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Short communication

Nanosecond electric pulse-induced calcium entry into chromaffin cells

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ABSTRACT

Electrically excitable bovine adrenal chromaffin cells were exposed to nanosecond duration electric pulses at field intensities ranging from 2 MV/m to 8 MV/m and intracellular calcium levels ($[Ca^{2+}]_i$) monitored in real time by fluorescence imaging of cells loaded with Calcium Green. A single 4 ns, 8 MV/m pulse produced a rapid, short-lived increase in $[Ca^{2+}]_i$, with the magnitude of the calcium response depending on the intensity of the electric field. Multiple pulses failed to produce a greater calcium response than a single pulse, and a short refractory period was required between pulses before another maximal increase in $[Ca^{2+}]_i$ could be triggered. The pulse-induced rise in $[Ca^{2+}]_i$ was not affected by depleting intracellular calcium stores with caffeine or thapsigargin but was completely prevented by the presence of EGTA, Co^{2+}, or the L-type calcium channel blocker nitrendipine in the extracellular medium. Thus, a single nanosecond pulse is sufficient to elicit a rise in $[Ca^{2+}]_i$ that involves entry of calcium via L-type calcium channels.

1. Introduction

Nanosecond duration electric pulses of high electric field intensity (MV/m) induce intracellular responses in the absence of conventional electroporation of the plasma membrane [1,2]. Most notable is the release of calcium from internal stores [3–5], a response that can in turn activate cellular processes such as apoptosis, which has implications for cancer therapy [6,7].

Because the response of particular cell types to nanoelectropulses depends on the pulse rise time, pulse duration, number of pulses, and electric field intensity [8], the delivery of nanosecond pulses potentially could be tailored to achieve selectivity, not only with respect to the cellular response desired but also the cell type being targeted [9]. However, before this potential is realized, an understanding of how diverse cell types respond to nanoelectropulses is essential. The goal of the present work was to extend studies of ultrashort, high-field pulse effects to include electrically excitable neurosecretory chromaffin cells that release catecholamines via a mechanism triggered primarily by calcium entry into the cell via voltage-gated calcium channels, rather than by release of calcium from intracellular stores. Our approach was to monitor $[Ca^{2+}]_i$ in cells exposed to 4 ns duration electric pulses with field intensities ranging from 2 MV/m to 8 MV/m. The results show that a single pulse is sufficient to induce a pronounced transient increase in $[Ca^{2+}]_i$ that involves a different mechanism from that described to date for non-excitable cells. A preliminary report of this work has been previously presented [10].

2. Methods

2.1. Preparation of chromaffin cells

Chromaffin cells were isolated from fresh bovine adrenal medullas using the method described by Waymire et al. [11] and maintained in suspension culture as previously described [12].

2.2. Fluorescence microscopy of nanoelectropulsed cells

Chromaffin cell aggregates that form in suspension culture were dissociated into clusters of ten cells or less, including single cells [12,13], and the cells were loaded with the calcium fluorescent indicator dye Calcium Green-1-AM (Invitrogen; $Ex_{480\ nm}$ and $Em_{535\ nm}$) at 1 μM in RPMI 1640 medium (Irvine Scientific) for 1 h at 37 °C. After resuspension in dye-free RPMI 1640, the cells were transferred to a microfabricated electrode chamber [14] that was positioned on the stage of a Zeiss Axiovert 200M epifluorescence microscope. Images were captured and analyzed with a LaVision Imager QE camera and software and a MOSFET, saturable-core, transformer-switched, fast-recovery diode pulse generator [15] that was mounted on the microscope stage delivered 4 ns pulses (200–800 V) directly to the microchamber electrodes in ambient

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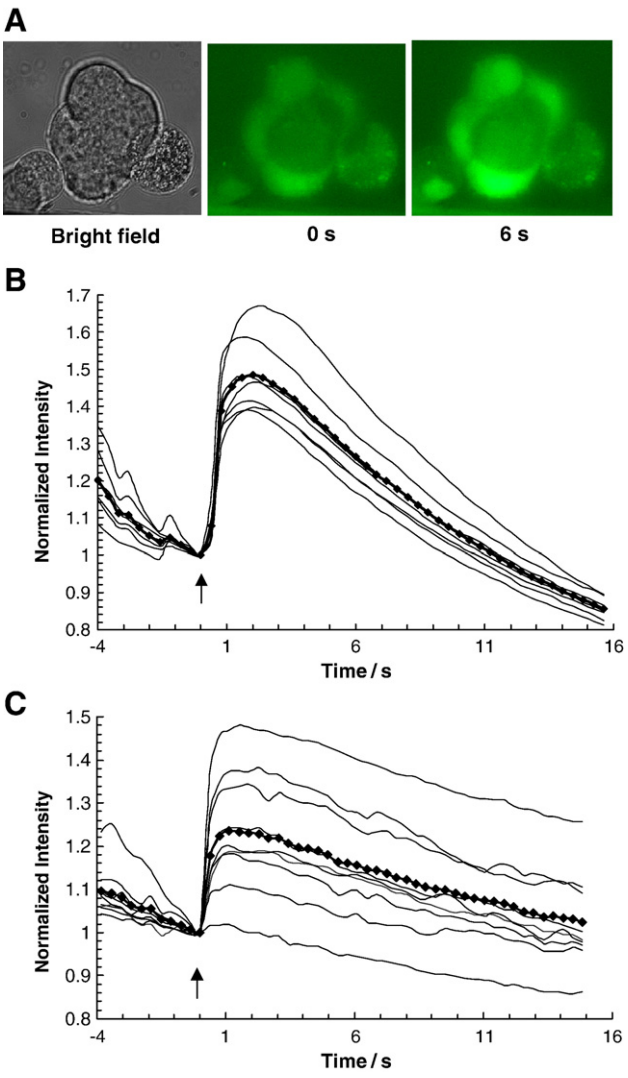

Fig. 1. Fluorescence imaging of $[Ca^{2+}]_i$ in chromaffin cells exposed to a single 4 ns, 8 MV/m pulse. (A) Photomicrographs of cells before (bright field and 0 s) and 6 s after the pulse was applied. (B) Calcium traces for the cells shown in (A); (C) calcium traces for cells from a separate, similar experiment. In (B) and (C), and in subsequent figures, the lines with symbols are mean values, and the arrows indicate when the pulse was applied.

atmosphere at room temperature. Details of this experimental apparatus are given in Sun et al. [16]. Fluorescence intensity curves were generated from a set of sequential images of cells from each experiment. All fluorescence data have been normalized to the intensity value at the point where the first pulse was applied, and each fluorescence curve represents the integrated response over the area of an individual cell. Background fluorescence was measured in several cell-free regions of the visual field. Because direct addition of reagents to the cells in the microelectrode chamber was not feasible, the response of the cells to addition of the nicotinic receptor agonist dimethylphenylpiperazinium (DMPP; Sigma) was obtained separately by introducing 0.4 μL of a 5 mM solution of the drug into a cover glass chamber containing 200 μL of the cell suspension. YO-PRO-1 was obtained from Invitrogen.

3. Results and discussion

3.1. Effect of 4 ns pulses on $[Ca^{2+}]_i$

As shown in Fig. 1, application of a single 4 ns, 8 MV/m pulse induced a rapid, marked rise in $[Ca^{2+}]_i$ that was maximal by 1 to 2 s and transient, with $[Ca^{2+}]_i$ returning to baseline values typically by 15 to 45 s. A second 4 ns, 8 MV/m pulse delivered before $[Ca^{2+}]_i$ reached baseline values produced only a slight additional elevation of $[Ca^{2+}]_i$ (Fig. 2A) and a maximal rise in $[Ca^{2+}]_i$ was again observed when another pulse was delivered more than 30 s after the calcium response from the previous pulse had returned to baseline (Fig. 2B). Consistent with this latter finding was that multiple pulses delivered at repetition rates from 0.5 Hz to 1 kHz did not elicit a greater rise in $[Ca^{2+}]_i$ than that elicited by a single pulse (data not shown). These results indicate that delivery of only a single 4 ns pulse is sufficient to elicit a maximal increase in $[Ca^{2+}]_i$ and that multiple pulses delivered to the cells at high repetition rates are no more effective for increasing $[Ca^{2+}]_i$ than a single pulse. They further show that a second increase in $[Ca^{2+}]_i$ can be elicited after $[Ca^{2+}]_i$ returns to resting levels, suggesting that calcium clearance mechanisms most likely involving both cell extrusion and uptake into internal storage sites are functional and that as long as a sufficient period of time elapses between pulses, the cells can respond fully to another nanoelectropulse stimulus.

The response of chromaffin cells to a nanoelectropulse was highly reproducible across experiments that included cells at different times in culture as well as cells from different preparations, and was also observed in both single and aggregated cells. In addition, the magnitude of the response was dependent on field intensity, with the rise in $[Ca^{2+}]_i$ progressively decreasing as field intensity decreased from 8 MV/m to 2 MV/m (data not shown). Although direct comparisons of the calcium response of the cells to a single nanoelectropulse and to the nicotinic receptor agonist DMPP were not feasible, the magnitude of the increase in $[Ca^{2+}]_i$ elicited by a pulse

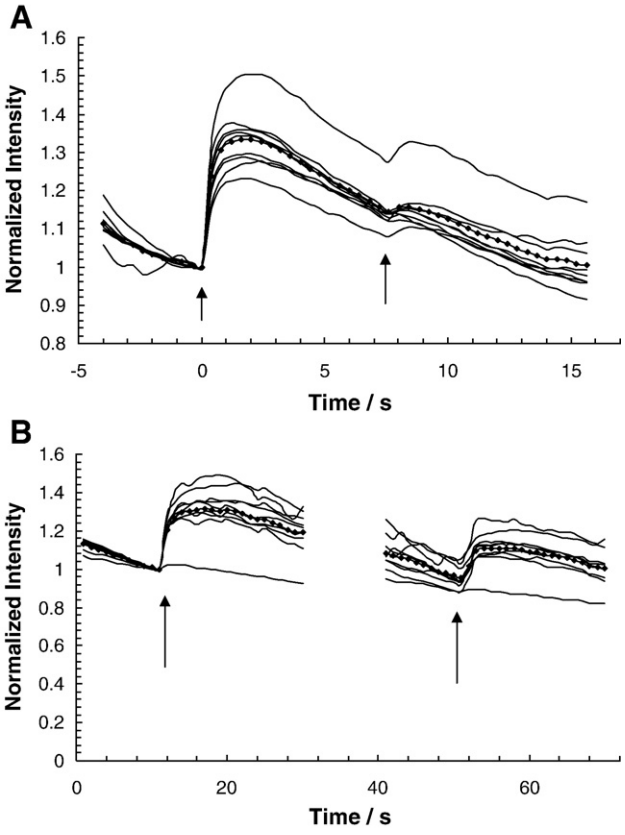

Fig. 2. Response of chromaffin cells to multiple applications of 4 ns pulses. (A) A second 8 MV/m pulse was applied 8 s after the first 8 MV/m pulse; (B) A second 8 MV/m pulse was applied 40 s after the first 8 MV/m pulse, with cell illumination discontinued between pulses.

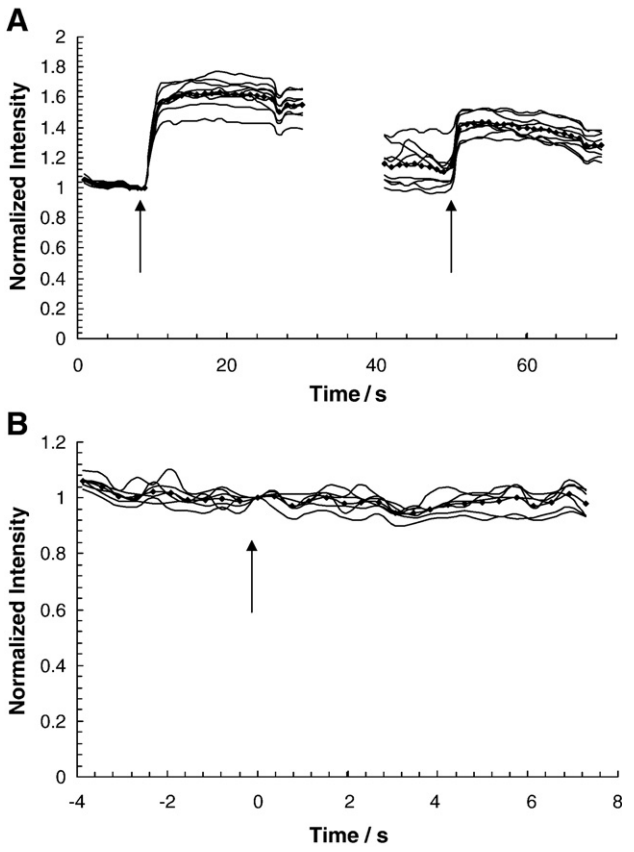

Fig. 3. Response of chromaffin cells to 4 ns, 8 MV/m pulses. In (A) the cells were incubated with 10 mM caffeine before and during the application of a single 4 ns, 8 MV/m pulse. A second 8 MV/m pulse was applied 40 s after the first 8 MV/m pulse, with cell illumination discontinued between pulses. In (B), 20 μM nitrendipine was present in the extracellular medium.

at a field intensity of 8 MV/m was at least as great in magnitude as that elicited by nicotinic receptor activation.

3.2. Inability of calcium store depletion to block the rise in $[Ca^{2+}]_i$

Experiments to determine whether the pulse-stimulated increase in $[Ca^{2+}]_i$ was due to release of calcium from internal stores consisted of incubating chromaffin cells prior to and during pulsing with drugs that cause a depletion of stored calcium. These drugs include caffeine (10 mM), which empties calcium from ryanodine-sensitive endoplasmic reticulum stores [17], and thapsigargin (2 μM), which prevents calcium from accumulating in stores by blocking endoplasmic reticulum Ca^{2+}-ATPase [18]. As shown in Fig. 3A, neither the response to a single 4 ns, 8 MV/m pulse nor to a subsequent pulse applied 40 s after the first pulse was prevented by caffeine-induced calcium store depletion. However, the rise time of the pulse-induced increase in $[Ca^{2+}]_i$ appeared to be faster and the calcium response was more sustained when caffeine was present. Treatment of the cells with thapsigargin also did not prevent the increase in $[Ca^{2+}]_i$ elicited by the nanoelectropulse (data not shown). Thus, a single nanosecond duration electric pulse elicits a rapid rise in $[Ca^{2+}]_i$ in chromaffin cells that does not involve mobilization of calcium from internal stores. This finding contrasts with that observed for non-excitable cells, such as Jurkat and HL-60 cells, where application of a single nanosecond electric pulse elicits a rise in $[Ca^{2+}]_i$ that can be prevented by agents that deplete calcium stores [3–5,14]. Taken together, these results suggest that for excitable chromaffin cells, internal organelle membranes are not the primary target of the nanoelectropulse with respect to increasing $[Ca^{2+}]_i$.

3.3. Chelating extracellular calcium or blocking plasma membrane calcium channels prevents the rise in $[Ca^{2+}]_i$

In contrast to the results observed with calcium store depleting agents, chelating calcium in the extracellular medium by adding EGTA to a final concentration of 5 mM prevented the rise in $[Ca^{2+}]_i$ (data not shown), indicating that the source of calcium was from outside the cell. To explore this result further, cells were pulsed in the presence of Co^{2+} (1 mM), a non-selective blocker of calcium channels. The response of the cells to the nanoelectropulse was again blocked (data not shown). Pulsing the cells in the presence of nitrendipine (20 μM), a dihydropyridine that is a selective antagonist of L-type voltage-sensitive calcium channels, also prevented the rise in $[Ca^{2+}]_i$ (Fig. 3B), suggesting that L-type calcium channels are involved in the pulse-induced entry of calcium into the cells. These results indicate not only that the plasma membrane appears to be the immediate target of the pulse but also that calcium entry into the cell occurs via a specific type of plasma membrane voltage-sensitive calcium channel. Our findings therefore broaden the range of responses that have been identified for nanoelectropulse effects on biological cells to include, as a primary response, changes in the conductance of a specific dihydropyridine-sensitive, voltage-dependent plasma membrane ion channel that gates calcium. With respect to bovine chromaffin cells, this observation is particularly intriguing since these cells express three other types of plasma membrane voltage-sensitive calcium channels (N- and P/Q-type channels [19,20]) that account for 80% of the whole cell calcium current [21]. Therefore, if the pulse-induced rise in $[Ca^{2+}]_i$ results from a pulse-driven depolarization of the chromaffin cell plasma membrane [22] that activates the voltage-dependent L-type channels, then one might expect N- and P/Q-type channels to open as well since these channels are not only present in much greater abundance relative to L-type channels but also activate at membrane potentials similar to those for L-type channels [23]. Also worthy of note is that our pulse exposures appear to have no effect on a constitutively active, voltage-insensitive plasma membrane cation channel that is responsible for influx of calcium into bovine chromaffin cells under resting conditions [24]. One possible explanation for the apparent selectivity of the pulse-induced effects on calcium entry into the cells is that the very short duration of the high intensity electric field has a direct electroperturbating effect only on L-type channels. This possibility is currently being explored.

3.4. Lack of evidence of plasma membrane electroporation after a single 4 ns pulse

The observation that application of multiple pulses to chromaffin cells does not result in additive increases in $[Ca^{2+}]_i$ suggests that

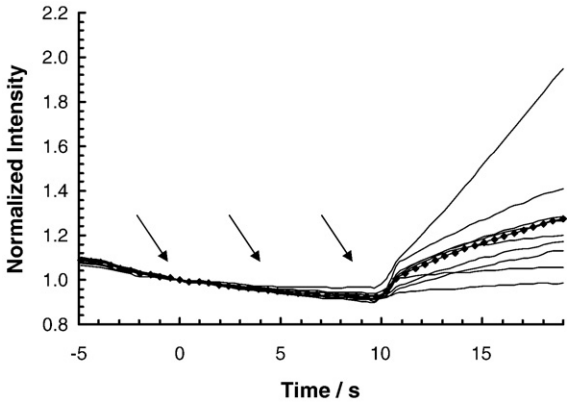

Fig. 4. Effect of single and multiple applications of 4 ns, 8 MV/m pulses on the influx of YO-PRO-1 into chromaffin cells. A single pulse was delivered at 0 s and at 5 s, and 50 pulses were delivered at 10 s, as indicated by the arrows.

nanoelectropulse treatment does not produce significant poration of the chromaffin cell plasma membrane, or that any pores produced are not calcium-conductive. To assess plasma membrane integrity directly, nanoelectropulses were delivered to the cells in the presence of the fluorochrome YO-PRO-1 (5 μM), a compound that is excluded from cells with intact membranes [25] and which has been used as an indicator of plasma membrane permeabilization after nanosecond pulse exposure [26]. As shown in Fig. 4, detectable uptake of the dye into the cells occurred after the application of fifty 4 ns, 8 MV/m pulses, but not after single pulses. Thus, plasma membrane electroporation does not appear to be a significant factor in the chromaffin cell response to the application of a single pulse.

4. Conclusion

This study is the first to show that for an electrically excitable cell, a single 4 ns electric pulse causes entry of calcium into the cell via voltage-sensitive L-type channels. Because L-type channels in chromaffin cells are associated with the exocytotic machinery that is located at the plasma membrane and responsible for triggering catecholamine release, an obvious question is whether the calcium that enters the cells with each nanoelectropulse attains sufficiently high subplasmalemmal levels to have physiological significance. A determination of whether catecholamine release is stimulated in response to a single pulse is underway since the results of these experiments will have implications for predicting effects of nanosecond electric pulse exposure on the behavior of other excitable cells that use calcium entry via L-type calcium channels for eliciting and controlling cellular responses.

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