Active Generation and Propagation of Ca\(^{2+}\) Signals within Tunneling Membrane Nanotubes

Ian F. Smith,†* Jianwei Shuai,§ and Ian Parker†‡

†Department of Neurobiology and Behavior and ‡Department of Physiology and Biophysics, University of California, Irvine, California; and §Department of Physics, Xiamen University, Xiamen, China

ABSTRACT  A new mechanism of cell-cell communication was recently proposed after the discovery of tunneling nanotubes (TNTs) between cells. TNTs are membrane protrusions with lengths of tens of microns and diameters of a few hundred nanometers that permit the exchange of membrane and cytoplasmic constituents between neighboring cells. TNTs have been reported to mediate intercellular Ca\(^{2+}\) signaling; however, our simulations indicate that passive diffusion of Ca\(^{2+}\) ions alone would be inadequate for efficient transmission between cells. Instead, we observed spontaneous and inositol trisphosphate (IP\(_3\))-evoked Ca\(^{2+}\) signals within TNTs between cultured mammalian cells, which sometimes remained localized and in other instances propagated as saltatory waves to evoke Ca\(^{2+}\) signals in a connected cell. Consistent with this, immunostaining showed the presence of both endoplasmic reticulum and IP\(_3\) receptors along the TNT. We propose that IP\(_3\) receptors may actively propagate intercellular Ca\(^{2+}\) signals along TNTs via Ca\(^{2+}\)-induced Ca\(^{2+}\) release, acting as amplification sites to overcome the limitations of passive diffusion in a chemical analog of electrical transmission of action potentials.

Received for publication 15 December 2010 and in final form 10 March 2011.

*Correspondence: ismith@uci.edu

Cells have long been known to employ gap junctions and synapses to communicate with their neighbors. A recent study (1) described a new route of cell-cell communication via tunneling nanotubes (TNTs; membrane protrusions, a few hundred nanometers in diameter, that physically link cell bodies over distances of tens of micrometers). These membrane tubes have been observed in diverse cell types in vitro and in vivo, contain F-actin, and are characteristically distinct from other cellular protrusions in that they lack contact to the substratum. TNTs have been shown to transfer membrane-bound components such as lipids and proteins between cells, to permit transfer of organelles such as mitochondria, and to facilitate intercellular transfer of pathogens such as bacteria, HIV-1, and prion (2). TNTs have also been shown to mediate transmission of intercellular Ca\(^{2+}\) signals (3,4) in a manner that is analogous to the well-established intercellular transmission of Ca\(^{2+}\) waves via gap junctions but enables transmission between cells that are not in intimate contact.

To investigate whether passive diffusion of Ca\(^{2+}\) ions along TNTs might be sufficient to enable cell-cell communication, we simulated diffusion between two cells connected by TNTs of different radii and lengths containing 100 μM immobile cytosolic Ca\(^{2+}\) buffer (see Fig. S1 in the Supporting Material). At 10 s after a large (10 μM) step increase in cytosolic free [Ca\(^{2+}\)] in one cell, the Ca\(^{2+}\) flux (current) from the end of a 30 μm TNT with a typical diameter of 200 nm was <1 fA. Given that openings of a single inositol trisphosphate receptor (IP\(_3\)R) channel Ca\(^{2+}\) passing a current of ~100 fA (5) generally fail to trigger Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) (6), passive diffusion alone appears to be inadequate for robust intercellular transmission of Ca\(^{2+}\) signals via TNTs.

We therefore looked for evidence of active Ca\(^{2+}\) signaling within TNTs by employing cultured SH-SY5Y neuroblastoma and HEK cell lines in which we had previously characterized Ca\(^{2+}\) signaling mechanisms. TNTs were present in both cell types and displayed properties consistent with those previously reported (2). They were suspended in the medium above the base of the imaging dish (Fig. 1A), contained F-actin but little or no tubulin (Fig. 1B), attained lengths as great as 70 μm, and allowed interchange of mitochondria between cells, demonstrating cytosolic continuity between the TNT and cell body (Movie S1). Of note, in the context of Ca\(^{2+}\) signaling, the TNTs contained extensions of the endoplasmic reticulum (ER; Fig. 1C) and expressed type 1 IP\(_3\)Rs along their length (Fig. 1D). The IP\(_3\)R channel mediates liberation of Ca\(^{2+}\) ions sequestered in the ER, and its opening is promoted by IP\(_3\) and cytosolic Ca\(^{2+}\), leading to regenerative CICR (7) that may remain localized as Ca\(^{2+}\) puffs or propagate as a Ca\(^{2+}\) wave (8).

To investigate whether TNTs display localized Ca\(^{2+}\) signals independently of their connected cells, we incubated cells with membrane permeant-esters of the Ca\(^{2+}\) indicator Fluo-4, caged iIP\(_3\), and the slow Ca\(^{2+}\) buffer EGTA (9). Fig. 2A illustrates a TNT that was initially quiescent but generated recurring localized transient fluorescence Ca\(^{2+}\) signals after the iIP\(_3\) was photoreleased by a UV flash that illuminated the entire imaging field. These localized fluorescence signals had a mean amplitude ΔF/F₀ of 0.43 ± 0.05,
mean duration (at half-maximal amplitude) of 68 ± 25 ms, and spatial spread (full width at half-maximal amplitude) of 1.1 ± 0.11 μm (n = 7 sites). Except for being essentially constrained along one spatial dimension, local Ca$^{2+}$ events in TNTs thus closely resemble the IP$_3$-mediated Ca$^{2+}$ puffs that arise from discrete clusters of IP$_3$R within the bodies of many cell types (6,8). Moreover, local events in TNTs were spaced 4–6 μm apart (Fig. 2 A), in similarity to the distribution of puff sites in the cell body (6).

We also observed spontaneous Ca$^{2+}$ events along TNTs (n = 16) even without photorelease of IP$_3$ and in cells that were not loaded with caged iIP$_3$. Fig. 2 D shows an instance in which Ca$^{2+}$ signals initially remained localized but a localized event subsequently triggered a regenerative wave of Ca$^{2+}$ that propagated in a saltatory manner across neighboring release sites with a velocity of ~8 μm s$^{-1}$. The localized signals persisted in the absence of external Ca$^{2+}$ (medium with zero added Ca$^{2+}$ and 1 mM EGTA), indicating that they involve a liberation of intracellular Ca$^{2+}$ and not an influx of extracellular Ca$^{2+}$. Moreover, SH-SY5Y and HEK293 cells lack ryanodine receptors (RyRs; the other major class of ER Ca$^{2+}$ release channels), and signals were inhibited by 20 mM caffeine, an IP$_3$R antagonist but RyR agonist (9/12 TNTs with spontaneous events in control: 3/14 after caffeine). Thus, the spontaneous events also appear to primarily involve IP$_3$Rs rather than RyRs, and may arise because of endogenous basal IP$_3$ within TNTs.

The observation of active, local IP$_3$-mediated Ca$^{2+}$ signals within TNTs suggests that they may facilitate Ca$^{2+}$ wave propagation between cells. We investigated this possibility by focusing a small (~8 μm diameter) UV spot onto one cell of a pair bridged by a TNT to evoke Ca$^{2+}$ liberation. This technique achieved a highly selective stimulation of the illuminated cell in that closely adjacent cells (not connected via TNTs) failed to respond, whereas in our hands an approach using local mechanical stimulation was confounded by intercellular Ca$^{2+}$ waves mediated by extracellular release of ATP. As shown in Fig. 3, local uncaging of iIP$_3$ evoked

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**FIGURE 1** TNTs between cultured SH-SY5Y cells. (A) Cells stained with the fluorescent membrane dye Di-8-ANEPPQ showing a TNT suspended ~6 μm above the coverglass. Images were obtained focused at the coverglass (bottom) and 6 μm higher (top). (B) Immunofluorescence staining for tubulin (green) and F-actin (red, phalloidin 647). (C) SH-SY5Y cell transfected 24 h previously to express ER-GFP showing the presence of ER in a TNT. (D) Immunofluorescence staining of type 1 IP$_3$Rs along a TNT. All scale bars are 20 μm. Images are representative of ≥8 TNTs.

**FIGURE 2** IP$_3$-evoked (A–C) and spontaneous (D) local Ca$^{2+}$ events along TNTs. (A) Monochrome image shows resting fluo-4 fluorescence in a TNT bridging two HEK cells. Asterisks indicate locations of local Ca$^{2+}$ events. (B) Linescan image derived by measuring Ca$^{2+}$-dependent fluorescence along the TNT (abscissa) as a function of time (ordinate). The arrow indicates when a photolysis flash was delivered. (C) Numbered traces show fluorescence signals measured from corresponding regions of the TNT marked in A. (D) Linescan image from a TNT bridging SH-SY5Y cells illustrating spontaneous local Ca$^{2+}$ signals and their coordination to generate a propagating Ca$^{2+}$ wave (marked by the white diagonal line).

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Biophysical Journal 100(8) L37–L39
sive cycles of Ca\(^{2+}\) release, diffusion, and CICR, these channels may serve as active amplification sites to promote propagation of Ca\(^{2+}\) signals along TNTs. Opening of IP\(_3\)R channels absolutely requires IP\(_3\) as well as Ca\(^{2+}\) (7). Our simulations (Fig. S2) indicate that passive flux of IP\(_3\) (which diffuses faster than Ca\(^{2+}\) (10)) from a stimulated cell could rapidly elevate [IP\(_3\)] within a TNT so as to allow active wave propagation, but would only slowly raise [IP\(_3\)] throughout the enormously larger volume of a connected cell. This may explain why connected cells often gave only small Ca\(^{2+}\) signals or failed to respond. Nevertheless, active transmission of Ca\(^{2+}\) waves along TNTs may be an efficient physiological mechanism for communication across a network of connected cells that are primed by a small basal elevation of cytosolic [IP\(_3\)]. We further speculate that other cell types may use an analogous mechanism mediated by RyRs that exhibit CICR without requiring a further second messenger.

**SUPPORTING MATERIAL**

Methods, model simulations, a reference, two figures, and four movies are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00317-1.

**ACKNOWLEDGMENTS**

This work was supported by grants from the National Institutes of Health (GM 40871 to I.P., and GM65830 to I.P. and J.S.) and the National Natural Science Foundation of China (30970970), and the Specialized Research Fund for the Doctoral Program of Higher Education (20090121110028 to J.S.).

**REFERENCES and FOOTNOTES**