (1997) in granule cells from cerebellum and suggest that in our model, LS labels acidic synaptic vesicles and that its fluorescence is dependent upon the vesicular pH. We next performed experiments with PKC activator and inhibitors using LS to monitor pH-dependent fluorescence variation. As previously observed with LT, PMA (100 nM) induced a significant increase in LS fluorescence after 20 min of incubation with the drug (Fig. 6A, second panel) if compared to a control nerve terminal (Fig. 6A-first panel). At lower PMA concentration (10 nM), there was no statistically significant increase in fluorescence at nerve terminal (data not shown). Calphostin C (100 nM) alone did not alter LS labeling in cell terminals (Fig. 6A-third panel). However, PMA was unable to increase LS fluorescence when cells were pre-incubated with calphostin C for 10 min (Fig. 6A-fourth panel). We have also performed experiments where the PKC activator and inhibitors were exposed to bipolar cells after LS loading and the same results were acquired for both conditions. We used DIC images to monitor ruffling of the membrane surface and accumulation of vacuoles in the periphery of the terminal as observed elsewhere (Holt et al., 2003). No major alterations in the surface membrane were observed after PKC activation with PMA for 30 min experiments (data not shown). This result indicates that, at least for 30 min, no detectable new acidic compartments (i.e., vacuoles or endosomes) were formed in

the cell terminals surface. We explored the effects of PMA using the broad spectrum PKC inhibitor, staurosporine (100 nM). No differences in fluorescence levels were observed between terminals that were stained with LS alone or in the presence of PMA plus staurosporine (Fig. 6B). 4alpha-PMA (100 nM), the inactive analog of PMA, did not increase LS fluorescence compared to control conditions (Fig. 6B). These results show that activation of PKC increases LS fluorescence and consequently PKC phosphorylation might modulate synaptic vesicles acidification in retinal bipolar cells.

4. Discussion

In the present work we used the fluorescent probes LT and LS to optically detect synaptic vesicle acidification in retinal bipolar cell terminals. LT can be employed to visualize acidic vesicles (Giner et al., 2007; Stenovec et al., 2007) whereas LS is more suitable for visualizing pH-dependent changes in vesicle acidification.

We examined whether intracellular signaling pathways that are known to control synaptic plasticity by presynaptic effects can alter LT fluorescence. We found that activation of PKC by phorbol esters increased LT fluorescence and that this effect was antagonized by the PKC inhibitor calphostin C. In contrast, drugs that activate or inhibit PKA or CaMKII did not affect LT fluorescence, suggesting that control of synaptic vesicle acidification might be selectively regulated by PKC. We confirmed that PKC activation increased synaptic vesicle acidification in a pH-dependent fashion using LS. We observed that activation of PKC by phorbol esters increase LS fluorescence and that this effect was antagonized by PKC inhibitors calphostin C and staurosporine. Furthermore, 4alpha-PMA did not produce significant alterations in LS fluorescence.

PKC activation has been demonstrated to facilitate transmitter release in a number of neurons (see Byrne and Kandel, 1996) and this potentiation is mainly due to the phosphorylation of proteins involved in the release process distal to Ca^{2+} entry, presumably those involved in vesicle dynamics (Majewski et al., 1997; Majewski and Iannazzo, 1998). One of the modulatory effects of the PKC activation in the secretory facilitation is on neurotransmitter release potentiation. Aspirin, for example, activates PKC in rat hippocampal isolated nerve terminals which subsequently enhances Ca^{2+} influx and increases glutamate release (Wang, 2006). The phorbol ester PMA potentiates glutamate release from goldfish bipolar cells by an increase in the amount of releasable transmitter, but this occurs through a step downstream to Ca^{2+} entry (Minami et al., 1998; Tachibana, 1999). Bipolar cells have two components of transmitter release: a fast, initial component and a larger but slower component (von Gersdorff et al., 1998). It is only the slower component that is potentiated by PKC (Minami et al., 1998). Since the slow component of transmitter release may reflect the recruitment of synaptic vesicles to the ribbon-type active zones, it has been suggested that the potentiation of transmitter release by PMA application may be due to the modification of the recruitment process of vesicles towards synaptic ribbons or the increase in the number of vesicles attached to synaptic ribbons (Mennerick and Matthews, 1996; von Gersdorff et al., 1996). However, more recently it has been shown that PKC activation potentiated the slow component of transmitter release and increased the number of synaptic vesicle fusion events specifically outside the ribbon regions in bipolar neurons (Midorikawa et al., 2007).

PKC activation also is involved in actin cytoskeleton morphology in bipolar cells since phorbol esters accelerated growth of F-actin. However, F-actin disruption with cytochalasin D did not affect the cycling of synaptic vesicles (Job and Lagnado, 1998).

Moreover, PKC could enhance transmitter release by regulating the size of the releasable pool of vesicles. Stevens and Sullivan (1998) have shown that phorbol esters increased the size of the readily releasable pool and accelerated the refilling of empty sites at glutamatergic hippocampal synapses, contributing to synaptic potentiation. PKC also increased the pool size of the releasable vesicles in bipolar cells, an increase more restricted to the slow component of exocytosis and without significant effect on endocytosis (Berglund et al., 2002).

Thus, PKC acts in distinct targets in order to facilitate transmitter release and it could participate in other steps prior to vesicle fusion that are not well characterized, including those that involve neurotransmitter uptake and synaptic vesicle acidification. In this study, we report that PKC modulates synaptic vesicle acidification in bipolar cells and these results might have implications in neurotransmitter release facilitation acquired by PKC activation. Glutamate transport into synaptic vesicles is driven by $\Delta\mu\text{H}^+$ and it seems that the $\Delta\Psi$ has the primary role for glutamate uptake (Maycox et al., 1988; Tabb et al., 1992) and accumulation into vesicles, whereas ΔpH is essential to antagonize efflux and retain glutamate inside vesicles (Wolosker et al., 1996). We propose that changes in vesicle acidification may participate in neurotransmitter release, perhaps by decreasing glutamate leakage from synaptic vesicles, which would contribute to transmitter release potentiation. There are many levels of control of vesicular storage and filling, including vesicular transporter number, cytoplasmic concentration of transmitter and other molecules that act on vesicular chloride ion channels or chloride binding sites on VGLUTs (see Erickson et al., 2006). Synaptic vesicle acidification might be another way to indirectly adjust vesicular neurotransmitter content and release.

Our results suggest that calcium influx into the bipolar cell terminal may activate PKC and thus lead to increased acidification of synaptic vesicles. This may then lead to increased levels of glutamate inside bipolar cell synaptic vesicles. Mechanisms that modulate calcium influx into terminals may thus indirectly control synaptic strength through a modulation of quantal size. Our findings thus reveal a new mechanism for potentiating glutamatergic synapses.

Searching for phosphorylation and dephosphorylation protein target in bipolar terminals synaptic vesicles is beyond the scope of this work, however, three candidates are certainly the most conspicuous targets: the vesicular chloride channel (CLC-3), V-H⁺ATPase and VGLUT. Although a recent proteomic analysis failed to detect chloride channels in rat brain purified synaptic vesicles (Takamori et al., 2006), a previous work have shown that a member of the chloride channels family (CLC-3) is highly expressed in rat brain and retina (Stobrawa et al., 2001). In fact, CLC-3 knock-out mice are blind (Stobrawa et al., 2001). Vesicular acidification is increased by chloride uptake (Maycox et al., 1988) and if Cl⁻ channels contribute to synaptic vesicle acidification, we would expect that a blocker of this channel would decrease LS fluorescence. However, we did not observed any changes in LS fluorescence at bipolar synaptic vesicles in the presence of the Cl⁻ channel blocker NPPB (data no shown), so maybe Cl⁻ channels might not regulate synaptic vesicle acidification in retina bipolar cell terminals.

One candidate that could control synaptic acidification by phosphorylation is the V-H⁺ATPase. Despite the remarkable diversity of functions that V-H⁺ATPase serve, knowledge of their regulation and in particular their integration into cellular signaling networks is limited (Forgac, 2000). Although it is possible to identify PKC consensus sites for phosphorylation by sequence analysis, the phosphorylation of V-H⁺ATPase subunits by protein kinases has not been demonstrated so far (Hong-Hermesdorf et al., 2006). Phosphorylation may directly alter the activity of the V-H⁺ATPase (Arai et al., 1988)

enhancing proton pump and consequently increasing ΔpH . Therefore, PKC phosphorylation of V-H⁺ATPase could constitute a possible mechanism involved in increasing LS fluorescence in bipolar cells.

Another potential phosphorylation target for PKC is the vesicular glutamate transporter (VGLUT), which belongs to a family of the type I phosphate transporter that presents three isoforms (VGLUT1-3). VGLUT1 and VGLUT2 have a complementary distribution in rodent adult brain (Freneau et al., 2001; Herzog et al., 2001; Hallberg et al., 2006). Membrane topology analysis of VGLUT2 revealed potential sites of phosphorylation by PKC and casein kinase II, which could regulate transport activity and trafficking (Jung et al., 2006; Fei et al., 2007). Glutamate-induced increase in acidification was detected in synaptic vesicles (Maycox et al., 1988) and in microvesicles from bovine pineal glands (Moriyama and Yamamoto, 1995), suggesting that an increase in VGLUT transport activity would cause an increase in ΔpH . Indeed, VGLUT1 and PKC colocalize in ON bipolar terminals in rodent retina (Johnson et al., 2003). Thus, VGLUT phosphorylation by PKC could be another mechanism for increasing LS fluorescence in retinal bipolar cells.

In conclusion, this work shows that intracellular signaling mechanisms can regulate synaptic vesicles acidification. Considering that ΔpH is essential to antagonize glutamate efflux, we propose that such an increase in acidification via PKC phosphorylation will likely promote glutamate retention inside synaptic vesicles.

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Abbreviations

CALP	calphostin C
LS	LysoSensor Green®
LT	LysoTracker Red®
PMA	phorbol 12-myristate 13-acetate
STAU	staurosporine
V-H⁺ATPase	electrogenic vacuolar H ⁺ -ATPase
$\Delta\mu\text{H}^+$	transmembrane electrochemical gradient
ΔpH	chemical gradient
$\Delta\Psi$	electrical potential
4α-PMA	4 α -phorbol 12-myristate 13-acetate

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Fig. 1.

LysoTracker stains preferentially retinal bipolar terminals. (A) DIC image of a representative bipolar Mb cell (left). The same cell was stained with LT (100 nM) for 10 min (right). (B) Graphic showing increased LT fluorescence levels in cell terminals compared to cell bodies ($*P < 0.05$) after 10 min of LT exposure. $n = 7$ cells. Scale bar = 10 μM .

Fig. 2.

PKA and CAMKII do not change LysoTracker fluorescence in retinal bipolar terminals. (A) Panel showing representative cell terminals stained with LT 100 nM (first panel) in association with NH₄Cl 30 mM (second panel); SP-cAMP 100 nM (third panel), RP-cAMP 100 nM (fourth panel), and KN93 1 μ M (fifth panel). Scale bar = 10 μ M. Upper row, DIC images; lower row, fluorescence images. (B) Graphic showing that cells exposed to NH₄Cl were less stained with LT compared to control (* P < 0.05; n = 8) whereas SP-cAMP (P > 0.05; n = 10), RP-cAMP (P > 0.05; n = 9) and KN93 (P > 0.05; n = 9) did not cause significant changes in fluorescence in retinal bipolar terminals if compared to their controls.

Fig. 3.

Activation of PKC increases LysoTracker fluorescence in retinal bipolar terminals. (A) Panel showing representative cell terminals of different cells stained with LT 100 nM (first panel) and exposed to PMA 100 nM (second panel); calphostin C 100 nM (third panel) and calphostin C + PMA (fourth panel). Scale bar = 10 μ M. Upper row, DIC images; lower row, fluorescence images. (B) Bar graph showing that PMA increased LT fluorescence compared to control (* $P < 0.05$; $n = 8$) while cells exposed to calphostin C ($P > 0.05$; $n = 9$) and calphostin C + PMA ($P > 0.05$; $n = 9$) did not have their fluorescence changed if compared to their controls.

Fig. 4. LysoSensor staining of retinal bipolar terminals. (A) DIC image of a representative bipolar Mb cell (left). Same cell after staining with LS (100 nM) for 20 min (right). (B) Graphic showing increased LS fluorescence levels in cell terminals compared to cell bodies ($*P < 0.05$) after 20 min of dye exposure. $n = 11$ cells. Scale bar = 10 μM .

Fig. 5. LysoSensor staining is dependent on transmembrane electrochemical gradient ($\Delta\mu\text{H}^+$). (A) Representative cell terminals stained with LS 100 nM (first panel), pre-incubated with methylamine 10 nM (second panel), NH₄Cl 30 mM (third panel) and bafilomycin 500 nM (fourth panel). Scale bar = 10 μM. Upper row, DIC images; lower row, fluorescence images. (B) Graphic showing that all cell terminals pre-incubated with these pharmacological agents for 10 min and maintained in the medium during LS staining showed a decrease in fluorescence compared to their controls (* $P < 0.05$; $n = 8$ for methylamine, $n = 10$ for NH₄Cl, $n = 10$ for bafilomycin).

Fig. 6.

Activation of PKC increases synaptic vesicles acidification in retinal bipolar terminals. (A) Representative bipolar cells terminals stained with LS 100 nM (first panel), pre-incubated with PMA 100 nM (second panel); calphostin C 100 nM (third panel) and calphostin C + PMA (fourth panel). Upper row, DIC images; lower row, fluorescence images. Scale bar = 10 μ M. (B) Bar graphic showing that cells exposed to PMA increased LS fluorescence (* $P < 0.05$; $n = 9$) while the group of cells exposed to calphostin C ($P > 0.05$; $n = 9$), calphostin C + PMA ($P > 0.05$; $n = 9$), staurosporine ($P > 0.05$; $n = 10$), staurosporine + PMA ($P > 0.05$; $n = 9$) and 4alpha-PMA ($P > 0.05$; $n = 8$) did not cause a significant change in fluorescence compared to their controls.