ACCELERATED PUBLICATION

Cholesterol-dependent partitioning of PtdIns(4,5) P_2 into membrane domains by the N-terminal fragment of NAP-22 (neuronal axonal myristoylated membrane protein of 22 kDa)

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A myristoylated peptide corresponding to the N-terminus of NAP-22 (neuronal axonal myristoylated membrane protein of 22 kDa) causes the quenching of the fluorescence of BODIPY[®]-TMR-labelled PtdIns(4,5) P_2 in bilayers of 1-palmitoyl-2-oleoyl phosphatidylcholine containing 40 mol % cholesterol and 0.1 mol % BODIPY[®]-PtdIns(4,5) P_2 . Both fluorescence spectroscopy and total internal reflectance fluorescence microscopy

revealed the cholesterol-dependent nature of PtdIns(4,5) P_2 -enriched membrane-domain formation.

Key words: cholesterol, fluorescence, membrane raft, neuronal axonal myristoylated membrane protein of 22 kDa (NAP-22), PtdIns(4,5) P_2 , total internal reflectance fluorescence (TIRF) microscopy.

INTRODUCTION

NAP-22 (neuronal axonal myristoylated membrane protein of 22 kDa) is a protein found in neurons that is important for neuronal sprouting and plasticity [1]. In addition to the high abundance of this protein in nerve terminals, it is also present in significant amounts in kidney, testis and lymphoid tissue, in part as N-terminal fragments of this protein [2]. A protein with a high sequence similarity to NAP-22, and likely to have very similar properties, is CAP-23 (cortical cytoskeleton-associated protein of 23 kDa) [3]. Along with GAP-43 (growth-associated protein of 43 kDa) and MARCKS (myristoylated alanine-rich C-kinase substrate), CAP-23 accumulates in rafts, where it co-localizes with PtdIns(4,5) P_2 [4]. All three of these proteins have clusters of cationic amino acid residues. At least for MARCKS, this feature has been shown to be responsible for sequestering of PtdIns(4,5) P_2 into domains [5,6]. It has also recently been demonstrated that a peptide segment of caveolin will promote the formation of membrane domains containing both cholesterol and PtdIns(4,5) P_2 [7]. The basis for domain partitioning into lipid rafts remains poorly understood. In particular, one would not, *a priori*, expect that PtdIns(4,5) P_2 would be a constituent of a membrane domain enriched in cholesterol, since (a) the polyunsaturated acyl chains in PtdIns(4,5) P_2 are not very miscible with cholesterol [8], and (b) negatively charged lipids, such as PtdIns(4,5) P_2 , in general separate from cholesterol [9].

A powerful approach for studying supported planar lipid bilayers, TIRF (total internal reflectance fluorescence) microscopy, relies on the propagation of an evanescent wave to excite fluorophores within approx. 100 nm from an interface [10,11]. Since fluorophores distributed in the bulk of the solution are not excited by the evanescent excitation wave, the resulting images are largely free of any background fluorescence. In the present

study, we demonstrate by steady-state fluorescence and time-lapse TIRF microscopy that the myristoylated N-terminal fragment of NAP-22, myristoyl-GGKLSKKKKGYNVNDEKAK-amide, will sequester PtdIns(4,5) P_2 into domains in a cholesterol-dependent manner. This cholesterol dependence is also observed with PtdIns(3,4,5) P_3 , but not at all with PtdIns(3,5) P_2 . This finding provides an explanation for the observed co-localization of PtdIns(4,5) P_2 and cholesterol in membrane domains.

EXPERIMENTAL

Materials

NAP-22 peptide was from BioSource International (Hopkinton, MA, U.S.A.), phospholipids and cholesterol were from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and the fluorescent-labelled PtdIns P_2 , as well as the 200 nm FluoSpheres (F8811), were from Molecular Probes (Eugene, OR, U.S.A.).

Preparation of LUVs (large unilamellar vesicles) for fluorescence spectroscopy

POPC (1-palmitoyl-2-oleoyl phosphatidylcholine) films with cholesterol and 0.1 mol % BODIPY[®]-TMR (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-1,3-dimethyl-5-(4-methoxyphenyl)-2-propionyl-amino-hexanoyl-amino-hexanoyl)-labelled PtdIns P_2 s were prepared in chloroform/methanol (2:1) and hydrated with a 10 mM Hepes buffer, pH 7.4, containing 1 mM EDTA and 140 mM NaCl or as a function of salt concentration, with NaCl replaced by either 100, 200 or 300 mM KCl. A Lipex extruder [12] was used to convert the lipid suspension to LUVs and the concentration of lipid determined by phosphate analysis [13]. Care was taken to keep the probe-containing liposomal preparations protected from light.

Abbreviations used: BODIPY[®]-FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl-amino-hexanoyl; BODIPY[®]-TMR, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-1,3-dimethyl-5-(4-methoxyphenyl)-2-propionyl-amino-hexanoyl-amino-hexanoyl; CAP-23, cortical cytoskeleton-associated protein of 23 kDa; GAP-43, growth-associated protein of 43 kDa; LUV, large unilamellar vesicle; MARCKS, myristoylated alanine-rich C-kinase substrate; NAP-22, neuronal axonal myristoylated membrane protein of 22 kDa; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; PLC, phospholipase C; TIRF, total internal reflectance fluorescence.

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Fluorescence quenching

Fluorescence measurements were made in silanized glass cuvettes containing 2 ml of buffer at 25 °C. Peptide concentration was determined at an A_{275} . An amount of LUVs were added to the cuvette and then titrated with small aliquots of peptide solution. The fluorescence emission was corrected for the small volume change in the cuvette upon titration.

The excitation and emission monochromators were set at 542 nm and 571 nm respectively, with a 500 nm cut-off filter. The bandpass slits were set at 4 nm. The shutter was toggled only at the beginning of the recording of each emission scan to prevent photobleaching of the probe.

Hydrolysis of BODIPY®-TMR-PtdIns(4,5) P_2 with PLC (phospholipase C)

Approx. 5 nM recombinant human PLC- δ_1 (80 % pure) was added to LUVs composed of 0.1 mol % BODIPY®-TMR-PtdIns(4,5) P_2 with POPC and 40 mol % cholesterol in a buffer with the composition 100 mM KCl, 25 mM Hepes, 100 μ M EGTA, 102 μ M CaCl₂ (approx. 5 μ M free Ca²⁺) and 2 mM dithiothreitol, pH 7.0. The mixture was incubated at 37 °C for 60 min.

Preparation of supported bilayer for TIRF microscopy

A lipid film with 0.1 mol % BODIPY®-FL (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl-aminohexanoyl)-PtdIns(4,5) P_2 was hydrated with Hepes buffer, pH 7.4, containing 1 mM EDTA and 140 mM NaCl to give a lipid concentration of approx. 1 mg/ml and sonicated for approx. 20 min. It was then applied to the surface of freshly cleaved mica and the lipid allowed to fuse to the mica surface over a period of 10 to 20 min, with warming to 50 °C. The sample was cooled to approx. 30 °C and flushed with 10 mM Hepes/0.14 M NaCl/1 mM EDTA, pH 7.4, before imaging. Peptide in buffer was introduced by syringe transfer into the TIRF fluid cell, which had lipid bilayers already formed by *in situ* vesicle fusion to the mica surface.

TIRF Microscopy

The TIRF instrument comprised an Olympus Fluoview 500 confocal microscope equipped with an Olympus TIRF accessory [10 mW argon (488 nm) laser (Melles Griot, CA, U.S.A.); Olympus 60 $\times$ 1.45 NA TIRF objective]. The TIRF images were acquired simultaneously using a cooled charge-coupled-device camera (CoolSNAP HQ; Roper Scientifics, Tucson, AZ, U.S.A.) and ImagePro Plus (Media Cybernetics). All images were obtained as 1392 pixel $\times$ 1040 pixel data sets using a 300 ms exposure time, and horizontal and vertical binning values of 1 at a capture rate of approx. 1.7 frames/s. Image analysis was performed using National Institutes of Health ImageJ (developed at the U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/ij/>).

RESULTS

Steady-state fluorescence quenching shows cholesterol-dependent domain formation

Addition of the NAP-22 peptide to LUVs containing 0.1 mol % BODIPY®-TMR-PtdIns(4,5) P_2 results in quenching of the BODIPY® fluorescence (Figure 1A). Self-quenching of BODIPY®-TMR-PtdIns(4,5) P_2 by the MARCKS peptide has been shown to be caused by sequestering of the labelled lipid into domains [5]. While quenching caused by the NAP-22

Figure 1 Quenching by the NAP-22 peptide of the fluorescence emission of BODIPY®-TMR-PtdIns

(A) BODIPY®-TMR-PtdIns(4,5) P_2 . LUVs of POPC (□), POPC with 40 mol % cholesterol (6:4) (■), 1:1 POPC/POPE (1:1) (◆). (B) BODIPY®-TMR-PtdIns(3,5) P_2 . LUVs of POPC (□), POPC with 40 mol % cholesterol (6:4) (■). (C) BODIPY®-TMR-PtdIns(3,4,5) P_3 . LUVs of POPC (□), POPC with 40 mol % cholesterol (6:4) (●). Maximum emission intensity at 571 nm is plotted against the peptide-to-lipid molar ratio (P/L). LUVs were present at a concentration of 50 μ M and the BODIPY®-labelled lipids were 0.1 mol % of the total lipid.

peptide is cholesterol dependent (Figure 1A), this is not a consequence of differential binding affinity, since cholesterol has no effect on the binding of this peptide [14]. We further note that POPE (1-palmitoyl-2-oleoyl phosphatidylethanolamine), as well as cholesterol, can augment binding of the intact NAP-22 [14]; however, POPE has no effect on the extent of NAP-22-induced quenching of BODIPY®-TMR-PtdIns(4,5) P_2 (Figure 1A). This quenching phenomena is also not due to dissociation of the BODIPY® probe from the membrane, since sedimentation studies of sucrose-loaded vesicles revealed that almost all of the probe, at concentrations used in the fluorescence experiments, sedimented with the vesicles in the presence of NAP-22 peptide.

Figure 2 Fluorescence emission spectra of BODIPY®-TMR-PtdIns(3,4,5) P_3 as 0.1% of POPC with 40 mol% cholesterol as LUVs

The highest emission intensity corresponds to the lipid alone. Each subsequent spectrum represents an increment of 0.32 μ M NAP-22 peptide. The LUVs concentration is 50 μ M.

The specificity of this quenching was tested with other forms of phosphorylated PtdIns. BODIPY®-TMR-PtdIns(3,5) P_2 is only weakly quenched by the peptide (Figure 1B) compared with BODIPY®-TMR-PtdIns(4,5) P_2 in POPC with 40 mol% cholesterol (Figure 1A). In contrast, BODIPY®-TMR-PtdIns(3,4,5) P_3 (Figure 1C) behaves very much like BODIPY®-TMR-PtdIns(4,5) P_2 , both with and without cholesterol. The change in emission spectra of BODIPY®-TMR-PtdIns(3,4,5) P_3 with addition of peptide is shown (Figure 2).

The quenching of the BODIPY®-TMR-PtdIns(4,5) P_2 fluorescence by the NAP-22 peptide occurs over a range of cholesterol mole fractions (Figure 3). Cleavage of the labelled lipid with PLC- δ_1 to produce the corresponding diacylglycerol results in only a small loss of approx. 20% in the initial fluorescence intensity, but the quenching effect of the NAP-22 peptide is virtually completely eliminated (Figure 4). Note that the labelled lipid is only 0.1 mol% of the total lipid. The clustering of the labelled lipid by the NAP-22 peptide has a strong electrostatic component, given that 300 mM KCl significantly diminishes the effect (Figure 4).

TIRF microscopy imaging of membrane domains

In the absence of the NAP-22 peptide, the fluorescence in POPC bilayers is uniformly distributed (Figure 5), as it is with POPC in the presence of 40 mol% cholesterol (Figure 6). The bright, well-defined circular objects in these images are fluorescent 200 nm diameter FluoSpheres used as focusing aids during the initial setup of the microscope. The large periodicity ripples seen in Figures 5 and 6 are likely a consequence of interference fringing. Although the mica substrates were freshly cleaved prior to imaging, it is

Figure 3 Effect of cholesterol on the quenching of the fluorescence emission of BODIPY®-TMR-PtdIns(4,5) P_2 by the NAP-22 peptide

LUVs of POPC with no added cholesterol (■) or with 10 (◆), 30 (▲) or 40 (●) mol% cholesterol. Maximum emission intensity at 571 nm versus peptide-to-lipid molar ratio (P/L). LUVs were present at a concentration of 50 μ M.

Figure 4 Effect of 100 mM (▼), 200 mM (◆) or 300 mM (●) KCl on the quenching of the fluorescence emission of BODIPY®-TMR-PtdIns(4,5) P_2 by the NAP-22 peptide and hydrolysis of BODIPY®-TMR-PtdIns(4,5) P_2 to BODIPY®-TMR-diacylglycerol by PLC- δ_1 (■)

Maximum emission intensity at 571 nm is plotted against the peptide-to-lipid molar ratio (P/L). LUVs were present at a concentration of 200 μ M, containing 0.1 mol% probe.

possible that the surface of the mica contained within the TIRF excitation volume contains multiple surface steps and that this stepped structure may contribute to interference fringing and additional internal reflection of the TIRF excitation wave.

When a 0.5 mg/ml solution of the NAP-22 peptide replaced the peptide-free buffer over the bilayer, there was no change in the TIRF fluorescence pattern in the absence of cholesterol (results not shown). However, when the bilayer contained 40 mol% cholesterol, there was a dramatic and time-dependent change in the fluorescence pattern (Figure 7, and see also <http://www.BiochemJ.org/bj/379/bj3790527add.htm>). The previously uniform fluorescence was replaced by the formation of small, non-contiguous discrete irregularly shaped regions of non-fluorescence. These domains range in size from approx. 2 to 20 μ m². The domains are formed rapidly upon addition of the NAP-22 peptide, suggesting that the peptide was facilitating clustering of the PtdIns(4,5) P_2 into domains. Since the lipid bilayer contained 0.1 mol% BODIPY®-FL-PtdIns(4,5) P_2 , this would suggest that the dark domains that formed upon NAP-22 peptide addition

Figure 5 Representative TIRF microscopy image of cholesterol-free, 0.1 mol% BODIPY[®]-FL-PtdIns(4,5) P_2 containing POPC bilayers on mica in the absence of the NAP-22 peptide

The bright spots correspond to approx. 200 nm diameter FluoSpheres used as focusing aids. The relatively uniform fluorescence is indicative of even distribution of PtdIns(4,5) P_2 throughout the bilayer. Image size, approx. 45 μm $\times$ 45 μm . Image acquired in peptide-free buffer.

represent bilayer regions that are now depleted in PtdIns(4,5) P_2 . We cannot discount the possibility of self-quenching; however, the addition of NAP-22 peptide to a lipid bilayer containing the labelled PtdIns(4,5) P_2 , but lacking cholesterol, did not result in obvious domain formation as resolved by TIRF microscopy. The progression of domain formation was followed by capturing sequential images (with no rest time between images) over a period of approx. 2 min after exchanging the peptide-free buffer for one containing a solution of the NAP-22 peptide. The change in distribution of the fluorophore occurs rapidly and is largely complete within approx. 15 s. This is in agreement with the observed decline in steady-state fluorescence intensity after peptide addition.

DISCUSSION

Caroni and co-workers [1] have studied the importance of CAP-23, the chicken homologue of mammalian NAP-22, in re-organizing the actin cytoskeleton. This group found that CAP-23 is a major cortical cytoskeleton-associated protein and that sensory neurons lacking CAP-23 exhibited marked changes in neurite outgrowth that were similar to those observed with the administration of low doses of cytochalasin D [1]. It was concluded that CAP-23 together with GAP-43 promote the accumulation of the subplasmalemmal actin cytoskeleton [1]. This group also demonstrated that these two proteins, as well as the MARCKS protein, partitioned into membrane rafts together with PtdIns(4,5) P_2 . The formation of these PtdIns(4,5) P_2 -enriched rafts was dependent on a cluster of cationic residues found in each of these proteins [4]. Agents that mask PtdIns(4,5) P_2 mimic the action of these three proteins in recruiting peripheral actin and in cell spreading [4].

There is a known relationship between PtdIns(4,5) P_2 and the actin cytoskeleton [15,16]. It has recently been shown that dep-

Figure 6 Representative TIRF microscopy image of 40 mol% cholesterol in POPC bilayers containing 0.1 mol% BODIPY[®]-FL-PtdIns(4,5) P_2 on mica in the absence of the NAP-22 peptide (upper panel)

The bright spots correspond to approx. 200 nm diameter FluoSpheres used as focusing aids. Lower panel: line plot of relative fluorescence intensity in arbitrary units across the image as indicated. The broad circular features seen in the TIRF image are attributed to interference fringing/scattering of the TIRF excitation wave due to the stepped nature of the cleaved mica that is supporting the lipid bilayer. Image size, approx. 45 μm $\times$ 45 μm . Image acquired in peptide-free buffer.

letion of cholesterol from cells results in the reorganization of the actin cytoskeleton, resulting in increased mobility of membrane proteins [17]. This process is dependent on the loss or redistribution of PtdIns(4,5) P_2 [17]. PtdIns(4,5) P_2 stimulates actin polymerization by causing the dissociation of gelsolin-actin complexes [18], establishing a linkage between the plasma membrane and the cytoskeleton. More specifically, in the presence of proteins such as NAP-22 or its myristoylated N-terminal fragments, PtdIns(4,5) P_2 will be sequestered into rafts that in turn would also be linked to the cytoskeleton, resulting in altered cell morphology and spreading, which is the basis of the physiological effects of NAP-22.

Figure 7 Representative TIRF microscopy image of 40 mol% cholesterol in POPC bilayers containing 0.1 mol% BODIPY[®]-FL-PtdIns(4,5) P_2 on mica acquired approx. 23 s after addition of the NAP-22 peptide (upper panel)

Lower panel: line plot of relative fluorescence intensity in arbitrary units across the image as indicated. A video showing rapid progression of the change in fluorescence distribution in the bilayer is available at <http://www.BiochemJ.org/bj/379/bj3790527add.htm>. Addition of the NAP-22 peptide resulted in clear formation of distinct fluorescent (bright) and non-fluorescent (dark) regions in the bilayer, consistent with the myristoylated N-terminus of NAP-22 facilitating PtdIns(4,5) P_2 clustering. Image size, approx. 45 $\mu\text{m} \times 45 \mu\text{m}$. The image capture rate is approx. 1.7 frames/s, with the first frame captured approx. 11 s after the addition of peptide. Asterisks indicate regions where domains are changing.

It has been suggested that sequestering of PtdIns(4,5) P_2 by proteins may be a consequence of electrostatic interactions between the cationic cluster on the protein and polyanionic lipids [6]. Compared with MARCKS, NAP-22 has fewer cationic residues in the cationic cluster, and thus may be less effective in promoting the clustering of PtdIns(4,5) P_2 . In the case of MARCKS, a peptide corresponding to the cationic domain can sequester PtdIns(4,5) P_2 both in the presence and absence of cholesterol [5]. However, in the present work we show a marked dependence of the clustering of PtdIns(4,5) P_2 on cholesterol. In the case of POPC membranes containing 0.1 mol% PtdIns(4,5) P_2 , there

is only a weak quenching of the fluorescence by the NAP-22 peptide (Figure 1), and no redistribution of fluorescently labelled PtdIns(4,5) P_2 is observed by TIRF microscopy. These results suggest that there was either partial clustering of the PtdIns(4,5) P_2 in the absence of cholesterol into domains that are too transient and/or too small to be observed by TIRF microscopy, or the peptide is quenching the fluorescence by a mechanism different from self-quenching of the fluorophore. Although we cannot distinguish between these two possibilities, the extent of quenching is much smaller with POPC alone than it is in the presence of cholesterol.

Domain formation between PtdIns(4,5) P_2 and the NAP-22 peptide appears to be electrostatically driven. The quenching effect is reduced by increased salt concentration and eliminated by removal of the anionic headgroup of the PtdIns(4,5) P_2 (Figure 3). At lower salt concentrations, the quenching of PtdIns(4,5) P_2 is less sensitive to changes in salt concentration. This is likely a consequence of non-electrostatic contributions, such as insertion of the hydrophobic myristoyl group or the tyrosine residue of the peptide into the membrane. Similar phenomena have been observed with MARCKS [19,20]. An electrostatic model has been proposed to explain how cationic peptides facilitate the formation of lipid domains enriched in anionic lipids [21]. There are several factors that determine the stability of these domains. Assuming that equilibrium existed between anionic lipids contained within these domains and those distributed randomly in the bilayer, a polycationic peptide will preferentially bind to the anionic lipids located in the domains, thus favouring domain formation. The entropy of mixing will oppose this segregation. In addition, counterions bound to the domains of charged lipids will result in an additional energy term that will be reduced by the binding of oppositely charged peptides. An analogy was drawn between this phenomenon and the discharging of a capacitor [21]. In the present system we have an additional component, namely that domain formation is facilitated by cholesterol. We suggest that the role of cholesterol is related to its effect of increasing line tension [22,23]. An increase in line tension will favour the growth of domains so as to minimize the interfacial boundary.

The sequestering of phosphorylated phosphatidylinositols exhibits some specificity. Interestingly, PtdIns(3,5) P_2 is not greatly affected by the NAP-22 peptide, either in the presence or absence of cholesterol. PtdIns(3,5) P_2 has been identified as a minor lipid component that is found in cell vacuoles [24–28]. There is therefore no reason to expect that this lipid would have any relationship to the function(s) of NAP-22 in cells. However, the structures of PtdIns(4,5) P_2 and PtdIns(3,5) P_2 are quite similar, yet one is sequestered by the NAP-22 peptide and the other not. There is an important structural difference between these two forms of PtdIns P_2 : the '4,5' isomer has the added phosphates on opposite sides of the inositol ring, whereas the '3,5' isomer has the phosphates on the same side of the ring. This is likely to affect its interactions with cationic peptides, as well as with other lipids. In contrast, PtdIns(3,4,5) P_3 , which has the same '4,5' phosphates as PtdIns(4,5) P_2 and behaves the same way with the NAP-22 peptide, is found in cholesterol-rich regions of cell surface membranes [29]. Thus the specificity for different forms of PtdIns P_2 is what would be expected on the basis of those forms that are found in rafts and have biological roles that may be modulated by NAP-22.

The present work illustrates a role for cholesterol in stabilizing domains of PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 . It shows how the myristoylated N-terminus of NAP-22 will promote the sequestering of these lipids into domains in a cholesterol-dependent fashion. The model helps to explain the physiological action of NAP-22, a protein found in rafts, in reorganizing the actin cytoskeleton.

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