Novel Inverted Triple Grease-Gap Isolation Chamber for Electrophysiological and Calcium Release Studies in Skeletal Muscle Fibers

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Abstract: A novel inverted triple grease-gap chamber for electrophysiological studies and photometric detection of calcium release in skeletal muscle fibers is described. The chamber allows the construction of grease seals displaying high electrical resistance and mechanical sta-

bility. The fiber mounting procedure is fast, simple and not skill demanding, and the preparation is highly stable and reliable. Electrophysiological and photometric data are presented to illustrate the chamber performance. [Japanese Journal of Physiology, 50, 457–462, 2000]

Key words: inverted triple Vaseline® gap chamber, excitation–contraction coupling, calcium release.

Gap isolation methods, initially developed for electrophysiological studies, have been used successfully in conjunction with optical probes to study calcium release in skeletal muscle fibers [1–5]. Single, double, and triple gap isolation chambers have been adapted to this end [2–5]. Until recently, these conventional chambers (i.e. “upright chambers”) were the only versions available for photometric studies [6, 7]. As the result of routine use, we have identified several inconveniences in upright chamber models. They are very prone to fluid level fluctuations, which usually lead to severe changes in electrical parameters, and decrease the preparation viability. These chambers do not allow for a reliable mechanical restraining of fiber shortening, one of the main factors that impairs grease seal stability. More importantly, although useful for conventional (“space averaged”) photometric studies of calcium release [4], they are not compatible with modern confocal techniques for calcium detection [6, 7]. For the case of double grease-gap chambers, this limitation has been recently overcome with the introduction of “inverted” models [6, 7]. Nevertheless, to our knowledge, no inverted triple grease-gap chamber has been designed to date. This is probably due to difficulties in devising and constructing experimental

compartments having the geometry and dimensions required for this voltage clamp method. Although double gap chambers are more generally used for voltage clamp studies in skeletal muscle fibers, triple gap chambers are better suited to this end. This mainly results from the fact that (1) triple gap voltage clamps are based on a potentiometric measure of the membrane potential, and (2) longitudinal intracellular current is prevented by grounding an experimental compartment containing a depolarized segment of the fiber ([1] and references therein). Consequently, a better space clamp condition is obtained, which is a primary requirement to perform accurate studies of voltage-dependent mechanisms (membrane currents, calcium release, etc). Besides surmounting the limitations of conventional chambers, an inverted triple grease-gap chamber would be highly desirable to combine the newly introduced method for fast voltage clamping of the transverse tubular system [8] with confocal techniques for calcium detection [7]. The aim of this work was to design and construct a practical inverted triple grease-gap isolation chamber, and to develop a simplified method for fiber mounting leading to a stable and reliable preparation.

The new chamber (Fig. 1) was constructed from a

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Fig. 1. The inverted triple gap isolation chamber.

Scaled diagrams and cross-sections are shown. **Panels 1 and 4** are the upper and lower views of the chamber.

Panels 2 and 3 are cross sections across the sites indicated by the double arrows. In these panels, the body of the chamber is presented in black. Experimental compartments are not represented in panel 3. A detailed view of the experimental compartments (A, B, C and E) is presented in

panel 5. In this panel, the white areas represent perforated excavations, whereas the black areas represent non-perforated areas of compartments A and B. A sketch of a muscle fiber (MF), grease seals (GS), ceiling coverslip (CCS) and adhesive tape (AT) is included to illustrate the complete preparation.

KP are potassium pools, M are holes for magnets, MCI is an inset for a metal plate, CSCI is an inset for the floor coverslip, SI and SO are the inlet and outlet solution ports. CC1–3 are concentric excavations and CRC is the area where the experimental compartments were machined. The scale represents 20.0 mm for panels 1–4 and 4.0 mm for panel 5.

single block of black plexiglas. Four pools (KP) and three concentric circular areas (CC1–3) were milled in the upper surface of the plastic block (Fig. 1, panels 1–3). The KP pools were used as KCl (125 mM) deposits, whereas excavations CC1–3 were made to reduce the thickness of the central region of the chamber (CRC) to $\sim 500 \mu\text{m}$ (Fig. 1, panel 3), and to provide enough clearance to facilitate the fiber mounting procedure. Two square concentric insets (MCI, CSCI) and four holes (M) were milled in the lower surface of the plastic block (Fig. 1, panels 2–4). MCI and CSCI allocated a magnetic metal plate and a coverslip, respectively. The coverslip served as a removable floor of the chamber. The metal plate and the coverslip were held in position by four magnets embedded in the M holes. The experimental compartments (named A, B, C and E; according to Hille and Campbell, 1976 [1]; see also [3, 4, 8, 9]) were milled in the CRC region as shown in detail in panel 5 of Fig. 1. In order to render practical the machining of the experimental compartments and to increase the mechanical resist-

ance of the thinner parts of the chamber, two design strategies were taken: (1) the experimental compartments were devised in such a way that the area of the non-milled regions among them was maximized, and (2) a thin layer of plastic ($\sim 100 \mu\text{m}$) was left in the upper part of most of the lengths of compartments A and B (Fig. 1, panel 5). These “roofs” also prevented fluctuation in the level of solutions in compartments A and B. The lateral compartments (C and E) were oval perforations, whereas compartments A and B were ducts of rectangular cross section. As can be seen in panel 5 of Fig. 1, the four compartments were communicated through gaps milled in the septa separating adjacent compartments. Because of this key feature of our design, grease seal construction was reduced to the easy, simple and reliable procedure of “filling” the gaps with grease. The muscle fiber was oriented parallel to the major axis of the lateral compartments, across the gaps, spanning from one lateral pool to the other as illustrated in panel 5 of Fig. 1.

The chamber preparation and fiber mounting proce-

ture were similar to that previously described for inverted double-gap chambers [7]. The main differences were due to the presence of an additional seal and the smaller dimensions of both the grease seals and central channels (A and B). Briefly, a silylated coverslip was tightly sealed with grease to the CSCI area. Shallow grease “pre-seals” ($\sim 20\ \mu\text{m}$ in height) were made on the floor coverslip, and the chamber was flooded with isotonic K_2SO_4 . Once relaxed, a segment of fiber bathed in isotonic K_2SO_4 was transferred, at slack length, to the chamber. The fiber was stretched at the desired length and affixed to the floor coverslip with adhesive tape. Starting with the middle one, the three seals were then constructed. The roof-lacking areas of channels A and B were covered with a piece of glass and the excess solution was removed, rendering the four compartments isolated. The ends of fiber at pools C and E were permeabilized with saponin ($100\ \mu\text{g}/\text{ml}$, 1 min) and then washed with ~ 20 volumes of a solution mimicking the intracellular milieu [3, 4, 7], which also contained a calcium dye. Finally, the chamber was placed on the stage of an inverted microscope (Nikon, Garden City, NY, USA) and the perfusion and electrical connections were made. On average, the chamber preparation and fiber mounting could be accomplished in ~ 30 min with a success rate close to 100%. Segments of single muscle fibers dissected from the semitendinosus muscle of *Bufo marinus* were used throughout. Toads were anaesthetized in a 15% ethanol solution and then sacrificed by pithing. The solutions and electrophysiological methods were as previously described [1, 3, 4, 7]. Experiments were performed at $20\text{--}21^\circ\text{C}$.

Transmembrane potential ($-V_m$) was measured as the difference of potential at compartment A minus the potential at the fiber interior (point D, Hille and Campbell [1]), which was held at virtual ground by an amplifier connected to compartment C. E was the current injection compartment, and B was connected to ground. Optical and electrical measurements were made at compartment A. Membrane action potentials (MAPs) were elicited with $200\ \mu\text{s}$ current pulses applied individually or in trains (10–100 Hz, 1–10 s). Voltage clamp pulses were 5–100 ms in length. Capacitative and leak currents were subtracted using the $p/-4$ protocol [1–3, 6, 9]. Pulse protocols were generated by commercial hardware and software (TL-1 125 MHz system and pClamp 5.0, respectively; Axon Instruments, Foster City, CA, USA). Conventional (global, spaced averaged) calcium detection techniques were as previously described [7]. Briefly, the light from a mercury arc-lamp (Nikon) was filtered as required and focused onto the muscle fiber using dry

($20\times$, $40\times$) and oil immersion objectives ($100\times$). Changes in calcium concentration were followed with the fluorescent calcium dyes Rhod-2 ($K_d = \sim 500\ \text{nM}$) and Oregon Green Bapta 488-5N (OGB-5N, $K_d = \sim 32\ \mu\text{M}$). Electrical and photometric recordings were low-pass filtered at 10 and 5 kHz, respectively, prior to being acquired.

The usefulness of the new chamber critically relies on the quality of the inter-pool grease seals, and the sealing between the chamber's bottom and floor coverslip. To assess the extent of electrical isolation between adjacent compartments, the spontaneous repolarization of the fibers and properties of the action potential were measured. After replacing the isotonic K_2SO_4 solution in compartment A with normal toad Ringer, the transmembrane potential (V_m) increased rapidly (~ 30 s) and spontaneously to about $-50\ \text{mV}$ ($-52.5 \pm 5.3\ \text{mV}$, $n=8$; mean $\pm$ SE). From this value, the injection of a small steady current ($38.5 \pm 14.2\ \text{nA}$, $n=8$; mean $\pm$ SE) sufficed to set V_m to $-100\ \text{mV}$. Once fully repolarized, the fibers regained their normal excitability. As can be seen in Fig. 2 (panel 1), the MAPs showed the typical waveform previously described for fast skeletal muscle fibers ([7] and references therein). As indicated by the values of the maximum speed of depolarization, the amplitude and overshoot of the MAP, $780 \pm 61\ \text{V/s}$; $154 \pm 1.9\ \text{mV}$; $55.3 \pm 1.1\ \text{mV}$ (mean $\pm$ SE, $n=10$), respectively, the electrical records were free of attenuation or low-pass filtering. The rest of the MAP parameters (e.g., early after potential ($22.5 \pm 4.2\ \text{mV}$; mean $\pm$ SE, $n=10$), repolarization speed ($-115.1 \pm 41.4\ \text{V/s}$; mean $\pm$ SE, $n=10$) and time to peak ($0.49 \pm 0.2\ \text{ms}$; mean $\pm$ SE, $n=10$)) were also similar to those previously recorded with conventional chambers ([1, 2, 4, 7] and references therein). These results demonstrate that the grease seals have high electrical resistance and negligible capacitance. In the absence of muscle fibers, the resistance between adjacent compartments, determined with a digital meter, was in excess of $20\ \text{M}\Omega$.

In order to perform accurate electrical and optical recordings with gap isolation chambers, grease seal resistance and fiber geometry and position should remain constant. To evaluate the mechanical stability of the seals and the preparation as a whole, muscle fibers stretched to a sarcomere spacing of $3.6\text{--}4.0\ \mu\text{m}$ were either stimulated with current pulses at frequencies of 10–100 Hz for varying periods of time or voltage clamped with large, long-lasting voltage pulses. An example of the electrical responses elicited by a long 50 Hz train is shown in panel 2 of Fig. 2. As can be seen in this figure, the electrical record is free of mechanical artifacts. The pre-stimulus potential quickly

Fig. 2. Electrical recordings in current and voltage clamp conditions. **Panel 1:** Sample records of electrical responses to pulses of varying amplitude (traces 1–7). Traces 1–6 are subthreshold responses. Trace 7 corresponds to a typical MAP. V_m was set to -100 mV. **Panel 2:** Electrical responses to a 50 Hz (9.5 s) train and a single pulse applied 0.3 s after the train end (asterisk), obtained in the same fiber from panel 1. Fiber diameter: $85\text{ }\mu\text{m}$; width of pool A: $200\text{ }\mu\text{m}$; sarcomere spacing: $4.0\text{ }\mu\text{m}$; [EGTA]: $200\text{ }\mu\text{M}$. **Panel 3:** Fast records of sodium currents elicited by 5 ms depolarizing pulses. Pulses were given in 10 mV steps from -90 to $+40$ mV. Only the first 2 ms of the records are shown. The holding potential was set at -100 mV. Fiber diameter: $90\text{ }\mu\text{m}$; membrane area at pool A: $5.18 \times 10^{-4}\text{ cm}^2$; sarcomere spacing: $3.9\text{ }\mu\text{m}$; [EGTA]: $100\text{ }\mu\text{M}$. **Panel 4:** I - V curve of the sodium current. Peak sodium currents from records shown in panel 3 are plotted as a function of voltage. The experimental points are shown connected by a segmented solid line. **Panel 5:** Currents through the potassium delayed rectifier. Potassium currents were recorded isolated by blocking the sodium current with TTX (100 nM). Currents were elicited by depolarizing pulses, applied in 10 mV steps from -90 to $+60$ mV. Fiber diameter: $100\text{ }\mu\text{m}$; width of compartment A: $200\text{ }\mu\text{m}$; membrane area at compartment A: $6.28 \times 10^{-4}\text{ cm}^2$; sarcomere spacing: $4.0\text{ }\mu\text{m}$; [EGTA]: $200\text{ }\mu\text{M}$. The holding potential was set at -100 mV. **Panel 6:** Voltage dependence of the potassium currents. Currents in panel 5 were measured at 50 ms from the onset of the pulse and are plotted as a function of the voltage.

reached a stable minimal value during the train, and more remarkably, the fiber repolarized fast at the end of the train. The amplitude and kinetics of the MAP also recovered rapidly after the end of the tetanic stimulation (not shown). Records obtained at higher frequencies or using large, long-lasting voltage clamp pulses (50–100 ms, up to $+40$ mV; data not shown) were also free of mechanical artifacts.

The mechanical stability of the preparation was also evaluated by performing extensive maneuvers of stage translation and focusing while using a $100\times$ oil immersion objective. During this procedure, there was no detectable change in the holding current or V_m , in-

dicating that the preparation was immune to pressure or shearing forces applied by the objective to the bottom of the coverslip through the immersion oil. The above results clearly demonstrate the mechanical stability of the preparation.

Another salient feature of the chamber is the stability of the flux of solution in compartment A (solution in compartment B was usually not exchanged). In our chamber, compartment A is a duct continuous with the perfusion inlet tubing, and only open at the outlet port. Thus, opposite to what very often occurs in conventional chambers, no fluid level fluctuation or drying of this compartment is possible in the present

chamber. Consequently, the fiber is never exposed to the air-liquid interface, and the access resistance remains constant. Solution flux can be adjusted over a wide range, stopped and restarted as desired without affecting the electrical recording or impairing the seals. Additionally, the small volume of pool A ($\sim 20 \mu\text{l}$) is especially suited for pharmacological assays of very expensive or scarce compounds.

Opposite to conventional chambers, the compartments of the new chamber have fixed dimensions. For this reason, it was important to evaluate if our voltage clamp circuit [3, 4] was able to effectively control the potential of the relatively large segment of fiber in pool A (typically $200 \mu\text{m}$ in length). As a highly demanding test, sodium currents were measured in the presence of a normal extracellular sodium concentration. Potassium currents were blocked by replacing internal potassium with cesium and adding 10 mM TEA to the Ringer. As can be seen in panel 3 of Fig. 2, for each voltage tested, the sodium currents exhibited typical fast activation and slow inactivation phases and only one maximum. Current peak and kinetics changed smoothly with voltage. The peak-current vs. voltage curve also showed normal voltage dependence (Fig. 2, panel 4). A typical voltage dependence of g_{Na} was also found (not shown). From data from six fibers, average values of $-55.5 \pm 1.8 \text{ mV}$ (mean $\pm$ SE), $60.1 \pm 2.9 \text{ mV}$ (mean $\pm$ SE) and $6.9 \pm 1.2 \text{ mA/cm}^2$ (mean $\pm$ SE), were found for the activation potential, reversion potential and peak of the sodium current, respectively. From this same set of experiments, values of $-25 \pm 2.3 \text{ mV}$ (mean $\pm$ SE) and $120 \pm 9.5 \text{ mS/cm}^2$ (mean $\pm$ SE) were calculated for half-activation potential and maximum sodium conductance, respectively. These values are in good agreement with previously reported data [9, 10]. The above results show that, for the diameter and length of fiber used, and even in the presence of a large and fast current, the space clamp and frequency response obtained with our method were similar to those obtained using conventional methods [9].

Our preparation also allowed recording of the delayed potassium current. Figure 2 (panel 5) shows a family of potassium currents obtained in the presence of $1 \mu\text{M}$ extracellular TTX. The potassium currents showed typical voltage dependence and kinetics [10]. A typical I - V curve was also obtained (Fig. 2, panel 6). The recording of this relatively labile current [10] is evidence of the preservation of the integrity of our preparation.

Global detection of calcium transients could be easily performed with the new chamber using dry ($20\times$ and $40\times$) and oil immersion objectives ($100\times$). As an

Fig. 3. Global detection of calcium release. Calcium release, reported by two different affinity calcium dyes (Rhod-2 and OGB-5N), is presented as calcium-dependent fluorescence changes. Data are shown in a normalized basis at two different time scales (**panels 1 and 2**) to better appreciate kinetics and signal to noise ratio differences. Records were obtained from two different fibers. Only the record of one membrane action potential is included because electrical records from both fibers were indistinguishable. Fiber diameter: $100 \mu\text{m}$ (Rhod-2) and $90 \mu\text{m}$ (OGB-5N); sarcomere spacing: $3.8 \mu\text{m}$ (Rhod-2) and $4.0 \mu\text{m}$ (OGB-5N); holding potential: -100 mV (both fibers); MAP overshoot: $+45 \text{ mV}$ (both fibers); width of pool A: $200 \mu\text{m}$ (Rhod-2) and $190 \mu\text{m}$ (OGB-5N); [Rhod-2]: $100 \mu\text{M}$; [OGB-5N]: $500 \mu\text{M}$; [EGTA]: $100 \mu\text{M}$ (both fibers).

example, calcium-dependent fluorescence transients elicited by action potential stimulation and recorded using a $100\times$ oil immersion objective are shown in Fig. 3 at two different time scales. The records (Rhod-2 and OGB-5N) are similar to previously reported data [4, 7]. Kinetics differences between both traces (Rhod-2 and OGB-5N) reflect differences in the dye reaction rate constants, whereas differences in signal/noise ratio are mainly due to differences in the quantum yield of the dyes. The time to peak was 4.85 and 12.2 ms , and the coupling delay was 1.55 and 1.92 ms for OGB-5N ($n=3$) and Rhod-2 ($n=2$), respectively. The time for half-decay of the fluorescence transient was 11.8 and 34.5 ms for OGB-5N and Rhod-2, respectively. The fluorescence changes shown corresponded to $\Delta F/F$ values of >10 for Rhod-2 and

~2.5 for OGB-5N. All of these values are concurrent with previously published data [4, 6, 8]. These results, obtained with a short working distance objective, clearly show that the chamber can also be used for high-resolution confocal detection of calcium. Optical measurements could also be made with an upright microscope provided a long working distance objective was used (20× Fluor, 0.75 NA, 0.66 WD, Nikon).

The chamber described here is, to our knowledge, the first inverted model for a triple grease-gap isolation chamber yet described. Our evaluation clearly showed that the new chamber allows for electrical recordings similar to those obtained with conventional chambers, which in addition, are free of problems related to fluctuation in solution level and fiber movement. Moreover, our data show that the new chamber is fully compatible with “global” and confocal techniques of calcium detection implemented in inverted microscopes, and easily adaptable to upright microscopes. Finally, the fiber mounting procedure is uncomplicated, fast and not skill demanding, and yields a very stable preparation. All of these features make our chamber a better choice to conventional chambers. In conclusion, for electrophysiological studies in skeletal muscle fibers, the new inverted triple-gap isolation preparation introduced in this paper is more reliable and stable than its upright counterpart versions, and has the additional advantage of being compatible with most (low and high-resolution) photometric techniques for calcium detection.

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