

MAINTENANCE OF ISOLATED SMOOTH MUSCLE CELLS OF THE CTENOPHORE *MNEMIOPSIS*

BY P. G. STEIN AND P. A. V. ANDERSON

*C. V. Whitney Laboratory and Department of Physiology, University of
Florida, Route 1, Box 121, St Augustine, FL 32086, U.S.A.*

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Progress toward understanding the physiology of smooth muscle has been slow for several reasons. In most smooth muscle tissues, the cells are extremely small, electrically coupled to one another, often spontaneously active and complexed with large amounts of inelastic connective tissue. The technical problems posed by these factors are numerous and have been reviewed extensively (Tomita, 1970; Anderson, 1977; Attwell & Cohen, 1977; Beeler & McGuigan, 1978). It is clear that the nature of the tissue severely limits the types of experiments which can be performed and the interpretation of any data obtained. Recent efforts to circumvent these problems have increasingly focused on the development of isolated, single cell models. Thus far, isolated cells from *Bufo* (Bagby *et al.* 1971; Fay & Delise, 1973; Singer & Walsh, 1980), *Mytilus* (Ishii & Takahashi, 1982), *Beroë* (Hernandez-Nicaise, Mackie & Meech, 1980; Hernandez-Nicaise, Bilbaut, Malaval & Nicaise, 1982; Nicaise, Hernandez-Nicaise & Malaval, 1982) and *Mnemiopsis* (Anderson, 1984; Stein & Anderson, 1984) have been studied. These preparations have eliminated many of the complicating variables mentioned above and they have allowed the first simplified examination of smooth muscle cellular physiology.

While single cell preparations offer many technical advantages over whole tissue preparations, they usually require a much greater period of time to prepare. Additionally, to avoid the problems arising from smooth muscle cell dedifferentiation in culture (Chamley-Campbell, Campbell & Ross, 1979), investigators generally use the cells within 24 h of isolation (Singer & Walsh, 1980; Warshaw & Fay, 1983). This short time over which the cells can provide reliable data means that new cells must be isolated daily. As the isolation procedure is lengthy, a sizeable percentage of each day must be devoted to cell isolation instead of experimentation. In an effort to increase the useable lifespan of smooth muscle cells isolated from *Mnemiopsis* and to reduce the percentage of time spent isolating cells, we have attempted to formulate a medium in which the cells may be maintained for an extended period of time. Our intent is that cells placed in this medium will retain electrical properties identical to those seen in freshly isolated cells for several days following isolation. At the same time, we hope to provide sub-optimal conditions for growth such that cells will not be placed in true culture, and the inherent problems of culturing smooth muscle cells (Chamley-Campbell *et al.* 1979) will be obviated.

Specimens of *Mnemiopsis* were collected from the intracoastal waterway, in the vicinity of the Whitney Laboratory and maintained in Kriesel chambers (Greve,

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1968) on a diet of plankton and brine shrimp nauplii. Following dissection, the smooth muscle cells were isolated from the surrounding mesogloea (Anderson, 1984) and the individual cell segments then placed in either the control medium or the presumptive maintenance medium. The control medium was a low Ca^{2+} artificial sea water (ASW) of the following composition (mmol l^{-1}): NaCl 476, KCl 9.7, MgCl_2 24.2, MgSO_4 27.0, NaHCO_3 2.3, CaCl_2 1.7. This Ca^{2+} concentration was chosen as a means of reducing the tendency of cells to contract while still imparting membrane stabilization. The pH of this solution was adjusted to 7.35 and tonicity was also adjusted to that of ambient sea water; in the vicinity of 960–980 mosmol l^{-1} . The maintenance medium consisted of this low Ca^{2+} ASW with the following additions: 1 mg ml^{-1} bovine serum albumin (BSA) (Sigma) to prevent the cells from adhering to the Petri dish; 1 mg ml^{-1} glucose, and 1 mg ml^{-1} glutamine (Gibco) as carbon- and nitrogen-containing sources of energy, 2% foetal calf serum (FCS) (Gibco) as a growth promoter, and 1% penicillin-streptomycin (Gibco) to minimize bacterial contamination. The pH and tonicity were adjusted as described above after addition of the supplements. Isolated cells were transferred to this maintenance medium using a mouth pipette and stored at 9°C until needed. Prior to recordings, cells were allowed to equilibrate at room temperature (22°C) for 30–60 min.

The basic electrical properties of the cells were examined using conventional single-electrode intracellular recordings. Thin-walled (Frederick Haer), 3 mol l^{-1} KCl-filled microelectrodes with impedances of 8–15 $\text{M}\Omega$ were used. The recorded signals were amplified using a conventional capacity compensated d.c. amplifier (Getting Instruments) equipped with a Wheatstone bridge circuit. A home-made virtual ground in the bath ground circuit was used to measure the magnitude of the injected currents. Recorded signals were displayed on a digital oscilloscope (Nicolet Instruments) and stored on a floppy disk for subsequent analysis. Hard copies of records were made on a Houston Instruments X-Y plotter.

When examined in the light microscope, freshly isolated cells may appear to be smooth and elongate (20–25 μm in diameter and up to several mm in length) or they may be partially or wholly contracted into coils. In either instance, the membrane of undamaged cells appears uniformly translucent and there is no evidence of the membrane swelling and apparent myofilament aggregation seen in damaged cells. Cells showing such obvious signs of damage typically disintegrate and disappear from the bath in less than 1 h. Cells lacking these signs of damage will remain viable for considerably longer; if placed in maintenance medium, these cells will appear essentially unchanged up to 7 days later, except that they tend to become shorter with time, approaching a spherical shape by the seventh day. As cells become more round, they lose the ability to contract in response to electrical stimulation.

Having established that cells will still be in evidence in the maintenance medium at the end of 7 days, it was next necessary to determine whether cells so maintained were electrically identical to freshly isolated cells in unsupplemented media. The passive electrical properties of the membrane were first examined using single electrode intracellular recording methods. The parameters examined included: the resting membrane potential E_R , input resistances (Z) [determined as the slopes of the I/V curves in both the hyperpolarizing (Z_h) and depolarizing (Z_d) directions from (E_R)], and the membrane time constant (τ) determined as described in Singer & Wal-

(1980). For freshly isolated cells in low Ca^{2+} ASW, the mean values for these parameters are: $E_R = -51.6 \pm 2.2$ mV (mean \pm s.e.m., $N = 12$), $Z_h = 27.2 \pm 4.7$ M Ω , $Z_d = 11.4 \pm 2.0$ M Ω and $\tau = 37.4 \pm 7.5$ ms. These, the control values, were then compared with the values obtained from cells in supplemented medium. Two-way analysis of variance (ANOVA) was used to assess differences in electrical properties with time or medium. ANOVA results were considered to differ significantly if $P < 0.05$. Fig. 1 is a summary of these data and shows the similarities in cell properties with variations in time and medium. As shown, none of the parameters changes drastically either as a function of time or medium, though gradual changes do occur. E_R diminishes slightly but insignificantly during the 7-day trial. Though Z_h shows a gradual increase with time, values do not differ significantly from controls. For the 7-day trial period, Z_d shows a parallel gradual increase and becomes statistically different from controls at day 4. τ diminishes gradually with time and begins to differ from control values at day 5. All of the noted differences for the passive parameters arose as a function of time in storage and none could be attributed to the difference in media.

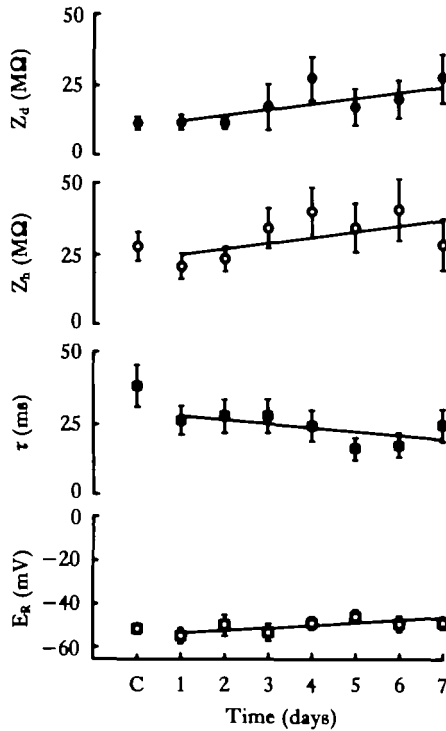


Fig. 1. The passive electrical properties of *Mnemiopsis* cells vs time. Resting potential (E_R) (mV, \square), input impedance (M Ω) depolarized (\bullet) and hyperpolarized (\circ) from E_R and membrane time constant (τ) (ms, \blacksquare). Symbols represent means, error bars represent the s.e.m. Lines connecting points are linear regressions determined by the least squares method. Two-way analysis of variance (ANOVA) was used to determine that resting E_R and Z_h did not differ significantly ($P > 0.05$) from controls for days 1 to 7, though Z_d and τ began to differ from controls ($P < 0.05$) by the fourth and fifth days respectively. In no case were differences attributable to supplemented media itself. As shown, the parallel gradual increase in both Z_h and Z_d during the 7-day period indicates that the rectifying properties of the membrane remain intact while the overall input impedance is increasing slightly. C, control values.

A typical action potential from a freshly isolated cell is shown in Fig. 2A. With $1.7 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ present, the mean amplitude is $67.6 \pm 2.6 \text{ mV}$ (mean \pm s.e.m., $N = 12$), and at half-maximal amplitude, duration is $6.5 \pm 0.4 \text{ ms}$ (mean \pm s.e.m.). The rate of depolarization during the spikes, dV/dt , is $24.1 \pm 1.7 \text{ V s}^{-1}$ (mean \pm s.e.m., $N = 12$) and the potential generally overshoots zero by $16.0 \pm 3.5 \text{ mV}$ (mean \pm s.e.m.). Action potentials recorded from cells on days 1 to 7 were essentially

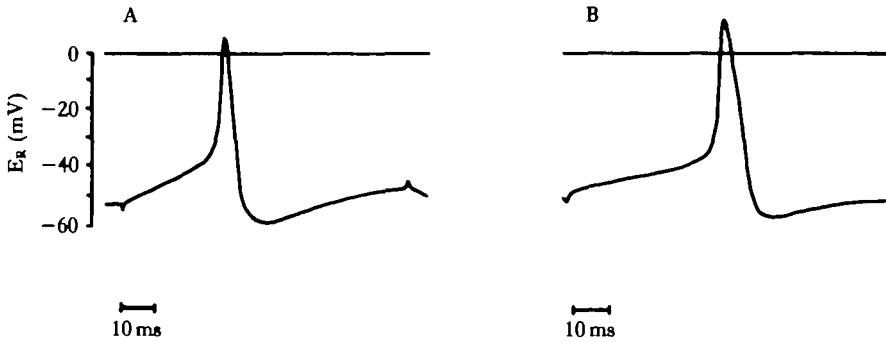


Fig. 2. Action potentials recorded using single intracellular electrodes in freshly isolated (A) and 7-day-old *Mnemiopsis* smooth muscle cells (B) in supplemented medium. The similarity in resting E_R , amplitude, dV/dt and duration of these events indicates that cells retain their basic electrical properties for up to a week in supplemented medium at 9°C .

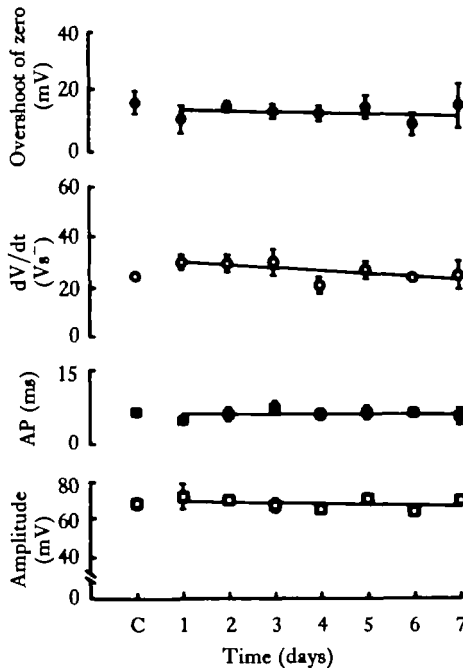


Fig. 3. The active electrical properties of *Mnemiopsis* cells vs time. Action potential amplitude (mV, \square), duration of action potential at half-maximal amplitude (AP, ms, \blacksquare), dV/dt (V s^{-1} , \circ) and overshoot of zero (mV, \bullet) are shown. Amplitude was constant throughout the 7-day period and equal to control ($P > 0.05$). dV/dt did not differ with time but was slightly elevated in supplemented medium ($0.05 > P > 0.02$). Spike duration was the same as that recorded in controls during the first 6 days, while overshoot of zero remained similar to control values for the first 4 days. C, control values.

the same as those in unsupplemented, low Ca^{2+} ASW (Fig. 2A, B). A closer analysis of the action potential waveforms confirms this. As Fig. 3 illustrates, the properties of the action potentials – amplitude, duration at half-maximal amplitude, overshoot of zero and rate of rise of the spike (dV/dt) – each remained fairly constant throughout the 7-day trial. Amplitude did not vary with medium or time during the week. Overshoot of zero and duration at half-maximal amplitude were like control cells for the first 4 and 6 days, respectively. While dV/dt remained constant for cells in the supplemented medium throughout the week, dV/dt for these cells was found to be somewhat higher than in controls ($0.05 > P > 0.02$). This was the only parameter showing an effect attributable to the supplemented medium.

These results taken together indicate that *Mnemiopsis* cells can be maintained for several days longer than their normal *in vitro* lifespan by the simple media modifications described above. The constancy of the measured electrical parameters, both passive and active, indicate that fundamental cellular processes are constant during the period in question. The stability of E_R during this time is an important indicator of cellular viability as it reflects the ability of the cell to maintain both the ionic gradients and the conductance ratios for specific ions which are the determinants of resting potential. The parallel, gradual increases in Z_h and Z_d , indicate that while Z is increasing, the mechanisms responsible for outward-going rectification continue to operate fully through the seventh day. As τ , Z_h , Z_d and E_R do not differ significantly from controls for the first 4 days following isolation, it is concluded that supplemented medium does not alter the fundamental passive properties of these cells for this period.

Similarly, action potential amplitude, dV/dt , duration and overshoot of zero are reflections of the electrochemical gradients for the ions involved, the membrane conductance of the ions and the temporal relationship between the different ionic currents. As amplitude, duration and overshoot are constant and like the controls up to the fourth day, it is reasonable to conclude that the mechanisms regulating the gradients, conductances and temporal relations of currents must also be patent for this period. dV/dt does not vary with time, although it is consistently higher in the supplemented medium. Though the basis for such an increase is unknown, this difference does not compromise the value of stored cells for experimental purposes. dV/dt is a reflection of both the electrochemical gradients for Na^+ and Ca^{2+} , the ions responsible for the spike depolarization (Anderson, 1984) and of the ability of the membrane to conduct the currents carried by these ions. As dV/dt is slightly but significantly elevated in supplemented medium, this difference must reflect an enhancement of the inward, depolarizing current rather than a medium-induced reduction in function.

Given the patency of the membrane and the continued ability of the cells to contract upon stimulation, the slow change in morphology which occurs with time is probably due to a contraction resulting from the continued workings of patent contractile elements. Gradual contraction is a possible indication of 'latched' smooth muscle cross-bridge formation (Aksoy, Murphy & Kamm, 1982; Dillon & Murphy, 1982) in the absence of any significant internal or external resting forces opposing cell shorten-

ing. The overall conclusion to be drawn from these studies is that cells in supplemented

medium may be used for electrophysiological studies for up to 4 days following isolation. During this period of time, the membranes appear to behave electrically like those of freshly isolated cells. Additionally, individual cells may exhibit properties like those of freshly isolated cells beyond this time, even though the mean values for a group of cells may begin to differ from the control cells. For these reasons, supplementation of the medium significantly extends the period of time during which the cells can be studied *in vitro*. Increasing the *in vitro* lifespan of *Mnemiopsis* cells four-fold will allow a proportionate increase in what can be learned regarding the electrical properties of isolated smooth muscle cells.

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