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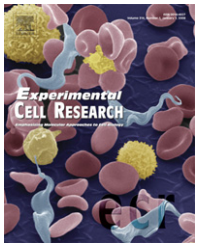

Research Article

Heterogeneity and timing of translocation and membrane-mediated assembly of different annexins

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ABSTRACT

Many cell types, including neurons and epithelial cells, express a variety of annexins. Although the overall function has only been partially unravelled, a dominant feature is the formation of two-dimensional assemblies under the plasma membrane in a calcium-dependent manner. Here we show that fluorescently tagged annexins A1, A2, A4, A5, and A6 translocate and assemble at the plasma membrane and the nuclear envelope, except annexin A2, which only attaches to the plasma membrane. All annexins have different response times to elevated calcium levels as was shown by the translocation of co-expressed proteins. Fluorescence recovery after photobleaching revealed the static nature of all annexin assemblies. Analysis of the assemblies by Foerster resonance energy transfer (FRET) using acceptor bleaching demonstrated mostly annexin-specific self-assembly. Heterogeneous assembly formation was shown between annexins A5 and A1, and A5 and A2. The formation of homo- and heterogeneous annexin assemblies may play an important role when high increases in calcium occur, such as after disruption of the plasma membrane.

Introduction

Annexins are a family of calcium-regulated proteins with a well-defined architecture [1]. Most annexins have four conserved calcium/phospholipid-binding repeats, but some (annexin A6) are made of eight units [2,3]. The calcium binding sites significantly differ from those of other calcium-binding proteins [4,5]. In particular, annexins recognize negatively charged surfaces of phospholipid-containing membranes, using calcium as a mediator between protein carboxy groups and acidic phospholipids [6–8]. Upon binding of calcium many annexins translocate to various cellular membranes, with the plasma membrane being the preferred target [9–13]. The affinity for calcium varies between annexins [14,15]. Annexins A5 and A1 may also interact with hydrophobic parts of the lipid bilayer [16,17]. At the membrane, several annexins (i.e. annexins A4, A5 and A6) initially form trimers which subsequently constitute two-dimensional assemblies coating the respective membrane [11,18–27]. Annexins are therefore thought to be involved in calcium-dependent processes where extensive membrane rearrangement is required, i.e. endo- or exocytosis [1,28].

The vertebrate annexin family consists of 12 subtypes. For many of them, the formation of lateral assemblies was shown on model membranes [1,2]. While the above mentioned trimer formation leads to fairly regularly shaped 2-dimensional patches of proteins, annexins A1 and A2 showed more amorphous membrane-bound protein clusters [29,30]. Recently we demonstrated self-association for annexin A4 in living cells by using *Aequorea* fluorescent protein fusions [11].

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With the help of two differently labelled (ECFP and EYFP) annexin A4 molecules transfected into one cell we showed that annexin A4 packing in assemblies at the plasma membrane is sufficiently tight to exhibit substantial Foerster resonance energy transfer (FRET). From this initial experiments the question arises whether other annexins, especially those known to form amorphous clusters, are behaving similarly to annexin A4 and whether there are differences in translocation/assembly kinetics. In addition, in this study we wanted to explore the intriguing possibility that two annexins may form mixed assemblies or that clustering might occur in a spatially segregated way.

Materials and methods

Construction of plasmids

The cDNA for human annexins A1, A2, A5, and A6 were obtained from RZPD (Berlin, Germany). To construct ECFP–annexin and EYFP–annexin fusions for the above-named proteins, annexins were amplified by PCR. The resulting products of annexins A1 and A2 were digested with *Sal* I and *Bam*H I, annexin A5 was treated with *Eco*R I and *Bam*H I and annexin A6 with *Sal* I and *Age* I. The products were inserted into the pEYFP-N1 and pECFP-N1 vectors (Clontech, Palo Alto, CA; ECFP variant used was described in [31]). mDsRed and EGFP-labelled annexins were obtained by exchange of ECFP or EYFP from the constructs described above with mDsRed or EGFP sequence using *Age* I and *Not* I restriction enzymes. pECFP-N1–annexin A4 and pEYFP-N1–annexin A4 constructs were cloned previously [11]. The mPlum–annexin A4 fusion was a kind gift of Michael W. Davidson (NHMFL, Tallahassee, FL).

Cell culture and transfection

N1E-115 cells were passaged and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.1 mg/ml primocin. For imaging experiments, cells were plated in 35 mm MatTek chambers (Ashland, MA) and transfected with FuGENE 6 reagent (Roche, Mannheim, Germany) at 50% confluency. Transfections were performed in Opti-MEM (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were washed 12–24 h after transfection and incubated with imaging medium (20 mM HEPES, pH 7.4, 115 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, 2 g/l D-glucose) at 37 °C under a 5% CO_2 atmosphere for about 1 h before imaging.

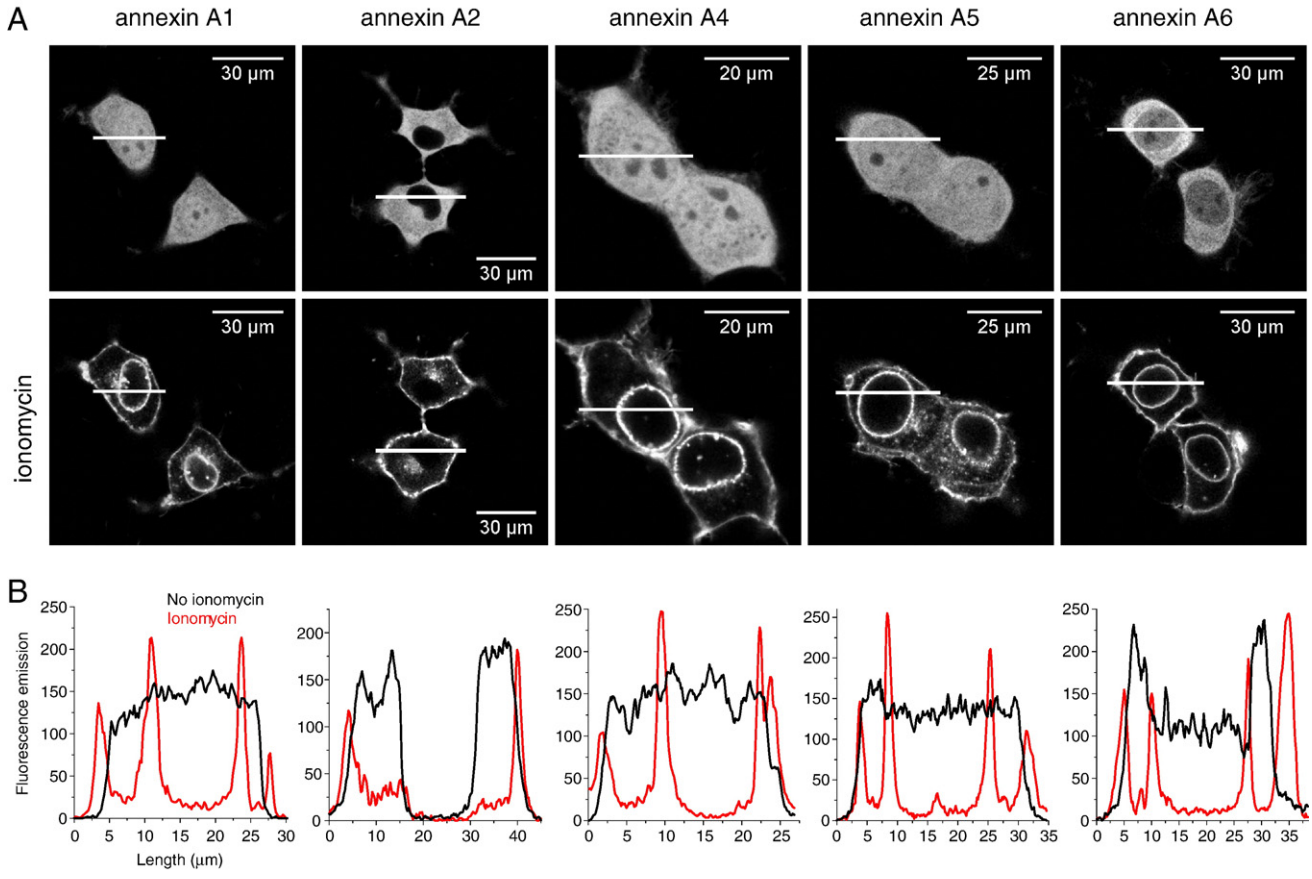

Fig. 1 – Localization of different annexins in N1E-115 cells. (A) Complete translocation of annexin–EYFP fusions to the plasma membrane and the nuclear envelope 5 min after addition of 10 µM ionomycin. Note that annexin A2 is absent from the nucleus and does not translocate to the nuclear membrane. (B) Topographic cuts from the cell images before (black trace) and after (red trace) ionomycin treatment demonstrate the dramatic change in all annexin distributions. Relatively low levels of annexin A6 in the nucleus prior to translocation are reflected by lower fluorescence intensity at the nuclear membrane after translocation.