Calcium concentration threshold and translocation kinetics of EGFP-DOC2B expressed in cultured Aplysia neurons

Guy Malkinson, Micha E. Spira*

Department of Neurobiology, The Life Sciences Institute, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Received 1 August 2005; received in revised form 10 October 2005; accepted 17 October 2005

Available online 21 November 2005

Abstract

The double C2 domain protein family (DOC2) is characterized by two calcium-binding domains (C2). Upon binding to calcium, the affinity of the protein to phospholipids is significantly increased, leading to translocation of the protein from the cytosol to the plasma membrane. These properties, and the binding domain of DOC2B to Munc13, suggested that DOC2B could play a role in augmentation and potentiation of synaptic release. Nevertheless, the level of the free intracellular calcium concentration ([Ca2+]i) which triggers its translocation under in vivo conditions, is not known.

Using cultured Aplysia neurons that express rat EGFP-DOC2B, we found that the [Ca2+]i increment necessary to induce EGFP-DOC2B translocation is approximately 200 nM in the bulk of the cytoplasm. The rate of EGFP-DOC2B recruitment to the plasma membrane is slower than the [Ca2+]i elevation rate, while the detachment of EGFP-DOC2B from it is faster than the calcium removal. The extent of EGFP-DOC2B translocation to the plasma membrane reflects local submembrane [Ca2+]i.

Our observations are consistent with the view that DOC2B can participate in the regulation of neurotransmitter release. It should be noted that EGFP-DOC2B could be used as a tool to map sub-membrane calcium dynamics under physiological conditions.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: EGFP-DOC2B; Calcium; Aplysia

1. Introduction

The double C2 domain protein family (DOC2) is characterized by two calcium-binding domains (C2). The C2 domain is found in a large group of over fifty proteins including, for example, synaptotagmin I, Rabphilin3-A, PKC and Munc-13 (for review[1]). Upon binding to calcium, the affinity of the protein to phospholipids is significantly increased, leading to the translocation of the protein from the cytosol to the plasma membrane.

DOC2 is a 400 amino-acid long protein, with no predicted transmembrane domain[2]. So far three DOC2 isoforms have been found: DOC2A, specifically expressed in neuronal cells, and DOC2B and DOC2C, which are expressed ubiquitously. Whereas DOC2A and DOC2B were shown to interact with Munc13-1 and Munc18, proteins that are involved in synaptic neurotransmitter release[3–5], DOC2C does not show Ca2+-dependent phospholipid-binding activity, and was thus suggested to serve a role in negative regulation of Munc13-1[6].

It has been demonstrated in HEK293 cells that application of phorbolster (PE) can cause DOC2B translocation to the plasma membrane, and that this translocation is mediated by the interaction of DOC2B and Munc13-1[7]. Overexpression of full-length DOC2 was shown to enhance Ca2+-dependent exocytosis of growth hormone in PC12 cells[8]. The possibility that DOC2B is involved in neurotransmitter release is supported by a recent study showing that membrane depolarization by potassium chloride (KCl) is sufficient to cause translocation of DOC2B to neuronal plasma membranes[9]. GST-DOC2B fusion proteins were used to measure the calcium-dependent affinity of DOC2B to phospholipids in a cell free system. Under these conditions the half-maximal
value of calcium concentration to induce DOC2B translocation was found to be 5 μM. The half-time of the association and dissociation rates of DOC2 with the plasma membrane were studied by potassium depolarization of cultured cells. The values obtained were approximately 2 and 8 s, respectively [9]. The above-mentioned properties of DOC2 suggest that it may play a role in slow components of synaptic events, such as augmentation and potentiation (reviewed in [10,11]).

Two aspects, which are required to evaluate the potential physiological role of DOC2 proteins, were not addressed to date. The first is the [Ca2+]i threshold which triggers the following events, such as augmentation and potentiation (reviewed in [9]). The above-mentioned properties of DOC2 suggest that it may play a role in slow components of synaptic events, such as augmentation and potentiation (reviewed in [10,11]).

The values obtained were approximately 2 and 8 s, respectively [9]. The above-mentioned properties of DOC2 suggest that it may play a role in slow components of synaptic events, such as augmentation and potentiation (reviewed in [10,11]).

For the study we expressed EGFP-DOC2B in identifiable cultured Aplysia neurons and examined the relationships between the [Ca2+]i, and EGFP-DOC2B translocation from the bulk of the cytoplasm to the plasma membrane.

2. Methods

2.1. Cell culture

Neurons B1 and B2 from the buccal ganglia of Aplysia californica were isolated and maintained in culture as previously determined [12–14].

The cultures were maintained in Leibovitz’s L-15 Medium (Gibco-BRL, Paisley, Scotland) supplemented for marine species by the addition of 12.5 g/l NaCl, 6.86 g/l (s) glucose, 3.15 g/l anhydrous MgSO4, 344 mg/l KCl, 192 mg/l NaHCO3, 5.7 g/l MgCl2·6H2O and 1.49 g/l CaCl2·2H2O, 10% filtered hemolymph (obtained from Aplysia fasciata). Penicillin, streptomycin and amphotericin B (Biological Industries, Kibbutz Beit Haemek, Israel) were added to reach a final concentration of 100 units/ml, 0.1 mg/ml and 0.25 μg/ml, respectively. Confocal imaging was done in artificial seawater (ASW) composed of: NaCl 460 mM, KCl 10 mM, CaCl2 11 mM, MgCl2 55 mM, HEPES 10 mM, adjusted to pH 7.6.

2.2. Fluorescence microscope imaging of fura-2

The fluorescent microscope system used consisted of a Zeiss Axiosvert microscope equipped with a 75 W Xenon arc lamp. The objective lens used was a Zeiss 40×/0.75 NA Plan-Neofluar objective. Images were collected with an intensified CCD video camera (Hamamatsu) digitized directly at 512 × 512 pixels with a PC hosted frame grabber (Imaging Technologies), stored as computer files and processed using a software package written in our laboratory. The final images were prepared using commercially available software (Adobe Photoshop).

Fura-2 imaging was done as previously described [15–18]. Briefly, the cell body was impaled with 5–10 MΩ micropipettes filled with 2 M KCl and 10 mM fura-2 potassium salt (Molecular Probes). The indicator was loaded into the neurons by pressure injection. The neurons were loaded to a final indicator concentration of 25–75 μM. Imaging was performed after the dye had equilibrated throughout the main axon (approximately 30 min).

Calcium concentration imaging was done by real-time averaging of 16 video frames. Images were grabbed using a filter set, composed of 340 ± 5 and 380 ± 5 nm band pass excitation filters, set in a computer-controlled, lambda-10 filter changer, a dichroic mirror with a cut-off of 395 nm, and a 510 ± 10 nm band pass emission filter. As the axons autofluorescence was negligible compared to the fluorescence intensity of the Ca2+ indicators, background images at excitation wavelength of 340 and 380 nm were obtained from regions near the axon. Ratio images of the fluorescent intensities were obtained by dividing each pixel in the 340 nm fluorescence images by the corresponding pixel in 380 nm images.

The fura-2 ratio values were converted to free intracellular Ca2+ concentration by means of a calculated curve as previously developed by Grynkiewicz et al. [15]. To that end the parameters Rmax and K0 of the system were determined with a Ca2+ free calibration solution. Rmax and K0 were determined in a Ca2+ saturated calibration solution. A dissociation constant of fura-2 and Ca2+ (K0) of 760 mM was used as previously determined by Ziv and Spira [16].

2.3. Fura-2 and DOC2B imaging

Imaging of the [Ca2+]i, and EGFP-DOC2B translocation from a single neuron, while stimulating it to fire trains of action potentials, was done as follows: A neuron was first alternatingly imaged at 340 and 380 nm for several seconds prior to the experiment. Then alternating imaging proceeded at 380 nm (for calcium) and at 490 nm (for EGFP-DOC2B) during the generation of action potentials and after it, at rates of approximately 0.5 s⁻¹. During the resting periods between the stimuli the axon was again ratio imaged at 340 nm/380 nm. The initial images obtained by excitation wavelength of 340 nm obtained prior to the stimuli (referred to as 340i) were used off-line to produce ratio values of 340 nm/380 nm for the above-described series of 380 nm/490 nm images that were taken during the experiment. To assess the underestimation of the ratio value introduced by the use of 340i nm images obtained prior to the stimuli, we applied the exact same train of stimuli to the same neuron and estimated the [Ca2+]i, using the 340 nm/380 nm ratio imaging as described [15,16,19]. The values reported in the study were corrected for this underestimation. At the range of 140–170 mM free intracellular calcium concentration, the procedure used introduces an underestimation of the [Ca2+]i, by 40 ± 20 mM. Since bleaching of fura-2 takes place during the course of the experiments, the bleaching...
was corrected using a value obtained from the same experiment. Fura-2 and EGFP-Doc2B images were analyzed offline using NIH ImageJ software (Bethesda, MD) by selecting 1–3 rectangular areas of interest (AOI). For EGFP-Doc2B analysis, pairs of AOIs were chosen: each pair consisted of an AOI surrounding the plasma membrane area and an AOI of the exact same size, in the cytosolic region adjacent to it. The ratio of the averaged fluorescent intensity of the membranal AOI divided by the average cytosolic signal was calculated. For Fura-2, only the cytosolic regions were analyzed as described above.

Since EGFP-Doc2B expression levels varied between different cells, and also exhibited a certain degree of variability in their response to calcium elevations, we also used logistic regression analysis [20] (SPSS software, SPSS, Chicago) to estimate the probability of Doc2B to undergo translocation to the membrane in response to different [Ca\(^{2+}\)].

2.4. Confocal-microscope imaging of EGFP-Doc2B, rhod-2; RH237

The system used for confocal imaging consisted of an Olympus microscope IX70 and a Bio-Rad Radiation 2000/AGR-3 confocal imaging system. The objective used was an Olympus planApo 60× 1.4 NA oil objective.

Images of EGFP-Doc2B fusion proteins were acquired by excitation at 488 nm (argon laser), the emitted fluorescence collected at 500–560 nm.

For calcium imaging using rhod-2 (Molecular Probes) the cell body was impaled with 10–15 MΩ microelectrode filled with 2 M KCl and 10 mM rhod-2 potassium salt (Molecular Probes). The indicator was loaded into the neurons by pressure injection. The neurons were loaded until a vague indicator signal (a few gray levels) was detectable in the axon. Imaging was performed after the dye had equilibrated throughout the main axon (approximately 30 min). The dye was imaged with the 543 nm laser line, and collected with the 590/70 band-pass filter.

RH237 (a gift from Dr. R. Hildesheim, The Weizmann Institute) was added to a final concentration of 7.4 μM in the experimental plate. It was imaged with the 488 nm laser-line and collected with the 610 LP filter.

Ionomycin (Sigma) was prepared as stock solution in DMSO and diluted in ASW to a final concentration of 50 μM in the micropipette. Care was taken to ensure that no contact was made by the micropipette and the plasma membrane.

Phorbol 12,13-dibutyrate (PDBu) (Sigma) was prepared as stock solution in DMSO and diluted to the final concentration of 1 μM in the experimental dish.

All images were then collected and processed using laser-Sharp and laserPix BioRad software, respectively. The figures were prepared using Adobe Photoshop software. For calculation of the rhod-2 F0, the first 500 points were averaged.

2.5. EGFP-Doc2B mRNA preparation and injection

mRNAs encoding EGFP-Doc2B (a gift from Dr. M. Verhage and Dr. A.J. Groffen, Amsterdam, The Netherlands) were in vitro transcribed using recombinant transcription system as described by our laboratory [21]. The transcribed mRNAs were pressure injected into the cytoplasm of the cultured neurons 4–12 h after plating. For the injections 0.5–5 μg/μl mRNA was prepared in 80 mM KCl. We estimate the injected volume to be approximately 10% of the cell’s body volume. Throughout the injection the input resistance and transmembrane potential of the neuron were recorded by the injection micropipette. At the end of the injection the micropipette was pulled out of the cell.

3. Results

3.1. Expression and translocation of heterologous Doc2B in cultured Aplysia neurons

We began by examining whether rat Doc2B could be expressed in cultured Aplysia neurons, and if so, whether it can translocate from the cytosol to the plasma membrane in response to elevation of the [Ca\(^{2+}\)], and PDBu (phorbolester) application.

To that end, cultured buccal neurons were pressure injected with mRNA encoding EGFP-Doc2B as described earlier by our laboratory [21]. We found that EGFP-Doc2B could be imaged as early as 4 h after mRNA injection (Fig. 1). Doc2B expressing neurons developed normally in culture and no effects on the passive or excitable membrane properties were recognized. In rested neurons EGFP-Doc2B is homogeneously distributed in the cytoplasm, and is not present in the nucleus. To examine whether physiological stimuli that elevate [Ca\(^{2+}\)], induce Doc2B translocation to the plasma membrane, we loaded the neurons with the calcium indicator rhod-2. Upon intracellular stimulation, a rise in the [Ca\(^{2+}\)], was detected (Fig. 1 compare (A2) to (B2)), accompanied by the translocation of Doc2B to the membrane (Fig. 1 compare (A2) to (B2)). After the termination of the stimulus, the [Ca\(^{2+}\)] recovered (Fig. 1(C2)) and the EGFP-Doc2B translocated back to the cytosol (Fig. 1(C2)).

To better visualize the translocation of EGFP-Doc2B to lipid membranes, we labeled the plasma membrane and subcellular organelles with the styryl dye RH237 [22]. On-line confocal imaging of EGFP-Doc2B translocation in response to the trains of action potentials generated by an intracellular microelectrode (Fig. 1(D)) revealed that Doc2B translocated to both the plasma membrane and intracellular organelles. However, not all organelles were labeled by EGFP-Doc2B (Fig. 1(E2)). Interestingly, as noted earlier [9] the translocation of EGFP-Doc2B to the membrane is not uniform and showed focal accumulations (Fig. 1(E2) arrowheads).

We next examined whether EGFP-Doc2B translocates from the cytosol to the plasma membrane in Aplysia neurons
Fig. 1. DOC2B translocates to the plasma membrane and to intracellular membranes. A buccal neuron expressing EGFP-DOC2B was loaded with the calcium indicator rhod-2. Generation of ten consecutive trains of action potentials by a sharp intracellular electrode (D), led to a rise in $[Ca^{2+}]_i$ (A2 to B2) and EGFP-DOC2B translocated to the plasma membrane (A1 to B1). Both EGFP-DOC2B and $[Ca^{2+}]_i$ recovered to baseline levels (C1 and C2 correspondingly). To image the distribution of EGFP-DOC2B in the plasma membrane and intracellular organelles, a buccal neuron was labeled with the styryl dye RH237 (red) (E1 to E3). The cell was stimulated to fire action potentials leading to EGFP-DOC2B translocation to the plasma membrane and to some of the RH237 labeled organelles (E2, yellow, indicating co-localization). Note the accumulations of EGFP-DOC2B fluorescent signal in patches of the plasma membrane (arrows), its translocation to intracellular organelles (arrowheads), and the intracellular organelles to which DOC did not translocate (asterisks).

in response to the application of PDBu. To that end, cells expressing DOC2B were first stimulated to generate action potentials to confirm that EGFP-DOC2B translocation can be imaged. Then, 1 μM PDBu was applied to the bathing solution, and the DOC2B signal was monitored for up to 45 min. No EGFP-DOC2B translocation was imaged. Since in cultured Aplysia neurons synaptic PE facilitates transmission at concentrations of 20–100 nM [23], we conclude that PDBu has no effects on DOC2 translocation in this preparation. As a Munc-13 EST was identified in Aplysia neurons (Prof. E. Kandel, personal communication), this observation suggests that the specific molecular domains that link Munc-13 and DOC2B in a phorbolester dependent manner [3,7,24] are not expressed in Aplysia neurons. In the following we thus focused on the analysis of the relations between DOC2B translocation and $[Ca^{2+}]_i$ kinetics.
3.2. EGFP-DOC2B translocation to the plasma membrane follows calcium concentration gradients

The uneven "patchy" distribution of EGFP-DOC2B in the plasma membrane (Fig. 1(E2)) can represent either heterogeneities in the distribution of voltage gated calcium channels, or the formation of submembrane local calcium gradients due to uneven distribution of buffers, or differences in the affinity of EGFP-DOC2B to plasma membrane components. Use of fura-2 ratio imaging, rhod-2 or fluo-4 in cultured Aplysia neurons, revealed that stimulation of the neurons by trains of action potentials elevates the [Ca\(^{2+}\)] homogeneously in the axon’s cytosol (Figs. 1(A2–C2) and 2(B2); see also Ziv and Spira [16]). It was thus impossible to use classical water-soluble calcium-indicators to differentiate between the above hypotheses. We therefore examined whether local elevation of the [Ca\(^{2+}\)], along plasma membrane patches that are not labeled by EGFP-DOC2B following membrane depolarization, can be labeled by EGFP-DOC2B if the calcium concentration is locally elevated. For the experiment (n = 4), a neuron expressing EGFP-DOC2B was loaded by rhod-2 (Fig. 2(A2) and (A2)). The neuron was then stimulated to fire a train of action potentials (not shown) leading to elevation of the [Ca\(^{2+}\)], and EGFP-DOC2B translocation (Fig. 2(B1)) and (B2) correspondingly. We focused in this experiment on a patch of plasma membrane that was not labeled as intensely as the surrounding plasma membrane by EGFP-DOC2B (Fig. 2(B1) rectangle, and (D1)). Local ionomycin applications to this patch of membrane led to a local elevation of the [Ca\(^{2+}\)] (Fig. 2(C2)) and localized EGFP-DOC2B translocation to the plasma membrane (Fig. 2(C1) and (D2)). It should be noted that while the [Ca\(^{2+}\)] gradients declined

![Image of experimental setup with labeled regions](image-url)
over a distance of tens of microns (Fig. 2(C2)), the EGFP-DOC2B translocation to the plasma membrane was by far more restricted (Fig. 2(C1)).

This observation is consistent with the view that the translocation of EGFP-DOC2B to the plasma membrane is affected by submembrane [Ca2+]i gradients and thus may be used to map the density of voltage gated calcium channels on the plasma membrane and the ensuing local submembrane calcium levels with much higher fidelity than conventional water soluble calcium indicators that rapidly diffuse in the cytosol [19].

Interestingly, EGFP-DOC2B also forms membrane patches in response to bath application of ionomycin (not shown). These observations suggest that in addition to local calcium concentration gradients, the local composition of the plasma membrane may also affect the differential localization of EGFP-DOC2B.

3.3. Kinetics of DOC2B

The kinetics of DOC2B translocation was studied by Groffen et al. [9] in response to potassium-induced membrane depolarization. The half-time for the on rate was found to be 2.05 ± 0.11 s and that of the off rate 7.9 ± 0.6 s. Here, we examined the kinetics in cultured neurons in response to trains of action potentials generated by intracellular stimulation. To that end we used the single-line mode scan of the confocal microscope. This mode of imaging allows for a sampling rate of 166 Hz. For the experiments we first imaged the EGFP-DOC2B translocation by conventional confocal microscope scanning and then selected a membrane patch that revealed a clear increase in DOC2B fluorescence. Calcium was imaged simultaneously with rhod-2.

Next, we set the system to line scan this location (Fig. 3(A) and (B)). The translocation of DOC2B is presented as the ratio of the fluorescent intensity in the membrane and the axoplasmic domain. The calcium level is given as the relative intensity as measured in the axoplasm (ΔF/F0). In all experiments the initial rate of rise of the [Ca2+]i was significantly faster than that of EGFP-DOC2B translocation to the membrane. As soon as the train of action potentials ends, both the [Ca2+]i and the EGFP-DOC2B signal declines. Nevertheless, in most experiments the rate of calcium removal from the axoplasm was slower than that of EGFP-DOC2B. In some experiments the rates of decay were identical.

3.4. [Ca2+]i threshold for DOC2B translocation

Earlier studies of DOC2B translocation established that potassium depolarization is sufficient to induce EGFP-
DOC2B translocation in intact cells. The calcium concentration, which induces cytosol-to-lipid translocation, was determined so far using cell-free liposome systems [9, 25]. In these experiments the threshold for EGFP-DOC2B translocation was found to be in the micromolar range, the half maximal translocation was estimated to be close to 8 μM and saturation at approximately 100 μM.

The [Ca^{2+}]_i threshold for EGFP-DOC2B translocation in intact cells was nevertheless not determined. To determine the [Ca^{2+}]_i threshold, we simultaneously imaged the [Ca^{2+}]_i using fura-2 [16, 17, 19] and EGFP-DOC2B. For the experiments, neurons expressing EGFP-DOC2B were loaded with fura-2 penta-potassium by pressure injection into the cytosol. After a rest period of approximately 30 min to allow for the distribution of the indicator, the neurons were stimulated by rectangular depolarizing pulses to generate trains of action potentials with increased frequencies and the calcium concentration. Buccal neurons expressing EGFP-DOC2B were loaded with fura-2. The cells were then stimulated to generate an action potential, which elevates the [Ca^{2+}]_i at cytoplasmic domains [9] . It should be noted that while we could not detect the cytosolic-to-lipid translocation, whereas an increment of 272 ± 71 nM did not induce translocation, whereas an increment of 272 ± 66 nM induced it.

To estimate the probability of DOC2B to undergo translocation to the membrane in response to different [Ca^{2+}]_i, we performed logistic regression analysis. The response is described by the following equation: 

$$ y = \frac{1}{1 + e^{-\left(\frac{\delta}{\text{fit}}\right)}}, \quad r^2 = 0.727. $$

The graph of Fig. 5 depicts 163 observations collected from five preparations in which the relations between [Ca^{2+}]_i and EGFP-DOC2B translocation were mapped at one to three different regions along the axon. It reveals that the 50% probability to observe EGFP-DOC2B translocation to the membrane occurs at a [Ca^{2+}]_i increment value of 217 nM.

These results suggest that the [Ca^{2+}]_i threshold for EGFP-DOC2B translocation is lower by about an order of magnitude than the [Ca^{2+}]_i values estimated in the liposome preparation [9]. It should be noted that while we could not detect the translocation of EGFP-DOC2B in response to single action potentials, the [Ca^{2+}]_i threshold implies that a single action potential, which elevates the [Ca^{2+}]_i at cytoplasmic domains that surround the opening of calcium channels to 10–100 μM [11, 28], is expected to recruit DOC2B to the plasma membrane. Whether such local translocation is of physiological significance is not known (see Section 4).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{The relation between EGFP-DOC2B translocation and the increments in calcium concentration. Buccal neurons expressing EGFP-DOC2B were loaded with fura-2. The cells were then stimulated to generate an increasing number of action potentials, and the changes in EGFP-DOC2B distribution (upper three traces) and [Ca^{2+}]_i (lower three traces) were imaged by a fluorescent microscope. The grabbed images were analyzed by selection of three pairs of equal areas of interest (AOIs). Each pair consisted of one AOI including mainly the plasma membrane, and the other, which included only the adjacent axoplasmic region. All AOIs were then analyzed for EGFP-DOC2B signal and for fura-2 signal. For the EGFP-DOC2B signal, each trace represents the ratio between the averaged fluorescent membranal signal (F_m) and the averaged cytosolic signal (F_c). For the fura-2 signal, each trace represents the cytosolic fluorescent levels within the axoplasmic AOI in ΔF/F_0. The matching pairs of EGFP-DOC2B translocation and calcium transient are indicated by identical colors.}
\end{figure}
4. Discussion

Using cultured Aplysia neurons to express rat EGFP-DOC2B, we estimated the calcium concentration threshold to induce EGFP-DOC2B translocation from the bulk of the cytoplasm to the plasma membrane and analyzed the translocation kinetics. Our main finding is that the [Ca\(^{2+}\)] increase, which induces detectable EGFP-DOC2B translocation from the bulk of the cytosol to the plasma membrane, is in the range of 200 nM. The calcium-induced EGFP-DOC2B translocation is concentration-dependent and can be used to map submembrane calcium concentrations gradients under physiological conditions.

The translocation of EGFP-DOC2B from the cytoplasm to the membrane is always significantly lower than the rate of [Ca\(^{2+}\)] elevation. EGFP-DOC2B detaches from the plasma membrane at a faster or equal rate to the decrease in the [Ca\(^{2+}\)].

Our observations confirm earlier studies that DOC2B translocation to the plasma membrane does not occur uniformly. Through the use of local ionomycin micro ejections (Fig. 2) we found that the localization of EGFP-DOC2B fluorescence intensity in the plasma membrane follows the point source of ionomycin application (Fig. 2). Thus, it is likely that the observed heterogeneities of DOC2B mirror the distribution of [Ca\(^{2+}\)], micro domains that cannot be detected by the use of conventional calcium indicators. Since patches of membrane reveal intense EGFP-DOC2B fluorescence following bath application of ionomycin, it is also conceivable that lipid rafts of high affinity to ionomycin or DOC2 may also influence its distribution. These conclusions could also account for the observations that while EGFP-DOC2B can be detected in subcellular organelles, many organelles are not labeled by EGFP-DOC2B following membrane depolarization.

In view of the low calcium concentration threshold for EGFP-DOC2B translocation and the relation between the [Ca\(^{2+}\)] and the amount of EGFP-DOC2B translocation to the plasma membrane, we suggest that EGFP-DOC2B can be used to map calcium concentration gradients near the plasma membrane under physiological conditions.

A number of studies suggested a regulatory role for DOC2 proteins in Ca\(^{2+}\)-induced exocytosis [1,24,29–32]. As the half-time for DOC2B translocation was in the range of 2 s, it was suggested that DOC2 proteins could regulate slow aspects of neurotransmitter release such as augmentation (∼1 s) and potentiation (∼10 s) [9]. While we have not studied the involvement of DOC2B in synaptic physiology, our results have some potential implications in that respect. Since the threshold of EGFP-DOC2B translocation is in the sub micromolar range, it is reasonable to assume that limited quantities of DOC2 proteins are rapidly recruited to the plasma membrane, at the sites of calcium influx, during the generation of a single action potential. The duration during which DOC2 is recruited should last for seconds, for as long as the calcium concentration in the cytoplasm is above threshold.

The amount of recruited DOC2 would depend on the concentration of DOC2 within the domain in which the calcium concentration is above threshold and the duration during which the calcium concentration remains above threshold.

Thus, the localization of DOC2 proteins to strategic presynaptic sites is expected to start during the arrival of an action potential to the presynaptic terminal, and last for as long as the local calcium concentration is higher than the threshold. Thus, the frequency and duration of action potentials as well as the density and properties of the voltage gated calcium channels would define the effective DOC2 density in a given patch of plasma membrane. Given this, it is possible that DOC2 proteins could serve not only slow aspects of neurotransmitter release, but also fast events of evoked release.

Acknowledgments

This study was supported by grants from the Israel National Science Foundation (556/01) and the USA-Israeli Binational Science Research Foundation (2000354). Parts of the work were conducted at the Charles E. Smith Family Laboratory for Collaborative Research in Psychobiology. We thank Dr. E. Shapira for technical help in preparing mRNAs and Dr. D. Malkinson (Haifa University) for statistical advice. M.E. Spira is the Levi DeViali Professor in Neurobiology.

References


