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Use of patch-clamp technique to study voltage-independent cationic channels in normal and dystrophic skeletal muscle fibers.

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1. OBJECTIVE

The objective of this SOP is to describe the procedures and provide the main guidelines for performing electrophysiological recordings by means of the patch clamp technique to investigate the cation channels involved in the increase in sarcolemmal permeability to calcium observed in dystrophin-deficient muscles and therefore in altered calcium homeostasis. Importantly, this is a specific application of a wide technical approach, since eletrophysiological recordings allow to monitor function and possible disease-related dysfunction of virtually all sort of ion channels in the cell type of interest. Indeed, a certain number of studies, using different electrophysiological approaches, have been carried on in dystrophin-deficient animals. The reason resides in the role that membrane ion channels play as sensors of either voltage and metabolism changes, mechanical stress or injury in striated and cardiac myofibers. The results obtained with this approach contributed to better understand the link between dystrophin deficiency and tissue dysfunction. However, this SOP focuses on the technical procedures that allow a more direct investigation of the voltage-independent calcium channels that are considered nowadays as primarily related to the disease. This approach may potentially yield to a better understanding of the pathogenic events involved in the disease and an identification of the possible targets for pharmacological intervention.

2. SCOPE AND APPLICABILITY

The scope of the technique is to investigate the occurrence, biophysical properties and possibly the pharmacological sensitivity of subsets of voltage independent calcium permeable channels in single native muscle fibers of dystrophic animals and to verify possible changes due to the pathology and/or to any sort of therapeutic approach. Due to problems that will be briefly described in chapter 3, the technique is not at the moment suitable for screening therapeutic compounds in dystrophic fibers; however it may be used to verify if long term treatment with specific and promising compounds/approaches may yield or not to amelioration in channel biophysics (Squire et al., 2002; Rolland et al., 2006; De Luca et al., 2008). The investigation described in this SOP will hopefully allow to identify the molecular identity of the channels possibly contributing to the pathology and then important as drug target. This might lead in the future to apply HTS automated patch clamp analysis on the channel of interest, heterogously expressed, to screen and develop potentially effective compounds. The most frequently used method for studying cellular electrophysiological properties by patch clamping requires the use of microelectrode pipettes and is based on the establishment of a high-resistance seal between the pipette and the cellular membrane. This method can be used in either of two modes: current clamp (to record the changes in membrane potential resulting from an electrical/chemical stimulus) or

voltage clamp (to record current flowing through specific channels in response to a voltage/chemical/mechanical stimulus). Different configurations of the patch clamp technique allow current recordings from the entire membrane (whole cell – not applicable to adult myofibers due to cell geometry) or from a restricted channel population present in the patch under the pipette mouth (cell-attached, inside-out, or outside-out). All of these configurations have advantages and disadvantages, as well as specific scientific use and the relevant technical details can be found in specialized texts.

3. CAUTIONS

As already mentioned, different electrophysiological properties of C57 and *mdx* fibers have been compared so far: resting membrane potential, action potentials, voltage operated Na^+ and Ca^{2+} channels, Cl^- conductances, acetylcholine receptors….(for review, see Allard, 2006). The most obvious difference was found in the open probability of voltage-independent nonspecific cationic channels. Therefore, the present protocol is focused on detection of these channels, keeping in mind that this SOP is not exhaustive of all the potential investigation that the technique allows.

Voltage independent calcium channels in dystrophic fibers have been first described by Steinhardt & collaborators (Fong et al., 1990) and Franco-Obregon & Lansman (for review, see Lansman and Franco-Obregon, 2005). However, their gating mechanisms, molecular identity and involvement in the physiopathology of Duchenne muscular dystrophy are still under evaluation. Therefore, this protocol is valuable mostly for basic research aims (also when evaluating the outcome of a therapeutic interventions in dystrophic animals) while it should be used with extreme caution for drug screening.

The poor information about the molecular identity of the channel involved, along with the complex mechanisms underlying calcium homeostasis in myofibers, should also lead to an extreme caution. In particular the technique and the specific recordings on one channel subset can be informative on the altered calcium homeostasis in dystrophin-deficient fibers only if properly paralleled by other technical approaches**.**

Apart this general concern, technical advantages and disadvantages can also be underlined:

ADVANTAGES

The patch clamp recordings allow to directly monitor channel activity and/or expression in dystrophic myofibers and the direct investigation in the function of proteins that may be direct target of drugs with potential therapeutic interest. These are standardized techniques may lead to highly repetitive results and can represent the functional cellular approach to biochemical and/or imaging data.

DISADVANTAGES

1) the techniques are highly specialized and requires a strong background in channel biophysics. Training for acquiring skills in performing both proper recording and analysis, can be long, especially for single channel recordings
2) Costs. An electrophysiological set-up is expensive
3) Time. Experiments are time consuming for both execution and analysis
4) Numbers. When measuring channel activity from native fibers, a quite large number of fibers have to be sampled to have a condition representative of the whole tissue
5) When using freshly dissociated fibers, a certain bias can be introduced by the different sensitivity to collagenase treatment between normal and dystrophic fibers, as this may lead to fiber selection and consequently to problems in evaluation and interpretation of the results.

As mentioned, data relative to channel function have to be paralleled by concomitant approaches to evaluate the real relation between channel dysfunction/pathology/ drug efficacy.

4. MATERIALS

Electrophysiological set-ups require sophisticated equipment, including high-precision manipulators, low-noise amplifiers, anti-vibration tables, and specialized software. Highly specialized pipette pullers are also needed to obtain glass pipettes of a specific shape and dimension so as to allow proper “contact” with the membrane.

A detailed description of patch-clamp techniques is beyond the scope of this brief account. For general description, see Sakmann and Neher, 1995.

5. METHODS

A. Isolation of skeletal muscle fibers

For patch clamp recordings, the muscle must be enzymatically and mechanically treated to obtain isolated fibers with collagen-free membranes (Vandebrouck *et al*., 2002; Rolland *et al*., 2006). Isolated fibers can be used soon thereafter, or they can be placed in culture. Freshly dissociated fibers maintain the typical properties of adult differentiated muscle fibers, whereas cultured myofibers may lose some of their original properties as a result of de-differentiation and denervation. Thus, for *ex vivo* analysis of a drug treatment, freshly dissociated fibers are preferred.
A detailed procedure to obtain isolated fibers from Flexor Digitorum Brevis muscle or from Interossei muscles is provided in the “Calcium Homeostasis” protocol.

B. Patch-clamping

As anticipated above, proper and sophisticated equipment is commercially available from different brands for patch clamp recordings. Quality and performance are generally comparable between brands. Thus the details provided throughout have to be taken simply as suggestion and/or information about more commonly used equipment.

- Cells are transferred to specific chambers for patch-clamp recordings equipped with proper system for solution exchange and temperature control and placed on the stage of an inverted phase contrast microscope, e.g. an Axiovert S-100 (Zeiss) or similar ones.
- Patch electrodes (borosilicate glass capillaries, usually 1.5 mm in diameter) are pulled using an adequate puller (Zeitz-Instruments, Narishige, Kopf, Utter instruments,…) to obtain a pipette resistance of 2-5 M.
- Three axis high precision micromanipulators (Narishige, etc) allow placing the patch pipette (connected via Ag/AgCl electrode to the pre-amplifier and to the electronic apparatus) on the surface of the fiber. A negative suction applied through electrode holder then leads to the formation of the high resistance seal between glass and membrane.
- Solutions:
  The intrapipette solution contains (mM): $CaCl_2$ 110, Hepes 10 and DIDS (4.4'-diisothyocyanate stilbene-2.2' disulfonic acid) 0.01, pH 7.2. DIDS is used to block possible chloride conductances. Ca^{2+} of the intrapipette solution can be replaced by Mn^{2+}, Ba^{2+} or other cations (Sr^{2+}…) for studying the permeability to these ions.
  The geometry of the muscle fibers does not allow a good clamp of the plasma membrane potential so a trick consists in using a potassium aspartate bathing solution to render null this latter; then the patch potential would strictly follow the applied voltage command. The bathing solution contains (mM): K-aspartate 150, $MgCl_2$ 5, EGTA 10 and HEPES 10 (pH 7.4). The osmolarity of each solution may be adjusted to 300-330 mosmol.l^{-1} by adding glucose.
- Single channel activity can be recorded at a constant holding potential of - 60 mV and at room temperature, by using a patch clamp amplifier (from Heka, Axon instruments, A-M systems, Dagan, Bio-Logic,…).
- Current records are typically filtered with a Bessel filter at 3 kHz and digitized at 10 kHz; and corresponding data are analyzed using the "amplifier associated software" (Pulse-Fit, Fit-master, Clampfit, …).

Dependence of the channel activity to store-depletion and stretch sensitivity can be tested by treating the fibers with 1 µM thapsigargin and/or 10 mM caffeine and by applying suction pressure on the pipette (-20 to -100 mmHg), respectively. Other pharmacological tools for channel characterization can be used according to the experimental need.

6. EVALUATION AND INTERPRETATION OF RESULTS

Electrophysiological recordings point to a greater activity of voltage-independent cation-permeable channels in dystrophin-deficient fibers. The channels involved have been reported to be stretch/mechano-sensitive (Lansman et al., 2005; Yeung et al., 2005; Ducret et al., 2006), store-dependent (Vandebrouck et al., 2002), and growth factor-sensitive (Iwata et al., 2003; Rolland et al., 2006).

The patch clamp recordings allow monitoring of channel occurrence, open probability, and unitary conductance. In general, dystrophic muscle fibers show a greater occurrence and open probability of these voltage-independent channels. Additional properties can be measured such as activation and inactivation kinetics, current amplitude, and open and closed state time.

The gating mechanisms (store-dependence and stretch sensitivity) and the pharmacological profile of these channels has led to the suggestion that they might be constituted of TRP proteins (Vandebrouck et al., 2002) but other possibilities exist.

The possibility that more than one channel type may be present and involved, along with the different biophysical and gating properties, do not allow to provide detailed reference values for the SOP. The reader should be aware of this aspect and refer to specific literature in the field.

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