

Imaging SNAREs at work in 'unroofed' cells – approaches that may be of general interest for functional studies on membrane proteins

T. Lang¹

Department of Neurobiology, Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany

Abstract

When cultured cells are subjected to a brief ultrasound pulse, their upper parts burst, but the basal plasma membranes with their embedded membrane-protein complexes remain intact. Such two-dimensional, paraformaldehyde-fixed plasma membrane sheets have been used in the past to visualize the morphology of the inner plasmalemmal leaflet by electron or light microscopy. More recently, fluorescence microscopy of unfixed native membranes has been applied to study SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) function. For instance, biochemical reactions of the plasmalemmal SNAREs with soluble fluorescent SNAREs, patching of SNARE and raft domains, and online monitoring of SNARE-mediated membrane fusion has been performed. The results obtained with the membrane sheet system have added some novel aspects to our understanding of the regulation of neuronal exocytosis. Surprisingly, SNAREs are concentrated in cholesterol-dependent microdomains that are different from membrane rafts. SNAREs in such domains are highly reactive, and define sites for vesicle exocytosis. Secretory granules that fuse on the membrane sheets are retrieved intact in a dynamin-dependent process, suggesting that the 'kiss-and-run' mechanism is not a reversed SNARE reaction, but is driven by a biochemically different mechanism. So far, studies of this type have focused on neuronal exocytosis; however, the method might be widely applicable. Data obtained with this system are derived from a 100% pure plasma membrane preparation that is only several seconds old, and membrane proteins are studied in their natural microenvironment that is defined by local lipid composition and putative bound proteins. Hence this approach yields results that most probably reflect the situation in a live cell.

Introduction

The plasma membrane is a busy part of the cell that contains a universe of receptors, ion channels and sites at which vesicles bud or fuse. How can the cell assure that the right proteins interact with high speed and efficiency at the right time, or avoid inefficient cross-talk between proteins that drive non-related dynamics? Most probably, proteins are embedded in pre-assembled supra-molecular structures that represent 'hot spots' for individual biological processes. A detailed analysis of the organization and reactivity of individual components of such large complexes would significantly advance our understanding of plasma membrane function; however, transmembrane proteins cannot be isolated without massive perturbation of their local environment that may also lead to disassembly of the complexes as such. One exception may be the production of plasma membrane sheets, by adsorbing cells strongly to a substrate and then 'unroofing' them in a manner so that only the substrate-supported basal plasma membrane remains (Figure 1). It has been reported

that cells can be 'unroofed' either by a squirt of buffer or by a gentle blast from an ultrasonic probe (for a review, see [1]).

This technique has already been applied for several decades for viewing the inner leaflet of the plasma membrane by electron microscopy (e.g. [2]; for a review, see [1]) or using light microscopy for visualizing plasmalemmal clathrin-coated pit assembly [3] or insulin-activated GLUT4 translocation to the plasma membrane (e.g. [4]). More recently, the plasma membrane sheet assay has been applied to study aspects such as the reactivity of membrane proteins [5] and, in addition, has been used as a cell-free assay for neuronal exocytosis [6,7].

These studies have focused on the neuronal SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) that comprise a superfamily of proteins that are essential for eukaryotic membrane fusion (for reviews see, e.g., [8,9]). They vary greatly in size and structure, but all have at least one SNARE motif that generally is in close proximity to a membrane anchor. For each fusion step in the cell, a specific set of SNAREs is required. For synaptic vesicle exocytosis, the neuronal SNAREs syntaxin 1A and SNAP-25 (25 kDa synaptosome-associated protein) at the plasma membrane and synaptobrevin 2 at the vesicle membrane form a ternary complex that pulls the opposing membranes closely together, leading finally to membrane fusion.

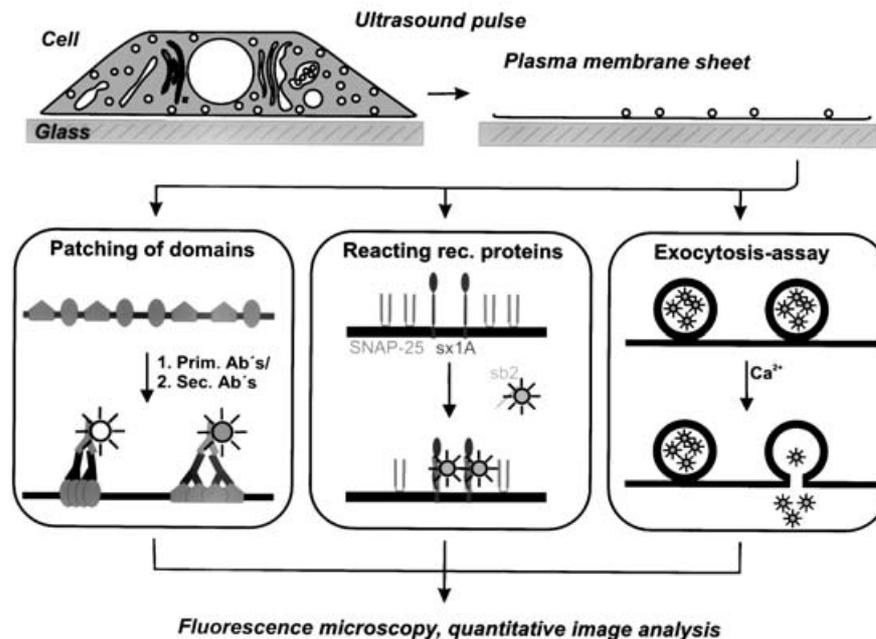
Key words: fluorescence microscopy, plasma membrane sheet, SNARE, syntaxin cluster, vesicle exocytosis.

Abbreviations used: GFP, green fluorescent protein; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

¹e-mail tlang@gwdg.de

Figure 1 | Approaches to using the plasma membrane sheet system for functional studies on membrane proteins

Abbreviations: Ab, antibody (Prim., primary; Sec. secondary); sx1A, syntaxin 1A; sb2, synaptobrevin 2; rec., recombinant.



Most of our knowledge about SNAREs comes from studies that use the soluble parts of the SNAREs in solution, detergent extracts or reconstituted SNAREs in artificial liposomes. However, under such conditions the SNAREs have been removed from their natural environment, and observed biochemical properties may not reflect the situation in a live cell. This review discusses the methodological advantages provided by the plasma membrane sheet system in the context of results obtained by studying SNARE-protein organization and function.

Plasma membrane sheets are ideal for the microscopic study of membrane protein biochemistry

Plasma membrane sheets can easily be generated by applying a gentle 100 ms ultrasound pulse to a cell either in ice-cold buffer or at room temperature. The plasma membranes have several advantages with regard to biochemical studies of the inner leaflet of the membrane and suitability for microscopic analysis. Put simply, an 'unroofed' cell is a fresh, 100% pure, biochemically accessible plasma membrane matrix that can be positioned into the optical plane of an objective, and the entire membrane sheet can be analysed by taking only a single image. Figure 1 shows different experimental approaches that may be followed after generation of plasma membrane sheets. All of these approaches have been applied to study SNARE function, and will be discussed in more detail.

Spatial organization of SNAREs

When SNAREs were visualized on membrane sheets, it was found that they were not distributed homogeneously,

but concentrated in spots. Several SNAREs were analysed, including syntaxin 1 and SNAP-25 [10], and syntaxin 4 and SNAP-23 [11]. SNAREs were visualized using either indirect immunofluorescence or green fluorescent protein (GFP) tagging. All SNAREs were found to be concentrated in several 100 nm-large microdomains, although SNAP-25 spots appeared less discrete. The SNARE cluster disintegrated upon cholesterol depletion [10,11], and photolabelling of syntaxin 1 with a cholesterol derivative, but not with a phosphatidylcholine derivative, indicated that cholesterol is part of the syntaxin cluster [10]. Secretory granules were associated with this syntaxin 1 cluster, and dispersion of the SNARE domains by cholesterol depletion using either β -methylcyclodextrin [10] or the cholesterol synthesis blocker lovastatin [12] strongly inhibited exocytosis, indicating that syntaxin-cluster integrity is of functional importance.

Are the specialized fusion sites membrane rafts?

The experiments described so far suggested that SNAREs in the plasma membrane are concentrated in cholesterol-dependent microdomains. Such microdomains could be membrane rafts, i.e. microdomains containing cholesterol, sphingomyelin and specific proteins with glycosylphosphatidylinositol anchors [13]. Membrane rafts are insoluble in Triton X-100 and float to a low density during density-gradient centrifugation. However, the SNARE domains apparently were not classical membrane rafts, since they were soluble when proteins were induced to form micelles in 1% Triton X-100 at a protein concentration of several mg/ml and Triton X-100 was included in the step gradient

during centrifugation [10]. When less stringent conditions were applied, SNAREs were found to float together with raft markers on the top of a density gradient ([10,12]; T. Lang, unpublished work).

In addition to the solubility criteria, co-patching experiments can also be performed to address whether a specific protein is localized to rafts [14]. In this type of study, epitopes of a raft marker and the protein of interest are artificially cross-linked with antibodies, leading to aggregation of the two types of protein when both localize to rafts. Conversely, proteins segregate if they are in different membrane entities. The process can be monitored with a fluorescence microscope when fluorescently labelled antibodies are used. So far, co-patching studies have been performed mostly on intact cells, allowing no biochemical access to the inner leaflet of the plasma membrane. However, this does not account for membrane sheets. To clarify if SNAREs are in rafts, co-patching studies were performed with antibodies directed against the raft marker Thy-1 and the intracellular part of syntaxin 1. The two signals were found to segregate, indicating that the clusters containing syntaxin 1 are different from the classical Thy-1 membrane rafts [10].

Are SNAREs in the live cell reactive?

As outlined in the introduction, complex formation between syntaxin 1A and SNAP-25 at the plasma membrane and synaptobrevin 2 at the vesicle membrane is supposed to drive fusion of regulated secretory vesicles with the plasma membrane. However, so far it has been unclear if the reactivity of the plasmalemmal SNAREs syntaxin 1A and SNAP-25 is tightly regulated, or if they are constitutively active. Tight regulation is likely, since several dozens of binding proteins have been reported for syntaxin 1A, e.g. Munc18, which has been suggested to block syntaxin 1A in a closed conformation, making it impossible for the SNARE motif to participate in SNARE-complex formation [15]. As illustrated in Figure 1, this question has been addressed using the membrane sheet assay. Native membranes were reacted with soluble fluorescently labelled synaptobrevin 2 to ascertain whether it forms a complex with syntaxin 1A and SNAP-25 in the membrane sheet [5]. Hence quantified incorporated fluorescence is the readout for the amount of reactive SNAREs. When the plasma membrane sheets were imaged, fluorescence could be detected at highest sensitivity, because the preparation is two-dimensional and no out-of-focus light needs to be filtered out, avoiding the extensive loss of fluorescent light that occurs with confocal microscopy. Hence fluorescence can be quantified accurately, and the images also provide spatial information about the localization of the proteins. It was found that synaptobrevin 2 integrated readily in the native membranes specifically at sites where the syntaxin cluster resided. The reaction was saturable, and was blocked in the presence of the neurotoxins BotNT/C and BoNT/E, which cleave syntaxin 1A and SNAP-25 respectively, indicating that both SNAREs are required for the reaction. Interestingly, the

membrane sheets displayed different biochemical properties after incubating them for 10 min at 37°C. First, much less synaptobrevin 2 was incorporated; in addition, syntaxin 1A, which is almost quantitatively cleavable by BotNT/C on fresh membrane sheets, became cleavage resistant. Further analysis showed that both effects were due to SNARE-complex formation between endogenous syntaxin 1A, SNAP-25 and synaptobrevin 2, all present in the native membrane. In a live cell, these proteins are apparently constitutively dissociated, probably by the activity of *N*-ethylmaleimide-sensitive factor [16]. The results showed that SNAREs in the native membrane are highly reactive. Most interesting from a technical point of view is the fact that the biochemical properties of isolated native plasma membranes change quickly after preparation, and indicate that it is important to perform studies on membrane proteins on preparations that are not more than a few minutes old.

Monitoring of exocytic/endocytic events using plasma membrane sheets

After cell disruption, the cortical network and secretory vesicles also remain attached to the glass-adsorbed plasma membrane. Secretory granules can be visualized either by using the acidic lumen marker Acridine Orange [17] or by expression of GFP-tagged peptide hormones [18]. Holroyd and co-workers [7] used both markers for visualization of secretory granules on plasma membrane sheets, and observed that, upon addition of Ca²⁺, MgATP and rat brain cytosol, granules released Acridine Orange completely, but GFP only partially. This was because granules were retrieved intact before complete emptying ([7]; see also a study on intact cells [19]), and only the smaller Acridine Orange was washed out completely during the transient opening ([6,7]; for studies on intact cells, see [17]). It has been debated whether the fast, direct retrieval of fusing secretory vesicles – also termed the ‘kiss-and-run’ mechanism of exocytosis – is due to a reversible SNARE-complex formation or is mediated by a biochemically independent reaction, e.g. by a dynamin-dependent fission machinery [20,21]. In the study by Holroyd et al. [7], retrieved granules were shown to have endocytosed a fluid-phase marker that was added during stimulation. In the presence of peptides that interfere with dynamin function, the retrieval mechanism was strongly inhibited, demonstrating that direct granule retrieval in this case (maybe identical to the ‘kiss-and-run’ mechanism) is not due to reversible SNARE-complex formation, but is a biochemically distinct process.

Conclusions

The plasma membrane sheet system is a unique tool for studying the organization and function of membrane proteins using microscopic methods. Rapid isolation of the plasma membranes ensures that findings reflect the situation in a live cell. This is supported by the observation that SNARE reactivity in isolated native membranes decreases after a few minutes. The method also provides data derived from

pure plasma membranes, which is impossible to achieve with conventional membrane-preparation techniques. Plasma membrane sheets have so far been prepared using many cell types; however, the yield is low (approx. 100 membrane sheets from 50 000 cells), requiring either cell lines or primary cultures from tissue that is available in large amounts (e.g. bovine chromaffin cells [22]). To summarize, the method allows biochemical studies on a high-quality level, in the sense that protein complexes or biochemical reactions in pure and fresh plasma membranes can be studied at high optical resolution and in a quantitative manner.

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