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Calcium current in growth balls from isolated *Helix aspersa* neuronal growth cones

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Abstract. Growth cones were severed from their neurites in primary cultures of *Helix aspersa* neurons. Following isolation, growth cones rolled up into 5–10-μm-diameter spheres, which remained attached to a poly-L-lysine or lectin-coated glass coverslip. Whole-cell-configuration patch-clamp recordings from isolated growth cones revealed inward calcium currents upon block of outward currents with internally perfused CsCl. Up to 50 μM tetrodotoxin did not affect this current. In 20-μm-diameter spheres, a peak current of 1.2 nA was reached within 3 ms under voltage-clamp conditions for a 60-mV pulse from a holding potential of −50 mV. Channel density calculations averaged to approximately one channel per square micrometer. A two-phase inactivation was evident under voltage-clamp steps from −50 mV to +15 mV. The growth balls described can be internally perfused and voltage clamped to measure ionic currents involved in growth cone function.

Key words: Calcium channels – Growth cones – Patch clamp – Molluscan neurons – Cell culture

Introduction

Neuronal growth cones play an essential role in brain development and nerve regeneration. Growth cones serve as the site of new membrane incorporation, exhibit functional transmitter release, and are actively involved in target recognition and synapse formation. Understanding the manner in which these activities are controlled is of fundamental importance. Hitherto, studies of ionic currents in growth cones have been hampered by the small size and flat shape of the structure. Previous approaches enabled membrane potential recordings from growth cones (Grinvald and Farber 1981; Meiri et al. 1981), but did not allow systematic and quantitative current measurements under voltage-clamp control. Moreover, for a meaningful analysis of growth cone currents, they must be separated from somatic and axonal current contamination. Here we describe a novel approach which allows us to manipulate growth cones into isolated spheres which we call "growth balls". These "growth balls" can be internally perfused and voltage clamped to measure ionic currents involved in growth cone function. We utilize this approach to give here a first-time description of calcium currents under voltage-clamp conditions from growth cone membranes.

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Materials and methods

All measurements were performed on growth cones obtained from 2–5-day-old cultures of *Helix aspersa* neurons. *Helix* ganglia were treated with 1.35% neutral protease (Boehringer, Mannheim, FRG) at 37°C for 90 min. Single neurons were then isolated by mechanical dissociation of the ganglia and plated on glass coverslips. To obtain high adhesiveness and large growth cones, the coverslips were pretreated with 10 μg/ml poly-L-lysine (> 300,000 M.W.), concanavalin A, or *Helix aspersa* lectins (Sigma, St. Louis, MO, USA). Acid-cleaned glass coverslips were soaked in these solutions from 2–24 h at room temperature and then washed 3 times with distilled water and once with Leibovitz-15 medium (Gibco, New York, NY, USA). Cells were grown in Leibovitz-15 medium with appropriate salt concentrations. Final concentration of salts was (mM): NaCl 80, KCl 4, $CaCl_2$ 7, $MgCl_2$ 5. This medium was supplemented with glucose 10 g/l, fetal calf serum 4% (Gibco), penicillin (Teva, Jerusalem, Israel) 100 U/ml, streptomycin (Teva) 0.1 mg/ml, and Fungizone (amphotericin B; Squibb) 0.25 μg/ml. Cells grown in this culture medium were kept in 35-mm Petri dishes at 26°C. Neurons up to 150 μm in diameter attached and sprouted neurites overnight.

Growth cones were isolated by pressing the shaft of a slender glass micropipette at the point where the growth cone narrows to form a neurite. Sufficient pressure at this point disrupted the continuity between the growth cone and the neurite.

In order to record the total ionic current flowing through the membrane of a growth ball, we used the whole-cell patch-clamp configuration (Hamill et al. 1981) in conjunction with a single-electrode voltage-clamp amplifier provided with a variable switching speed of up to 10 kHz (Almost Perfect Electronics, Basel, Switzerland). Lower-amplitude whole-cell-configuration currents of the growth ball and single-channel recordings were monitored with a patch-clamp amplifier with a 10-GΩ feedback resistor (Yale, New Haven, CN, USA). For specific recording solutions, see results section and legends to figures.

Results

Upon severance of a growth cone from its neurite in primary cultures of *Helix* neurons, both the proximal and distal cut

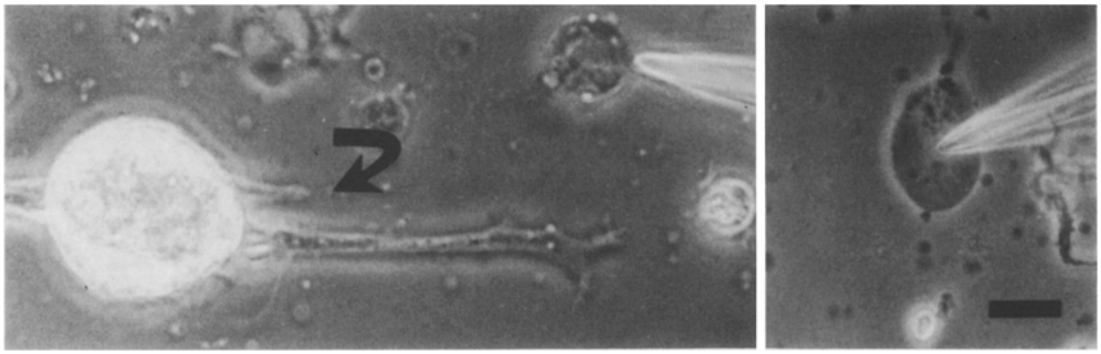

Fig. 1. Growth balls with attached patch electrodes. A growth ball is obtained by severance of a growth cone from its neurite, whereupon the growth cone rolls up and the neurite retracts, as indicated by *arrow* in left frame. *Right frame* shows a phase micrograph of a growth ball from another neuron. Neurons were plated 3 days prior to growth cone manipulation on glass coverslips coated with 10 μg/ml of either poly-L-lysine, concanavalin A, or *Helix aspersa* lectins. Cells were cultured in Leibovitz-15 medium with appropriate salt concentration. *Horizontal bar* = 10 μm

ends sealed rapidly. The proximal neurite, now separated from its growth cone, often showed retraction. The growth cone responded by rolling up to form a sphere (Fig. 1). Invariably, the resultant growth balls remained attached to the substrate. Occasionally, several filopodia remained extended from the growth ball. Intracellular events taking place during the rolling-up process are not clear, but may reflect the release of tension between neurite and growth cone (Bray 1984). These "growth balls", 5–20 μm in diameter, readily formed GΩ seals with fire-polished glass capillaries. This enabled direct measurement of single ionic channels and ionic currents flowing through the entire growth ball by utilization of the whole-cell patch-clamping configuration. This approach enabled us to look at the ionic currents of a growth cone without any contamination from axonal or somatic sources. Moreover, the spherical shape of the growth ball is ideal for a space clamp and accurate calculations of current and channel densities.

Under current-clamp conditions, these growth balls were capable of generating action potentials (Fig. 2A). Voltage-clamp recordings from single growth balls ($n = 15$) showed an early inactivating inward current and a slower outward current (Fig. 2B).

Our main interest was in calcium currents, since these have been implicated as essential for various functions of a growth cone, including new membrane incorporation (Anglister et al. 1982) and the capability to release transmitter (Hume et al. 1983; Young and Poo 1983). Based on reports of calcium-dependent action potentials (Grinvald and Farber 1981; Meiri et al. 1981), one would expect to find calcium currents in growth cones. Recent abstracts, however, have been inconclusive in establishing the presence of calcium channels in growth cones (Belardetti et al. 1984, 1986; Greenberg and Spector 1984). Belardetti et al. (1986) report on tetrodotoxin (TTX)-sensitive Na^+ currents and outward K^+ currents only, recorded from *Aplysia* RUQ neuron growth cones in culture, while Greenberg and Spector (1984) describe the dominance of Na^+ and K^+ channels with the "possible presence of small (< 1 pA) currents that may represent Ca^{2+} channels" in growth cones of neuroblastoma cells.

Since it appeared that the density of calcium channels was low in these growth cones, we directed our initial efforts toward whole-cell-configuration recordings ($n = 10$). To isolate possible calcium currents in growth balls, they were internally dialyzed with a solution containing 120 mM CsCl, 5 mM EGTA, and 5 mM HEPES, pH 7.4. The external medium contained sodium-free solutions with 10 or 40 mM $CaCl_2$ (Fig. 3A and B, respectively). As seen in Fig. 3, inward calcium currents were detected under these conditions. This calcium current peaked at 10 mV and had a reversal potential at 65 mV for the 10 mM external calcium solution. Such values are expected of fully open channels in asymmteric ionic concentrations (Reuter and Scholz 1977; Hagiwara and Byerly 1981; Fenwick et al. 1982). TTX was added to ensure that the calcium current was not flowing through Na^+ channels. Application of up to 50 μM TTX did not block this current. In a 20-μm-diameter growth ball, a peak current of 1.2 nA was reached within 3 ms for a 60-mV depolarizing pulse from a membrane holding potential of −50 mV. The calcium current showed two phases of inactivation (Fig. 3A). The average calcium current densities calculated from peak current values and growth ball surfaces areas ranged from 0.3–1.2 pA per square micrometer. Estimates of channel densities based on a unitary channel current of 0.5 pA at −20 mV and 40 mM Ca^{2+} (Brown et al. 1982) averaged approximately one channel per square micrometer.

Discussion

Recordings of ionic currents from the isolated rounded growth cones described here have several advantages over previous approaches. The spherical shape makes it more amenable to single-channel recordings than the thin, flat shape of a growth cone, although recording from the latter is feasible and has recently been described (Belardetti et al. 1986; Greenberg and Spector 1984; Cohan et al. 1985; O'Lague et al. 1985). Furthermore, the spherical shape is ideal for a spatial clamp. A more important advantage offered by the growth ball is the feasibility of current isolation at the macro level from somatic and axonal current contamination. Taken together with the further advantage of internal perfusion, this approach presents favorable conditions for quantitative studies of the effect of various factors on ionic currents in neuronal growth cones.

Utilizing the growth ball form, we measured voltage-dependent calcium currents, as shown in Figs. 2 and 3. The

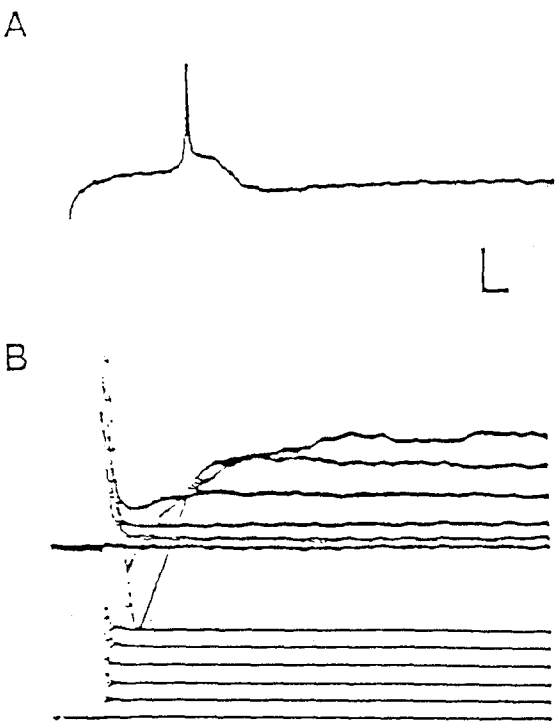

Fig. 2. **A** An action potential recorded from an isolated growth cone under current-clamp conditions. Records were obtained in the whole-cell configuration with a single-electrode voltage-clamp amplifier. Hard glass capillaries were used, and seals were held for up to 30 min. Recordings were carried out with the culture medium as the bath solution. Pipette solution: 135 mM KCl. Calibration: 7 mV, 25 ms. **B** Voltage-clamp recordings of a 10-μm-diameter growth ball (shown in Fig. 1, left frame). An early inactivating inward current is seen, followed by a noninactivating outward current. Pipette and bath solutions: same as in **A**. Holding membrane potential: −70 mV. Calibration bar: 35 mV, 160 pA, 5 ms

current traces showed complex voltage-dependent activation and current decay as the command voltage was raised from −20 to +40 mV. The biphasic current decay evident at +15 mV was indiscernible at +40 mV, where only the slow decay was evident. In other systems, such behavior has been taken to suggest the existence of two separate currents (Bossu et al. 1985).

Calculations of calcium channel densities on a growth cone fell within the range reported for other cells. Cultured rat cardiac cells, for example, have 0.1 − 0.5 calcium channels and chromaffin cells 5 − 15 channels per square micrometer (Reuter 1983). Our calculated value is also within the range reported from *Helix* somata 3 μm^{2-} (Lux and Brown 1984). This trend, showing a similar distribution of calcium channels on the somata and growth cones, has also been found in PC12 cells (Streit and Lux 1986). The calculated value of about one channel per square micrometer on a growth cone may, however, be misleading, since clustering of channels may considerably increase the local density to form calcium channel "hot spots" (Huang 1985).

Does the same multiplicity of calcium channel types found on cell bodies (Nowycky et al. 1985) exist on growth cones? If yes, what is the role of the various channel types in the various functions of a growth cone? Are there calcium channel clusters on growth cones? These questions can now be approached by combining single-channel recordings with the "growth ball" configuration described here.

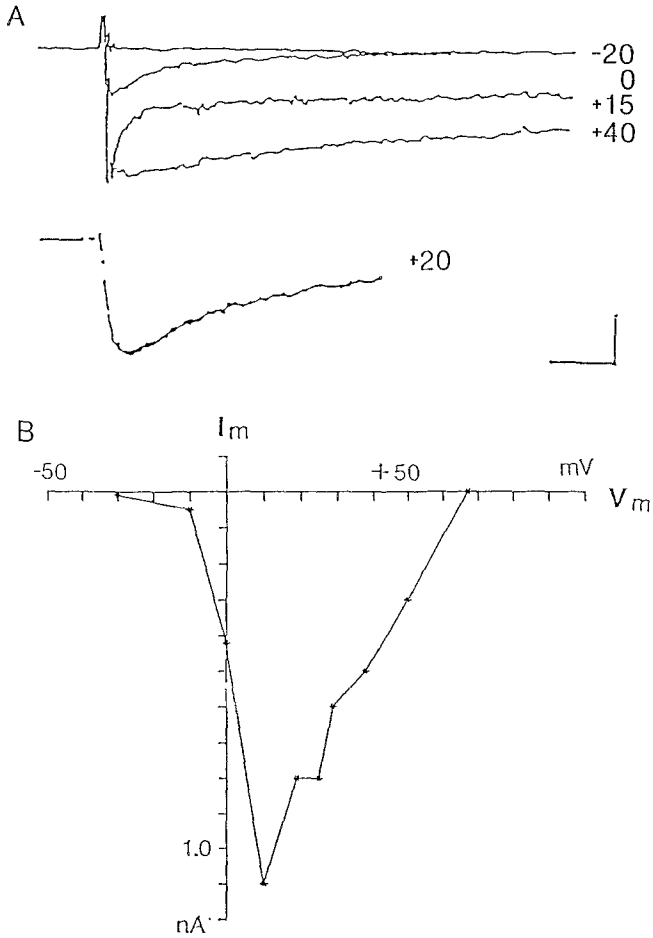

Fig. 3A,B. Calcium currents from a growth ball. **A** Currents recorded from a growth ball under whole-cell patch-clamp configuration. Upper 4 traces were obtained with a patch-clamp amplifier with a 10 GΩ feedback resistor, while the bottom trace, recorded from another growth ball, was obtained with a single-electrode voltage-clamp amplifier, as described in legend to Fig. 2, to attain higher current levels. Bath solution contained (mM): $CaCl_2$ 10, $MgCl_2$ 5, 4-AP 5, Tris-HCl 20, TEA-Cl 35, 50 μm TTX, pH 7.4. Pipette solution contained (mM) CsCl 120, EGTA 5, HEPES 5, pH 7.4. Holding potential: −50 mV. Calibration upper 4 traces: 40 pA, 16 ms; bottom trace: 400 pA, 8 ms. **B** Voltage-current curve of a calcium current recorded from the growth ball shown in the right-hand frame of Fig. 1

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