Visualizing ion channel dynamics at the plasma membrane

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Cardiac ion channels are surprisingly dynamic in nature, and are continuously formed, trafficked to specific subregions of plasma membrane, inserted in the plasma membrane, and removed to be degraded or recycled. Because of these movements, which affect channel availability, ion channel function is dependent on not just channel biophysical properties but channel trafficking as well. The development of molecular techniques to tag proteins of interest with fluorescent and other genetically encoded proteins, and of advanced imaging modalities such as total internal reflection microscopy (TIRF), have created new opportunities to understand the intracellular movement of proteins near the plasma membrane and their dynamics therein. In this article we present approaches for ion channel biologists to the use of fluorescent and nonfluorescent fusion proteins, techniques for cloning and expression of fusion proteins in mammalian cells, and imaging techniques for live-cell high-resolution microscopy. For illustration, original data are presented on creation of a stable cell line capable of inducible expression of connexin 43 tagged to green fluorescent protein and its distribution viewed with both wide-field epifluorescence and TIRF microscopy. With revolutionary advances in fluorescence microscopy, ion channel biologists are now entering a new realm of studying channel function, which is to understand the mechanisms of channel trafficking.

KEYWORDS: Ion channel; Trafficking; Plasma membrane; Fusion proteins; Fluorescence microscopy; TIRF; Gap junctions; Connexin 43

(Heart Rhythm 2008;5:S7–S11)

Introduction

Acting as the primary gatekeepers of cellular charge and osmolarity, ion channels are one of the most important classes of transmembrane proteins expressed in biological cells. They play a central role in cell survival and orchestrate key physiological functions including the propagation of action potentials through the nervous system and myocardium.[1] The expression and localization of specific ion channels, not only in certain cell types, but also in precise domains within the plasma membrane itself, is essential for the normal function of many tissues.[2–4] Their disruption, through either genetic mutation or acute pathological states such as ischemia, often leads to serious diseases primarily of the nervous system and heart.[5,6] Although the existence of membranous ion channels was first proposed over 50 years ago,[7] substantial gaps remain in our understanding of ion channel basic biology. The patch-clamp technique has dominated the field of ion channel research since its introduction by Neher and Sakmann in 1976.[8] Although this technique is the gold standard for biophysical properties of channels and groups of channels, it is more limited in the information it can provide regarding the dynamic nature of intracellular ion channel movements within and on the surface of cells. The development of novel therapeutics aimed at treatment of disorders associated with altered ion channel expression and localization will depend on further elucidation of ion channel trafficking.

Gap junctions are a class of intercellular channels that form low-resistance conduits between the cytoplasms of adjacent cells via serial coupling of connexin hemichannels.[9-11] Although gap junctions are relatively nonspecific in distinguishing which molecules they allow to pass, their location at the intercalated discs of the myocardium permits rapid transmission of excitation between adjacent cardiomyocytes in an organized and polarized fashion. Action potentials are propagated throughout the working myocardium in this manner, facilitating well-orchestrated and efficient contractile function.[11] Altered regulation and localization of gap junctions are an intrinsic part of the molecular basis of the arrhythmias of sudden cardiac death[12-14] and have been implicated in dyssynchronous cardiac function during congestive heart failure.[15] However, despite the importance of gap junctions in normal physiology and ischemic heart disease, much is unknown about their regulation. We recently found that gap junctions can be directly and specifically targeted to the cell-cell interface by dynamic microtubules.[4] Through the use of advanced imaging techniques such as total internal reflection (TIRF) microscopy and fluorescent recovery after photobleaching (FRAP) in combination with fluorescently tagged fusion proteins, it is possible to study the dynamic behavior of gap junction repopulation in real time.[4]

Supported by the National Institutes of Health, National Heart, Lung, and Blood Institute, and GlaxoSmithKline Research and Education Foundation (Dr. Shaw). **Address reprint requests and correspondence:** Dr. Robin Shaw, 505 Parnassus Avenue, M1181, Division of Cardiology, University of California at San Francisco, San Francisco, California 94143-0124. E-mail address: shawrm@medicine.ucsf.edu.

1547-5271/$ -see front matter doi:10.1016/j.hrthm.2008.02.015

The recent refinement of live-cell imaging techniques, together with the development of spectrally distinct fluorescent protein tags, has opened the door to a new era of basic cell biology research. This article discusses those tools and approaches that are currently available and relevant to the study of ion channel dynamics in living cells. Topics covered include choice of fusion protein tag, cloning approach, expression system, and methods of imaging and acquisition. Supported by complementary assays based on biochemical and genetic techniques, exploitation of these recent advances in live-cell imaging will enhance our understanding of the basic molecular mechanisms underlying ion channel trafficking and dynamics.

Fluorescent fusion protein tags

The cloning of the green fluorescent protein (GFP) gene of the jellyfish *Aquoria victoria* in 1992 led to its rapid exploitation as a reporter gene and development as a valuable tool for studying protein behavior in living cells when expressed as a genetic fusion product.[16,17] As a result of cloning additional fluorescent proteins (FPs) from other marine organisms and the genetic modification of these and GFP, an increasingly broad range of spectrally distinct fluorescent proteins is now available. It should be noted, however, that use of these proteins can be complicated by multimeric tendencies, suboptimal brightness, variable photostability, and artifactual changes to the protein of interest. Care should therefore be taken when choosing FPs as fusion tags depending on their intended application.[18,19] In general, the brightest FPs emit in the green and yellow wavelengths of the spectrum, whereas those emitting in blue and red show fainter fluorescence. The development of red FP fusion tags was also hindered by their oligomeric nature. A monomeric form of DSRed is available, but the superior photostability of mCherry and the recently described TagRFP render these the 2 FPs of choice in the visible red spectrum.[18,20] Based on our own experience, we would recommend the use of the FPs EGFP, EYFP or Venus, and mCherry or TagRFP for the study of ion channels in the green, yellow, and red wavelengths, respectively. The markedly lower brightness shown by blue FPs and those in the far-red spectra such as mPlum make the imaging of single ion channels difficult, although these proteins still have considerable value when encoded as genetic reporters.

An exciting addition to the field of FPs are those whose fluorescent properties can be manipulated in real time by the researcher. Photoactivatable and photoswitchable FPs such as PA-GFP and Kaede permit the study of protein dynamics in a manner comparable to pulse-chase experiments. PA-GFP fluorescence increases 100 times, and Kaede experiences an emission shift from green to red on exposure to 413 nm and 350 nm excitation, respectively.[21,22] Therefore, the movement of a specific subset of fusion proteins within the cell can be tracked over time after activation/switching without contaminating background from those fusion proteins not exposed to the lower-wavelength excitation.

Nonfluorescent fusion tags for secondary labeling and fluorescent live cell imaging

Despite the convenience of using genetically encoded FP fusion tags, their physical properties can potentially interfere with the structure, trafficking, and function of the protein of interest. Several alternative strategies exist that involve the use of smaller nonfluorescent fusion tags (typically <30 residues, compared with 229 residues for GFP), which then can be fluorescently labeled in a secondary step before imaging. The FLAG (DYKDDDDK) and HA (influenza virus hemagglutinin epitope; YPYDVPDYA) affinity tags typically used in biochemical assays can be used to introduce foreign epitopes either internally or at a terminus of the protein of interest. Fluorophores are subsequently attached specifically via monoclonal antibodies, or more elegantly, via Fab fragments of monoclonal antibodies.

Enzymes that catalyze posttranslational modifications of proteins can also be exploited in the specific fluorescent labeling of ion channels on the cell surface. Two such methods, *Escherichia coli* BirA biotin ligase and *Bacillus subtilis* Sfp phosphopantetheinyl transferase, are adapted from bacterial systems and provide efficient, high-affinity labeling of target proteins with minimal background. BirA biotinylates a lysine within a 15-residue acceptor peptide permitting subsequent labeling with streptavidin conjugated fluorophores.[23,24] One drawback of biotinylation is that the avidin family of proteins exist as multivalent tetramers that could lead to aggregation of tagged (biotinylated) protein and as such, elegant steps have been taken to overcome this caveat.[25] A recent method, termed phosphopantetheinylation, exploits Sfp phosphopantetheinyl transferase in the covalent attachment of coenzyme A-linked fluorophores to a serine within an 11-residue motif termed the *ybb*R tag. This procedure, which involves fewer steps than that involving BirA, does not lead to aggregation of target ion channels, and the covalent bond provides a high level of stability in comparison to affinity tag-antibody binding.[26]

A popular tag used for indirect fluorescent labeling is the tetracysteine motif (CCPGCC), which, unlike the techniques mentioned above, can also be used to label intracellular proteins. Biarsenical dyes, available in both red and green wavelengths, will only fluoresce on binding to the small tetracysteine motif, providing a method to time protein localization without attaching large GFP-type fluorophores to the proteins.[27,28] For dynamic studies, the tetracysteine motif method may not have the quantum efficiency for very rapid live cell imaging of protein movements.

Considerations for fusion protein cloning and expression

The imaging laboratory often requires the generation of a wide range of constructs expressing fluorescent fusion proteins of various colors. Traditional restriction cloning methods can prove time consuming and cumbersome when generating a variety of expression vectors. It can be helpful to use site-specific recombination cloning technology such as