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Selective block of tunneling nanotube (TNT) formation inhibits intercellular organelle transfer between PC12 cells

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ABSTRACT

Organelle exchange between cells via tunneling nanotubes (TNTs) is a recently described form of intercellular communication. Here, we show that the selective elimination of filopodia from PC12 cells by 350 nM cytochalasin B (CytoB) blocks TNT formation but has only a weak effect on the stability of existing TNTs. Under these conditions the intercellular organelle transfer was strongly reduced, whereas endocytosis and phagocytosis were not affected. Furthermore, the transfer of organelles significantly correlated with the presence of a TNT-bridge. Thus, our data support that in PC12 cells filopodia-like protrusions are the principal precursors of TNTs and CytoB provides a valuable tool to selectively interfere with TNT-mediated cell-to-cell communication.

1. Introduction

Intercellular communication plays a central role in the physiology of multi-cellular organisms, and the exchange of molecular information is achieved by a variety of cellular mechanisms. Recently, a new principle of cell-to-cell communication based on the de novo formation of thin membrane channels between mammalian cells was documented [1]. These channels, referred to as tunneling nanotubes (TNTs), were shown to permit the direct intercellular transfer of endosome-related organelles and other cellular components [1]. Subsequently, TNT-like nanotubes were found to connect other cell types [2–4] and the transfer of mitochondria [5,6], plasma membrane components [1,7,8], calcium ions [8] as well as viral proteins [9,10] along TNT-like bridges was demonstrated. From these studies and the recent demonstration that TNT-connectivity exists in vivo between immune cells of the corneal stroma [11], it emerges that TNT-mediated communication has the potential to play an important role in many physiological processes of multi-cellular organisms.

TNT-like structures appear to form by two different mechanisms that may vary with cell type: by the directed outgrowth of a filopodium-like protrusion toward a neighboring cell or by dislodgement of attached cells after a certain required interaction time [3,4]. In contrast to filopodia, TNTs between cultured cells have no contact to the substratum but hover freely in the intercellular space. Filopodia along with lamellipodia are F-actin-based protrusive structures attached to the substratum of cultured cells with migratory and exploratory functions [12]. Filopodia have a rodlike shape and are filled with bundles of parallel actin filaments, whereas lamellipodia are flat protrusions with a meshwork of actin filaments. In contrast to lamellipodia, filopodia possess a so-called tip complex consisting of different anti-capping proteins and F-actin nucleators [13–15]. Interestingly, treatment with cytochalasin B/D in the nanomolar range has been an elegant way to specifically block filopodia formation in neuronal cell types [16,17]. The exact mode of action is not known, but it is presumed that at low concentrations cytochalasin B/D specifically compete with Ena/VASP proteins at the filopodial tip and thus, block the addition of actin monomers [16,17].

In this study, we investigated the effects of nanomolar concentrations of cytochalasin B (CytoB) on TNT formation and stability as well as on organelle exchange in PC12 cell cultures.

Abbreviations: TNT, tunneling nanotube; WGA488, Alexa Fluor® 488 wheat germ agglutinin; WGA633, Alexa Fluor® 633 wheat germ agglutinin; CTB, CellTracker™ Blue CMAC; CTG, CellTracker™ Green CMFDA; DiD, Vybrant® DiD cell-labeling solution; DiI, Vybrant® DiI cell-labeling solution; CytoB, cytochalasin B; DMSO, dimethylsulfoxide

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2. Materials and methods

2.1. Reagents and cell culture

Alexa Fluor® 488 wheat germ agglutinin (WGA488), Alexa Fluor® 633 wheat germ agglutinin (WGA633), CellTracker™ Blue CMAC (CTB), CellTracker™ Green CMFDA (CTG), Vybrant® DiI cell-labeling solution (DiI) and Vybrant® DiD cell-labeling solution (DiD) were purchased from Molecular Probes (Invitrogen Detection Technologies, Carlsbad, CA, USA). CytoB was obtained from Sigma Aldrich (Sigma–Aldrich Corp., St. Louis, MO, USA). For all experiments, singularized PC12 cells (rat pheochromocytoma cells, clone 251, [18]) were plated at a density of 6×10^4 cells/cm^2 and cultured as described [1].

2.2. Imaging and FACS analysis

Confocal and wide-field microscopy were performed with a Leica TCS SP5 (Leica Microsystems GmbH, Wetzlar, Germany) and a Zeiss Axiovert 200M (Carl Zeiss, Jena, Germany) or an Olympus IX70 microscope (Olympus Optical Co., Europe GmbH, Hamburg, Germany), respectively, as described [19]. Flow cytometry analysis of organelle transfer was performed on a FACSCalibur™ flow cytometer (Becton–Dickinson, Mountain View, CA, USA) as described [19].

2.3. Analysis of organelle transfer

For quantitative analyses of TNT structures and intercellular organelle transfer, cocultures of CTB or CTG stained cells (acceptor population) and DiD stained cells (donor population) were plated at a ratio of 1:1. Notably, the DiD staining was performed 1 day before the start of the coculture. One hour after plating, the medium was removed to eliminate cell debris, and new medium containing 350 nM CytoB [1:1000 dilution from a 350 μM stock in dimethylsulfoxide (DMSO)] was added. The control cells were cultured without DMSO since additional experiments indicated that a 1:1000 dilution of DMSO had no effect on TNT number or organelle transfer (data not shown). Analysis of organelle transfer was performed at 2 and 24 h after cell plating. For microscopy analysis, WGA488 was added to the cells to permit cell segmentation and TNT detection (see Section 2.6). Image stacks covering the whole cellular volume were acquired by wide-field microscopy for the CTB, DiD and WGA488 channels using excitation wavelength of 400 nm, 633 nm and 488 nm, respectively. For FACS analysis, DiD-labeled donor cells and CTG-labeled acceptor cells were analyzed at 633 nm and 488 nm excitation wavelength, respectively.

2.4. Analysis of phagocytosis

Cell debris was generated by mechanical lysis of DiI-stained PC12 cells using a custom-made CellCracker. Then, a post-nuclear supernatant was prepared by centrifugation at 3000×*g* for 10 min. The supernatant was split into two equal portions, one was supplemented with 350 nM CytoB, and both portions were added to plated cells, respectively. As a control for the analysis algorithm, cells without added cell debris were incubated in the absence of CytoB in parallel. After 2 h of incubation, the different cell populations were washed, further incubated in growth medium in the absence or presence of CytoB and imaged 24 h later. Directly before the start of imaging, the growth medium was replaced by Tyrode's solution (2 mM $CaCl_2$, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCb, 30 mM glucose, 25 mM HEPES, pH 7.4, 2 mM bromphenol blue) [20]. Notably, bromphenol blue quenches extracellular fluorescence signals and thus guarantees that only internalized signals are quantified. 3D image stacks for DiI fluorescence were acquired at 555 nm excitation wavelength using wide-field microscopy. Cell segmentation was performed on the same channel using the watershed method since the cells appeared as dark regions surrounded by brighter extracellular space (Fig. 4A). Phagocytosed cell debris was detected using adaptive thresholding.

2.5. Analysis of fluid-phase endocytosis

WGA633 was added to plated cells and incubated for 30 min at 37 °C in the presence or absence (control) of 350 nM CytoB, or at 0.7 °C in the absence of 350 nM CytoB. Subsequently, WGA488 was added and stacks of 20 focal planes spanning the whole cellular volume were recorded by wide-field microscopy at 488 nm and 633 nm excitation wavelength. The data were processed by 3D automated image segmentation using the WGA488 channel (see Section 2.6). The rate of endocytosis was determined by a quantitative analysis of the WGA633 signal within the cell regions. Signals were calculated by subtracting the mean signal intensity inside the cells from the mean signal intensity outside the cells, both applied to the WGA633.

2.6. Quantitative image processing

3D image stacks of WGA488-stained cells acquired by wide-field fluorescence microscopy were used for cell segmentation by applying automated marker construction [21] and the watershed algorithm by immersion [22]. The TNTs were detected using the method in [23], except from the edge detection, which was replaced by adaptive thresholding [21]. Every detected cell was labeled as either CTB positive, DiD positive or neutral, in the following referred to as 'CTB' cells, 'DiD' cells or 'unstained' cells, respectively. The labeling of the cells was accomplished by converting the CTB and DiD channel into binary images by thresholding. For the CTB channel, a global thresholding was suitable since the images are homogenous, but the DiD channel required an adaptive thresholding for the binarization due to the varying signal intensity. Both thresholding techniques convert the respective channels into binary images with pixel values '1' and '0'. A cell was classified as CTB or DiD positive, if it had at least $4/3\pi r^3$ associated '1'-pixels in the respective channels, where r is the smallest allowed radius of an organelle, which was estimated to 153 nm. A cell positive for both CTB and DiD was said to be double positive and those cells were of special interest since DiD organelles have obviously been transferred.

We defined four groups of cells, A, B, C and D, shown in Fig. 5A: (A) double positive cells with a TNT to a DiD cell, (B) acceptor cells with a TNT to a DiD cell, (C) double positive cells without a TNT to a DiD cell and (D) acceptor cells without a TNT to a DiD cell. From these definitions it follows that $A \subseteq B$ and $C \subseteq D$ since every double positive cell is also an acceptor cell. Furthermore, let $\{A \cup B \cup C \cup D\} \cap G = \varnothing$ where G is the set of acceptor cells with at least one direct donor neighbor. Thus, these cells were excluded from the analysis to minimize the influence of neighbor cells.

The TNT number was counted and directly compared between the 24-h control and 24-h CytoB condition. The overall transfer in the 24-h time point in the presence of CytoB was normalized by subtracting the transfer in the 2-h time point. Let $n(x,y)$ be the *number of cells* in group x at time point y and define the transfer rate at time point y as $tr(y) = (n(A,y) + n(C,y))/(n(B,y) + n(D,y))$. Then, the transfer rate TR between the time points was computed as $TR = (tr(24\text{ h}) - tr(2\text{ h})) * 100\%$. The correlation between TNT connected cells and transfer was also computed. This analysis was performed by comparing group A/B to group C/D after subtracting the transfer rate $tr(24\text{ h})$ (Fig. 5B). This normalization