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Following Tracks of Hemichannels

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Abstract

It has been suggested that plasma membrane-bound hemichannels perform physiological and pathophysiological functions *per se*. Such functions require the presence of hemichannels on the cell surface and their accessibility to the extracellular environment for at least some limited period of time. We have previously shown that hemichannels can be labeled by means of antibodies directed to an external loop domain of connexin (Cx) 43 (1). We now provide evidence that trafficking of hemichannel vesicles can be visualized upon binding of a labeled homophilic peptide corresponding to a region of the first extracellular loop (EL1) of Cx43.

In vivo imaging was performed after labeling hemichannels from the extracellular site with a mimetic peptide tagged with a fluorochrome (Alexa-546). Using a Cx43-CFP transfected HeLa cell line for incubation with the mimetic peptide, a significant number of double-labeled vesicles were found inside the cells. This double labeling indicates that a portion of Cx43 within the cell had accessed the cell surface as hemichannels where it bound to the peptide and was subsequently endocytosed. Pulse labeling with the peptide showed a decrease in the number of dual-labeled vesicles over time, indicating degradation and/or concurrent recycling of hemichannel vesicles.

Keywords

Connexin hemichannels; gap junction; trafficking

INTRODUCTION

Hemichannels or connexons constitute the two halves of functional gap junctions. After oligomerization in the trans Golgi network (2) and transport to the plasma membrane, hemichannels are destined to pair with a partner connexon in order to form functional gap junction channels. However, recent evidence from various laboratories indicates that unapposed hemichannels in the plasma membrane provide functions *per se*, which are independent from those performed by apposing hemichannels/gap junction channels (3). Specifically, Cx43 hemichannels have been suggested to form large conductance anion channels in myoblasts (4) and to constitute a class of “suicide channels” in macrophages (5). Cx43 hemichannels have been considered responsible for dye uptake and increased cell death upon metabolic inhibition of astrocytes (6) and cardiac myocytes (7). Additionally, Cx43 hemichannels have been reported to provide pores for ATP release (8) as well as glutamate and aspartate efflux in astrocytes (9). These divergent functions attributed to gap junctional hemichannels are controversial (10), since some of these effects are now

attributable to other types of channels and can also be elicited from astrocytes in Cx43 null-mutant animals.

MATERIALS AND METHODS

To generate improved tools for hemichannel detection and isolation we developed labeled mimetic peptides and antibodies, both of which bind to external loop domains of Cx43. One peptide, here referred to as RD4, constitutes a short homophilic sequence from the first external loop of Cx43. The peptide was conjugated to a fluorophore (Alexa 546, Molecular Probes, Leiden, Netherlands) and HPLC purified to separate the conjugated fraction from non-conjugated remnant. The antibody recognizes a 20 amino acid domain from the second external loop of Cx43 and binds with high affinity to this peptide in surface plasmon resonance experiments (data not shown). Details of the antibody production and specificity have been described elsewhere (1).

To evaluate peptide and antibody binding to un-apposed external loop domains different cell types were incubated with these probes. Firstly, *HeLa* cells transfected with a Cx43-CFP construct were exposed to the peptide under tissue culture conditions for 30 min, washed with culture medium (DMEM), and imaged using an inverted microscope equipped with a humidified and gassed incubation chamber. Secondly, primary astrocytes were used for peptide studies under *in vitro* conditions. After incubation with the peptide, sites of Cx43 expression were visualized by subsequent immunostaining of fixed and permeabilized (100% ethanol) cells using a monoclonal anti-Cx43 antibody (Zytomed, Berlin, Germany).

To minimize the amount of non-specific antibody uptake after ethanol permeabilization, that is, the fraction of unspecific fluid phase-absorbed endocytotic vesicles, astrocytes were rinsed overnight at 4°C in PBS.

Localization of the labeled homophilic peptide and Cx43-CFP fluorescence was analyzed by *in vivo* imaging. Quantification and co-localization were determined by tracing yellow fluorescent vesicles, resulting from superimposed red (peptide) and green (CFP) fluorescence, over the course of 20 frames (exposure time 3 sec. without intervals) of recorded images. Primary astrocytes subjected to immunolabeling were studied by conventional and confocal laser microscopy and the amount of co-localization was assessed by on screen-quantification using Metamorph software. All experiments were done in triplicates and statistical significance was determined through Origin® software.

RESULTS AND DISCUSSION

Binding of Labeled Mimetic Peptides to Hemichannels

Expression of Cx43-CFP in stably transfected HeLa cells resulted in intense fluorescent vesicles of uniform size (approximately 150 nm), which apparently represent secretory hemichannel vesicles (11). Besides these secretory vesicles, distinct vesicles of variable diameter were detected which increased in number over time and are considered to be endocytosed gap junctions in form of annular gap junction vesicles (12). In addition, surface labeling was consistently found, indicating successful insertion of the Cx43-CFP fusion protein into the plasma membrane.

After incubation of Cx43-CFP transfected HeLa cells with the labeled mimetic peptide, two additional classes of vesicles became apparent. (a) red fluorescent vesicles carrying fluid phase-absorbed peptides, which constitute the predominant endocytosed fraction, and (b) vesicles carrying both the red fluorescence of the mimetic peptide and the Cx43-CFP fluorescence which resulted in a yellow signal when superimposed (Figure 1). One tentative

interpretation of this co-localization is that part of the plasma membrane-bound hemichannels was labelled with the mimetic peptide by homophilic binding and subsequently endocytosed. We rarely found co-localization of both signals within the plasma membrane. In all likelihood, the lack of plasmalemmal co-localization may be due to low-level signals derived from peptide-labeled plasma membrane bound hemichannels. Weakness of the signal can be explained by either dilution of hemichannels through lateral diffusion within the plasma membrane and/or competitive effects during binding of the peptide to the hexameric connexon complex.

Quantification of the dual-labelled vesicles exhibited a clear time-dependent decay in number (Figure 2A), reaching zero levels four hours after incubation.

Control studies with a randomized peptide containing the same amino acids as the homophilic peptide, resulted in significantly lower co-localization. In contrast to the mimetic peptide the randomized form did not reveal a complete disappearance of co-localizing vesicles over time (Figure 2B). Two possibilities are suggestive to explain co-localization of the signals with the randomized peptide. First, superimposition of inbound and outbound vesicles within the optical plane cannot be resolved when following vesicle movements over 20 frames and/or, secondly, collision and fusion of both vesicle types might occur.

To minimize the fraction of vesicles with fluid phase-absorbed peptides we used a different experimental paradigm. Primary astrocytes were incubated with the peptide using the same time schedule as for HeLa cell experiments. After *in vitro* labeling, astrocytes were treated with 100% ethanol which both fixes the cells and leads to the permeabilization of cell membranes. By extensive rinsing with PBS, differentiation was improved due to wash out of a majority of the unbound fluid phase peptide fraction.

Confocal imaging of primary astrocytes exposed to the labeled mimetic peptide resulted in a significant higher number of chimeric vesicles as compared to incubation with the randomized peptide (compare Figures 3A, B). These data indicate that part of the plasmamembrane-bound unapposed hemichannels are accessible from the external milieu and are subject to endocytotic re-uptake. It remains to be clarified whether this re-uptake is part of a distinct metabolic pathway of plasma membrane-bound unapposed hemichannels or if it represents the cellular response to peptide binding with subsequent internalization, thereby cleansing the peptide-hemichannel complex from the cell surface.

Binding of External Loop Antibodies to Plasma Membrane-Bound Hemichannels

To further peruse the accessibility of external loop domains of PMB hemichannels from the external environment we used an affinity purified antibody which is directed to amino acids 186 to 206 of the second external loop. In addition to already published data (1) we extended our studies to astrocytes derived from homozygous Cx43 null mice ($-/-$), which served as negative controls. Wildtype as well as Cx43 ($-/-$) astrocytes were briefly (5 min) incubated with the antibody, then rinsed in PBS and fixed in 2% paraformaldehyde/PBS. Fixation was followed by secondary labeling with an IgG-Alexa-488 goat anti-rabbit antibody. Images were obtained by conventional and confocal laser microscopy using appropriate excitation wavelengths in combination with differential interference contrast to visualize cell structures.

In wildtype astrocytes, intensive labeling occurred preferentially in membrane domains of filopodia and at sites of unapposed plasma membranes (Figure 4). The observed spotted distribution is consistent with a local concentration of plasma membrane-bound hemichannels in the form of plaques rather than a random distribution. This is in contrast to the distribution of hemichannels in transfected HeLa cells, where fluorescence was observed

within the entire plasma membrane. A reasonable explanation for this difference in plasma membrane-bound hemichannel distribution in these cell types is the much less pronounced cellular polarity in tumor cells than in primary astrocytes.

When astrocytes from Cx43 (–/–) mice were incubated with the antibody the cells were devoid of any labeling although in some locations vesicular labeling was also observed in macrophages, mostly in the form of microglial cells. This effect can be explained by endocytotic uptake of immunoglobulins by these highly active cells during *in vitro* incubation with antibodies.

In summary, our data obtained using novel molecular probes (homophilic peptides and site-specific antibodies) indicate that plasma membrane-bound hemichannels are accessible from the external environment. Endocytotic uptake of hemichannels labeled with homophilic external loop peptides is suggestive for a specific metabolic cycle of hemichannels, but alternative explanations include cleansing of channel-peptide complexes from the cell surface through reuptake and degradation.

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Figure 1. Single frame of *in vivo* imaged Cx43-CFP transfected HeLa cells exposed with the mimetic external loop peptide. Note the three classes of vesicles corresponding to secretory hemichannel vesicles (green), fluid phase absorbed peptide (red), and co-localizing Cx43-CFP/mimetic peptide (yellow). A small fraction of the green fluorescent vesicles could be endocytosed vesicles from gap junction plaques. (See Color Plate X).

Figure 2.

Quantification of co-localization of Cx43-CFP and the mimetic peptide (left) of *in vivo* imaged HeLa cells. The mimetic peptide shows a time-dependent decrease, reaching zero levels at 4 hr. The randomized peptide (right) shows reduced co-localization.

Figure 3.

In vivo labeling of primary astrocytes with the mimetic (left) and the randomized peptide (right). Astrocytes were counterstained with a Cx43 specific antibody to localize Cx43 presence. Vesicles were counted per cell. Each column indicates the average ($\pm$ SD) of vesicles obtained from confocal laser images of 10 cells (done in triplicate). Note that the mimetic peptide shows a significantly ($p < 0.005$) higher number of co-localizing vesicles. The fraction of peptide-Alexa546 vesicles is equal in both mimetic and randomized peptide experiments due to basal fluid phase absorption.

Figure 4.

Superimposed phase contrast and immunostained images of primary astrocytes. Staining was obtained with an antibody directed to a peptide domain of the second external loop of Cx43. (A) shows the wildtype astrocytes with immunoreactivity in a number of cells. (B) indicates a culture from Cx43 ($-/-$) mice devoid of immunolabeling in astrocytes. Only a single macrophage (rounded phenotype) is labeled which apparently has digested the IgG under the incubation conditions.