Culture Conditions for the Production of Porcine Myotubes and Myoballs

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ABSTRACT: The existence of a method for culturing porcine muscle cells would greatly facilitate the development of new breeding criteria for stress resistance and growth regulation in pig breeding. Also, many effects of nutritional or pharmacological components that influence animal performance could be studied first in muscle cell preparations. Therefore, we developed a specific procedure to culture porcine skeletal muscle cells from well-established methods for murine and human muscle cell culturing. Best results were obtained by isolating satellite cells from muscle tissue removed postmortem after normal slaughter procedure, using enzymatic dissociation. The satellite cells were allowed to proliferate for 3 to 5 d in a culture medium composed of 83% Ham's F-12 medium, 15% fetal calf serum (FCS), and 2% chick embryo extract (CEE). Well before reaching confluence, the cells were transferred to collagen-coated dishes filled with Dulbecco's modified Eagle's medium containing 5% horse serum (HS) for the differentiation to multinucleated myotubes. Also, 5% FCS can be used instead of HS. Besides the fusion to myotubes, the presence of voltage-sensitive Na+ channels is regarded as a specific feature of the muscle phenotype of the cells. To perform electrophysiological experiments of good quality, myotubes were converted into freely floating "myoballs." Voltage-clamp experiments in the whole-cell mode showed transient inward currents that had kinetics and voltage dependences very similar to those of the Na+ currents in human myoballs. The porcine Na+ currents were almost completely blocked by 1 μmol/L of tetrodotoxin, indicative of the presence of the adult form of the Na+ channel. We conclude from this electrophysiological evidence that the cells cultured according to our method are really of muscular phenotype.

Key Words: Cell Culture, Pigs, Muscle Tissue

Introduction

The negative correlation between extensive muscle growth and stress resistance is a basic problem in pig production. Pig breeds with a high degree of muscular hypertrophy, such as Pietrains, show rather high preslaughter death loss; in addition, the incidence of PSE pork is substantially elevated in stress-susceptible pigs (Eichinger, 1982). Even after many years of selection against the halothane reaction (Eikelenboom and Minkema, 1974), partly in combination with creatine kinase testing, the stress problem is still significant (Wolf-Schweroin and Kallweit, 1991). Before new breeding criteria can be introduced, the specific pathways coupling the porcine stress syndrome to extreme muscle growth have to be understood. Especially, the transmembranal transduction of growth and of stress signals seems to provide new insights into the pathophysiology of the porcine stress syndrome (Iaizzo et al., 1991) and the development of poor meat quality. Cell cultures of porcine skeletal muscle would be the ideal preparation for such studies.

Cell culture is extensively used for the investigation of mammalian skeletal muscle (Inestrosa, 1982). Basic physiological properties of skeletal muscle, such as the electrical characteristics of the sarcolemma, have been successfully studied in cultured muscle cells from mice, rats, and humans (Witkowski, 1986). In earlier attempts in our laboratory, the application of standard culture methods to pig skeletal muscle...
achieved limited success because high amounts of non-fusing fibroblast-like cells contaminated the cultures. Therefore, we have substantially modified the usual techniques of muscle culturing to provide reliable cell cultures and appropriate single cells for experimentation. The quality of the final culture cells will be demonstrated by the characterization of the voltage-dependent sodium channels, key elements for the physiological function of membrane excitability in muscle.

The results were reported at the 37th International Congress of Meat Science and Technology. After we submitted this paper, a method for culturing porcine muscle cells was also published by Doumit and Merkel (1992).

**Experimental Procedures**

**Cell Culture**

Shortly after slaughtering six German Landrace pigs ranging in age from 3 d to 3 mo we took samples of approximately .5 g from the supraspinatus muscle and stored them overnight in an ice-cooled bath in Hank's salt solution with 2 mmol/L of HEPES, pH 7.4. We then dissected the tissue mechanically into small pieces. For comparison, we also dissected tibialis anterior muscles from adult female mice of the Balb/c strain and cultured them just as the pig muscle except that we did not store them overnight. We dissociated the muscle fragments by enzymatic treatment, incubating them from 60 to 90 min at 36°C in a shaker with 1.5 mg/mL of collagenase (#17149, Serva, Heidelberg, FRG) and 2 mg/mL of protease (Type X, Sigma, St. Louis, MO) dissolved in Ham's F-12 medium. The pH value was adjusted to 7.4 with 2 mol/L of HEPES. After the muscle fragments were to a great extent disintegrated and the solution had become cloudy, we filtered the suspension through a nylon gauze (pore size 20 μm) to remove remaining tissue fragments. Afterward we centrifuged the suspension and resuspended the pellet in growth medium. We counted the cells in a “Neubauer” chamber, leaving out the smallest type of cells, which probably were erythrocytes. Then we seeded them without preplating in a density of 1,000 to 10,000/cm² in a growth medium with the following composition: 83% Ham's F-12 medium (Biochrom KG, Berlin, FRG), 15% fetal calf serum (FCS; Gibco, Eggenstein, FRG), and 2% chick embryo extract (CEE; Gibco). The cells were allowed to grow under these conditions during the next 3 to 5 d. To stimulate differentiation, we detached the cells from the bottom by trypsin treatment (.03% trypsin in PBS (L-2133, Biochrom)) and reseeded them on collagen-coated dishes (calf skin collagen, Sigma) in a density of 5 × 10⁴ cells/cm². The culture medium, called differentiation medium, had the following composition: Dulbecco's modified Eagle's medium (DMEM) with either .5% FCS or 5% horse serum (HS; Gibco). As an alternative method, we seeded the cells from the beginning in a 1:1 mixture of Ham's F-12 medium and CMRL medium (Biochrom) with 2% FCS and 2% HS, a condition which is usually used in our laboratory for growth and differentiation of human muscle cells (modified from Probstle et al., 1988). All media contained 50 μg/mL of gentamycin and were renewed every 2 to 3 d.

As a quality criterion of the muscle cultures, we estimated the ratio of the nuclei assembled in the multinucleated cells and the total number of nuclei in a culture. For this, we counted the number of nuclei in the myotubes and the number of the non-fused cells in randomly chosen microscopic fields of view. For each estimation we counted approximately 200 nuclei in five to seven fields of view.

For electrophysiological experiments, we detached the cultures from the bottom of the culture dish by trypsin treatment and resuspended them in culture medium. During the next 4 to 6 h the elongated muscle cells formed spherical, floating myoballs. In some cultures the detachment from the bottom occurred spontaneously. We then transferred the myoballs to dishes with hydrophobic bottoms (Petriperm, Haereus, Hanau, FRG).

**Clonal Cultures**

In two experiments we seeded some of the dissociated cells in low density of 100 to 200 cells/6-cm dish. This condition gave rise to clonal cultures. To stimulate differentiation in the clones, we replaced the growth medium after 9 to 12 d by the differentiation medium without detaching the cells with trypsin.

**Electrophysiology**

The “external solution” in the experimental dishes contained (in millimoles/liter) 140 NaCl, 3.5 KCl, 1.0 CaCl₂, 1.0 MgCl₂, and 2 HEPES (pH 7.4). For whole-cell recording of Na⁺ currents, we sucked the cells onto patch pipettes pulled from hematocrit capillaries with tip diameters of approximately 5 μm (resistance 300 to 500 kΩ). The pipettes were filled with “internal solution” containing (in millimoles/liter) 140 CsCl₂, 1.4 MgCl₂, 10 EGTA, and 10 HEPES. A home-made amplifier served for conventional whole-cell recordings. The Na⁺ currents were elicited with square, depolarizing voltage pulses going from a holding potential of −85 mV to variable test potentials. For the investigation of the voltage dependence of activation (v-curves), we applied a cyclic pulse program with each cycle consisting of a constant 100-ms prepulse to −135 mV and an 8-ms test pulse that was varied from −65 mV to +31 mV in 4-mV steps. For the investigation of the voltage dependence of inactivation of the Na⁺ channels (h∞ curve), we applied a cyclic pulse program with each cycle consisting of a 100-ms
conditioning pulse to \(-135\) mV, a 32-ms prepulse that was varied between \(-135\) and \(-19\) mV in 4-mV steps, and a constant test pulse to \(-20\) mV. The resulting Na\(^+\) currents were low-pass-filtered (10 kHz) and digitized at 40 kHz. For data reduction, we fitted Boltzmann distributions to the \(h_\infty\) curves and to the falling phase of the \(v\)-curves and extracted the inflection point and slope of each of these curves from the analysis. From the Na\(^+\) current transients, we determined the time constants of activation and inactivation, \(\tau_1\) and \(\tau_2\) as described by Pröbstle et al. (1988) and originally defined by Hodgkin and Huxley (1952). All experiments were performed at 21°C. In some experiments, we used the Na\(^+\) channel blocker tetrodotoxin (TTX, Sigma) to characterize the type of Na\(^+\) channel. Application and washout of TTX occurred by a shift of the pipette with the attached myoball between two parallel troughs placed in the experimental chamber. The troughs were the end of a theta-glass capillary from which a 2-mm-long, 30° wedge was ground off perpendicular to the partition. External solution with 1 pmol/L of TTX and without TTX was flushed through the two barrels of the capillary.

Results

Cell Cultures

The yield from 300 to 500 mg of fresh pig muscle was 2 to 4 \(\times\) 10\(^6\) mononucleated satellite cells. The efficiency of plating was between 10 and 20%. Storage of the tissue overnight in ice-cooled Hank's solution did not affect the yield and the viability of the cells. We obtained the best proliferation when the cells were seeded in a density of 10,000 cells/cm\(^2\) in growth medium. Many cells were floating in the medium when the cultures were allowed to reach confluence, which occurred between 4 and 6 d after plating. Further cultivation of confluent cultures in growth medium resulted in a dense lawn of mononucleated cells. Myotube formation was then scarce.

We obtained a higher yield of myotubes when we detached the cells from the bottom before they reached confluence (Figure 1a) and reseeded them in differentiation medium (5% HS) on a collagen-coated culture dish (Figure 1b). The formation of myotubes was extensive during the next 7 d (Figure 1c, d). After this period of development, nearly 50% of the nuclei of a culture had assembled in myotubes (Figure 1d). Such cultures were best suited for myoball production and electrophysiological measurements.

During the following week, the remaining non-fusing cells, probably fibroblasts, continued to proliferate slightly, so that the myotubes were packed in a dense network of mononucleated cells. Many of the myotubes detached spontaneously from the bottom and rounded up to myoballs of 20 to 100 \(\mu\)m in diameter (Figure 2a). This procedure led to a successive substitution of myotubes by unfused fibroblast-like cells in long-term cultures. The proliferation of the non-fusing fibroblast-like cells was considerably slower when the cells were kept in the differentiation medium with only .5% FCS. However, under these conditions the myotubes looked unhealthy, showing intracellular granulate-like and vesicle-like inclusions (Figure 2b).

Myotube formation was very rare in cultures with the alternative medium for differentiation consisting of a 1:1 mixture of Ham's F-12 medium and CMRL with 2% FCS and 2% HS, which we normally use to establish human muscle cultures (not shown).

Seeding in low density of 100 to 200 cells per dish led to the growth of 10 to 20 cell clones each containing 1,000 to 10,000 cells after 9 to 12 d. The cells in a single clone looked morphologically homogeneous in many cases and, 3 to 5 d after switching to the differentiation medium, myotube-forming clones could be distinguished from non-fusing clones (not shown).

After having observed many clonal muscle cultures, we could predict myotube-forming precursor cells by morphological criteria. Myotube-forming precursor cells tended to have a more spherical and compact
shape and a brighter appearance in the phase contrast display than non-fusing fibroblast-like cells, which were larger, more spread, and darker (Figure 2c, d). This was especially evident in uncoated culture dishes and became less obvious in collagen-coated dishes with higher cell densities (Figure 1b).

**Sodium Currents**

All tested pig myoballs showed inward currents between 2 nA and 60 nA during depolarizing square voltage pulses (see Methods). The currents must have been carried by sodium ions, because of the ionic composition of the intracellular (CsCl) and extracellular (NaCl) solutions used, and because of their direction of flow and equilibrium potential (Figure 3b). Figure 3a shows a typical set of Na⁺ currents induced by depolarization from a holding potential of -85 mV to potentials between -65 and +31 mV. Significant activation of the current is first achieved at -41 mV and half-maximum amplitude occurs at -25 mV (Figure 3a). Figure 3b illustrates the current-voltage relation of the peaks of the transients of Figure 3a. Boltzmann distributions fitted to the falling phase of many of these curves resulted on average in an inflection point at $-27.4 \pm 2.6$ mV (mean ± SD, $n = 10$), a value that is very similar to that determined from human myoballs under similar conditions (Table 1).

The Na⁺ currents were nearly completely inhibited by 1 μmol/L of tetrodotoxin (Figure 3c), which is a specific feature of the adult type of the Na⁺ channel. For the determination of the kinetic parameters of activation and inactivation of single Na⁺ current transients, $\tau_a$ and $\tau_i$, we selected a cell that contained only TTX-sensitive Na⁺ channels. The semilogarithmic plots of $\tau_a$ and $\tau_i$ against the membrane potential were not linear (Figure 3d).

The voltage dependence of inactivation of the Na⁺ channels is illustrated in Figure 4. A variable prepotential preceding the test potential leads to an inactivation of Na⁺ channels, which results in a more decreased amplitude of the Na⁺ currents the more positive the prepotential is. Figure 4a shows the original current transients, Figure 4b the corresponding $h_a$ curve. In all cases, the fit with a Boltzmann distribution was appropriate. On average, the inflection point of the fitted $h_a$ curve was at $-62.7 \pm 3.7$ mV (mean ± SD, $n = 13$), a value that is very similar to that of the adult Na⁺ channel in human skeletal muscle (Table 1).

In one experiment we recorded Na⁺ currents from myoballs that had spontaneously detached. Their properties were not different from those of myoballs that we had detached by trypsin treatment (Table 1).

For comparison, we determined corresponding data on Na⁺ channels from mouse myoballs in a different series of experiments. The murine muscular Na⁺ currents were distinct from human and porcine currents because their current-voltage relation and their $h_a$ curves were shifted by 10 to 15 mV in the negative direction (Table 1).

**Discussion**

To develop porcine skeletal muscle cultures with a high proportion of intact myotubes, we varied each major step of the methods established for the culturing of muscle from other mammalian species. For the preparation of the mononucleated satellite cells, we tested a dissociation procedure originally developed for mouse muscle (Hauschka et al., 1979) and another protocol introduced for human muscle (Yasin et al., 1977; Pröbstle et al., 1988). The two methods differed considerably in their composition of the digestive enzymes and in the duration of the exposure of the tissue to these enzymes. We found that both methods gave about the same yields, indicating that the enzymatic isolation of precursor cells was not a critical step in porcine muscle cell culture.

For past proliferation of mononucleated cells, we found that our established method for human myoballs (Pröbstle et al., 1988), that is, constant growth...
in a medium with few mitogens, was not satisfactory for pig muscle because of an overwhelming growth of fibroblasts. We therefore started the culture in a medium with a high mitogen content for a quick production of many myoblasts, and later continued in a medium with few mitogens for the differentiation of these myoblasts into myotubes (Blau and Webster, 1981; Linkhart et al., 1981). By this method the excessive growth of fibroblasts was avoided. The advantage of the latter method lay in the fact that the formation of myotubes from myoblasts was so fast that the growth period could be terminated before too many fibroblasts had developed. This advantage was essential for porcine muscle cultures, whereas it is not important for human (Yasin et al., 1977; Pröbstle et al., 1988) or rat (Boldin et al., 1987) muscle cultures.

In the early stages, the morphological appearance of the pig myoblasts (Figure 2a) is very similar to that of mouse myoblasts, which were intensively studied in clonal cultures (Linkhart et al., 1981) and are distinct from human myoblasts (Blau and Webster, 1981).

The tendency of single pig myoblasts and myotubes to detach from the culture dish at the stage of confluence is a feature so far not observed by us with any other species. When the pig myoblasts or myotubes were floating they degenerated within a few days. This feature was very convenient when a certain number of myoballs was needed for experimentation on consecutive days: the daily harvest of floating cells allowed us to make use of the same culture for a week or so. When we wanted to have a large number of myoballs at the same time, we detached the whole lawn before confluence and transferred the cells to collagen-coated culture dishes. The collagen seemed to prevent detachment of the myotubes and, at the same time, to limit the proliferation of fibroblasts. It seemed to be essential for the differentiation of the myoblasts but not to be important for proliferation. It is possible that other components of extracellular matrix as fibronectin, laminin, or type IV collagen have beneficial effects on the porcine muscle cultures (Sanderson et al., 1986).

Figure 3. Sodium currents recorded from pig myoballs. [a] Family of Na⁺ inward currents, induced by square voltage pulses going from a holding potential of -85 mV to test potentials varying between -65 and +31 mV in 4-mV steps. Every other trace is plotted. The responses to three selected test pulses (to -33, -25, -9 mV) are labeled. [b] Current-voltage relation of the Na⁺ currents; current maxima are plotted against the test potential. [c] Na⁺ current, measured in external solution (control) and after the application of 1 μmol/L of tetrodotoxin (TTX). [d] Semilogarithmic plot of the Hodgkin-Huxley conditions of activation and inactivation (τₚ and τₘ) of the current transients shown in [a].
Figure 4. Voltage dependence of inactivation of Na⁺ currents from pig myoballs. (a) Family of Na⁺ inward currents, induced by square voltage pulses of 8 ms duration to a test potential of -20 mV. Each test pulse was preceded by a conditioning prepulse of 32 ms duration, which varied between -135 and -19 mV in 4-mV increments. Every other trace was plotted. The transients corresponding to three selected prepotentials (-87, -71, -55 mV) are labeled. (b) Voltage dependence of inactivation of the Na⁺ currents (hᵣ curve) in external solution. The maxima of the current traces shown in [a] were plotted against the prepotential. A Boltzmann curve was fitted to the data points. More than 95% of the Na⁺ currents recorded from this cell were blocked by 1 μmol/L of tetrodotoxin.

The presence of Na⁺ channels in the porcine muscle cells can be considered a second characteristic of muscle development, because Na⁺ channels are specifically expressed during muscle differentiation (Sherman and Catterall, 1984) and are not present in the non-excitable fibroblasts (Gray et al., 1986). Thus, we conclude that the cells selected for electrophysiological measurements were differentiated muscle cells originating from the myotubes.

The Na⁺ inward currents of the pig myoballs were in most characteristics very similar to human Na⁺ currents measured with the same technique (Table 1; Trautmann et al., 1986). The similarity of the inactivation characteristics for pig and human channels is particularly striking when one considers that the curves for the voltage-dependence of activation and inactivation of murine Na⁺ channels are situated at considerably more negative potentials (Table 1). Consequently, the time constants for activation (τₐ) and inactivation (τᵢ) of single Na⁺ current traces are at a given voltage smaller in mouse than in human and pig myoballs (Table 1, right column). Although the literature is abundant on channel data from adult human muscle fibers, we refrain from a comparison, because the Na⁺ channel characteristics are highly influenced by the recording technique applied, the

Table 1. Values of the potential dependence of steady-state Hodgkin-Huxley conditions for activation and inactivation and time constants for the activation and inactivation of single Na⁺ current transients

<table>
<thead>
<tr>
<th>Species/condition</th>
<th>Conditions of activation</th>
<th>Conditions of inactivation</th>
<th>Activation/inactivation kinetics</th>
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<tr>
<td></td>
<td>U_m, mV</td>
<td>V_m, mV</td>
<td>U_h, mV</td>
</tr>
<tr>
<td>Pig (21°C)</td>
<td>-27.4 ± 2.6</td>
<td>-6.34 ± 0.87</td>
<td>-62.7 ± 3.7</td>
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<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 13)</td>
</tr>
<tr>
<td>Pig, floating cells (21°C)</td>
<td>-26.1</td>
<td>-5.49</td>
<td>-66.2</td>
</tr>
<tr>
<td>Human (24°C) b</td>
<td>-33.9 ± 4.1</td>
<td>-5.74 ± 1.21</td>
<td>-65.9 ± 4.6</td>
</tr>
<tr>
<td>Mouse (21°C)</td>
<td>-45.6 ± 7.5</td>
<td>-7.1 ± 1.4</td>
<td>-81.0 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>(n = 13)</td>
<td>(n = 13)</td>
<td>(n = 15)</td>
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The values for U_m, V_m, U_h, and V_h were obtained by fitting Boltzmann distributions to the hᵣ curves and to the falling phase of the v-curves. U_m, U_h are the potentials at which the curves have their points of inflection. V_m, V_h are the slopes of the curves at the potentials U_m or U_h. τ_m (-20 mV) and τ_i (-20 mV) are the time constants for activation (τ_m) and inactivation (τ_i) of Na⁺ currents induced by test pulses to -20 mV. (n) is the number of cells tested.

From Probstle et al. (1988).
Agnew, 1989). Furthermore, the uniformity and the position of the \( h \), curves of the \( Na^+ \) currents recorded from pig myoballs (Table 1) may indicate that the adult type of the \( Na^+ \) channel is dominant in our cultures. The \( h \), curves of embryonic types of \( Na^+ \) channel are situated much more negative than those of the adult forms in rats (Ruppersberg et al., 1987) and humans (Pröbstle et al., 1988). We conclude from the electrophysiological data that, under the culture conditions that we used, the adult form of the \( Na^+ \) channel is mainly expressed, and that pig \( Na^+ \) channels are more similar to human than to murine \( Na^+ \) channels.

An interesting application of porcine muscle cultures would be the use for the carrier detection of porcine stress syndrome (Iaizzo et al., 1991). The myotubes of carriers of the defective gene are by analogy likely to show an increased sensitivity to halothane and caffeine because myoballs from human carriers of the corresponding condition of malignant hyperthermia show this susceptibility (Ruppersberg and Rüdel, 1988).

Implications

We have developed a method for the isolation and culture of porcine skeletal muscle cells and have shown that the cultured cells can be used for electrophysiological experiments. This method, which is based on the use of pig muscle collected postmortem from slaughtered animals, can be adopted by any laboratory familiar with cell culture. Growth characteristics and genetic dispositions, which are of major interest in the production of livestock, can be studied at the cellular level. Pigs are also used as models in pharmacology and human medicine. Cell culture can reduce the number of experiments with animals and might even provide information otherwise not obtainable.

Literature Cited


