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Abstract

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ABSTRACT

Carbon-based nanoprobes are attractive for minimally-invasive cell interrogation but their application in cell physiology has thus far been limited. We have developed carbon nanopipettes (CNPs) with nanoscopic tips and used them to inject calcium-mobilizing messengers into cells without compromising cell viability. We identify pathways sensitive to cyclic adenosine diphosphate ribose (cADPr) and nicotinic acid adenine dinucleotide phosphate (NAADP) in breast carcinoma cells. Our findings demonstrate the superior utility of CNPs for intracellular delivery of impermeant molecules and, more generally, for cell physiology studies. The CNPs do not appear to cause any lasting damage to cells. Their advantages over the commonly used glass pipettes include smaller size, breakage and clogging resistance, and potential for multifunctionality such as concurrent injection and electrical measurements.

1. INTRODUCTION

Since the discovery of carbon nanotubes [1] and the fabrication of nanoscale carbon pipes [2], we and others have sought to create carbon-based, one-dimensional cellular probes [3-9] for, among other uses, intracellular delivery, probing, sensing, and nanosurgery. Carbon nanotube-based probes are minimally invasive to cell membranes and organelles and offer superior electrical, thermal, and mechanical properties [10, 11] compared with their glass counterparts. Several one-dimensional, nanotube-based probes developed recently [5-8] have been used for cell interrogation, but difficulties in probe fabrication and concerns about nanotube toxicity have hindered the use of carbon-based nanoprobes in cell physiology studies. To address these shortcomings, we have developed carbon nanopipettes (CNPs) capable of probing cells and injecting fluids without compromising cell viability [9]. In this report, we show that CNPs are superior to their glass counterparts for fluid delivery. We demonstrate the utility of CNPs in cell

physiology studies by injecting calcium-mobilizing messengers into cells to discover signaling pathways in breast cancer cells.

2. BACKGROUND

Intracellular, calcium-mobilizing messengers are charged, cell-impermeant molecules, produced in response to a variety of external cues, which couple cell surface stimulation to the release of calcium from intracellular calcium stores [12]. The endoplasmic reticulum is an established calcium store housing calcium channels sensitive to the messenger inositol trisphosphate (IP_3) and, in some cells, to cADPr. More recent studies have identified lysosomes as calcium stores which appear to express calcium channels for the messenger NAADP [13], although this is still controversial [14]. Unregulated release of stored calcium has been implicated in several diseases, including cancer [15]. However, to date, only IP_3 signalling has been investigated in cancerous cells. Here, we use CNPs developed and fabricated in-house to inject calcium-mobilizing messengers into SKBR3 cells derived from a human breast carcinoma. By monitoring the cytosolic calcium concentration changes during messenger injection, we study the calcium release mechanisms of these cells.

3. EXPERIMENT

CNP Fabrication

CNPs suitable for microinjection (Fig. 1) were fabricated using processes described previously [9]. Quartz capillaries (Q100-70-7.5, Sutter Co.) were filled with 18mg Iron (III) Nitrate (Fisher) dissolved in 25ml isopropyl alcohol and allowed to air dry for 24 hrs at room temperature. The catalyst-laden capillaries were pulled with a Sutter P-2000 micropipette puller into very fine-tipped, blunt taper micropipettes (Fig. 1a). A carbon film was deposited on the

inner catalyzed surface by chemical vapor deposition (900°C) in an Argon/Methane environment (300/200sccm, resp.). The tips were then wet-etched with 6:1 buffered hydrofluoric acid (BHF, Transene) at room temperature for 4 min to remove the quartz exterior and expose a short length of the interior carbon nanopipe (5-15 μm length, 30 nm wall thickness). Hundreds of CNPs with tip outer diameters ranging from 200 to 400 nm (Fig. 1b) were fabricated concurrently without any assembly. Although their tip diameters can be decreased to tens of nanometers, we used relatively larger CNPs in this study to facilitate fluid transport and manipulation under light microscopy.

Cell Culture

SKBR3 (GPR30-positive breast cancer cells) were purchased from ATCC. Cells were grown in McCoy's 5A modified media supplemented with 10% fetal serum. The cells were plated on 12 mm cover slips 48 h prior to the calcium measurements.

Cytosolic Ca2+ Measurement

Cytosolic Ca2+ measurements were performed as described previously [16]. Cells were incubated with 5 μ M Fura-2 AM (Molecular Probes, Eugene, OR, USA) in Hanks' balanced salt solution (HBSS) at room temperature for 45 min in the dark, washed thrice with dye-free buffer, and then incubated for another 45 min to allow for complete de-esterification of the dye. The cover slips were subsequently mounted in a custom-designed bath, which was positioned on the stage of an Eclipse TE 2000-U Nikon inverted microscope equipped with a Roper Scientific CCD camera (Optical Apparatus Co., Ardmore, PA, USA). Cells were routinely superfused with HBSS at a flow rate of 0.5 ml/min. Fura-2 fluorescence (emission = 510 nm), following alternate excitation at 340 and 380 nm with a Sutter LB10W32 filter wheel, was acquired at a frequency

of 0.33 Hz. The ratio of the fluorescence signals (340 nm/380 nm) was converted to Ca2+ concentrations [17]. Images were acquired and analyzed using Metafluor software.

Microinjection

Control injections were performed with intracellular solution containing 20mM HEPES, 110mM KCl, 10mM NaCl, pH 7.2 and was filtered (0.2um) before use. In the experiments, messenger molecules were diluted in intracellular solution to a final concentration of 10 μM. For glass micropipette injection, IP3 and NAADP were injected with Eppendorf Femtotips I (tip dimensions, 1.0/0.5 μm OD/ID) and cADPr with Eppendorf Femtotips II (tip dimensions, 0.7/0.5 μm OD/ID). For CNPs and glass micropipettes, injection settings were adjusted (compensation pressures 30-60 hPa, injection pressures 60-120 hPa, 0.4 sec duration) to inject ~1% of cell volume as determined by visualizing fluorescent dye injection. Resulting injections were estimated to produce 100 nM messengers inside cells. Concurrent calcium imaging and injection were performed on a Nikon Eclipse TE2000-U inverted microscope as described above. Eppendorf Transferman NK2 and Femtojet were used, respectively, for micromanipulation and microinjection.

4. RESULTS AND DISCUSSION

We studied the response of SKBR3 cells when injected with intracellular solution and with the calcium-mobilizing messenger IP_3 (Fig. 2). Figures 2a and b depict the cytosolic calcium concentrations as functions of time in response to injections of intracellular solution and IP_3 with CNPs and glass micropipettes, respectively. First, CNPs and conventional glass micropipettes (Eppendorf, 0.7-1.0 μm tip outer diameters) were used to inject intracellular solution into SKBR3 cells. Basal intracellular calcium concentration was not significantly affected either by

the penetration of the probes through the cell membrane or by the injection of intracellular solution alone (Fig. 2a and b, blue circles).

Next, CNPs were used to inject IP_3. The IP_3 injection (resulting in approximately 100 nM IP_3 inside the cell) evoked a prompt increase in intracellular calcium concentrations (Fig. 2a, black squares) above basal levels followed by decay back to the basal levels. The signals were highly reproducible (Fig. 2a, the black squares feature modest deviations from the mean). The return to basal calcium concentrations 3 min after injection indicates the IP_3-mediated calcium increases are transient. These observations are significant since sustained increases in cytosolic calcium concentration are a sensitive indicator of cytotoxicity [18]. Thus, our data illustrates that CNP use does not compromise cell viability or unexpectedly alter basal cytosolic calcium.

The IP_3-evoked responses following CNP injection (Fig. 2a, black squares) were similar in kinetics and magnitude to those evoked by the injection of IP_3 with glass micropipettes (Fig. 2b, black squares). As expected, IP_3 responses were forestalled by cell pre-treatment with the IP_3 receptor antagonist, Xestospongin C (XeC, 10 μM for 15 min) (Fig. 2b, red triangles). Additionally, IP_3-evoked responses were only observed in injected cells. Figure 2c shows a CNP positioned tens of microns above a Fura-2-loaded SKBR3 cell. Figures 2d and e show, respectively, the ratiometric image of Fura-2 fluorescent signals immediately before and after the CNP injected IP_3 into the cell on the right. A cell directly injected with the messenger stimulated calcium release (Fig. 2e, white color) while cells that were not injected (left cell) remained at basal calcium levels (Fig. 2d and e, blue-green color).

To determine whether additional calcium release pathways are present in SKBR3 cells, we used CNPs to inject cADPr and NAADP. Figure 3 depicts the cytosolic calcium concentrations as functions of time following the injection of the two messengers. As shown in Figures 3a and c, injection by CNPs of either messenger evoked clear calcium signals, providing the first direct

evidence for IP_3-independent calcium release pathways in SKBR3 cells. As with IP_3, the cADPr-and NAADP-evoked calcium signals following injections with CNPs (Fig. 3a and c, black squares) were indistinguishable from those elicited by glass micropipettes (Fig. 3b and d, black squares).

To identify the mechanisms of messenger-evoked calcium release, cells were pre-treated with the V type ATPase inhibitor, bafilomycin-A1 (BAF, 1 µM for 60 min). As expected, depleting acidic calcium stores with BAF had no effect on cADPr-mediated calcium signals (Figure 3b, red triangles). In marked contrast, the prevention of NAADP-mediated calcium signals in BAF pre-treated cells (Figure 3d, red triangles) suggests that NAADP targets acidic calcium stores in breast cancer cells, as is shown in several non-transformed cells [19]. The effects of cADPr were blocked by cell pre-treatment with the ryanodine receptor antagonist, Ryanodine (Ry, 10 µM for 15 min). This confirms cADPr's involvement in releasing calcium via the ryanodine receptor (Figure 3b, blue circles). The partially inhibited response of Ry-treated cells to NAADP (Figure 3d, blue circles) confirms that, in breast cancer cells, acidic store Ca^{2+} release by NAADP triggers ryanodine receptors in the ER to produce global Ca^{2+} signals [20].

In the process of discovering calcium signaling pathways in breast cancer cells with CNPs, we recognized significant advantages for using CNPs for microinjection instead of glass. First, unlike their glass counterparts, the carbon tips of CNPs can bend or buckle without fracture [9]. During injection experiments, we observed less instances of tip breakage with CNPs than with commercial glass injectors. Second, CNPs were less prone to blockage than the commercial glass injectors despite their smaller tip diameters. In practice, a single CNP was used to inject roughly 4 times the number of cells than injected with a single glass injector. Third, compared to glass, CNPs were more readily visible under light microscopy due to their dark color. This feature substantially facilitated microinjection by providing more accurate control of the location

and depth of the tip for penetrating the cell membrane. Better control resulted in more efficient microinjection by causing less cell damage and death (~75% success rate with CNPs as opposed to ~40% success rate with glass). We also expect CNPs to cause less damage to cell membranes and organelles than their glass counterparts because of their smaller diameters. Although not studied here, CNPs' electrical conductivity [9] allows concurrent fluid transport and electrical signal measurement.

In our studies, we did not observe any adverse impact resulting from short duration penetration of carbon nanopipes into cells. Recently, Poland et al. [21] have reported, however, that prolonged exposure to long carbon nanotubes introduced into the abdominal cavity of mice caused asbestos-like pathogenicity.

5. SUMMARY

We have used carbon nanopipettes (CNPs) to effectively deliver calcium-mobilizing messengers into live cells without compromising viability, thereby highlighting the utility of these probes for cell physiology and pharmacology studies. We have shown, for the first time, the existence of functional calcium channels sensitive to cADPr and NAADP in breast cancer cells, both of which might therefore contribute to altered calcium signaling during tumor progression. We have found that NAADP acts on a ryanodine-insensitive channel located on an acidic calcium store, the activation of which is intimately coupled to the opening of ryanodine receptors on the endoplasmic reticulum. We identified several advantages for using CNPs for microinjection over commercial glass injectors such as smaller size, higher durability, easier spatial control, and resistance to clogging. In the future, we will take advantage of the electrically conductive hollow carbon tip to simultaneously inject fluids and measure electrophysiological signals, such as membrane potentials.

ACKNOWLEDGEMENTS

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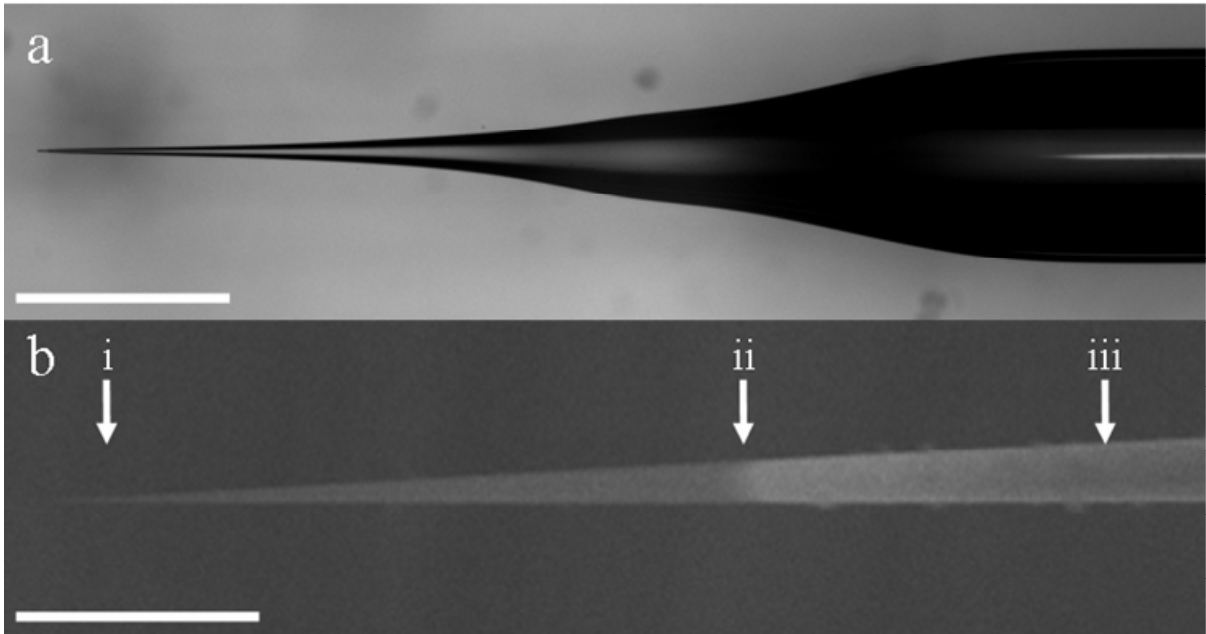
Figure 1: CNP for microinjection. (a) Image of a very fine-tipped, blunt taper CNP. Scale bar, 1mm. (b) SEM micrograph (10KV) of the very fine tip of a blunt taper CNP showing the nanoscopic carbon tip (i), the end of the exposed carbon tip (ii), and the quartz micropipette exterior (iii). Scale bar, 10 μm.
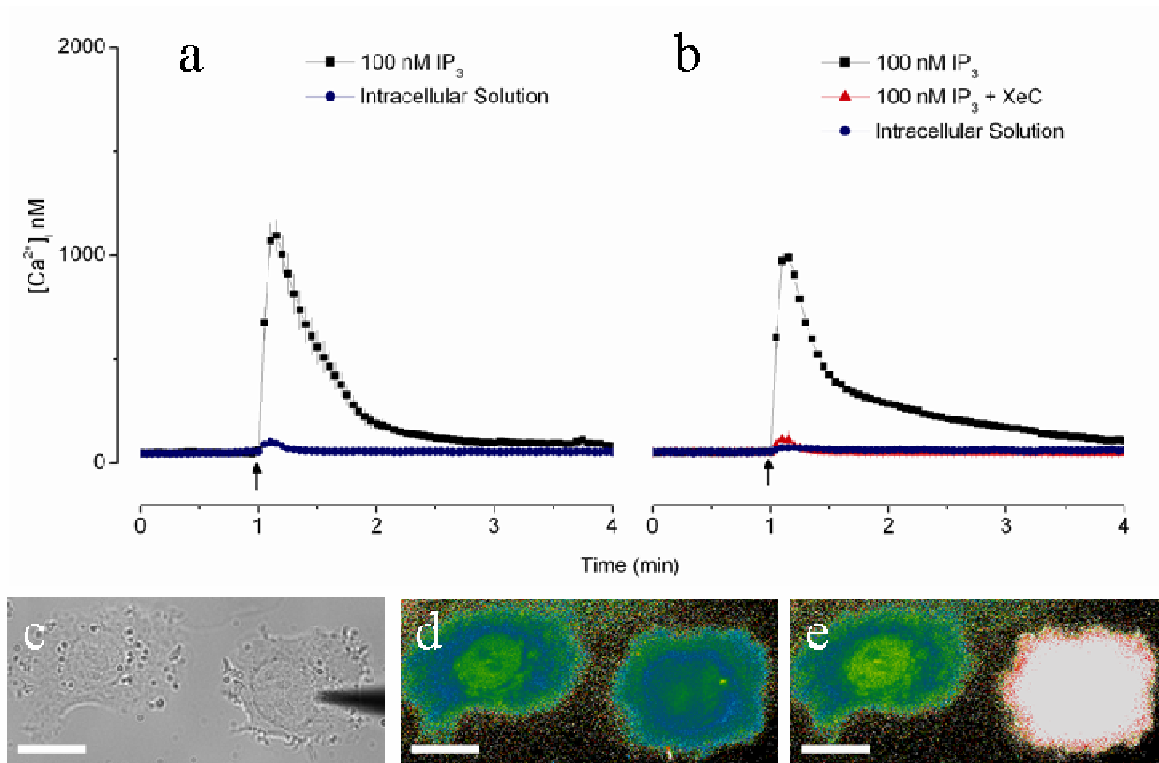
Figure 2: Calcium response to IP_3. (a) Time-course of changes in intracellular calcium concentration upon injection (arrow) of intracellular solution (blue circles) and IP_3 (black squares) using CNPs. Results are means +/- S.E.M of 6 cells. (b) Time-course of changes in intracellular calcium concentration upon injection (arrow) of intracellular solution (blue circles) and IP_3 either in the absence (black squares) or presence (red triangles) of XeC using conventional glass micropipettes. Results are means +/- S.E.M of 6 cells. (c) Brightfield image of an IP_3-filled CNP (dark feature above right cell) positioned tens of micrometers above a SKBR3 cell prior to injection. (d-e) Images of intracellular Fura-2 fluorescence ratios 2 sec before (d) and 1 sec after (e) injection of IP_3 (only the right cell is injected). Scale bars, 10 μm.
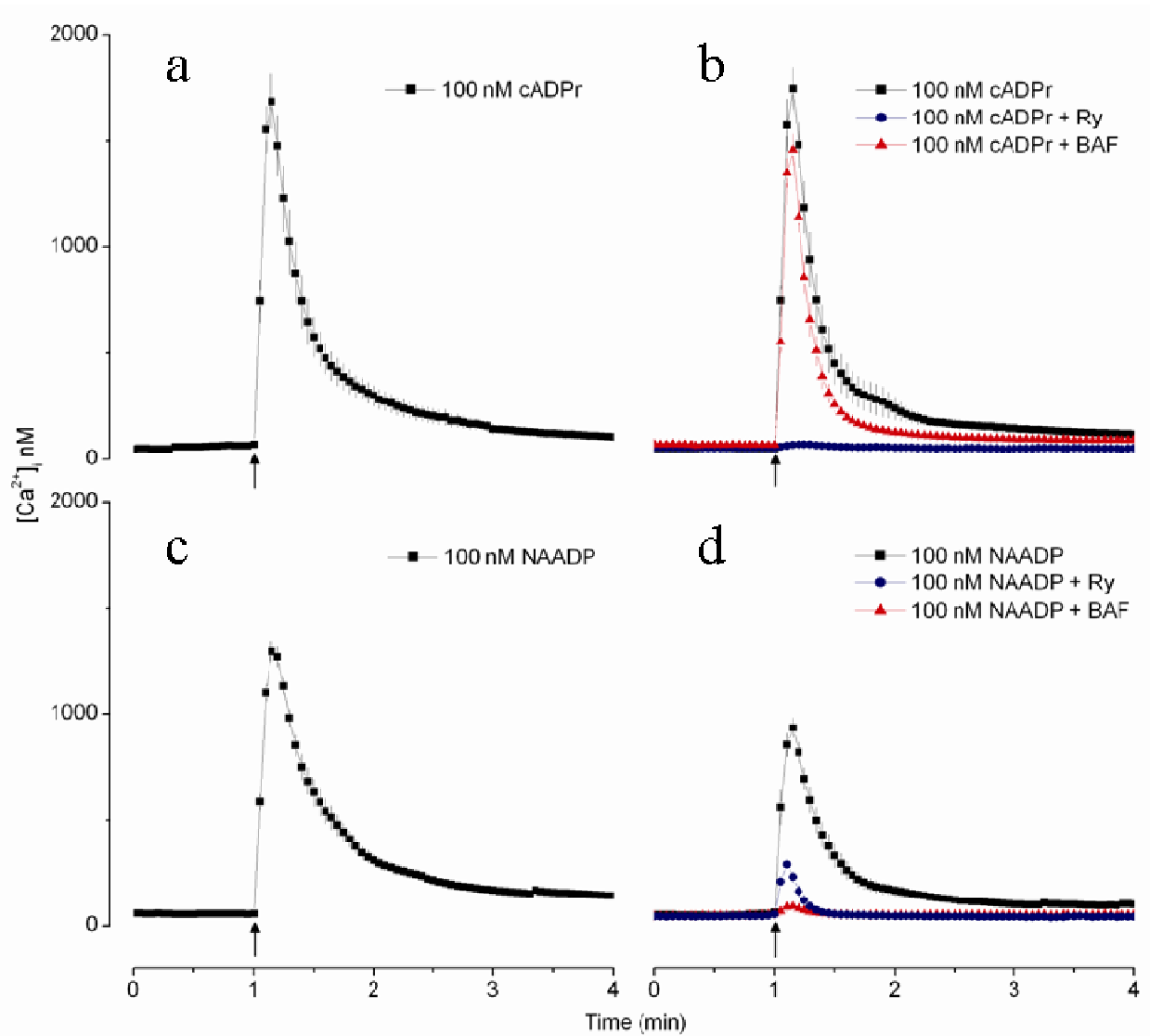
Figure 3: Identification of new intracellular calcium release pathways in SKBR3 cells. Time-course of changes in intracellular calcium concentration upon injection (arrows) of cADPr (a-b) and NAADP (c-d) using CNPs (a, c) and glass micropipettes (b, d). In some experiments, cells were pre-treated with Ry (b and d, blue circles) or BAF (b and d, red triangles). All results are means +/- S.E.M of 6 cells.