TRPM7 facilitates cholinergic vesicle fusion with the plasma membrane

Sebastian Brauchi*, Grigory Krapivinsky, Luba Krapivinsky, and David E. Clapham†

Department of Cardiology, Howard Hughes Medical Institute, Children’s Hospital Boston, and Department of Neurobiology, Harvard Medical School, Enders Building 1309, 320 Longwood Avenue, Boston, MA 02115

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TRPM7, of the transient receptor potential (TRP) family, is both an ion channel and a kinase. Previously, we showed that TRPM7 is located in the membranes of acetylcholine (ACH)-secreting synaptic vesicles of sympathetic neurons, forms a molecular complex with proteins of the vesicular fusion machinery, and is critical for stimulated neurotransmitter release. Here, we targeted pHluorin to small synaptic-like vesicles (SSLV) in PC12 cells and demonstrate that it can serve as a single-vesicle plasma membrane fusion reporter. In PC12 cells, as in sympathetic neurons, TRPM7 is located in ACH-secreting SSLVs. TRPM7 knockdown by siRNA, or abolishing channel activity by expression of a dominant negative TRPM7 pore mutant, decreased the frequency of spontaneous and voltage-stimulated SSLV fusion events without affecting large dense core vesicle secretion. We conclude that the conductance of TRPM7 across the vesicle membrane is important in SSLV fusion.

PC12 | pHluorin | transient receptor potential channels | single-vesicle fusion

Synaptic vesicles mediate the release of neurotransmitters into the intercellular cleft in response to an action potential. After releasing their contents, synaptic vesicles are taken up, refilled, stored, and reused in a new cycle (1, 2). Synaptic vesicle fusion and recycling is a highly orchestrated process regulated by many proteins (2). Ion channels have been proposed to function in synaptic vesicle membranes (e.g., refs. 3–5), but evidence for their role in successful fusion is lacking.

The process of synaptic vesicle fusion has been studied extensively for >50 years, with much of this work focused on central nervous system (CNS) synaptic vesicles. Studies of secretory vesicles suggest that several themes in CNS synaptic vesicular trafficking and fusion are likely to be common to other types of vesicular trafficking. These include the participation of protein families of the SNARE apparatus in vesicle approximation and initiation of fusion, the targeting roles of small G proteins, and the presence of transporters that accumulate vesicular cargo. Much less well understood are the fusion pore, cargoes destined for insertion into the plasma membrane, and vesicular membrane elements that control vesicle size, tonicity, and voltage.

During our studies of most of the 28 mammalian transient receptor potential (TRP) channel proteins, we found that several family members are commonly localized to vesicles of different sizes but also can function in the plasma membrane. These include TRPC5 (6), TRPV5 (7), TRPM7 (8, 9), and TRPML1 and TRPML3 (10). As with any vesicular membrane protein that incorporates into the plasma membrane, the question arises as to whether the protein also had some functional role in the vesicle in which it had been transported, which seems a particularly important question for TRP ion channels because their mechanisms of activation in native cells generally are not known, and many are increased in the plasma membrane in response to a triggering event (11).

TRPM7 belongs to the TRP ion channel family; conducts monovalent cations, Ca2+, and Mg2+; and is ubiquitously expressed (11, 12). The TRPM7 C terminus is enzymatically active and structurally homologous to other protein kinases except for the inclusion of a zinc-finger domain (13). This kinase phosphorylates TRPM7 (14) and other substrates [e.g., annexin 1 (15) and myosin heavy chain (16)], but its physiological role is not known. The pore of TRPM7 was proposed to be a major entry mechanism for Mg2+ ions and, in this role, critical for cell survival (17). TRPM7 is localized in the membrane of sympathetic neuronal acetylcholine (ACH)-secreting synaptic vesicles as part of a molecular complex of synaptic vesicle-specific proteins and its permeability is critical for sympathetic neurotransmitter release (9). Down-regulation of the TRPM7 channel conductance (using siRNA or dominant negative form of the channel) resulted in the attenuation of the neurotransmitter quantal size and probability of neurotransmitter release in response to neuronal stimulation (9). This last observation, relying on measurement of excitatory postsynaptic potential/ stimulation coupling, suggested that TRPM7 conductance might affect synaptic vesicle fusion with the plasma membrane. Rat pheochromocytoma PC12 cells have been used to study regulation of neurotransmitter release (18–21). These cells synthesize and store dopamine (DA) in large dense-core vesicles (LDCV) and ACh in small synaptic-like vesicles (SSLV) and release these neurotransmitters by Ca2+-dependent exocytosis (22). Morphologically and biochemically, SSLVs are closely related to neuronal ACh vesicles (23, 24).

The pH-sensitive GFP variant, pHluorin (25) targeted to a lumen of secreted vesicles has been used as a reporter of synaptic function and vesicular fusion (26–29), including single-vesicle fusion events (30–33). We targeted pHluorin to the acidic lumen of ACh-secreting synaptic vesicles by using the vesicular ACh transporter (VACHT), which is expressed exclusively in synaptic vesicles containing ACh (24, 34). Here, we show that endogenous TRPM7 and expressed VACHT-pHluorin were localized to ACh-secreting SSLVs but not to the monooamine-secreting LDCVs in PC12 cells. VACHT-pHluorin-transfected cells exhibited spontaneous and transient flashes of fluorescence, reflecting single-vesicle fusion events. The frequency of these events was increased by cell depolarization. TRPM7 knockdown by the siRNA, or abolishing channel activity by expression of the dominant negative TRPM7 (dnTRPM7) pore mutant, prevented spontaneous and voltage-stimulated vesicle fusion.


The authors declare no conflict of interest.

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*Present address: Laboratorio de Fisiología Sensorial, 407-D, Instituto de Fisiología, Facultad de Medicina, Universidad Austral de Chile, Campus Isla Teja, Valdivia, 511-0566, Chile.
†To whom correspondence should be addressed. E-mail: dclapham@enders.tch.harvard.edu.

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fusion events. We conclude that the conductance of TRPM7 across the vesicle membrane is required for vesicle fusion.

Results

Localization of TRPM7 in PC12 Cells. Previous experiments demonstrated that TRPM7 localized to the membrane of synaptic vesicles in sympathetic neurons and colocalized with synaptophysin in the neuromuscular junction. By comparison, TRPM7 was not detected in synapses of glutamergic hippocampal neurons (9). Speculating that TRPM7 might be expressed in synaptic vesicles carrying positively charged cargo, we examined cholinergic vesicles. PC12 cells contain two major types of secretory vesicles, monoamine-secreting LDCVs, and ACh-secreting SSLVs (34). To determine which vesicles contained TRPM7, we fractionated PC12 postnuclear supernatants on a sucrose gradient, where LDCVs and SSLVs are well separated (35). Fig. 1A shows that TRPM7 colocalized with synaptophysin and VAChT (markers of SSLVs) but was absent from fractions containing secretogranin II (SGII)-labeled LDCVs. Note that TRPM7 did not comigrate with the plasma membrane marker Na,K-ATPase (Fig. 1A), indicating that the majority of cellular TRPM7 was expressed in SSLVs. Confocal imaging of PC12 cells labeled with TRPM7 antibody also demonstrated that TRPM7 is located in small (~0.25-μm diameter) vesicles or vesicular aggregates (Fig. 1B).

Targeting pHluorin to SSLVs. Because TRPM7 localized predominantly to SSLVs, we targeted pHluorin to the membrane of these vesicles using VAChT, an ACh transporter exclusively localized to SSLVs in PC12 cells (35). VAChT is a 12-transmembrane domain protein with six extramembranous loops protruding into the vesicular lumen (36). The C terminus of VAChT is critical for synaptic vesicle targeting (35), but removal of the first two transmembrane domains does not disrupt vesicular targeting (37). Based on these data, we incorporated pHluorin into the putative first intraluminal loop of VAChT. Sucrose density gradient fractionation of the PC12 cells stably transfected with the VAChT-pHluorin construct showed that pHluorin was expressed in SSLVs (Fig. 1A). Exposure of VAChT-pHluorin expressing cells to a membrane-permeant buffer (pH 7.4) potently (10-fold) enhanced pHluorin fluorescence (data not shown), confirming that pHluorin is in a low pH luminal environment.

Vesicular Fusion Events in PC12 Cells Recorded with VAChT-pHluorin. PC12-expressed VAChT-pHluorin appears in the periplasmic membrane space in discrete structures (vesicles) as small as ~0.25 μm (Fig. 2A, supporting information [SI Movie 1]). Both immobile and mobile vesicles were observed in live cell imaging. Infrequently, bright, discrete, short-lived (<10-s) flashes of fluorescence were observed (Fig. 2A Insets, SI Movie 1). These events were interpreted as SSLV fusion to the plasma membrane resulting in exposure of the quenched luminal pHluorin to the more alkaline extracellular media, enhancing pHluorin fluorescence. The fusion kinetics were faster than the 100-ms time resolution of the recordings (Fig. 2B, SI Movie 1).

If the flash decay is approximated as a first-order process, three types of events can be distinguished based on the decay kinetics (Fig. 2). We separated these into fast flashes that vanish in <1 s (τ = 0.68 ± 0.28 s), slowly dissipating flashes (τ = 3.25 ± 0.42 s), and sustained bright objects (lifetime >30 s). Fast flash intensities decayed without a change in the size of the object, whereas slowly dissipating flashes diffused into the plane of the membrane (see SI Movie 1). Field stimulation of cells increased the number of flashes, consistent with earlier observations of stimulus-dependent SSLV fusion and ACh release from PC12 cells (23). Similar to a previous study of synaptic vesicle fusion in hippocampal neurons (32), we interpret the fast events as transient vesicle fusion followed by pore closure and vesicle reacidification. Similarly, slowly dissipating flashes represented complete vesicle fusion accompanied by equilibration of vesicle proteins with the cell surface (28). The sustained events were not further analyzed.

TRPM7 Conductance Affects SSLV-Plasma Membrane Fusion. Because TRPM7 localized to the membrane of SSLVs, we next examined...
whether TRPM7 affected SSLV fusion with the plasma membrane. Knockdown of TRPM7 protein using siRNA substantially decreased the frequency of SSLV fusion events, whereas transfection with control siRNA had no effect (Fig. 3A). These experiments suggest that ion conduction via TRPM7 is critical for SSLV fusion with the plasma membrane.

Overexpression of TRPM7 in PC12 cells did not significantly affect SSLV fusion frequency (Fig. 3A). dnTRPM7 or wild-type (wt) channel overexpression did not affect intravesicular pH in the time frame of fluorescence reporting.

To test whether the dependence of fusion frequency on TRPM7 was specific to SSLVs, we measured LDCV-mediated norepinephrine release in TRPM7 siRNA-transfected PC12 cells. These measurements demonstrate that TRPM7 knockdown did not affect spontaneous and stimulated LDCV-mediated secretion (SI Fig. 6). We conclude that TRPM7 down-regulation specifically affects SSLV fusion to the plasma membrane.

The Kinase Function of TRPM7 Affects SSLV Mobility. To test whether TRPM7 kinase activity affected SSLV fusion, we overexpressed the TRPM7 kinase-dead mutant (TRPM7-KA) (38, 39). As shown in the Fig. 3A, TRPM7-KA expression in PC12 cells did not alter vesicular fusion frequency. However, we noticed that vesicular mobility was higher in TRPM7-KA-transfected cells (SI Movie 2). Calculated diffusion coefficients for wt, dnTRPM7, and TRPM7-KA transfected cells (0.28 ± 0.05, 0.24 ± 0.04, and 0.31 ± 0.02 μm²/s⁻¹, respectively) were not statistically different. To quantify mobility during a 300-frame recording, mobility (D values, see Materials and Methods) was calculated for each pixel (Fig. 4A). Cells expressing the TRPM7-KA mutant contained a significantly larger fraction of the highly mobile SSLVs and a substantially smaller fraction of low-mobility SSLVs (Fig. 4B and C). Cells in which endogenous TRPM7 was decreased (siRNA-transfected) also displayed an increased proportion of the highly mobile vesicles. In contrast, no significant change in vesicle mobility was recorded in TRPM7- or dnTRPM7-overexpressing cells (Fig. 4C). Thus, inhibition of TRPM7 kinase activity significantly increased the mobility of juxtacellular SSLVs in PC12 cells.

Discussion
The motivation for this study was to develop a more direct measure of fusion events for TRPM7-containing vesicles. TRPM7 is present in synaptic vesicles of sympathetic neurons and alteration of its function as a channel affects the postsynaptic
response. Detailed analysis of the altered postsynaptic responses suggested that both the size of neurotransmitter quanta and release probability were diminished by TRPM7 knockdown or dnTRPM7 expression (9). Here, we show that TRPM7 is predominantly in SSLVs in PC12, and its function is crucial to SSLV fusion to the plasma membrane.

The vesicle luminal pH reporter pHiH2rin, in combination with total internal reflection fluorescence (TIRF) microscopy, has been extensively validated as a direct assay of acidic vesicle fusion with the membrane (26, 40–42). Fast changes in pHiH2rin fluorescence is relatively specific for vesicular content exposure to the alkaline extracellular environment. Combining VACHT-tagged pH2rin with TIRF provides a high signal-to-noise ratio because the VACHT reporter is localized strictly to SSLVs, and TIRF narrows detection to within ~250 nm of the imaged membrane. Using these tools and TRPM7 functional modifications, we found that abrogation of the TRPM7 pore significantly decreased fusion frequency. In contrast, modification of the kinase of TRPM7 did not alter vesicle fusion rates. How might TRPM7 affect vesicle fusion?

Manipulation of TRPM7 channel activity did not affect the relative proportion of transient and complete fusion events (fast, slow, and sustained flashes). TRPM7 down-regulation also did not affect fusion pore closing time or the kinetics of dissociation of the fusion pore from the membrane. These data suggest that TRPM7 does not participate in the regulation of the fusion pore itself. Limitations in time resolution did not allow us to evaluate the effect of TRPM7 on pore formation kinetics. Based on these and our previous results (9), we hypothesize that TRPM7 channel conductance is critical to the early steps of vesicle fusion: docking, priming, or pore formation.

When membrane proteins fuse with the plasma membrane, the former vesicular surface faces the extracellular space and the cytoplasmic surfaces remain cytoplasmic. The vesicular membrane itself differs from the plasma membrane; P(4,5)P2 is excluded from vesicles but abundant in the plasma membrane (43, 44). Two properties of TRPM7 are relevant to these points: (i) acid pH potentiates inward divalent conductance (45) and (ii) PIP2 binding activates TRPM7 (46). If TRPM7 gating in vesicles is similar to those on the plasma membrane, TRPM7 should be activated on contact with inner plasmalemmal P(4,5)P2. In this model, TRPM7 acts as a coincidence detector, opening only when the vesicle pH is low and P(4,5)P2 in the plasma membrane is in the cytoplasmic domains of TRPM7 (e.g., just before fusion). TRPM7 might contribute at the ion exchange between the vesicular and cytoplasmic spaces, an event that we speculate is crucial to vesicular fusion.

Finally, we noticed that TRPM7 kinase-dead expression increased vesicle mobility. Multiple experiments have demonstrated that synaptic vesicles and juxta membrane secretory vesicles are mobile, and that inhibition of protein phosphorylation alters synaptic vesicle mobility (47–51). The possibility that the kinase of TRPM7 phosphorylates synaptic vesicle proteins affecting vesicle behavior warrants further investigation.

Materials and Methods

cDNA Constructs. The rat VACHT coding cDNA sequence was amplified from a rat brain cDNA library and subcloned into EcoRI/XbaI sites of the pcDNA3.1 vector (generously provided by G. Miesenbock, Sloan-Kettering Cancer Center, New York) was amplified by PCR with primers containing PSHai restriction sites and ligated into a unique VACHT PSHai site (base 217 of the coding sequence). N-FLAG-tagged wt mouse TRPM7 (NP_0767245), dnTRPM7 (9), and the kinase-dead mutant K1646A (TRPM7-KA) (39) were subcloned into a RpTracer-CAG bicistronic vector. pTracer-CAG was made from pTracer-CMV2 (Invitrogen) by replacing its GFP sequence with mRFP (kindly provided by Roger Tsien, University of California, San Diego) (52). The CMV promoter upstream of the multiple cloning site was replaced by a CAG promoter sequence (53). siRNA was designed to the unique region of rat TRPM7 (732–772 of AF375874; ref. 9). Nonsilencing double-stranded RNA (Ambion) was used as a negative control.

PC12 Cell Fractionation, Western Blotting, and Immunofluorescence. PC12 cell postnuclear supernatants were obtained as described in ref. 35. For equilibrium density fractionation, a postnuclear supernatant (3–5 mg of protein) was loaded onto a continuous 11-ml 0.6–1.6 M (20–46%) sucrose gradient buffered with 20 mM Hepes (pH 7) containing protease inhibitor mixture (Complete, Roche) for sedimentation at 40,000 rpm (SW41 rotor) at 4°C for 3 h. Twelve 1-ml fractions were collected from the bottom of the tube, and 30 μl of each fraction was analyzed by Western blotting with the appropriate antibody (TRPM7 antibodies were described in ref. 9). SIgG antibody was provided by R. Fisher-Colbrie (University of Innsbruck, Austria). Commercial antibodies included rabbit Na,K-ATPase (ABR), mouse synaptophysin and FLAG antibodies (Sigma), and goat VACHT antibody (Santa Cruz Biotechnologies). For immunofluorescence, cells were fixed with 4% formaldehyde, permeabilized with 0.05% Triton X-100, and immunostained with TRPM7 antibody as described (9, 46). Control experiments with the antibody preabsorbed with antigenic peptide confirmed the specificity of immunostaining. Images were acquired on an Olympus Fluoview-1000 confocal microscope (> x60, 1.4 N.A. objective). Basal and Ba2+–stimulated [3H]norepinephrine secretion from PC12 cells was performed as described in ref. 54.

Cell Solutions. PC12 (American Type Cell Type Cell Collection) cells were grown on glass coverslips coated with poly-L-lysine in DMEM/F12 media supplemented with penicillin/streptomycin, 15% horse serum, and 5% fetal bovine serum. PC12 cells stably transfected with the VACHT-pHiH2rin construct were selected and propagated in the culture media containing 0.4 mg/ml G418. Cells were transfected with Lipofectamine 2000 (Invitrogen) and cultured for 3–5 days. FLAG-TRPM7 protein expression was tested by Western blot and immunofluorescence (FLAG tag). Cells expressing RFP from RpTracer-CAG were selected for imaging.

PC12 cells were imaged in standard external solution consisting of: 140 mM NaCl, 5 mM KCl, 4 mM CaCl2, 2 mM MgCl2, 10 mM glucose, 5 mM EGTA, and 5 mM NaHepes (pH 7.4). To increase intravesicular pH, cells were bathed in an external solution in which 50 mM NaCl was replaced by 50 mM NH4Cl.

TIRF Microscopy. Cells were imaged by using an objective-based TIRF microscope. A 488-nm solid-state diode laser and a 532-nm diode pump laser were focused onto a single-mode optical fiber and transmitted via the rear illumination port of an Olympus IX70 microscope. Digitally synchronized mechanical shutters (Vincent Associates) controlled exposure times. Laser light reflected from a double dichroic mirror (Z488S/532RPC; Chroma Tech) passed through a high-numerical aperture objective (> x60, N.A. 1.45, oil; Olympus) and was totally internally reflected by the glass-water interface. Fluorescence transmission was passed through an HQ515–30nm (Chroma) filter for enhanced GFP, or a D620–60nm (Chroma) filter for DsRed2, and collected by a cooled-CCD (ORCA ER II; Hamamatsu). Under our experimental conditions, fluorescent objects were within 250 nm of the glass–water interface (6).

GFP fluorescent images were acquired at 100-μs intervals with MetaMorph software (Universal Imaging). Cells were photographed during a 30- to 40-s period (300 frames) in TIRF mode before the recordings to augment the signal-to-noise ratio. Cells were field-stimulated at 20 Hz with square, 1-ms, 10-V/cm pulses across the chamber. Recording under field stimulation always followed an initial 30-s recording of spontaneous activity, and in most cases a third 30-s period of spontaneous activity was recorded after the stimulation period.

Image Analysis. Thirty-second image sequences were analyzed with ImageJ version 1.39c (National Institutes of Health). The background fluorescence was subtracted, and fusion events were identified as changes in intensity above a certain level (at least two times nonfused vesicle average intensity) and counted. The number of events was normalized to cell area. The number of fusions per pixel2 was normalized by the averaged activity of vector-transfected cells. To calculate fluorescence kinetics, regions of interest (ROIs) were established on previously identified fusion spots, and the fluorescence time course of fusion events was recorded. A single exponential decay function was fitted to the fluorescence relaxation process, and time constants (~) were calculated. To determine the diffusion constant, we tracked single fluorescent particles (nonfused vesicles) and calculated the mean square displacement (MSD) (55) and the diffusion coefficient (D ~ MSD/4t).

To quantify vesicle mobility, we consider only the likelihood that a particle moved. The fluorescence signal was filtered to select near-membrane, nonfusing vesicles. The low threshold was set at 2 standard deviations above the mean of the camera and photon noise. The upper threshold was set to remove
transient flashes. Thresholded images were converted into binary format events. For each pixel of the image sequence, the mobility function ($\delta$) was calculated by using the following equation:

$$
\delta = \frac{1}{n} \sum_{i=1}^{n-1} \left( f_i - f_{i+1} \right)^2
$$

where $f$ is the amplitude value of each pixel (1 or 0) in the binary image, $i$ is a frame number, and $n$ is total number of frames. The processed image reflects object mobility pixel by pixel (“mobility image”) where each pixel represents a color-coded $\delta$ value. In mobility distribution histograms, $\delta$ values are presented as a fraction of the theoretical maximum ($\sim 1$). These values were obtained for each cell, normalized, averaged, and presented in probability distribution plots (number of pixels per total pixels).

Data Analysis. ANOVA and Bonferroni tests were used in all cases. We analyzed 9–12 independent experiments and >100 events for every condition. The analysis was performed with GraphPad Prism 5.01 (GraphPad Software). Data plot analysis and curve-fitting were done with Origin 7 (Microcal Corp.). Figures were designed with The GIMP 2.2 (GNU General Public License).

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Supporting Information

Files in this Data Supplement:

SI Movie 1
SI Figure 5
SI Figure 6
SI Movie 2
**Fig. 5.** TRPM7 activity does not affect fusion event fluorescence amplitude. Amplitude histograms for events recorded in mock-, TRPM7dn-, and TRPM7 siRNA-transfected cells. Fifty events were analyzed for each condition; binning = 2. Kolmogorov-Smirnov and ANOVA-Bonferroni statistical tests showed no significant difference between amplitude distributions. Average fluorescence amplitude was measured in a small area near the center of the flash event; relative flash amplitude was calculated as: $A = \frac{F(\text{peak} - \text{background})}{F(\text{base} - \text{background})}$, where $F(\text{base})$ is fluorescence amplitude of an area averaged over 20 acquisitions before the flash, and $F(\text{background})$ is the average signal amplitude in a region outside the cell. Normalization allowed us to exclude variations in pHluorin expression level and TIRF excitation efficiency between different cell regions and different cells.
**Fig. 6.** TRPM7 levels do not affect exocytotic catecholamine release. Two siRNAs previously shown to knock down TRPM7 protein in PC12 cells [Krapivinsky G, Mochida S, Krapivinsky L, Cibulsky SM, Clapham DE (2006) The TRPM7 ion channel functions in cholinergic synaptic vesicles and affects transmitter release. *Neuron* 52:485-496] depleted of TRPM7. Norepinephrine release (see Materials and Methods) from TRPM7-depleted cells was compared with controls in Ca\(^{2+}\) and Ba\(^{2+}\)-containing media. Error bars indicate ± SEM.

**SI Movie 1**

**Movie 1.** GFP fluorescent images were acquired (100-ms intervals for 30 s) of a PC12 cell expressing VaChT-pHlorin by using TIRFM (λ 600, N.A. 1.45, oil; Olympus). Cells were prebleached (30% laser output power) during a 30- to 40-s period (300 frames) in TIRF mode before the recordings were made to augment the signal-to-noise ratio.
Movie 2. GFP fluorescent images were acquired as described in the legend to Movie 1. (Left) Mock-transfected cell. (Right) Cell expressing TRPM7-KA. Images were acquired at 100 ms per frame for 20 s; playback at 20 frames per s is twice the real-time frame rate.