Neuroligin-2 dependent conformational activation of collybistin reconstituted in supported hybrid membranes

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The assembly of the postsynaptic transmitter sensing machinery at inhibitory nerve cell synapses requires the intimate interplay between cell adhesion proteins, scaffold and adaptor proteins, and γ-aminobutyric acid (GABA) or glycine receptors. We developed an in vitro membrane system to reconstitute this process, to identify the essential protein components, and to define their mechanism of action, with a specific focus on the mechanism by which the cytosolic C terminus of the synaptic cell adhesion protein Neuroligin-2 alters the conformation of the adaptor protein Collybistin-2 and thereby controls Collybistin-2 interactions with phosphoinositides (PtdInsPs) in the plasma membrane. Supported hybrid membranes doped with different PtdInsPs and 1,2-dioleoyl-sn-glycero-3-(N-(5-aminooxy-1-carboxypentyl)iminodiacetic acid)succinyl]nickel salt (DGS-Ni) to allow for the specific adsorption of the His6-tagged intracellular domain of Neuroligin-2 (His-cytNL2) were prepared on hydrophobically functionalized silicon dioxide substrates via vesicle spreading. Two different collybistin variants, the WT protein (CB2SH3) and a mutant that adopts an intrinsically ‘open’ and activated conformation (CB2SH3/W24A-E262A), were bound to supported membranes in the absence or presence of His-cytNL2. The corresponding binding data, obtained by reflectometric interference spectroscopy, show that the interaction of the C terminus of Neuroligin-2 with Collybistin-2 induces a conformational change in Collybistin-2 that promotes its interaction with distinct membrane PtdInsPs.

Synaptic signaling between neurons is based on the presynaptic release and postsynaptic sensing of neurotransmitters. In the mammalian brain, inhibitory synaptic signaling relies on the neurotransmitter γ-aminobutyric acid (GABA), which is detected by specific postsynaptic GABA_A receptors that operate as ligand-gated Cl– channels. The clustering of these receptors in the postsynaptic plasma membrane, in direct apposition to the presynaptic transmitter release site, ensures fast signal transduction, so that GABA release induces a hyperpolarization of the postsynaptic membrane and reduced excitability (1–6). Aberrant assembly and function of GABAergic synapses are the cause of multiple brain diseases (7–12).

A defined protein machinery is required for GABA_A receptor clustering at many GABAergic postsynaptic sites (Fig. 1A). At the core of this machinery is the cell adhesion protein Neuroligin-2 (NL2) (13, 14), which interacts with presynaptic Neurexins (15, 16) and within postsynapses with the scaffolding protein Gephyrin (17, 18) and the adaptor protein Collybistin (CB). Previous studies showed that CB binds to phosphoinositides (PtdInsPs) via its pleckstrin homology (PH) domain (19–22). On binding to PtdInsPs, CB serves as an adaptor to connect Gephyrin to the plasma membrane. This triggers Gephyrin oligomerization and the subsequent clustering of GABA_A receptors (14, 23, 24).

The adaptor protein CB, a guanine nucleotide exchange factor (GEF), is expressed in several splice variants that differ in their N- and C termini (CB1-CB3) and the presence or absence of a regulatory src homology 3 (SH3) domain (25). All CB variants contain a DH (Dbl homology) domain, which has GEF activity, and a C-terminal PH domain. Multiple studies demonstrated that mutations in CB cause neuronal dysfunction in various brain diseases. For example, an arginine to histidine exchange at position 290 within the DH domain affects the intramolecular interaction within the DH-PH tandem domain and thereby reduces the affinity of the PH domain to PtdIns[3]P, which is correlated to epileptic symptoms (21). Similarly, an arginine to tryptophan exchange at position 338 perturbs PH domain binding to PtdInsPs and causes a form of X-linked intellectual disability (26). These and other findings demonstrate that binding of the PH domain of CB to PtdInsPs is regulated by intramolecular interactions and of pivotal importance for the assembly of inhibitory postsynapses.

Previous studies (14) indicated that the most abundantly expressed, full-length, SH3-domain-containing CB isoform 2, CB2SH3, adopts a closed conformation (Fig. 1B), in which the SH3, DH, and PH domains interact intramolecularly and thus render the protein inactive, as is the case with the homologous GEFs Asaf1 and Asaf2 (27, 28). Disrupting this intramolecular interaction, e.g. by disabling the intramolecular binding sites in the CB2SH3/W24A-E262A mutant (14), leads to a more open and active conformation, so that the PH domain can bind PtdInsPs. It has been hypothesized that in the biological context of the synapse the cytosolic NL2 C terminus binds the SH3 domain of CB2, thus activates it, and allows its interaction with plasma membrane PtdInsPs (29). However, this type of NL2-dependent CB2 activation has so far only been inferred from data obtained with corresponding knock-out neurons or with cultured neurons that express NL2 or CB2 variants that are either

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unable to interact or constitutively active. Clear molecular insights into the process have been lacking.

The present study was conducted to obtain direct molecular evidence of an NL2-mediated activation of CB2, leading to increased CB2 binding to plasma membrane PtdInsPs. To this end, we established an in vitro membrane system to reconstitute this putative key step in the development of inhibitory synapses. Specifically, we used supported hybrid membranes (SHMs) on hydrophobically functionalized silicon dioxide substrates via spreading of small unilamellar vesicles (SUVs). We showed recently that SHMs are superior to supported lipid bilayers as they provide a more homogeneous distribution of PtdInsPs (30). SHMs were doped with PtdInsPs serving as receptor lipids for CB2, whereas 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] nickel salt (DGS-NTA(Ni)) was added to specifically adsorb the intracellular domain of NL2 (His-cytNL2) via its His6-tag (Fig. 1C). By means of reflectometric interference spectroscopy we were able to analyze the lipid-binding behavior of WT CB2SH3 and the intrinsically activated CB2SH3/W24A-E262A mutant (14) in the absence or presence of His-cytNL2. Our data show that the interaction of the C terminus of NL2 with CB2 induces a conformational change in CB2 that promotes its interaction with membrane PtdInsPs.

Results

Formation of supported hybrid membranes

In a first step, we produced lipid membranes on a silicon support to investigate and quantify the binding capability of CB2 to PtdInsPs in the presence or absence of the intracellular NL2 C terminus. We used a previously published protocol (30) to generate lipid monolayers composed of POPC and doped with different PtdInsPs. By using silicon dioxide substrates functionalized with 1,1,1-trimethyl-N-(TMS)silanamine (HMDS), to which small unilamellar vesicles (SUVs) were fused (Fig. 1C), a possible asymmetric distribution of PtdInsPs between the two lipid leaflets is prevented (30). Lipid monolayers composed of POPC and doped with 3 mol % of PtdIns[3]P, PtdIns[4,5]P2, or PtdIns[3,4,5]P3 on HMDS were prepared. The three PtdInsPs were chosen based on previous studies that had highlighted the involvement of these specific PtdInsPs as CB regulators in the formation of inhibitory synapses (19, 22, 31).

The spreading process of SUVs after HMDS functionalization was monitored in a time-resolved manner by reflectometric interference spectroscopy (RIFS). A characteristic time trace of the formation of a supported hybrid membrane (SHM) is depicted in Fig. 2A. Adsorption and spreading of the SUVs results in an increase in optical thickness (ΔOT) reaching a maximum at around 20 min. After monolayer formation, the system was rinsed with buffer B to remove excess lipid material and to adjust appropriate conditions for protein binding. ΔOTSHM (Fig. 2A) is used as a quality parameter for the SHM preparation. For all three PtdInsPs doped POPC monolayers, ΔOTSHM values of 2.77–2.80 nm were obtained, in good agreement with the expectation of a lipid monolayer on top of the HMDS monolayer. The mean values of ΔOTSHM were (2.77 ± 0.11) nm for PtdIns[3]P (n = 24), (2.80 ± 0.07) nm for PtdIns[4,5]P2 (n = 31) and (2.77 ± 0.10) nm for PtdIns[3,4,5]P3 (n = 22), showing that the PtdInsP species does not influence the final monolayer thickness (Fig. 2B).

To follow the binding of the cytosolic domain of NL2 (His-cytNL2) via its His6-tag to the membrane, we additionally prepared lipid monolayers composed of POPC, doped with 3 mol % of PtdInsP and 3 mol % of DGS-NTA(Ni). Again, ΔOTSHM were readout from the RIFS experiments (Fig. 2C). Mean values of ΔOTSHM were (2.74 ± 0.05) nm for DGS-NTA(Ni)/PtdIns[3]P (n = 38), (2.79 ± 0.05) nm for DGS-NTA(Ni)/PtdIns[4,5]P2 (n = 45) and (2.75 ± 0.07) nm for DGS-NTA(Ni)/PtdIns[3,4,5]P3 (n = 32). These results demonstrate that the addition of DGS-NTA(Ni) to the lipid composition does not alter the monolayer quality as deduced from the measured monolayer thickness. Only in case of the SHM composed of POPC and DGS-NTA(Ni), lacking a PtdInsP, a slightly larger mean ΔOTSHM value (3.0 ± 0.1 nm, n = 19) was found.

We next tested whether the PtdInsP lipid mobility in the monolayer is influenced by the presence of the Ni2+–loaded DGS-NTA-lipid. Sufficient lipid mobility is a prerequisite to ensure that a lateral interaction between CB2 and NL2 at the membrane interface is possible. To investigate the lateral lipid mobility, we replaced 10% of the PtdInsPs by the corresponding BODIPY®-TMR labeled phosphoinositides and performed

Figure 1. The postulated NL2-CB2 interaction at inhibitory synapses and the design of the SHM assay. A, Schematic drawing illustrating the GABAAR clustering machinery at inhibitory synapses. B, Recombinant CB2SH3 proteins (WT CB2SH3 or the constitutively active CB2SH3/W24A-E262A mutant) used in this study. C, Scheme of a supported hybrid membrane composed of an HMDS monolayer and a POPC monolayer (yellow) doped with DGS-NTA(Ni) (blue) and PtdInsPs (red) on a silicon dioxide substrate.
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Fluorescence recovery after photobleaching (FRAP) experiments. Fig. 3A shows a typical FRAP experiment of an SHM composed of POPC/DGS-NTA(Ni)/PtdIns[4,5]P₂/BODIPY®-TMR-PtdIns[4,5]P₂ (94:3:2.7:0.3, n/n) to an HMDS functionalized silicon dioxide surface at t = 0 min. The arrow indicates the time point of rinsing with buffer B. Box plots of ∆OTSHM of POPC monolayers doped with 3 mol% of PtdInsP. Box plots of ∆OTSHM of POPC monolayers doped with 3 mol% of PtdInsP and 3 mol% of DGS-NTA(Ni). The boxes extent from upper to lower quartile whereas the whiskers represent 1st and 99th percentiles. The medians are shown as horizontal lines and the means as red squares. DGS refers to DGS-NTA(Ni).

CB2 binding to phosphoinositides

Based on the established SHMs, we investigated the binding of CB2 to the different PtdInsPs in the absence (Fig. 5) and in the presence (Fig. 7) of bound cytNL2, whereas all other experimental conditions remain the same. Characteristic time traces of ∆OT upon SHM formation and CB2 addition (definition see Fig. 5B) to a POPC/PtdIns[3,4,5]P₃/DGS-NTA(Ni) (94:3:3, n/n) SHM in absence of cytNL2 are shown in Fig. 5A (CB2SH3) and B (CB2SH3/W24A-E262A). A protein concentration of 1 μM was chosen for these experiments to obtain sufficiently high protein coverage without wasting too much protein (Fig. S1A, B). Different lipid compositions were used to elucidate the binding properties of WT CB2SH3 and the CB2SH3/W24A-E262A mutant, which also allowed us to assess their nonspecific binding behavior. SHMs doped with only DGS-NTA(Ni) or with one of the three phosphoinositides were used or both receptor lipids were reconstituted simultaneously.

∆OT_{CB2} for the different lipid compositions are depicted in Fig. 5C for CB2SH3 and CB2SH3/W24A-E262A. In case of CB2SH3, a small increase in ∆OT of about 0.1 nm was observed independently of the chosen lipid composition [DGS-NTA(Ni) or...
Figure 3. FRAP experiment to access the mobility of PtdInsPs. A, Time lapse series of a FRAP experiment for an SHM composed of POPC/DGS-NTA(Ni)/PtdIns[4,5]P_2/BODIPY®-TMR-PtdIns[4,5]P_2, 94:3:2.7:0.3, n/n) at four different time points, with the bleached area indicated by a white circle. Scale bar: 5 μm. B, FRAP recovery curve for an SHM composed of POPC/DGS-NTA(Ni)/PtdIns[4,5]P_2/BODIPY®-TMR-PtdIns[4,5]P_2, 94:3:2.7:0.3, n/n). Box plots of the diffusion coefficients (D) in C and the mobile fractions (γ_0) in D of the three different labeled PtdInsP in the presence or absence of DGS-NTA(Ni). The boxes represent the S.E. whereas the whiskers show the S.D. The medians are shown as horizontal lines and the means as red squares. Significant differences are indicated by **p < 0.01 and ***p < 0.001.

Figure 4. Characterization of the different proteins used in this study. A, SDS-PAGE (left) and Western blotting overlay of chemiluminescence and marker image (right) of CB2_SH3. B, SDS-PAGE (left) and Western blotting overlay of chemiluminescence and marker image (right) of CB2_SH3/W24A-E262A. C, SDS-PAGE of His-cytNL2. M: Marker, 1: protein sample. The vertical black lines mark the splice borders.
PtdInsP or a combination thereof except for the POPC/PtdIns[4,5]P2/DGS-NTA(Ni) (94:3:3, \( n/n \)) membrane composition. The small \( \Delta OT \) values of about 0.1 nm indicate that there is no specific interaction between CB2SH3 and the lipids analyzed. They can in part be ascribed to changes in the refractive index \( n \) caused by the addition of protein to the aqueous solution.

On SHMs composed of POPC/PtdIns[4,5]P2/DGS-NTA(Ni) (94:3:3, \( n/n \)) a slightly larger \( \Delta OT \) of (0.24 ± 0.05) nm (\( n=4 \)) was found indicating an increased amount of adsorbed CB2SH3. We can only speculate that the electrostatics on the surface is slightly different owing to an interaction of DGS-NTA(Ni) with PtdIns[4,5]P2. This can alter the position of the PtdIns[4,5]P headgroup protruding more from the membrane surface (33), and thus induces electrostatically driven interactions. For CB2SH3/W24A-E262A significantly increased binding to the phosphoinositides was observed. Although there is a small nonspecific binding on DGS-NTA(Ni)-doped SHMs with \( \Delta OT \) of (0.20 ± 0.09) nm (\( n=3 \)), CB2SH3/W24A-E262A addition resulted in larger \( \Delta OT \) well distinguishable from the base line level, when only phosphoinositides were present. For PtdIns[3]P and PtdIns[4,5]P2 containing SHMs, \( \Delta OT \) was determined to be (0.28 ± 0.05) nm (\( n=7 \)) and (0.37 ± 0.07) nm (\( n=7 \)), respectively. The overall binding affinity given as a change in optical thickness at 1 \( \mu \)M protein concentration was largest for PtdIns[3,4,5]P3. Adsorption of CB2SH3/W24A-E262A led to \( \Delta OT \) of (0.67 ± 0.13) nm (\( n=4 \)). These results demonstrate that the CB2SH3/W24A-E262A mutant can interact with the phosphoinositides presumably because of an open conformation induced by the mutations. In contrast, the WT CB2SH3 apparently remains in a closed, inactive conformation, rendering the protein incapable of interacting with PtdInsPs.

The adsorption of CB2SH3/W24A-E262A in presence of DGS-NTA(Ni) resulted in \( \Delta OT \) of (0.35 ± 0.04) nm (PtdIns[3]P, \( n=8 \)), (0.57 ± 0.05) nm (PtdIns[4,5]P2, \( n=7 \)) and (0.43 ± 0.07) nm (PtdIns[3,4,5]P3, \( n=9 \)) showing that DGS-NTA (Ni) does not significantly influence the binding of the active mutant.

**Binding of His-cytNL2 to the SHMs**

To induce the postulated conformational change in CB2SH3 upon interaction with the cytosolic part of NL2, the two proteins need to interact with each other at the membrane interface. Thus, we investigated whether His-cytNL2 can be specifically bound via DGS-NTA(Ni) to the membrane. The specific adsorption of His-cytNL2 to DGS-NTA(Ni) doped membranes was measured by RIfS (Fig. S1C). For a concentration of 1.36 \( \mu \)M a specific binding with a mean change in \( OT \) of (0.69 ± 0.09) nm (\( n=4 \)) was observed (Fig. 6). To show the
Monolayer formation was initiated by the addition of SUVs (I). After rinsing with buffer B (II), His-WSH3 was capable of binding to PtdInsP-doped membranes in the absence of buffer B (IV), but CB2 was added (V, 1 NL2 (Fig. 7A). After cleavage, only a small change in \( \Delta OT_{NL2} \) was found for CB2 without any activation via cytNL2. CB2 was added (V in Fig. 7B) and one of the three PtdInsPs (III in Fig. 7B), either CB2SH3 or CB2SH3/W24A-E262A, which is expected to bind to phosphoinositide membranes, was added (V in Fig. 7B, and the change in optical thickness \( \Delta OT_{CB2*} \) was determined to account for binding to PtdInsPs and PtdIns[4,5]P2 containing SHMs, respectively (Fig. 7C). For comparison \( \Delta OT_{CB2*} \) was determined to be (0.12 ± 0.02) nm (n = 4). The difference in \( \Delta OT_{CB2*} \) was significant (p < 0.001) reduced amount of the CB2SH3/W24A-E262A mutant protein binds to the membrane surface in the presence of cytNL2 (Fig. 7C) compared with that found in its absence (Fig. 5C, lanes PtdInsPs & DGS). \( \Delta OT_{CB2*} \) was determined to be (0.12 ± 0.02) nm (n = 8) and (0.17 ± 0.02) nm (n = 5) for PtdIns[3]P and PtdIns[4,5]P2 containing SHMs, respectively (Fig. 7C). For comparison \( \Delta OT_{CB2*} \) was determined to be (0.35 ± 0.04) nm (PtdIns[3]P and 0.57 ± 0.05) nm (PtdIns[4,5]P2 (Fig. 5C) was measured. Only in case of PtdIns[3,4,5]P3 doped membranes the overall amount of adsorbed CB2SH3/W24A-E262A was not significantly influenced in the presence of cytNL2 with \( \Delta OT_{CB2*} \) = (0.37 ± 0.04) nm (n = 8) compared with (0.43 ± 0.07) nm (n = 9) in its absence (compare Fig. 5C and Fig. 7C). These results indicate that the natively unfolded cytNL2 occludes some of the PtdInsPs, possibly by ionic interactions. This inaccessibility of binding sites for CB2 leads to a reduction in binding capability of the CB2SH3/W24A-E262A mutant. Taken the reduced \( \Delta OT_{CB2*} \) values into account, one can calculate the fraction of remaining available binding sites (ABS) in the presence of cytNL2, defined as:

\[
\text{ABS} = \frac{\Delta OT_{CB2*} \text{ (mutant)}}{\Delta OT_{CB2*} \text{ (mutant)}}
\]

resulting in 34% (PtdIns[3]P), 30% (PtdIns[4,5]P2) and 86%
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(98x164,PtdIns[3,4,5]P_3). Thus, a direct comparison of CB2 WT adsorption in presence and absence of cytNL2 appears not to be reasonable as cytNL2’s potential to compromise the CB2-PtdInsP interaction has to be considered. Despite this fact, the trend that the amount of bound CB2SH3 in the presence of cytNL2 increases becomes obvious, if we compare the obtained \( \Delta OT_{CB2} \) and \( \Delta OT_{CB2*} \) values of CB2SH3 related to the corresponding values for CB2SH3/W24A-E262A that are measured under identical conditions and are set to 100%. This calculation leads to an increase of the relative amount of adsorbed CB2SH3 from 25% to 133% (PtdIns[3]P), from 42% to 153% (PtdIns[4,5]P_2) and from 9% to 68% (PtdIns[3,4,5]P_3).

**Discussion**

The present study provides direct molecular evidence of an NL2-mediated activation of CB2, leading to increased CB2 binding to plasma membrane PtdInsPs.

Previous studies led to the hypothesis that the cytosolic NL2 C terminus binds the SH3 domain of CB2 in the biological context of the postsynaptic membrane (14, 29). Using SHMs, which provide a homogeneous distribution of PtdInsPs (30), we were able to establish an *in vitro* molecular model that allowed us to investigate directly how the interaction of CB2 with the cytosolic part of NL2 influences its ability to bind to different PtdInsPs. Our results show that, without the membrane-associated part of NL2, the WT protein CB2SH3 does not bind to any of the three PtdInsPs tested. This agrees with previous studies indicating that CB2SH3 adopts a closed, autoinhibited conformation, in which the PH domain is not accessible for PtdInsP binding (14, 32). As previously shown (14), the mutant protein CB2SH3/W24A-E262A, in which intramolecular interactions between the C-terminal PH and N-terminal SH3 domains of CB are weakened, adopts a more open conformation, which allowed the protein to interact with all three PtdInsPs used in the present study. We have chosen PtdIns[3]P as one of the receptor lipids based on previous studies highlighting the high affinity of CB2 to this PtdInsP (19, 32). The other two PtdInsPs are the most abundant ones in the post synaptic plasma membrane (34, 35) and present during postsynaptic formation.

Whereas the interaction of CB with PtdIns[3]P allows the accumulation of CB and CB-associated proteins on early-endosomal membranes (22), the small GTPase TC10 binds to the PH domain of CB and induces a phospholipid affinity switch in CB, which allows CB to specifically interact with PtdInsP species present at the plasma membrane, such as PtdIns[4,5]P_2 and PtdIns[3,4,5]P_3 (31, 36). Similarly to the interaction of NL2 with CB2SH3, the TC10-CB2SH3 interaction was previously suggested to interfere with intramolecular interactions between the different domains of CB2SH3, leading to a transition toward an open state of CB, which allows the PH domain to specifically bind to PtdInsPs located at the plasma membrane (36).

In order to induce the postulated conformational change in CB2SH3 upon interaction with the cytosolic part of NL2, the two proteins have to interact with each other at the membrane interface. To bind the cytosolic part of NL2 to the membrane, we exploited a His_6-tag-DGS-NTA(Ni) strategy. Even though two oppositely charged lipids were inserted into the POPC matrix, a homogeneous SHM was produced, with laterally mobile lipids. However, the diffusion coefficients \( D \) of the BODIPY®-TMR PtdInsPs were reduced by about 50% compared with those without DGS-NTA(Ni). These diffusion coefficients are still in the range of those found in cellular plasma membranes of fibroblasts and epithelial cells with an average of \( D = (0.8 \pm 0.2) \mu m^2/s \) (37) and hence appear to be sufficient to allow for lateral protein-protein interactions at the membrane interface.

Our comparative analysis of the binding of CB2SH3 (WT and the W24A-E262A mutant) to PtdInsPs in the presence or absence of cytNL2 clearly indicates that the cytosolic C terminus of NL2 alters the conformation of CB2 and thereby controls CB2-interactions with PtdInsPs at the plasma membrane. In the absence of cytNL2, only the "open conformation-mutant", CB2SH3/W24A-E262A, efficiently interacted with PtdInsPs. In contrast, in the presence of cytNL2, similar amounts of WT CB2SH3 and the CB2SH3/W24A-E262A mutant bound to PtdInsPs. However, there is an overall decrease in the amount of CB2SH3/W24A-E262A bound to the three PtdInsPs in the presence of cytNL2, as compared with that in the absence of membrane anchored cytNL2. One cannot rule out that the W24A-E262A double-mutation of CBSH3 alters the specificity for the different PtdInsPs compared with the WT protein, as shown previously for CB and other proteins (38, 39). Moreover, in agreement with this hypothesis, a previous study indicated that a single (R290H) mutation in the DH domain of CB, which leads to epilepsy and intellectual disability in humans, alters the strength of intramolecular interactions between the DH and the PH domains of CB, thereby leading to a reduced PtdIns[3]P-binding of CB (21). Thus, whereas the interaction of WT CB2SH3 with endogenous activator-proteins, such as the cell-adhesion protein NL2 studied here or the small Rho-like GTPase TC10 (31), leads to a fine-tuned increase of the interaction of CB2 with certain PtdInsPs enriched at the plasma membrane, the "open-conformation mutant" CB2SH3/W24A-E262A might lead to a more general and less specific increase of CB binding to a broader range of PtdInsPs.

As regards WT CB2SH3, an interesting finding of our study is that for PtdIns[3,4,5]P_3 the \( \Delta OT_{CB2}^* \) (in the presence of cytNL2) was significantly increased, as compared with the \( \Delta OT_{CB2} \) (in the absence of cytNL2). For PtdIns[3]P and PtdIns[4,5]P_2, a similar trend toward increased \( \Delta OT_{CB2}^* \) was observed but did not reach significance, as compared with \( \Delta OT_{CB2} \). However, for both, WT CB2SH3 and the W24A-E262A mutant, it appears that the PtdInsP-accessibility is reduced upon His-cytNL2 binding to the membrane, resulting in an overall decrease in the amount of bound CB2 proteins. If this is assumed, we can relate the \( \Delta OT_{CB2}^* \) values obtained in the absence of cytNL2 to the changes in optical thickness for CB2SH3 in presence of cytNL2 and calculate an increase in the amount of bound CB2SH3 for all three PtdInsPs with a maximum increase for PtdIns[3,4,5]P_3. This agrees with a previous study, indicating that endogenous CB2 activators can induce a switch in the conformation of CB2, which allows enhanced interaction with plasma membrane-PtdInsPs (31). Thus, we conclude that in the presence of the cytosolic domain of NL2 a switch from a
closed to an open conformation of CB2 is induced, which enables CB2 to properly anchor at nascent inhibitory postsynapses enriched in PtdIns[4,5]P_2 and PtdIns[3,4,5]P_3. It is likely that this conformational switch is induced by the interaction of the N-terminal SH3 domain of CB2 with poly-proline sequences in cytNL2 (13, 40). As we only observed the water-soluble cytosolic domain of NL2, we moreover conclude that a dimerization of NL2 via the transmembrane domains, as it was observed for NL2 in vivo (41), is not required for CB2 activation.

**Experimental Procedures**

**Materials**

C_{16} derivatives and BODIPY®-TMR labeled derivatives of PtdInsPs (PtdIns[3]P, PtdIns[4,5]P_2, and PtdIns[3,4,5]P_3) were obtained as from Echelon Biosciences (Salt Lake City, UT). 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-[[N-(5-amino-1-carboxyptenyl)iminodiacid][succinyl]}nickel salt (DGS-NTA(Ni)) were purchased from Avanti Polar Lipids (Alabaster, Alabama). BL21(DE3) competent Escherichia coli cells were purchased from Invitrogen whereas BL21(DE3) Rosetta competent E. coli cells were from VWR International (Darmstadt, Germany). Chitin resin was obtained from New England Biolabs (Ipswich, MA) and NTA(Ni^{2+}) agarose (Protino^{®} from Macherey-Nagel (Düren, Germany). Antibodies specific for CB2 (1: 1000, Cat. No. 261 011) and NL2 (1: 1000, Cat. No. 129 213) were purchased from Synaptic Systems (Göttingen, Germany) whereas the His-tag antibody (1: 1000, ab18184) was obtained from Abcam (Cambridge, UK). Silicon wafers were purchased from Silicon Materials (Kaufering, Germany). 1,1,1-Trimethyl-N-(TMS)ilanamine (HMDS) was purchased from Silicon Materials (Kaufering, Germany). 1,1,1-Trimethyl-N-(TMS)ilanamine (HMDS) was purchased from Silicon Materials (Kaufering, Germany).

**Protein purification**

Proteins were recombinantly expressed in E. coli following previously described protocols (14, 42). Briefly, His-cytNL2 was obtained from transformed E. coli BL21(DE3) Rosetta cells containing the bacterial expression vector pETM11 (EMBL, Heidelberg, Germany). The plasmid was kindly provided by the group of Hermann Schindelin (Rudolf-Virchow-Zentrum, Würzburg, Germany). It encodes the intracellular domain of NL2 with an N-terminally fused histidine tag. First, the cells were grown to an OD_{600} = 0.8 in kanamycin (50 μg·ml^{-1}) containing LB medium. Protein expression was induced by addition of 0.5 mm isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation for ≥15 h at 15 °C, the cells were harvested by centrifugation (4,000 × g, 20 min, 4 °C), and cell lysis was performed using a microfluidizer (1 kbar, three cycles, ice cooled; LM10 processor, Microfluidics, Westwood, MA, USA). After centrifugation (70,000 × g, 30 min, 4 °C), the supernatant was applied to the equilibrated chitin resin column for 1 h. The resin was rinsed with 500 ml of wash buffer B (1 mg/ml NaCl, 20 mm HEPES, 2 mm EDTA, pH 8.0), and protein cleavage was induced by incubation with 50 mm DTT in buffer C (250 mM NaCl, 20 mM HEPES, 2 mM EDTA, pH 8.0) for >24 h. Finally, the protein was eluted with buffer C containing 5 mm DTT. Concentration and dialysis to buffer B (100 mM NaCl, 25 mM HEPES, pH 8.0) was performed by ultrafiltration using spin concentrators (Sartorius, Göttingen, Germany). Protein concentrations were determined by UV/Vis spectroscopy using extinction coefficients of ε_{280}(CB2_{siH3}) = 98,945 m^{2}·cm^{-1} and ε_{280}(CB2_{siH3/W24A-E262A}) = 93,445 m^{2}·cm^{-1}.

All proteins were analyzed by SDS-PAGE and Western Blots using a Gel-Imager (Azure c300, azure biosystems, Dublin, USA) for documentation.

**Substrate preparation**

Silicon substrates with a SiO_{2} layer thickness of 5 μm were cleaned two times with detergent solution followed by rinsing with ultrapure water in an ultrasonic bath for 15 min each. Afterward, the substrates were treated with O_{2}-plasma for 30 s and then exposed to hexamethyldisilazane (HMDS) as previously described in detail (30).

**Vesicle preparation**

A stock solution of POPC was prepared in chloroform at a concentration of 10 mg·ml^{-1}. Lyophilized PtdInsPs were dissolved in mixtures of chloroform/methanol/water to concentrations of 1 mg·ml^{-1}. Lipid stock solutions (0.4–0.8 mg of total lipid material) were mixed in a test tube preloaded with 100 μl chloroform at the desired molar ratio. Fluorophores were
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added as indicated. Organic solvent was evaporated with a gentle stream of nitrogen at 25 °C. To remove residual solvent, the lipid film was dried under vacuum for 3 h at the corresponding temperature. Lipid films were stored at 4 °C until use. A lipid film was rehydrated by adding 0.5–1.2 ml of spreading buffer (20 mM citrate, 50 mM KCl, 0.1 mM NaN₃, pH 4.8) and incubated for 30 min. Multilamellar vesicles (MLVs) were obtained by vortexing for 3 × 30 s at 5 min intervals. The MLV suspension was transferred to an Eppendorf cup and sonicated for 30 min using an ultrasonic homogenizer (Sonopuls HD2070, resonator cup; Bandelin, Berlin, Germany) to obtain small unilamellar vesicles (SUVs).

Reflectometric interference spectroscopy (RIfS)

RIfS is a label-free, noninvasive technique determining the optical thickness (n × d) of a thin layer by measuring white light interference. This interference is caused by partial reflection at interfaces whose distance is within the coherence length of white light (43). RIfS was employed to monitor the formation of SHMs and subsequent protein adsorption to receptor lipid containing membranes in a label-free and time-resolved manner. The experimental setup is described in detail elsewhere (44). Briefly, a Flame-S-UV/Vis spectrometer (Ocean Optics, Dunedin, FL, USA) was used to record interference spectra at intervals of 2 s. Data were evaluated applying a MATLAB (The MathWorks, Natick, MA, USA) tool following the work of Krick et al. (44).

Confocal laser scanning microscopy (CLSM)

CLSM images were taken with a confocal laser scanning microscope LSM 880 (Carl Zeiss Microscopy GmbH, Oberochen, Germany) equipped with a 40× objective (W Plan-Apochromat, NA = 1.0, Zeiss). BODIPY®-TMRRødPtdInsPs were monitored at 520–650 nm after excitation at 488 nm.

Fluorescence recovery after photobleaching (FRAP)

Fluorescence intensity in a region of interest (ROI) of a model membrane doped with one of the BODIPY®-TMRRødPtdInsPs (λ_bleach = 488 nm) was bleached by a short laser pulse, and the time-dependent fluorescence recovery was recorded with a frame rate of 3–4 frames s⁻¹. The diffusion coefficients and mobile fractions were calculated using a Hankel transformation (45).

Data availability

All data are contained within the article.

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Abbreviations—The abbreviations used are: GABA, γ-aminobutyric acid; NL2, Neuroligin-2; PtdInsPs, phosphoinositide; CB, Collybistin; PH, pleckstrin homology; GEF, guanine nucleotide exchange factor.

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