A Constitutive, Transient Receptor Potential-like Ca\(^{2+}\) Influx Pathway in Presynaptic Nerve Endings Independent of Voltage-gated Ca\(^{2+}\) Channels and Na\(^{+}/Ca^{2+}\) Exchange *\(^{S}\)

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Calcium levels in the presynaptic nerve terminal are altered by several pathways, including voltage-gated Ca\(^{2+}\) channels, the Na\(^{+}/Ca^{2+}\) exchanger, Ca\(^{2+}\)-ATPase, and the mitochondria. The influx pathway for homeostatic control of [Ca\(^{2+}\)]\(_{i}\) in the nerve terminal has been unclear. One approach to detecting the pathway that maintains internal Ca\(^{2+}\) is to test for activation of Ca\(^{2+}\) influx following Ca\(^{2+}\) depletion. Here, we demonstrate that a constitutive influx pathway for Ca\(^{2+}\) exists in presynaptic terminals to maintain internal Ca\(^{2+}\), independent of voltage-gated Ca\(^{2+}\) channels and Na\(^{+}/Ca^{2+}\) exchange, as measured in intact isolated nerve endings from mouse cortex and in intact varicosities in a neuronal cell line using fluorescence spectroscopy and confocal imaging. The Mg\(^{2+}\) and lanthanide sensitivity of the influx pathway, in addition to its pharmacological and short hairpin RNA sensitivity, and the results of immunostaining for transient receptor potential (TRP) channels indicate the involvement of TRPC channels, possibly TRPC5 and TRPC1. This constitutive Ca\(^{2+}\) influx pathway likely serves to maintain synaptic function under widely varying levels of synaptic activity.

Regulation of cytosolic [Ca\(^{2+}\)] in the presynaptic nerve terminal is critical to synaptic transmission. The primary means by which presynaptic [Ca\(^{2+}\)] is increased from basal resting levels is through activation of an array of local voltage-gated Ca\(^{2+}\) channels (VGCCs)\(^{6}\) (1) in response to the wave of depolarization of an invading action potential. In a variety of preparations, it has also been shown that Ca\(^{2+}\) release from internal stores in the presynaptic terminal, mainly through a ryanodine-sensitive pathway (via Ca\(^{2+}\)-induced Ca\(^{2+}\) release; CICR), contributes to regulation of synaptic neurotransmitter release (reviewed in Ref. 2), mainly as a function of action potential frequency or, in some cases, presynaptic receptor action (3–5). Following bursts of activity, partial store depletion may thus occur, necessitating the replenishment of the internal Ca\(^{2+}\) stores to restore resting homeostasis in the terminal. In addition, changes in external [Ca\(^{2+}\)] as a result of synaptic activity (6–10), typically dependent on firing frequency (11), often coupled with postsynaptic ionotropic receptor activation (12, 13) or postsynaptic depolarization (14), may alter presynaptic [Ca\(^{2+}\)] homeostasis, as will various pathological conditions (e.g. hypoparathyroidism, secondary to hypomagnesia).

In non-neuronal cells, various store-operated channels (SOCs) appear to function in the maintenance of internal Ca\(^{2+}\) stores, although they also contribute to regulated changes in cytosolic [Ca\(^{2+}\)], under some circumstances (15, 16). In the presynaptic terminal, the calcium-sensing receptor (CaSR) (17) and various TRPC channel subunits (18) have been detected, as has the neuronal calcium sensor-1 (NCS-1) (19), although the case for the latter has been controversial (20); however, a clear case for SOCs has not been forthcoming. Rather, the Na\(^{+}/Ca^{2+}\) exchanger was originally implicated as a possible pathway for Ca\(^{2+}\) entry following nerve activity, but despite an internal “Na\(^{+}\) load” with sustained activity, the inward-directed Na\(^{+}\) gradient still provides sufficient drive on the exchanger to favor Ca\(^{2+}\) efflux, even during nerve activity (Refs. 21–23; however, see Ref. 24). Thus, the Na\(^{+}/Ca^{2+}\) exchanger largely contributes to clearance of [Ca\(^{2+}\)]\(_{i}\), and not Ca\(^{2+}\) influx. In addition, the mitochondria (when present) and plasma membrane Ca\(^{2+}\)-ATPase appear to play dominant roles in resetting the final resting [Ca\(^{2+}\)]\(_{i}\) (25, 26). Here, we have identified a presynaptic calcium influx pathway for restoring homeostasis, revealed upon depletion of external calcium using isolated adult mature nerve endings from mouse cortex.

**EXPERIMENTAL PROCEDURES**

**Purification of Intact Isolated Presynaptic Nerve Endings (Synaptosomes)—** The protocol used in this study was approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee. The intact isolated presynaptic nerve endings were purified as described previously (27).
brief, cortices from adult C57/Bi6 mice (Jackson Laboratory, Bar Harbor, ME) were removed and placed into ice-cold 0.32 m sucrose. The cortices were homogenized in 0.32 m sucrose with a glass-Teflon tissue grinder. Synaptosomes were isolated using the Percoll step gradient method (28). The purified synaptosomes were washed with oxygenated HEPES-buffered saline (HBS, pH 7.4) containing 142 mM NaCl, 2.4 mM KCl, 1.2 mM KH2PO4, 1 mM MgCl2, 5 mM D-glucose, and 10 mM HEPES, plus 1 mM Ca2+. Under these conditions, the terminals are intact, closed structures, as they respire, maintain ionic gradients and functional ion channels, transport and secrete neurotransmitter in a Ca2+-dependent fashion (29). That they are fully intact functional structures is also evidenced by retention of fluorescent dyes (27, 30) and patch clamp recording (31).

**Cell Culture of NG108-15 Cells**—The neuronal cell line NG108-15 was cultured under differentiation conditions (32). In brief, the cells were plated onto glass coverslips coated with Cell-Tak (BD Biosciences) in Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum and 1 mM dibutyryl adenosine 3’5’-cyclic monophosphate for 3–5 days. Under these conditions, the cells extend long neurites, each with multiple, large presynaptic-like axonal varicosities, capable of Ca2+-dependent secretion of acetylcholine (33) and synaptic innervation of rodent myotubes (32). The presynaptic-like varicosities contain voltage-gated ion channels typical of presynaptic sites (34, 35), synaptic vesicles and mitochondria, and Ca2+ stores (35).

**Expression of shRNA Constructions in Differentiated NG108-15 Cells**—Transfection of mouse shRNA constructs into differentiated NG108-15 cells was accomplished using FuGene (Roche Diagnostics), with which we typically obtain 80–90% transfection efficiency as gauged by expression of green fluorescent protein. Effects of mouse TRPC1, TRPC5, and TRPC3 (as control) shRNAs were assessed after 3–4 days, using Ca2+ imaging and immunoblot analysis.

**Measurement of Relative Ca2+ Levels**—The purified synaptosomes or differentiated NG108-15 cells were loaded with 5 μM of various fluorescent Ca2+ indicator dyes (fluorometric studies: fura-2, fluo-4, mag-fluo-4, mag-fura-2, X-rhod-1, fura-2FF; confocal imaging: fluo-4) in HBS containing 1 mM Ca2+ using the Grynkiewicz equation (37). Hence, the data using fura-2 are presented simply as raw ratios. In addition, caffeine, which has been reported to quench certain fluorescent dyes (38), had no discernible quenching effect on fura-2 in synaptosome preparations. In experiments where recording at high time resolution was necessary, fluo-4 was used with 0.1-s sampling intervals. For the low affinity dye mag-fluo-4, emission was recorded at 515 nm in response to 490 nm. For the intermediate affinity dye X-rhod-1, fluorescent emission was recorded at 605 nm in response to 505 nm. For the high affinity dye fluo-4, fluorescence was recorded at 506 nm in response to 488 nm. For confocal imaging, the synaptosomes or differentiated NG108-15 cells were plated onto coverslips coated with Cell-Tak and then inserted into a rapid-exchange Warner perfusion system mounted on a Nikon Diaphot microscope attached to a Nikon PCM 2000 laser-scanning confocal imaging system. Fluorescent images were recorded in response to excitation at 488 nm. During the confocal imaging, the preparations were under constant perfusion at ~5 ml/min with HBS without or with Ca2+, as appropriate. Images were typically collected at 2- or 4-s intervals, with the first 5 consecutive images collected as a baseline. Each experiment corresponds to sequential images collected from a single preparation. The quantification of fluorescence intensities associated with individual synaptosomes or varicosities recorded in digitized images was calculated using MetaMorph (Molecular Devices, Downingtown, PA) and corrected for photobleaching based on the baseline images (typically <3%). Analysis was performed by an observer blind to the experimental conditions. Response to depolarization evoked by elevated extracellular K+ was used as a criterion for synaptosomal or varicosity viability. Data are presented as normalized responses (F/F0, where F0 is the fluorescence intensity associated with a given structure at t0). All reagents were used at or above maximal concentrations based on literature values (e.g. Ref. 39). Depletion was initially accomplished using 0.1 mM EGTA, final concentration. Note that the preparations were partially depleted prior to addition of EGTA, because of a final wash with nominally Ca2+-free HBS during preparation. For sequential depletion, EGTA was initially added at 0.1 mM and then following Ca2+ re-addition to 0.5 mM net concentration, EGTA was re-added to 0.8 mM concentration (see Fig. 1A). For experiments involving lanthanides (Gd3+ and La3+) at <10 μM, preparations were pre-depleted and then washed free of EGTA, due to the very high stability constants of the lanthanides for EGTA (40).

**Immunocytochemistry**—Immunostaining was performed as described (27, 41, 42). In brief, the synaptosomes or differentiated NG108-15 cells on Cell-Tak-coated coverslips were fixed with 4% paraformaldehyde in HBS for 30–45 min. The preparations were then permeabilized by incubation with Tris-buffered saline (50 mM Tris pH 7.2, 0.9% NaCl) containing 0.1% Triton X-100 for 30 min, followed by extensively washing with Tris-buffered saline. For double immunolabeling, the preparations were first incubated for 60–120 min at room temperature with affinity purified primary rabbit antibodies for specific TRP channels (1:100–200) and, in the case of the synaptosomes, a mouse monoclonal antibody for synaptophysin (clone SVP38;
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**FIGURE 1.** Ca\(^{2+}\) depletion leads to rapid influx of Ca\(^{2+}\) into isolated presynaptic terminals on re-addition of Ca\(^{2+}\). Representative time sequences of mouse cortical terminals loaded with (A) fura-2, (B), (G) fluo-4, (C) X-rhod-1, (D) fura-2FF, or (E) mag-fluo-4 and subjected to depletion of [Ca\(^{2+}\)], (−Ca\(^{2+}\); +EGTA) followed by re-addition of (mix) Ca\(^{2+}\) (+Ca\(^{2+}\)) and finally K\(^+\) depolarization (K\(^+\); +30 min) were recorded fluormetrically (A–E and G) or via confocal imaging (F and H). In F and H, synaptosomes in the confocal microscopic field (see inset in F for example sequence for an individual synaptosome) were identified by their size (0.5–2 μm) and their characteristic changes in Ca\(^{2+}\) levels obtained on K\(^+\)-induced depolarization. In G, the effect of variable depletion on subsequent Ca\(^{2+}\) influx was tested. In testing various concentrations of Ca\(^{2+}\), confocal imaging was used to minimize effects of external dye and to control the [Ca\(^{2+}\)], in the absence of EGTA. As shown in H, an E\(_{50}\) of ~0.1 mM net free Ca\(^{2+}\) was observed for Ca\(^{2+}\) following re-addition, whereas the E\(_{50}\) for K\(^+\) depolarization was ~0.5 mM net free Ca\(^{2+}\) (n = 3; see also, Ref. 99). F/F\(_{0}\), fractional change in fluorescence reflecting the relative change in [Ca\(^{2+}\)], ratio of fluorescence at alternating wavelengths.

with secondary antibodies. After extensive washing, stained preparations were mounted and imaging of the synaptosomes was performed using the confocal microscope, recording the fluorescence emitted in response to excitation at 488 (fluorescein) or 568 nm (rhodamine) as described (27, 41, 42). In particular, the black level was set using the control preparations incubated with secondary antibodies only, eliminating the low level of background fluorescence. This black level was used when imaging all subsequent samples stained with primary and secondary antibodies. For immunostaining of synaptosomes, merged images are shown; for immunostaining of the NG108-15 cells, images of fluorescence in the fluorescein channel are shown in grayscale. Positive immunostaining for TRP subunits in the synaptosomes was denoted by fluorescence contained within the margins of the staining for synaptophysin (see Ref. 44 for discussion). The specificity of the anti-TRPC antibodies was verified via immunoblotting of SDS extracts of synaptosomal preparations (18), visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences).

**Reagents**—The fluorescent Ca\(^{2+}\) indicator dyes were purchased from TefLabs (Austin, TX), with the exception of Fluo-4, which was purchased from Molecular Probes. The adhesive matrix Cell-Tak was from BD Sciences. Percoll was originally from Amersham Biosciences AB (Uppsala, Sweden). Ultrapure sucrose was from ICN Biomedicals (Aurora, OH). HEPES (ULTROL grade), KB-R7943, 1-oleoyl-2-acetyl-sn-glycerol, and ionomycin were from Calbiochem (San Diego, CA). CGP37157, SKF96365, and carbonyl cyanide p-trifluoromethoxyphenyldrazone were from Tocris (Ellsville, MO). ω-Conotoxin MVIIIC, ω-conotoxin GVIA, atagotox TK, and thapsigargin were from Alomone (Jerusalem, Israel). Anti-synaptophysin monoclonal antibody (clone SVP-38), flufenamic acid, ruthenium red, ryanodine, (−)-xestospongin C, cyclopiazonic acid, 2,5-di-(t-butyl)-1,4-hydroquinone, pertussin toxin, and caffeine were purchased from Sigma. All anti-TRPC antibodies were from Alomone Labs. Fluorescein-conjugated goat

1:500), which serves as a highly specific nerve terminal marker (43). After extensive washing with Tris-buffered saline, the synaptosomes were incubated for 60–120 min at room temperature with fluorescein-conjugated goat anti-rabbit IgG (1:500) and, in the case of the synaptosomes, rhodamine-conjugated donkey anti-mouse IgG (1:500) secondary antibodies in the presence of 10% goat serum in Tris-buffered saline. Control preparations were incubated without primary antibodies but

![Image](https://example.com/image.png)
anti-rabbit IgG antibody and rhodamine-conjugated donkey anti-mouse IgG antibody came from Jackson ImmunoResearch Laboratories (West Grove, PA). The mouse shRNA containing pLKO.1 constructs were obtained from the Drexel University RNAi Resource Center (under management of Dr. Aleister Saunders in the Department of Bioscience), having originally been obtained from the Open Biosystems Mouse ArrestTM shRNAmir Library (Huntsville, AL). Each construct was optimized for selective RNA knock-down. Separate constructs were engineered to co-express green fluorescent protein to assess transfection efficiency. All other chemicals were of the highest reagent grade.

Statistics—All experiments were independently replicated at least 3 times. Where indicated, the significance of the difference between mean values was determined by one-way analysis of variance followed by Scheffé F test or t test. Differences were considered significant when \( p \) was minimally <0.05.

RESULTS

**Ca\(^{2+}\) Depletion Reveals a Presynaptic Ca\(^{2+}\) Influx Pathway—**

Using a variety of fluorescent dyes with varying relative affinities for Ca\(^{2+}\), an increase in [Ca\(^{2+}\)]\(_{i}\) occurred on re-addition of Ca\(^{2+}\) to isolated mouse cortical nerve terminals following depletion of Ca\(^{2+}\) by incubation in Ca\(^{2+}\)-free HBS containing EGTA (Fig. 1), with responses evident at the level of individual terminals (Fig. 1F). This increase in [Ca\(^{2+}\)]\(_{i}\) following re-addition of Ca\(^{2+}\) to depleted synaptosomal preparations has been previously noted in numerous studies of synaptosomal Ca\(^{2+}\) regulation (e.g. Refs. 45–47). The time course for the increase in [Ca\(^{2+}\)]\(_{i}\) was fairly rapid, as seen using non-ratiometric Ca\(^{2+}\)-sensitive dyes (e.g. Fluo-4) at high time resolution (Fig. 1, B, F, and G; 0.1-s sampling interval). With direct addition of fluorescent dye as a measure of the time limitation for mixing (0.7 ± 0.17 s, mean ± S.D., \( n = 3 \)), the time course for the initial increase in [Ca\(^{2+}\)]\(_{i}\) measured for synaptosomes loaded with Fluo-4 was 1.55 ± 0.5 s (mean ± S.D., \( n = 18 \); binding of Ca\(^{2+}\) to Fluo dyes is diffusion-limited (48)). This time course was similar to that found for K\(^{+}\) depolarization-induced increases in [Ca\(^{2+}\)]\(_{i}\) (1.61 ± 0.13 s, mean ± S.D., \( n = 9 \)), measured after re-equilibration of the preparation with Ca\(^{2+}\). It was also similar to the increase observed in preparations twice-re-washed with HBS to remove any residual extracellular dye (2.0 ± 0.12 s, mean ± S.D., \( n = 3 \); Fig. S1A, inset). The time course for the initial depletion was slower (e.g. fluo-4, 3.1 ± 0.87, mean ± S.D.; \( n = 3 \); Fig. S1A, inset), but this response was highly variable. For the Ca\(^{2+}\)-dyes having relative lower affinities, fura-2FF and the less specific mag-fluo-4, the Ca\(^{2+}\) changes with depletion and re-addition will reflect a mixture of cytotoxic and compartmentalized Ca\(^{2+}\) (Fig. 1, D and E). Depletion in the presence of these dyes displayed a second, slower phase, as compared with the high affinity dyes. In contrast, X-rhod-1, a higher affinity Ca\(^{2+}\) dye, was loaded under conditions favoring localization to the cytosol (see Ref. 36) (Fig. 1C). Thus, there is an indication of Ca\(^{2+}\) present in a store/mitochondrial compartment(s) in addition to the cytosol.

Influx was confirmed using Mn\(^{2+}\) as the divalent cation in a typical fura-2 quench assay (Fig. 2; see also, Ref. 49). The initial rate of Mn\(^{2+}\) influx was not significantly affected following addition of Ca\(^{2+}\) (Fig. 2, inset). In addition, Ca\(^{2+}\) influx via this pathway could be detected within 1 s of depletion (partial) of the preparation, and had kinetics that did not change with longer periods of depletion (Fig. 1G) or with repetitive depletion (Fig. 1A). The increase in [Ca\(^{2+}\)]\(_{i}\), on re-addition of Ca\(^{2+}\) to depleted preparations was insensitive to prior treatment with pertussis toxin (see Ref. 50), nor was it affected by pretreatment with the permeant diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol (supplemental Fig. S1). Thus, the apparent influx pathway for Ca\(^{2+}\) into the depleted nerve terminal has kinetic features of a channel activity independent of a pertussin toxin-sensitive or DAG-coupled pathway; however, receptor regulation of this pathway is not excluded by these results.

**Ca\(^{2+}\) Influx Revealed by Ca\(^{2+}\) Depletion Is Independent of VGCCs, Na\(^{+}/Ca\(^{2+}\) Exchange, and Ca\(^{2+}\) Stores—**

Although K\(^{+}\) depolarization-induced increases in [Ca\(^{2+}\)]\(_{i}\), were invariably elicited following re-equilibration of the depleted preparations with Ca\(^{2+}\), the extent to which VGCCs are involved in the influx pathway revealed by Ca\(^{2+}\) depletion was assessed using VGCC blockers. In the presence of a mixture of peptide toxin blockers (agatoxin and conotoxins) that completely suppressed K\(^{+}\)-induced Ca\(^{2+}\) increases (27, 51), there was no effect on the Ca\(^{2+}\) influx pathway (Fig. 3A). In addition, depolarization in the presence of VGCC blockers prior to addition of Ca\(^{2+}\) back to depleted synaptosomes had no effect on the Ca\(^{2+}\) influx (Fig. 3B). Moreover, Sr\(^{2+}\), which is conducted poorly by classical SOCs but is carried by TRPC channels (52), could substitute for Ca\(^{2+}\) in the influx pathway (Fig. 3C), whereas Gd\(^{3+}\) had little if any effect, either as a SOC blocker (53) or cation carrier at \( \approx 10 \mu M \) (Fig. 3D). Ba\(^{2+}\), which also appears to be poorly conducted
by SOCs in many cases (54), could also substitute for Ca\(^{2+}\) (Fig. 3E). Mg\(^{2+}\) from 1 to 5 mM had no effect on the responses (not shown); however, reduced levels of external Mg\(^{2+}\) (<1 mM) resulted in a substantial increase in the extent (Fig. 3F; 153 ± 8% relative to 1 mM) and rate (Fig. 3F, inset) of the initial phase of Ca\(^{2+}\) influx, as did relatively high concentrations (≥25 μM) of the lanthanides La\(^{3+}\) (Fig. 3G; 173 ± 20% (mean ± S.E.) of control, \(p < 0.05; n = 3\)) or Gd\(^{3+}\) (Fig. 3D; 210 ± 43% (mean ± S.E.) of control, \(p < 0.05; n = 3\)), these effects being indicative of the presence of TRPC5 containing channels (55–57). Finally, the Ca\(^{2+}\) concentration dependence of the influx pathway has a distinctly lower apparent EC\(_{50}\) than that of the VGCCs (Fig. 1f).

To address other potential sources of Ca\(^{2+}\), blockade of the Na\(^{+}/Ca\(^{2+}\) exchanger was performed using the inhibitor KB-R7943 (58) and had no detectable effect when used at 5 μM. Block by KB-R7943 at 50 μM did result in an enhanced second phase of the Ca\(^{2+}\) influx after Ca\(^{2+}\) re-addition (Fig. 4A), indicating no direct involvement of the exchangers in the depletion-coupled influx per se but an involvement in the equilibration of [Ca\(^{2+}\)]\(_i\), as expected (59), wherein under normal external [Na\(^{+}\)], efflux of Ca\(^{2+}\) is favored. Furthermore, removal of external Na\(^{+}\) (substituted with N-methylglucamine), which should also facilitate Ca\(^{2+}\) entry via all of the Na\(^{+}/Ca\(^{2+}\) exchangers, had no significant effect on the depletion-coupled Ca\(^{2+}\) influx (Fig. 4B), although this result does not preclude conductance of Na\(^{+}\). Taken together, these results further support a voltage-insensitive channel being the mediator of this Ca\(^{2+}\) influx pathway, but not a SOC.

There is abundant evidence for the release of Ca\(^{2+}\) from ryanodine-sensitive stores in brain nerve terminals (for review, see Ref. 2), presumably from an endoplasmic reticulum compartment (60), having a significant impact on synaptic dynamics (61–63). However, we observed no detectable increase in [Ca\(^{2+}\)]\(_i\), after application of thapsigargin, caffeine, or ryanodine to synaptosomes equilibrated in normal [Ca\(^{2+}\)]\(_i\) (not shown), consistent

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**FIGURE 3.** Ionic analysis of the Ca\(^{2+}\) influx pathway revealed on Ca\(^{2+}\) depletion. Representative time sequences of mouse cortical terminals loaded with fura-2 were recorded fluorimetrically in the presence of VGCC toxin blockers (100 nM agatoxin TK, 500 nM ω-conotoxin GVIA, 500 nM ω-conotoxin MVIIIC) before (A) or after (B) K\(^{+}\) depolarization (blue trace). Responses were enhanced in the presence of 25 μM Gd\(^{3+}\) (D), low concentrations of Mg\(^{2+}\) (F), and 25 μM La\(^{3+}\) (G). Similar effects of La\(^{3+}\) and Gd\(^{3+}\) were also observed at 100 μM (not shown). Responses carried by 1 mM Sr\(^{2+}\) (C) or 1 mM Ba\(^{2+}\) in the presence of VGCC toxin blockers (E) were also measured. Note that K\(^{+}\) depolarization did not stimulate a significant increase in Sr\(^{2+}\) entry. (The \(K_v\) values for fura-2 for both Sr\(^{2+}\) and Ba\(^{2+}\) are higher than that for Ca\(^{2+}\).)
mean ± S.E., n = 3), consistent with the presence of a ryanodine receptor-linked Ca$^{2+}$ store involved at least in part, in CICR evoked via VGCCs (4, 66–68). During depletion or upon subsequent readdition of Ca$^{2+}$, sarco/endoplasmic Ca$^{2+}$-ATPase inhibitors (thapsigargin, cyclopiazonic acid or 2,5-di-(t-butyl)-1,4-hydroquinone) had no effect at concentrations typically

maximal (100 nM to 1 μM) for triggering Ca$^{2+}$ release from stores. On the other hand, relatively high concentrations of thapsigargin and cyclopiazonic acid induced an apparent small increase in basal calcium, more clearly evident using the cytosolic dye X-rod-1 (supplemental Fig. S2), followed by an enhanced rise in [Ca$^{2+}$], with Ca$^{2+}$ re-addition. The rise in [Ca$^{2+}$], with readdition and basal Ca$^{2+}$ during depletion were also enhanced after inhibiting the mitochondrion with micromolar carbonyl cyanide p-trifluoromethoxyphenylhydrazone (protonophore) (Fig. 5B), or after inhibiting the mitochondrial Na/Ca antiporter with 10 μM CGP37157 (Fig. 5C). (High micromolar concentrations of thapsigargin have been reported to directly release Ca$^{2+}$ from isolated mitochondria (69), here causing an overshoot of the second phase of the Ca$^{2+}$ influx.) In addition, pretreatment with ruthenium red, an inhibitor of the mitochondrial Ca$^{2+}$ uniporter (70), among other activities (39), led to a decrease (75 ± 7% of control; mean ± S.E.; n = 3; p < 0.05) in the depletion-coupled Ca$^{2+}$ influx (Fig. 5D). Moreover, no effect of xestospongin C, an inositol 1,4,5-trisphosphate receptor antagonist, was evident on re-addition of Ca$^{2+}$ to depleted synaptosomes (Fig. 5E). Together, these results confirm that the major site for Ca$^{2+}$ storage essential for presynaptic Ca$^{2+}$ homeostasis is the mitochondrion, with a contribution by a caffeine-sensitive (ryanodine receptor-linked) store. It is proposed that Ca$^{2+}$ released from such a store is rapidly taken up by the mitochondrion, most likely due to very close proximity of the endoplasmic reticulum Ca$^{2+}$ release sites to the mitochondria (71–73).

**Pharmacological and Immunocytochemical Evidence for TRPC Channels at Presynaptic Sites**—TRPC channel components have been identified in synaptosomal preparations (18), although definitive localization to presynaptic terminals was not confirmed. To consider whether functional TRPC channels may be involved in the Ca$^{2+}$ entry following Ca$^{2+}$ depletion, several inhibitors of TRP channels were used. The Ca$^{2+}$ influx revealed on depletion was significantly reduced (64 ± 6% of control; mean ± S.E.; n = 3; p < 0.01) following pretreatment with the non-selective cation channel inhibitor flufenamic acid (74), as was the K$^+$-induced Ca$^{2+}$ influx (Fig. 6A). In addition, SKF96365, another modulator of TRPC channels (39), strongly blocked the influx in response either to depletion (19 ± 6% of control; mean ± S.E.; n = 3; p < 0.01) or K$^+$ depolarization (Fig. 6B). Neither
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**FIGURE 6. TRPC channels in presynaptic terminals.** The effects of the TRP channel inhibitors flufenamic acid at 100 μM (A) and SKF96365 at 1 μM (B) were tested. C, localization of TRP channels to presynaptic terminals was determined using double immunostaining for the presynaptic marker synaptophysin (red) and individual TRP subunits (green), and was displayed as merged images. The percentages of synaptosomes with positive immunostaining for TRPC1 and TRPC5 over background were 50–80%. Also included is a panel (inset with TRPC1) of staining with secondary antibodies only, as background control (44). There was no significant immunostaining for TRPC4 or TRPC6 on isolated cortical terminals (not shown), although a prominent localization of TRPC4 to cortical neurons has been noted (94). There was also no positive staining for TRPC3 on synaptophysin-positive terminals was observed (Fig. 6C), and hence staining for TRPC4 or TRPC6 (data not shown). Staining for TRPC3 was similar to previous results (18); however, TRPC5 was rather weak.

Spermidine, 2-aminoethoxy phenylborane, nor 3,5-bis(trifluoromethyl) pyrazole had any effect (supplemental Fig. S3), each of these inhibitors typically having significant inhibitory activity toward SOCs, but displaying variable inhibition of certain TRP channels (16, 75–77). As noted previously, influx was also not affected by prior treatment with 1-oleoyl-2-acetyl-sn-glycerol (Fig. S1). These results, together with the previous data, are consistent with a store-independent pathway possibly involving TRP channels.

To assess more directly the presence of TRP channels, synaptosomal preparations were immunostained for individual subunits. Positive immunostaining for TRPC1 and TRPC5 but not TRPC3 or TRPV5 (control) in synaptosomal extracts detected via immunoblot analysis using rabbit anti-TRPC-specific antibodies (red channel), co-stained for actin (control) using a monoclonal anti-β-actin (green channel). Immunodetection for TRPC1, which displays multiple bands, and TRPC3 was similar to previous results (18); however, TRPC5 was rather weak.

that observed for synaptosomal preparations, likely due to equilibration with Ca\(^{2+}\) stores previously shown to be the dominant regulatory element within the varicosities (35), the latter also having a substantially larger volume (~1000 times) as compared with the synaptosome volume. The Ca\(^{2+}\) influx revealed on depletion was attenuated following expression of RNAi (shRNAmir) against mouse TRPC5, and to a lesser extent TRPC1, but not against TRPC3 (Fig. 8).

**DISCUSSION**

The most important intraterminal element responsible for presynaptic Ca\(^{2+}\) homeostasis is the mitochondrion (23), initially identified in a series of in-depth biochemical studies (78). Nearly all mature, active presynaptic terminals contain mitochondria, although their localization is highly dynamic (79), leading to their (transient) absence from some presynaptic sites in the brain (80). Nonetheless, they likely reside in close association with endoplasmic reticulum that is continuous with the endoplasmic reticulum in the axoplasm (71, 81). In addition, at the presynaptic membrane both the Na\(^{+}/\)Ca\(^{2+}\) exchanger and the Ca\(^{2+}\)-ATPase function in removing elevated Ca\(^{2+}\) after an impulse (Fig. 9), and hence are also essential to presynaptic Ca\(^{2+}\) homeostasis (78), as are VGCCs. However, substantial fluctuations in [Ca\(^{2+}\)] in the synaptic cleft may occur, particularly with high impulse frequency and postsynaptic depolarization (13), necessitating a mechanism to rapidly re-equilibrate intraterminal Ca\(^{2+}\) (82).

Here, we have identified a presynaptic Ca\(^{2+}\) influx pathway independent of Ca\(^{2+}\) stores, Na\(^{+}/\)Ca\(^{2+}\) exchange, and VGCCs that we propose operates in a constitutive fashion to maintain intraterminal Ca\(^{2+}\). Its putative constitutive behavior is supported by results of Mn\(^{2+}\) influx assay, wherein the Mn\(^{2+}\) influx was unaffected whether external Ca\(^{2+}\) was present or not (Fig. 2). On the other hand, the impact of this influx pathway on presynaptic homeostasis will be affected by the level of cytosolic [Ca\(^{2+}\)] achieved via VGCCs following presynaptic stimulation. Re-equilibration of basal presynaptic Ca\(^{2+}\) will thus involve all Ca\(^{2+}\) sources and pathways.

This Ca\(^{2+}\) influx pathway has properties distinct from a previously identified, outward rectifying non-selective cation channel activated in isolated terminals following reduction of [Ca\(^{2+}\)]\(_{o}\) via a putative G-protein-coupled pathway (31). The previously identified cation channel was strongly inhibited by
high micromolar to millimolar Mg\(^{2+}\) or Ca\(^{2+}\), and it was also blocked by Gd\(^{3+}\) and spermidine. These properties, together with outward rectifying behavior of the cation current, are more consistent with the TRPM7 channel (83, 84), which is strongly inhibited by physiological concentrations (1–2 mM) of intracellular Mg\(^{2+}\), perhaps in concert with Mg\(^{2+}\)-ATP via its kinase domain (85–87), and by activation of phospholipase C-coupled receptors (88, 89), and that appears to function in cellular Mg\(^{2+}\) homeostasis (90). In contrast, the presynaptic Ca\(^{2+}\) influx pathway identified here was strongly enhanced by lanthanides, enhanced in high micromolar Mg\(^{2+}\), and was completely insensitive to spermidine or other typical SOC inhibitors. The Mg\(^{2+}\) and lanthaneide sensitivity of the influx pathway, in addition to its ionic, pharmacological, and shRNA sensitivity, and the results of immunostaining for TRP channels indicate the involvement of TRPC channels, specifically TRPC5 and TRPC1 (55–57). The lack of effect of 1-oleoyl-2-acetyl-sn-glycerol is also consistent with this possibility (16, 91). Thus, although TRPM7 might appear to be an attractive candidate for the presynaptic Ca\(^{2+}\) influx pathway, because of its constitutive Ca\(^{2+}\) (and Mg\(^{2+}\)) permeability, the properties of TRPM7 would indicate otherwise, particularly the strong suppression of cation inward currents by millimolar Ca\(^{2+}\) or Mg\(^{2+}\), as noted previously. In contrast, the properties of TRPC5 containing channels fit better with the pharmacological and ionic sensitivity of the presynaptic Ca\(^{2+}\) influx pathway revealed following external Ca\(^{2+}\) depletion.

Previous studies have identified TRPC1, TRPC4, and TRPC5 channels in cortical synaptosome preparations (18), TRPC1 in axonal processes of neonatal hippocampal neurons (55), and TRPC5 in growth cones (92). TRPC1 has been found throughout the brain, including cortex (55). TRPC5 has also been noted in cortical regions (93), although it appears to be predominantly
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localized to hippocampus. (TRPC4 appears to be expressed in many brain regions, including cortex (94), and although no significant immunostaining in cortical terminals was found in the present study, it cannot be completely excluded.) Although the precise roles for TRP channels at presynaptic sites are largely unknown, it is proposed that TRPC1 and TRPC5 combine (55) to form a constitutively active Ca\(^{2+}\) influx pathway in brain presynaptic nerve terminals, revealed upon depletion of internal Ca\(^{2+}\) as a consequence of lower [Ca\(^{2+}\)]\(_o\) in the synaptic cleft and functioning in nerve terminal Ca\(^{2+}\) homeostasis following return of [Ca\(^{2+}\)]\(_o\) to normal values.

It remains to be determined the extent to which this presynaptic Ca\(^{2+}\) influx pathway is regulated and also the degree to which it is Ca\(^{2+}\) selective, with some significant Na\(^+\) permeability likely present, despite the lack of a pronounced effect of Na\(^+\)-free conditions (Fig. 4). As for specific regulators, NCS-1, present in presynaptic terminals (19), has been suggested to interact and functionally enhance TRPC5 containing channels in growth cones (95). Thus, the influx pathway in mature presynaptic terminals may exist in an activated state. It is also possible that several other processes regulate this pathway (56), including phosphorylation of TRPC (96), and activation of TRPC via lysophospholipids (97) and/or STIM1 (98). It is also possible that several other processes regulate this pathway (56), including phosphorylation of TRPC (96), and activation of TRPC via lysophospholipids (97) and/or STIM1 (98). It is also possible that several other processes regulate this pathway (56), including phosphorylation of TRPC (96), and activation of TRPC via lysophospholipids (97) and/or STIM1 (98).

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