Design and performance of an electrical stimulator for long-term contraction of cultured muscle cells

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Excitability in muscle cells manifests itself as contractility and may be evoked by electrical stimulation. Here we describe an electrical stimulator device applicable to cells seeded on standard multiwell plates and demonstrate how it effectively stimulates synchronous contraction of skeletal muscle C2C12 cells without damaging them. The electrical stimulator of cultured cells (ESCC) consists of two connection cards and a network of platinum electrodes positioned in such way that each well in a row is uniformly stimulated. The ESCC may produce a range of outputs based on the stimulation parameters it receives from a commercial pulse generator and can be placed in a standard cell incubator, allowing for long-term stimulation as required for biochemical and molecular biological assays. We show that a 90-min stimulation of C2C12 myotubes at 50 V, 30 ms of pulse duration, and 3 Hz of frequency enhances glucose metabolism and glycogen mobilization while oppositely modulating the activity ratio of glycogen metabolizing enzymes. Thus, we demonstrate that long-term electrical stimulation of C2C12 myotubes with the ESCC results in contractility and metabolic changes, as seen in exercising muscle.

INTRODUCTION

Excitation of muscle or neural cells may be evoked by electrical stimulation. Continuous electrical stimulation of isolated cells in culture provides an excellent model for the study of the ensuing biochemical and molecular changes, excluding the complex set of factors participating in vivo. Excitability in muscle cells causes contractility, and skeletal muscle cells do not contract unless they are stimulated either via the nerve or electrically. Excitation-contraction has many short- and long-term effects on muscle cell functionality. Electrical stimulation in vivo causes immediate effects on glucose metabolism, such as the activation of glucose uptake and glycogenolysis in both fast-twitch and slow-twitch muscles (1–3). However, chronic increases in contractile activity caused by low-frequency electrical stimulation induce a much more profound change in metabolic and transcriptional phenotypes, which leads to the transformation of fast-twitch glycogenolytic muscle fibers into slow-type oxidative fibers (4).

Currently, no device is capable of simultaneously and uniformly stimulating a large number of independent cultured muscle cells so that they will contract over long periods of time. An early attempt to develop such an in vitro model of electrical stimulation was reported by Brevet et al. (5) and later modified by others (6,7). In this setting, cells were cultured in 24-well plates. However, the designed electrical configuration was not easy to standardize because it did not deliver the same current intensity to every well. The device was composed of silver/silver chloride electrodes for the end wells and agar saline bridges for connecting internal wells. Due to their dimensions, saline bridges have lower conductivity and the voltage drop is higher than in culture media. In addition to being time-consuming to produce, disposable bridge electrodes have the disadvantage that solutes diffuse along the agar bridge and alter concentration gradients. Berger et al. (8) described an alternative system adaptable to 175 cm² tissue culture flasks that included large diameter graphite electrodes parallel to the long axis of the flask cover. This was a fairly

inflexible design. First, it required specially designed flasks for each sample of cells, making it difficult to generate a high number of replicates. Second, the large size of the flasks necessitated the preparation of large numbers of cells for each sample condition, which, depending on the source and nature of the cells, was not always feasible. Here we describe an original electrical stimulator of cultured cells (ESCC) suitable for continuous uniform stimulation of a large number of independent wells in standard multiwell culture plates that overcomes the limitations of previously reported systems. We demonstrate its application in the induction of contraction and consequent metabolic changes in C2C12 mouse skeletal muscle fibers.

MATERIALS AND METHODS

C2C12 Cell Culture

C2C12 cells were cultured in standard 6- or 24-well culture plates. Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and supplemented with 10% fetal bovine serum (FBS) was used as a growth medium for the myoblasts. Differentiation was induced by incubating cells in DMEM containing 25 mM glucose and supplemented with 10% horseradish peroxidase (HRP). Fused myotubes (ATCC, Manassas, VA, USA) were used 7 days after the induction of the differentiation. Media and serum were supplied by Invitrogen (Barcelona, Spain).

Preparation and Maintenance of the Electrical Stimulator of Cultured Cells System

The 6- or 24-well tissue culture plate is placed between two ESCC connection cards, which are attached to a transparent plastic support. Before immersion in the culture medium, platinum wire electrodes are sterilized with 70% ethanol and subsequently dried under ultraviolet light. The cards are connected by standard electrical cables to a Grass S-48 stimulator (Grass Instruments, Quincy, MA, USA) that generates variable electrical pulses (i.e., pulse duration, 10 μ s to 10 s; pulse fre-

quency, 0.01–1000 Hz; and voltage, 10 mV to 150 V). The ESCC system (24 cm × 16 cm × 5 cm) is placed inside a standard cell incubator (ThermoForma, Marietta, OH, USA).

Enzyme Activity Assays

Lactate dehydrogenase activity was measured spectrophotometrically in a reaction mixture containing 0.1 mM NADH and 0.3 mM pyruvate in 100 mM phosphate buffer, pH 7.4. To measure glycogen synthase and phosphorylase activities, frozen cell monolayers were scraped in 100 μ L of homogenization buffer (consisting of 10 mM Tris-HCl, pH 7.0, 150 mM KF, 15 mM EDTA, 600 mM sucrose, 15 mM 2-mercaptoethanol, 10 μ g/mL leupeptin, 1 mM benzamide, and 1 mM phenylmethylsulfonyl fluoride) and sonicated. The samples were centrifuged at 15,000 $\times$ g, and the supernatants were used for the determination of enzymatic activities. Glycogen phosphorylase activity was determined by the incorporation

of α -D-[U- 14 C] glucose 1-phosphate (Amersham Biosciences, Piscataway, NJ, USA) into glycogen in the absence or presence (active form/total activity) of the allosteric activator AMP (5 mM) as previously described (9). Glycogen synthase activity was measured by the incorporation of uridine diphospho-D-[U- 14 C] glucose (Amersham Biosciences) in the absence or presence (active form/total activity) of the allosteric activator glucose 6-P (10 mM) as previously described (10).

Metabolic Measurements

Glucose was measured enzymatically in a Cobas Fara II autoanalyzer (Roche, Basel, Switzerland) with the GlucoQuant kit (Roche). Lactate was measured enzymatically by standard techniques in the Cobas Fara II autoanalyzer. To measure the glycogen content, cell monolayers were scraped into 100 μ L of 30% KOH, and homogenates were boiled for 15 min. Homogenates were spotted onto 31 ET

paper (Whatman, Fungilab, Sant Feliu del Llobregat, Spain), and [14 C]-glycogen was precipitated by immersing the papers in ice-cold 66% ethanol. The papers were washed three times in 66% ethanol and once in acetone and dried at room temperature. Dried papers containing precipitated [14 C]-glycogen were suspended in scintillation fluid for a measurement of the incorporated radioactivity in a scintillation counter (Wallac 1209 Rack Beta; Wallac, Perkin Elmer, Gaithersburg, MD, USA).

Statistical Analysis

Data are presented as the mean ($\bar{x} \pm$ SEM). A Student's *t* test was used for statistical analysis. A value of *P* < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Description and Operation of the Electrical Stimulator of Cultured Cells

ESCC consists of two cards that are placed adjacent to the short axis of standard 24- or 6-well tissue culture plates (Figure 1A). These cards are connected to a commercial stimulator (Grass S-48) that generates variable electrical pulses. The connection cards distribute the electrical signal to up to six equivalent plug sites. To avoid electrolysis, in one of the cards, a capacitor (220 μ F) is connected in a series to the circuit to produce a signal with alternating polarity. The electrodes are made of platinum wire (0.25 mm $\varnothing$). The impedance of the platinum electrode (0.5 mm $\varnothing$) at 100 Hz was 75 Ω at 30°C. End-well electrodes are connected to the adjacent cards (Figure 1B). The portion of the electrode that is immersed in the culture medium has a semicircular shape that parallels the well walls. The dimensions of the arcs are 1 and 2.5 cm for 24- and 6-well electrodes, respectively. Internal or bridge electrodes (Figure 1B) are immersed in intermediate wells of every row and connect them, in a series of two by two, so that the plates are divided into equivalent independent rows. Connection of every row may be turned on or off by six switches that are incorporated into the connection

Figure 1. Image of the electrical stimulator of cultured cells (ESCC) system. (A) Global perspective of the ESCC device with the two connection cards (c) flanking a standard 6-well tissue culture plate. The internal components of the connection cards are indicated: the capacitor (Cp), switches (s), and plug sites for end-well electrodes (p). (B) Diagram of the ESCC system showing the design and position of the end-well electrodes connected to the connection cards and the internal bridge-well electrodes connected in series in a row of a 24-well plate.

cards (Figure 1A). Even though the pulse generator creates square-shaped monopolar pulses (Figure 2), the signal emerging from the connection cards is a bipolar pulse, with a quasi-square shape in the positive signal (Figure 2). The signal declines exponentially with time, with a time constant of 400 ms, giving a maximum decline of 7% for a pulse duration of 30 ms. The negative cycle has an exponential shape and a much lower intensity level, thus the excitation is induced by the positive cycle. The resistance of the culture medium in a 24-well plate filled with 1 mL was $227\ \Omega$ at 25°C and 100 Hz, while that of a 6-well plate filled with 4 mL was $297\ \Omega$. The homogenous electrical field of every row was confirmed experimentally by voltmeter (Philips, Eindhoven, The Netherlands) measurements within points B and C (Figure 2). When applying 20 V to the 24- or 6-well plate filled with saline media, the intensity of the current (root mean square for the positive cycle) for every row was 8.1 ± 0.1 and 21.6 ± 0.2 mA, respectively. Considering the impedance values of the electrodes and the saline content in the wells, we calculate that in the 24-well plate, 25% the voltage output generated by the stimulator decays in electrodes and 75% in the wells. The

electrical field within the well is not homogenous because it is a function of the electrode shape and decreases from the center toward the end of the diameter line perpendicular to the electrical field, with a maximal difference of 13% and 30% for the 24- and 6-well plates, respectively. In this diameter line, the estimated current density is $10.2\ \text{mA}/\text{cm}^2$ and $14.5\ \text{mA}/\text{cm}^2$ for the 24- and 6-well plate, respectively.

To test the applicability of the ESCC, a range of stimulation parameters were applied to C2C12 mouse skeletal myotubes, including voltages of up to 100 V, pulse durations of up to 100 ms, and pulse frequencies of up to 10 Hz. Observation under a microscope [(Figure 3 and video) (Video is available as supplementary material at the BioTechniques Web site at <http://www.BioTechniques.com/Jan04/SupplementaryMaterial/MarottaVideo.html>)] revealed that most cells in the monolayer contracted synchronously at voltages higher than 15 V and pulse durations longer than 1 ms. Stimulation at a frequency of 1 or 3 Hz caused most of cells in the monolayer to contract synchronously and in accordance with the pace-programmed conditions (see video). At frequencies higher than 10 Hz, no twitch responses were observed.

Because the ESCC system is lightweight, it can be placed in a standard cell incubator to maintain the cells in controlled conditions, allowing for medium to long-term stimulation periods. We then evaluated whether long-term electrical stimulation could disturb cell viability. To this end, we used stimulation conditions of 50 V, 30 ms, and 3 Hz, which caused effective contraction, and applied them for 90 min to the cells placed in the standard incubator. We assessed the release of lactate dehydrogenase into the medium at the end of the stimulation period. This activity is used as a marker of alterations in the integrity of the cell membrane. We found no difference in lactate dehydrogenase activity between media from resting and contracting cells (17.9 ± 0.6 and 17.3 ± 0.3 U/L, respectively). These data demonstrated that the device allows for medium to long-term stimulation periods without compromising cell viability.

Regulation of Glucose and Glycogen Utilization in Electrically Stimulated C2C12 Cells

We then studied changes in glucose metabolism in C2C12 myotubes following contraction. C2C12 myotubes were incubated in 25 mM glucose-containing medium to induce maximal glycogen accumulation, as glycogen is the main metabolic reservoir that provides the energy for contraction (11). The cells were then switched to a 5 mM glucose medium and electrically stimulated at 50 V, 30 ms, and 3 Hz or left unstimulated for 90 min. We measured the glucose clearance from the medium at the end of the stimulation period. Glucose removal was higher in electrically stimulated ($3.3 \pm 0.2\ \mu\text{mol glucose}/\text{mg protein}/\text{h}$) than in resting cells ($2.1 \pm 0.4\ \mu\text{mol glucose}/\text{mg protein}/\text{h}$).

Subsequently, we examined the glycolytic activity. Lactate production was measured in cells incubated with 5 mM glucose or in cells deprived of glucose during the stimulation period to indirectly evaluate glycogen mobilization. Lactate production in contracting cells was double that found in resting cells, independently of the presence of glucose in the incubation media (Figure 4A), which is consistent with the role

Figure 2. Electrical schema of the electrical stimulator of cultured cells (ESCC) system. PG is the pulse generator. Cp is the decoupling capacitor, which avoids any direct current flowing through the cell medium. The switches (S1–S6) allow for the selection of the well rows. S1, S3, S4, and S6 are used for the 4-row (24-well) plates, while S2 and S5 are used for the 2-row (6-well) plates. (A) The test points placed next to the switches allow monitoring of the applied voltage, while (B and C) the test points connected to the resistors R1–R6 allow the measurement of the resulting electrical current at each well row.

Table 1. Glycogen Synthase and Glycogen Phosphorylase Activities

	Total MGP Activity (mU/mg protein)	MGP Activity Ratio (-AMP/+AMP)	Total MGS Activity (mU/mg protein)	MGS Activity Ratio (-G6P/+G6P)
NCH stimulated	55.6 ± 1.4	0.76 ± 0.01	16.0 ± 0.2	0.025 ± 0.001
Electrically stimulated	52.1 ± 2.5	0.61 ± 0.03**	15.1 ± 0.4	0.038 ± 0.003*

C2C12 myotubes were incubated in a standard incubator at 37°C for 90 min, with Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose and nonstimulated or electrically stimulated with the electrical stimulator of cultured cells (ESCC) device at 50 V, with a pulse duration of 30 ms and a frequency of 3 Hz. Glycogen synthase (MGS) and phosphorylase (MGP) activities were measured at the end of the stimulation period as described in the text. Data are shown as means ($\bar{x} \pm$ SEM) of three independent experiments performed in triplicate. The significance of the differences between nonstimulated and electrically stimulated cells was * $P < 0.05$ and ** $P < 0.02$. G6P, glucose 6-P.

of lactate as a physiological marker of muscle contraction. Nevertheless, as expected, lactate release was at least 5-fold higher in glucose-incubated cells than in glucose-deprived cells, regard-

less of electrical stimulation.

To confirm whether glycogen mobilization was stimulated upon contraction, cell glycogen was prelabeled by incubation with 5 mM [U - ^{14}C] glucose

for 16 h. The cells were then incubated for 90 min with or without 5 mM glucose under electrical stimulation or nonstimulated conditions. At the end of this period, glycogen-associated radioactivity was 30% and 36% lower in contracting cells than in resting cells, respectively, with or without

glucose (Figure 4B), whereas glucose withdrawal caused a 20% reduction in nonstimulated cells.

The impact of contraction on the activity of glycogen synthase and glycogen phosphorylase, which are the rate-limiting enzymes for the synthesis and hydrolysis of glycogen, respectively, was examined. The total activity of either enzyme was unaltered by electrical stimulation of glucose-incubated cells (Table 1). Because both enzymes are interconverted by phosphorylation, which modulates their activation state in the opposite direction, we assessed the ratio of active form to total activity. We found that at the end of the 90-min stimulation period, the glycogen synthase activity ratio (with or without glucose 6-phosphate) was 52% higher in contracting cells than in controls. In contrast, the glycogen phosphorylase activity ratio (with or without AMP) was 24% lower in contracting fibers.

These results show that electrical stimulation of C2C12 myotubes by means of the ESCC system induces contractility and metabolic adaptations that imitate those occurring in exercising muscle (1,2). First, in spite of elevated glycogen stores, cell contraction stimulated glucose uptake and enhanced lactate production. Furthermore, we observed that contraction and glucose depletion acted as independent and additive factors to trigger glycogen mobilization. Second, we were able to correlate the stimulation of glycogenolysis with opposing effects on the activation state of glycogen synthase and glycogen phosphorylase, which were activated and inactivated, respectively. This pattern of changes was similar to that observed in vivo after glycogen-depleting exercise in the rat (12). In the energy-exhausted muscle, glyco-

Figure 3. Micrograph of contracting C2C12 myotubes. C2C12 myotubes were stimulated with the electrical stimulator of cultured cells (ESCC) device at 50 V, 30 ms of pulse duration, and 3 Hz of frequency. (A) Resting cells. (B) Contracting cells. Morphological differences in contracting myotubes are marked by arrows ($\times 200$).

Figure 4. Lactate production and glycogenolysis assays. C2C12 myotubes were incubated in a standard incubator at 37°C for 90 min in media without (white bars) or with 5 mM glucose (gray bars) and were nonstimulated or electrically stimulated with the electrical stimulator of cultured cells (ESCC) device at 50 V, with a pulse duration of 30 ms, and a frequency of 3 Hz. (A) Lactate concentration was measured in the culture medium at the end of the stimulation period. (B) Cells were preincubated for 16 h with 5 mM [U - ^{14}C] glucose (0.03 μ Ci/ μ mol) before the 90-min treatment. The radioactivity associated with glycogen was measured at the end of the stimulation period. Data are shown as the mean ($\bar{x} \pm$ SEM) of three independent experiments performed in triplicate. The significance of the differences between nonstimulated and electrically stimulated cells were * $P < 0.005$ and ** $P < 0.002$.

gen synthase is activated while phosphorylase is inactivated, compared to resting or preexercising muscle. Such changes are interpreted as a metabolic adaptation that drives a rapid recovery of glycogen after the termination of contraction.

Here we describe a new device, the ESCC, which is capable of simultaneously stimulating electrically a large number of independently cultured cells over long periods of time. The ESCC overcomes the limitations of previously reported systems. It is adaptable to cells cultured in 6- or 24-well standard tissue culture plates and generates a homogenous electrical field in every row of the culture plate. The ESCC system allows long-term stimulation in standard incubation conditions. Finally, it can produce a range of outputs based on the stimulation parameters it receives from the pulse generator. Therefore, this system may have a broad range of applications in excitable muscle (skeletal, smooth, or cardiac) or neural cells to study ensuing molecular or biochemical changes.

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