Journal of Muscle Research and Cell Motility **18**, 587–598 (1997)

Expression of ion channels during differentiation of a human skeletal muscle cell line

J. L. LIBERONA[1], P. CAVIEDES[1,2], S. TASCÓN[2], J. HIDALGO[2], J. R. GIGLIO[3], S. V. SAMPAIO[4], R. CAVIEDES[1*] and E. JAIMOVICH[1,2]

[1]*Departamento de Fisiología y Biofísica, Faculty of Medicine, Universidad de Chile, Santiago and* [2]*Centro de Estudios Científicos de Santiago, casilla 16443, Santiago 9, Chile,* [3]*Departamento de Bioquímica, Facultade de Medicina de Riberâo Preto and* [4]*Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Facultade de Ciências Farmacêuticas de Riberâo Preto, Universidade de Sao Paulo, Brazil*

Summary

An immortal, cloned cell line (RCMH), obtained from human skeletal muscle was established in our laboratory and shown to express muscle specific proteins. We measured ligand binding to ion channels, ion currents using whole cell patch clamp and intracellular calcium both in cells grown in complete media and in cells grown for 4–40 days in media supplemented with hormones and nutrients (differentiating media). Markers for differentiated muscle, such as the muscle isoform of creatine kinase and the cytoskeletal proteins α-actinin, α-sarcomeric actin, myosin and titin were present in early stages. Receptors for γ toxin from *Tityus serrulatus* scorpion venom, a specific modulator for voltage dependent sodium channels, were present (0.9–1.0 pmol mg^{-1} protein) during stage 1 (0–6 days in culture with differentiating media) and increased by 50% in stage 3 (more than 10 days in differentiating media). High and low affinity dihydropyridine receptors present in stage 1 convert into a single type of high affinity receptors in stage 3. Both intracellular calcium release and $InsP_3$ receptors were evident in stage 1 but ryanodine receptors were expressed only in stage 3. RCMH cells showed no voltage sensitive currents in stage 1. Between 7 and 10 days in differentiating media (stage 2), an outward potassium current was observed. Small inward currents appeared only in stage 3; we identified both tetrodotoxin sensitive and tetrodotoxin resistant sodium currents as well as calcium currents. This pattern is consistent with the expression of voltage dependent calcium release before appearance of both the action potential and ryanodine receptors.

Introduction

The goal of the present work was to study both the expression of ion channels and membrane potential–calcium release coupling during the process of differentiation of a permanent cell line of muscle origin. A cell line (RCMH) in permanent culture was established from biopsies of adult normal human skeletal muscle (Caviedes *et al*., 1992); these cells proliferate indefinitely in complete media containing 10% bovine serum and 2.5% foetal calf serum but have density inhibition of growth and maintain some differentiated markers. Multinucleated myotube-like structures and muscle specific proteins increase after transfer of RCMH to media containing 5% horse serum for a period of 48 h. Cells have high affinity receptors for α-bungarotoxin and for dihydropyridines at levels comparable to those of muscle cells in primary culture (Caviedes *et al*., 1992). A wide range of processes contribute to mechanical activation in skeletal muscle. Early events in excitation–contraction coupling involve several ion channels and receptors, namely nicotinic acetylcholine receptors at the neuromuscular junction, voltage dependent sodium and potassium channels at the sarcolemma and T-tubule membranes, voltage dependent dihydropyridine receptors in the T-tubule and one or more types of calcium release channel in the sarcoplasmic reticulum. On the other hand, voltage dependent calcium release may be related to mechanisms other than excitation–contraction coupling, i.e. processes related to myogenesis itself.

Expression of various ion channels have been studied both in rat embryo and in developing muscle cells *in vitro*: (1) dihydropyridine receptor density increases sharply upon development

*To whom correspondence should be addressed.

(Romey *et al.*, 1989, Kyselovic *et al.*, 1994); (2) L-type calcium currents parallel the onset of dihydropyridine receptor expression (Kidokoro, 1975; Romey *et al.*, 1989; Shimahara & Bournaud, 1991; Cognard *et al.*, 1993); (3) T-type calcium currents appear transiently during muscle development (Gonoi & Hasegawa, 1988; Rohwedel *et al.*, 1994) but not during primary culture differentiation (Cognard *et al.*, 1993); (4) sodium channels and sodium currents also increase during muscle development (Frelin *et al.*, 1983; Sherman *et al.*, 1983; Gonoi *et al.*, 1985; Weiss & Horn, 1986). In mammalian muscle, large shifts in tetrodotoxin (TTX) sensitivity occur during muscle development both *in vivo* and *in vitro* and the simultaneous use of ion flux and radioligand techniques has led to the definition of several sodium channel subtypes in developing muscle cells in culture (Frelin *et al.*, 1983, 1984); (5) potassium channel genes seem also to be developmentally regulated both in embryonic muscle and in a muscle cell line (Lesage *et al.*, 1992); (6) acetylcholine receptor channels also change during muscle development (Mishina *et al.*, 1986; New & Mudge, 1986); and (7) ryanodine receptor channels are seen only in mature muscle cells (Marks *et al.*, 1989; Airey *et al.*, 1991; Kyselovic *et al.*, 1994). Particularly intriguing are the temporal differences in the induction of dihydropyridine receptors and ryanodine receptors during skeletal muscle development (Kyselovic *et al.*, 1994), results that suggest that coupling between excitation and calcium release may differ in different developmental stages.

In order to use RCMH cells as a model for channel involvement in cell function, we explored both plasma membrane and intracellular ion channel expression as well as membrane potential induced calcium release during differentiation.

Materials and methods

Cell culture

Morphological and biochemical properties of a new muscle cell line (RCMH) have been reported elsewhere (Caviedes *et al.*, 1992). Cells were maintained in growth medium (GM) consisting of equal volumes of Ham F_{44} and Dulbecco's modified Eagle's medium (Sigma Chemical, St. Louis, MO), 10% bovine serum, 2.5% foetal bovine serum, 15 mM Hepes (pH 7.2), plus 50 mg l^{-1} streptomycin sulphate and 10 mg ml^{-1} sodium-penicillin-G. Cultures were incubated at 36° C, 100% humidity in an incubator with controlled 10% CO_2, 90% air atmosphere and total media changes performed every 3 days. All cultures were mycoplasma free as judged by standard bacteriological procedures.

Cell differentiation was induced using the method described by Olson and colleagues (1986); cultures were transferred to GM with 1% bovine serum and 10 μg ml^{-1} of both insulin and transferrin. In most experiments, differentiating media consisted of F12/D supplemented with 1% bovine serum and 1% stock supplement as described by Orozco and colleagues (1987). In these conditions, cells differentiated with little or no fusion occurring. In some experiments, fusion was attained after 2–4 days in 10% horse serum.

Immunocytochemical procedures

Cultures grown on glass coverslips were either frozen in liquid nitrogen or fixed for 15 min at −20° C, first with methanol and then with acetone. After rinsing in PBS, a drop of the diluted primary antibody was placed over the monolayer and biotinylated or unconjugated linking antibodies were added. Peroxidase anti-peroxidase (PAP) or streptavidin were used to visualize the antigen–antibody reaction. Muscle proteins such as myosin or α-actinin were analysed using polyclonal antibodies; α-sarcomeric actin and titin were analysed using monoclonal antibodies. Antibodies and PAP complexes were obtained from Sigma Chemical Co., St. Louis, MO, USA. Anti-human skeletal myosin, developed using myosin heavy and light chains was reported to specifically stain A-band of human and animal skeletal muscle but not smooth muscle. All antibodies used were reported to be specific for striated muscle. Control experiments in which the first antibody was replaced by normal serum from the corresponding animals were routinely performed.

Creatine phosphokinase isoenzymes

The creatine phosphokinase (CPK) isoenzymes were separated electrophoretically in agarose gels (0.8% v/v). The cells were lyophilized and then homogenized before electrophoresis. The samples were run at 50 V in a solution containing 0.1 M Tris–Acetic acid (pH 6.9) for 2 h. As isoenzyme standards, homogenate extracts from skeletal muscle (MM isoenzyme), cardiac muscle (MB isoenzyme) and rat brain (BB isoenzyme) were used. The gel was incubated at 37° C protected from light, in a medium containing 750 μM phosphocreatine, 500 μM glucose, 50 μM ADP, 125 μM AMP, 546 nM PMS, 240 μM $MgCl_2$, 50 IU hexokinase, 40 IU G6-PDH, 50 μM NADP, 9 μM TNBT and 0.1 M Tris–acetate buffer (pH 6.9). The coloured and insoluble TNBT–formazan complex reveals the CPK activity in the gels. The gels were fixed in methanol–acetic acid mixture and then dried over paper.

Iodination of TsTX-γ

Gamma-Toxin purified from *Tityus serrulatus* venom according to previously published procedures (Sampaio *et al.*, 1983), was iodinated using, with minor modifications, the chloramine-T method (Hunter & Greenwood, 1962). Briefly, TsTX-γ (50 μg) was incubated with 600 μCi of $Na^{125}I$ (Chilean Nuclear Energy Commission) in 90 μl of 10 mM Tris–HCl (pH 8.6). Four aliquots (10 μl) of a 10 mM solution of chloramine-T was added at 30 s intervals. The mixture was incubated for 20–30 min after addition of the last chloramine-T aliquot, and was immediately loaded on a SP-Sephadex C-25 (2 × 10 cm) column equilibrated and eluted with 50 mM NaH_2PO_2 buffer (pH 6.0) containing 160 mM NaCl. Under those conditions, all the toxin was adsorbed by the Sephadex matrix while the free iodine was

readily eluted. After passing 3–4 column volumes of buffer solution, the solution was changed to a mixture of 50 mM phosphate buffer (pH 6.0) with 500 mM NaCl. The elution of the labelled toxin was monitored by measuring the radioactivity and the absorbance at 260 and 280 nm in each fraction.

Binding experiments

Receptors for ion channel markers were measured using techniques previously described (Jaimovich *et al*., 1986). ^{125}I-TsTX-γ, ^{3}H-PN200-110, ^{3}H-Inositol(1,4,5)trisphosphare ($InsP_3$) and ^{3}H-ryanodine were used as markers for voltage dependent sodium channels (Barhanin *et al*., 1983; Norman *et al*., 1983; Arispe *et al*., 1988), voltage dependent calcium channels (voltage sensors) and calcium release channels respectively.

RCMH cells were washed three times with 5 ml PBS buffer and detached using a rubber policeman in ice-cold buffer (120 mM choline chloride, 2.54 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, 10 mM Tris–HCl pH 7.4).

Homogenization was performed in an Ultrasonic Homogenizer for 10–15 s. In the equilibrium binding, cell homogenates (0.1–0.2 mg ml^{-1}) were incubated in the same buffer with ^{125}I-TsTX-γ (0.5–10 nM) at room temperature by 30–40 min in the presence or absence of an excess of cold ligand (0.1 μM) to measure non-specific binding.

^{3}H-Ryanodine binding was measured in cell homogenates following the same protocol. The incubation medium contained 0.5 M KCl, 0.1 mM $CaCl_2$, 20 mM Hepes–Tris (pH 7.1) and 1 mM AMP-PNP or 0.5 mM ATP; samples were incubated with ^{3}H-Ryanodine (5–100 nM) for 90 min at 37° C, in the presence or absence of cold ryanodine (10 μM).

^{3}HPN200-110 binding was measured as previously described (Caviedes *et al*., 1992), in the presence or absence of nifedipine (2 μM) to measure non-specific binding.

^{3}H-$InsP_3$ binding was measured as follows: confluent plates were rinsed three times with PBS and homogenized by sonication for 10–15 s and incubated in a medium containing 50 mM Tris–HCl (pH 8.4) 1 mM EDTA, 1 mM 2Me-EtOH and variable concentrations of ^{3}H-$InsP_3$ (stock 1 μM^{3}H-$InsP_3$, specific activity 734 cpm pmol^{-1}) at 4° C for 30–40 min. Binding was stopped by centrifugation at 10 000 × g for 10 min (Heraus Biofuge 13), followed by aspiration of the supernatant. Pellets were dissolved in 1 M NaOH and the associated radioactivity was measured. The non-specific binding was determined in the presence of 2 μM $InsP_3$.

Patch clamp methodologies

The whole-cell (for macroscopic currents) configuration of the patch clamp technique was used (Hamill *et al*., 1981). The cells were visualized in an OLYMPUS CK-2 inverted microscope equipped with phase contrast optics. Glass electrodes (BLUETIP, Monoject Scientific, St. Louis, MO) were pulled in several stages using a Sachs-Sutter PC-84 programmable puller (Sutter Instruments Corp., San Francisco, CA), to yield open tip resistances of 1–8 MΩ.

Prior to recording, the cells were rinsed with 1 ml of saline with, unless otherwise indicated, the following composition (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES-Na or 120 NaCl, 5 KCl, 20 TEACl, 1 $MgCl_2$, 0.05 $CaCl_2$ or 150 TEACl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 Hepes-Na and 10 dextrose adjusted with NaOH to pH 7.4. The pipettes were filled with a normal intracellular solution consisting of (mM): 140 KCl, 1 $CaCl_2$, 11 EGTA-KOH, 10 HEPES-KOH, 10 dextrose (pH = 7.0), or with solutions of a different composition where indicated. Gigaohm seals (2–10 GΩ) were established between the patch pipette and the cell membrane by applying a soft suction pulse inside the pipette. After cancelling the fast capacity current transients using the null-bridge circuitry of our Axopatch-1C patch clamp amplifier (Axon Instruments, Foster City, CA), the whole cell configuration was achieved by applying a brief, stronger pulse of negative pressure. A minimum of 2 min was allowed for exchange of solution between the pipette and the cell, and to stabilize the membrane resting potential. Command voltage and current steps were generated by an AXOLAB digital-to-analog converter board (Axon) controlled by a customized program pClamp 5.5 (Axon) installed in a dedicated IBM PC-AT computer. Cell responses were sampled at rates of 10–50 KHz. All recordings were conducted at room temperature (20–22° C).

(1) *Current clamp*: passive properties were studied in current clamp mode, in response to hyperpolarizing pulses of 0.1 and 0.05 A amplitude, 200 ms duration. Membrane resistance was estimated as the maximal voltage amplitude in steady-state, divided by the applied current pulse amplitude. The membrane time constant (τ) was taken as the point in time corresponding to 0.632 of the maximal voltage amplitude. Alternative determinations of cell capacitance by integrating the capacitive spike in the voltage clamp configuration, which yielded the effective charge of the membrane (Q), which was related to the applied voltage, provided comparable results of capacitance to those obtained from the voltage trace in current clamp condition. Calculated cell capacitance (as $C = \tau R^{-1}$) was used to evaluate cell area (assuming 1 μF cm^{-2}) for calculation of ionic current density.

(2) *Voltage clamp*: the cells were clamped at −80 to −120 mV (more negative for very small currents), and depolarizing steps of 70 ms duration with increments of 10–20 mV were presented at 0.5 Hz. Capacitative and leak currents were compensated using on-line substraction with a conventional P/P/4 protocol (Hille, 1984). The records were composed of 1024 sample points, where the first segment (5 ms) was used to form the base line. The second segment (100 ms) was used to form the non-linear ion specific record of the current during the pulse, which is obtained by the addition of the transient during the pulse and the control transient of the current response to the corresponding hyperpolarizing pulses. Positive-feedback series-resistance compensation was not used. Voltage errors were calculated assuming a series resistance of twice the open tip resistance of the electrode, multiplied by the peak amplitude of the active membrane current. Only traces with voltage errors less than 3 mV were used. Peak currents were measured and corrected for cell area to obtain current density. External modulators of channel activity (tetrodotoxin) were applied externally with a pipette.

Calcium release

Intracellular calcium was measured in cells loaded with fluo3-AM (20–40 min in saline containing 5.4 μM fluo-3 AM, Molecular Probes). Confocal fluorescence images after 488 nm Ar laser excitation were acquired and analysed with an inverted microscope (Carl Zeiss Axiovert 145 M from the Centro de Equipamiento Mayor, Facultade de Ciencias, Universidad de Chile). Cells were grown in a round glass coverslip that was mounted as the bottom of a superfusion chamber in the microscope stage. The fluorescent images were collected every 0.2–1.0 s and analysed frame-by-frame with the data acquisition program of the equipment.

Chemicals

Reagents, culture media and antibodies were purchased from SIGMA Chemical (St. Louis, MO). Bovine and foetal calf serum were prepared from blood obtained at a local abattoir, clarified, sterilized and inactivated in our laboratory. ^{3}H-PN 200-110 (86 Ci m^{-1} mol) and ^{3}H-inositol (1, 4, 5) trisphosphate (21 Ci mmol^{-1}) were obtained from New England Nuclear (Boston), nifedipine was obtained from Bayer (Darmstad, Germany), ^{3}H-ryanodine was kindly provided by Dr J. L. Sutko (Reno, Nevada).

Results

We studied mononucleated cells growing in complete media as well as cells placed in differentiating media for up to 18 days. On the basis of both their morphology and expression of several markers, we can distinguish a less differentiated stage (stage 1) in which we classify both cells growing in complete media and cells incubated in differentiated media for less than 6 days. After 11 days in differentiating media, cells attained a distinct stage (stage 3) characterized by consistent expression of ion currents and channel markers. The intermediate, transitional stage (stage 2) includes cells with variable properties, incubated for 6–9 days in differentiating media, expressing only some of the differentiated markers.

Immunocytochemical and enzyme markers

Both dividing cells in complete media and non-fused cells 2–5 days in differentiating media (stage 1) display muscle specific markers. Figure 1A, shows the immunostained RCMH cells (stage 1) labelled with anti-α-sarcomeric actin antibodies conjugated with streptavidin-peroxidase. Immunostained granular bodies were evident through all the area of cell cytoplasm, while nuclei remained unstained. The anti-α-sarcomeric actin antibodies used are specific for skeletal and cardiac muscle and do not react with smooth muscle. Figure 1B shows the presence of titin by immunospecific staining with anti-titin antibodies conjugated with streptavidin-peroxidase. Again the cytoplasmic staining is evident, this antibody does not have cross-reaction with nebulin and is specific for striated muscle. Figure 1C illustrates the specific immunostaining against α-actinin in RCMH cells, detected with the same procedures (cells in stage 1). When non-immune serum replaced the specific antibody during the incubation procedure (Fig. 1D) or when an excess of externally added antigen was present (not shown), cells were not stained. The presence of creatine kinase, another muscle phenotype marker, was detected in cells in stage 1 by studying the CPK isoenzyme in a 0.8% agarose gel. Figure 1E shows staining for standard MM, MB and BB isoenzyme (lanes 1–3) and for an undifferentiated RCMH cell homogenate; it is clear that the MM or skeletal muscle isoenzyme is the only form detected in these cells. In general, differentiated cells (stage 3) were also positively stained for these markers; the activity of CPK MM isoenzyme increased in stage 3 as cells differentiated (not shown).

Receptors for TsTX-γ

Specific binding at equilibrium of ^{125}I-TsTX-γ toxin to RCMH cell homogenates in different stages of differentiation is illustrated in Figure 2. The Eadie–Scatchard analysis (see inset) shows one type of receptors both for undifferentiated cells in stage 1 (upper panel) and for differentiated cells in stage 3 (lower panel). Cells placed for 11 days in a culture medium that favours differentiation (supplemented medium) displayed a 50% increase in specific binding of ^{125}I-TsTX-γ toxin (Table 1). The presence of TsTX-γ toxin receptors in stage 1, where no sodium currents were seen (see below), suggests the existence of 'immature' or 'silent' channels in these cells; in order to test the possibility that part of the measured binding would correspond to intracellularly located 'immature' receptors, we compared binding to cell homogenates (stage 1) with binding to intact, trypsinized cells in the same stage (Table 1) and we got essentially the same figures. As TsTX-γ is a polar peptide that does not permeate the cell membrane, all receptors must be located at the extracellular face of the cell membrane.

Macroscopic currents

Using the whole-cell variation of the patch-clamp technique, we recorded macroscopic currents resulting from voltage clamp pulse protocols with normal Ringer's solution in the bath and using potassium as the major cationic component in the recording pipette. The type of current detected was a function of the cell's stage of differentiation; most cells dividing in complete media (stage 1) were electrically silent. Twenty cells in this stage were tested (Table 2) using the conditions described in the methods section; many others were tested in a variety of conditions which included 37° C tempera-

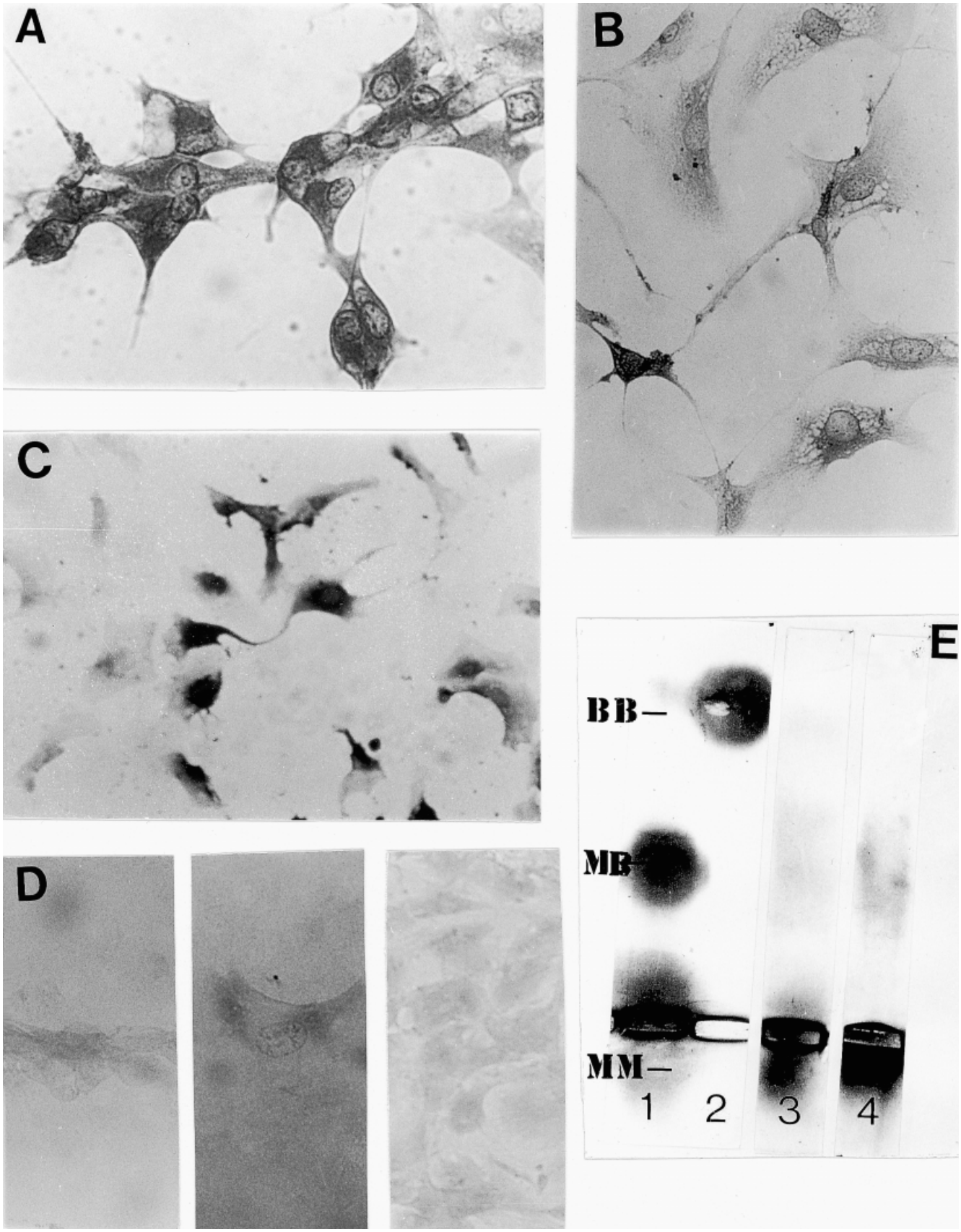

Fig. 1. (A) RCMH cells immunostained for α-sarcomeric actin. Cytoplasmic staining is evident. (B) Presence of titin in RCMH cells as shown by immunospecific staining. (C) Presence of α-actinin in RCMH cells, detected by the same procedures. (D) Control experiments for (A–C); the specific antibody was replaced by non-immune serum. (E) CPK isoenzymes in a 0.8% agarose gel. The figure shows the MM isoenzyme for undifferentiated RCMH cells in stage 1 (lane 4); migration of standards is indicated. Lane 1, cardiac muscle; lane 2, brain; lane 3, skeletal muscle.

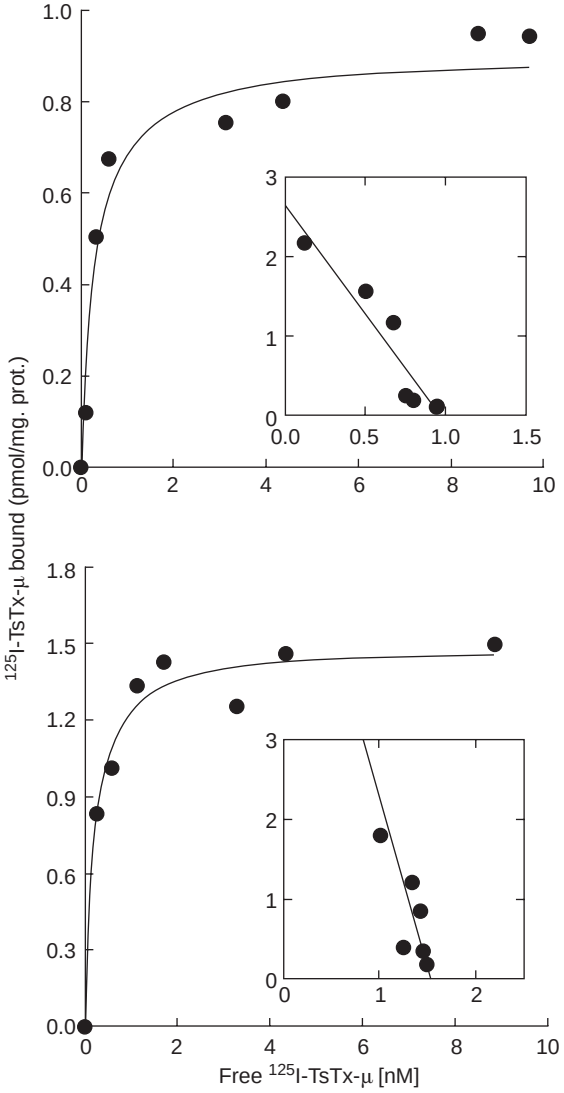

Fig. 2. Upper panel. Specific binding at equilibrium of ^{44}I-TsTX-γ to homogenates of RCMH undifferentiated cells in stage 1. The Eadie–Scatchard analysis (inset) shows one type of receptor. Curve-fitting gave the following values for the dissociation constant, $K_d = 0.32$ nM and maximal binding capacity, $B_{max} = 0.92$ pmoles mg^{-1} protein (prot.). Lower panel. Specific binding at equilibrium of ^{125}I-TsTX-γ to homogenates of RCMH cells exposed 10–12 days to a differentiating culture media (stage 3). The Eadie–Scatchard analysis shows one type of receptor. Curve-fitting gave the following values for $K_d = 0.20$ nM and $B_{max} = 1.49$ pmol mg^{-1} protein.

ture and gelatin or collagen coated dishes, with similar results. When cells were cultured in media with reduced serum and addition of various hormones and differentiating factors, two additional stages of differentiation could be characterized on the basis of ion currents (see Figs 3 and 6).

In stage 2 (6–9 days in differentiating medium), outward currents were recorded using potassium in the pipette. In some cells we found that these currents showed a biphasic time course at strong depolarizing test pulses, which could be attributed to either inactivation or blockade of a subclass of potassium channels. In general, the results are consistent with the presence of either rectifier or calcium activated potassium channels. The absence of fast inward currents in stage 2 contrasts with the presence of receptors for a specific sodium channel modulator in this and in the previous stage (see above). Stage 3 is characterized by the presence of fast inactivating inward currents (28 of 32 cells recorded) as depicted in Figure 3 (lower panel). Current density was estimated as 1.6 pA/pF for inward currents and 3 pA/pF for outward currents. The holding potential used varied in different cells in order to record the maximal current in each experiment. Development of ion currents during differentiation appears unrelated to cell fusion since records obtained in mononucleated cells did not differ from those obtained in fused, multinucleated cells. Either a significant increase in cell size or development of an open transverse tubular system can be discarded because we see no significant changes in membrane capacity nor in other passive electrical properties between stages 1, 2 and 3 of differentiation (Table 2).

Table 1. Binding of ^{125}I-TsTX-γ to RCMH receptors

Condition	*KD [nM]*	*B_{max} (pmol mg^{-1} prot.)*
Homogenates (Stage 1)	0.32 ± 0.08	0.93 ± 0.05
Intact cells (Stage 1)	0.21 ± 0.06	1.13 ± 0.08
Homogenates (Stage 3)	0.20 ± 0.05	1.49 ± 0.06

RCMH cells were washed with PBS solution, detached and homogenized as described in Materials and methods. ^{125}I-TsTX-γ was added to incubation media and the binding was followed by 30 min at room temperature. Intact cells were obtained with trypsin 0.1% (p/v) and 0.02% (p/v) EDTA in PBS (pH 7.0) for 3 min. Values are means ± SEM of at least three experiments.

Sodium currents are seen in isolation using caesium in the pipette and replacing calcium in the external medium (Fig. 4). The signals appeared to be composed mainly of fast transient currents. Two independent sodium current components can be distinguished by using TTX. Up to 40% of the peak inward current was blocked by 1 μM TTX (Fig. 4, lower record). The inset in Fig. 4 shows the voltage dependence for activation of the TTX resistant current (triangles) and of the TTX sensitive component (squares) obtained after subtraction of the lower record from the upper in Fig. 4. Membrane potential for 50% activation was −20 mV for the TTX sensitive and +30 mV for the TTX insensitive component.

Table 2. Active and passive electrical properties of RCMH cells during differentiation.

	Differentiation stage		
Parameter measured	*1*	*2*	*3*
Cells with outward current/cells tested	0/20	18/18	22/23
Cells with inward current/cells tested	0/20	0/18[a]	28/32
Membrane capacity (pF)	136 ± 25	91 ± 7	101 ± 14
Input resistance (MΩ)	218 ± 50	377 ± 128	314 ± 66
Time constant (ms)	29.5 ± 6.2	31.8 ± 10.2	51.1 ± 8.2

[a]In some cells, a small inward current can be seen under appropriate experimental conditions (Fig. 5)

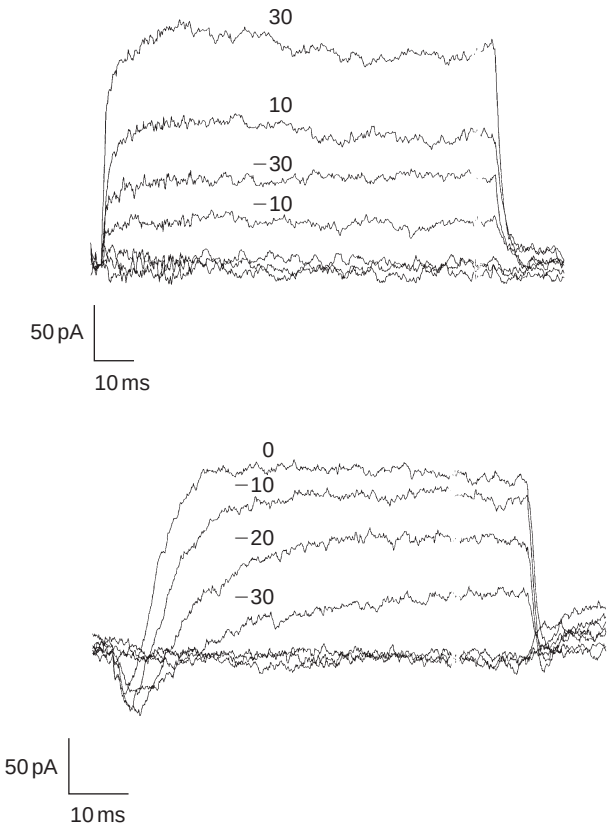

Fig. 3. Stage 2 (upper panel): RCMH cells cultured in differentiating medium for 7 days. Current traces obtained in whole cell voltage clamp conditions using K^+(no Cs^+) solution in the pipette. Outward current density; 3.8 pA/pF; holding level = −120 mV, current responses to steps of 10 mV are displayed. Stage 3: (lower panel). RCMH cells cultured in differentiating medium for 12 days. Inward current density; 1.6 pA/pF; outward current density: 3.6 pA/pF.

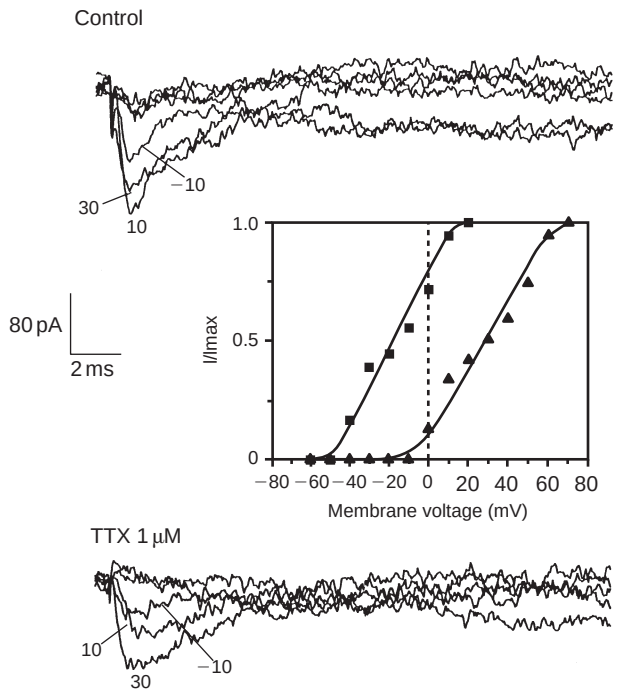

Fig. 4. Sodium current obtained in voltage clamp conditions in RCMH cells, using Cs^+ (no K^+) solution in the pipette and no Ca^{2+} added in the external medium. Upper record: recording of a cell cultured in differentiating medium for 30 days. V_{hold} = −100 mV, responses to 20 mV steps are shown (numbers indicate membrane potential during pulse). Lower record: Effect of 1 μM tetrodotoxin (TTX) applied in the bath to the same cell where the upper record was obtained. Forty per cent of the total peak current is abolished at 4 min in TTX. The extracellular solution consisted of (in mM): NaCl 120, TEA–Cl 20, $MgCl_2$ 1, HEPES–NaOH 10, dextrose 10 (pH 7.4); while the intracellular solution consisted of (mM): CsCl 120, TEA–Cl 20, EGTA–KOH 0.1, HEPES–NaOH 10, dextrose 10 (pH 7.0). The inset shows the voltage dependence for activation of the TTX-resistant current (triangles) and of the TTX-sensitive component (squares) obtained after subtraction of the lower from the upper record.

Dihydropyridine receptors and calcium currents (stages 1 through 3)

The presence of dihydropyridine receptors in undifferentiated (stage 1) RCMH cells (Caviedes *et al.*, 1992) was not accompanied by expression of voltage gated Ca^+ currents. At this stage, the binding isotherm can be fitted to two components; a minor, high affinity population and a relatively large component of low affinity receptors (Fig. 5, open circles). By contrast, in cells at stage 3 of differentiation, a single component of high affinity was apparent (Fig. 5 closed circles). From the Eadie–

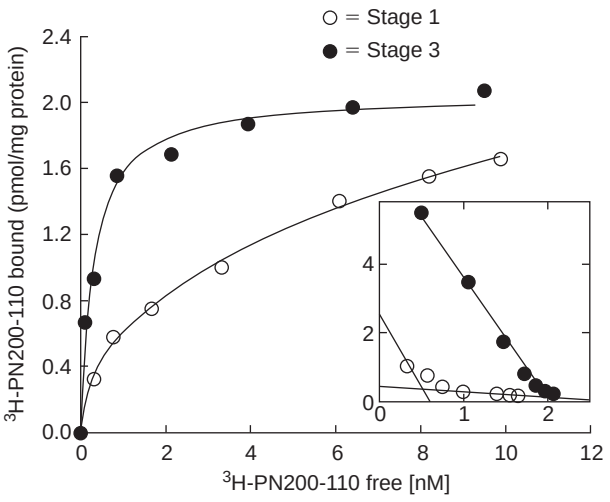

Fig. 5. Specific binding at equilibrium of ^{3}H-PN200-110 to RCMH cell homogenates exposed either to complete media (open circles, stage 1) or 10 days to a differentiating culture media (pmol mg^{-1} protein) (closed circles, stage 3). The Eadie–Scatchard analysis (inset) for stage 1 shows two types of receptor and a single type of receptor for stage 3. Curve fitting gave the following values: stage 1, high affinity component $K_d = 0.29$ nM, $B_{max} = 0.54$ pmol mg^{-1}; low affinity component $K_d = 12.8$ nM, $B_{max} = 2.62$ pmol mg^{-1}. For stage 3, $K_d = 0.28$ nM and $B_{max} = 2.04$ pmol mg^{-1}.

Scatchard plots in Fig. 5 it appears that, after differentiation, high affinity PN200-110 receptors were expressed at three- to four-fold higher levels than at stage 1, although with a similar K_d (0.28 nM). This increase was accompanied both by a reduction of the low affinity receptors and by expression of slow Ca^{2+} currents (see below).

Sustained inward currents with no apparent inactivation were present in stage 3 (Fig. 3); in order to test whether these inward currents are primarily carried by calcium ions, we performed experiments that exclude both Na^+ ions from the external saline and K^+ ions from the pipette, thus allowing the study of Ca^{2+} currents in isolation. After 8 days in differentiating medium (stage 2), a small transient inward current was apparent (Fig. 6, upper panel); this current was slower than those fast transient currents depicted in Figures 3 and 4 and has a different voltage dependence; the I/V curve (Fig. 6, inset) suggests three current components. After 18 days of differentiation (stage 3), a slow, non-inactivating current (Fig. 6, lower panel) becomes the main component. These Ca^{2+} currents may correspond to fast (T-type, activated at −60 to −40 mV), and slow (L-type, activated at 10–30 mV) currents; a third type of current (activated at −10 mV) such as that described in human muscle cells in primary culture (Rivet *et al.*, 1992) cannot be discarded.

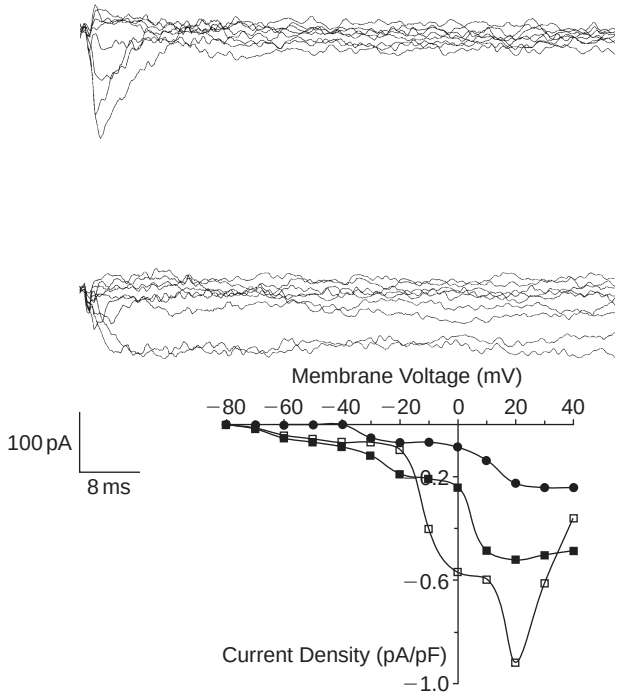

Fig. 6. Ca^{2+} currents in the RCMH muscle cell line. Upper panel shows currents in a cell (stage 2) after 8 days of differentiation (current density: 2 pA/pF), lower panel presents currents in stage 3, after 18 days of differentiation (current density 0.62 pA/pF). The extracellular solution consisted of (in mM): TEA–Cl 150, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES–KOH 10, dextrose 10 (pH 7.4); while the intracellular solution consisted of (mM): CsCl 150, $CaCl_2$ 0.45, EGTA–KOH 5, HEPES–KOH 10, dextrose 10 (pH 7.0). HL = −90 mV, steps of 20 mV are shown. Inset: I/V curves for the peak current of the experiment pictured in the upper panel (open squares) and for both the peak current (closed squares) and steady state current (closed circles) of the lower panel experiment.

Ins P_3 receptors ryanodine receptors and calcium release (stages 1 and 3)

Binding of ^{3}H-InsP_3 to RCMH cells reveals expression of InsP_3 receptors in undifferentiated cells (stage 1); the curve in Figure 7A (open circles) was fitted to a B_{max} of 3.9 pmoles mg^{-1} protein and a K_d of 100 nM. There was a minor change in InsP_3 binding after differentiation; if the same K_d (100 nM) is used, points can be fitted to a B_{max} of 4.6 pmoles mg^{-1} protein (closed circles). The ryanodine receptor, a specific marker for sarcoplasmic reticulum calcium release channels of striated muscle was also studied in these cells. Figure 7B shows the specific binding at equilibrium of ^{3}H-ryanodine to cell homogenates from RCMH cells both cultured in complete media (stage 1) and exposed to a differentiating culture medium (stage 3). Both the curve (closed circles) and the Eadie–Scatchard analysis (inset) for cells in stage 3 was fitted to one type of receptors with K_d = 2.95 nM and B_{max} = 0.73 pmoles mg^{-1} protein.

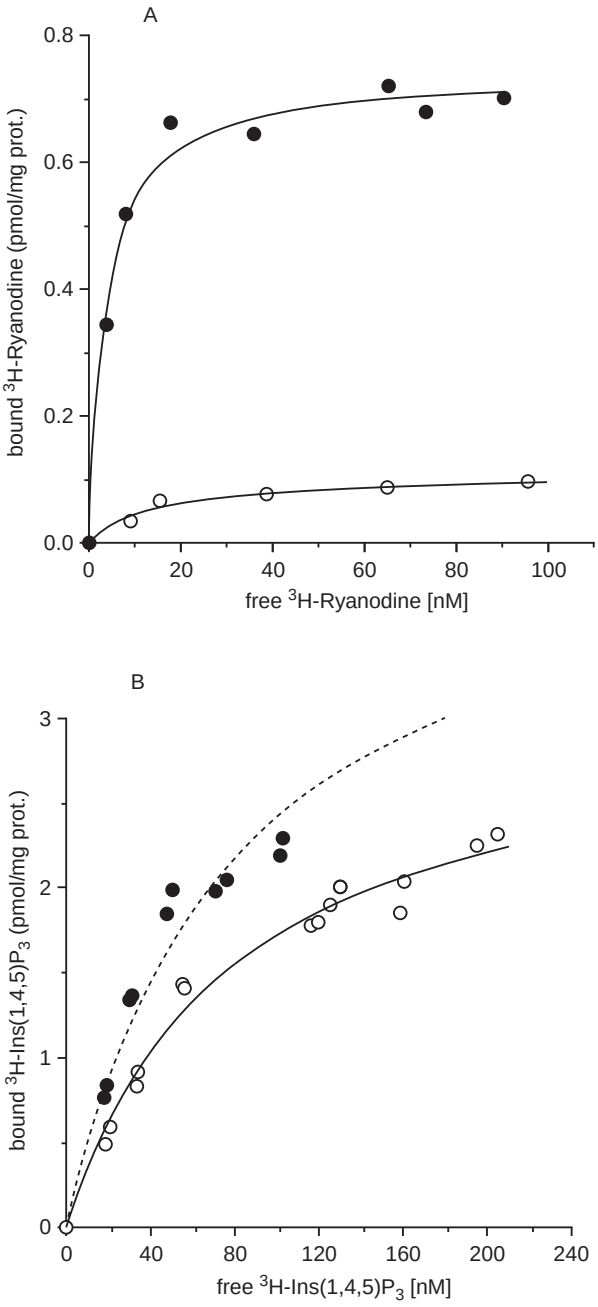

Fig. 7. (A) Specific binding at equilibrium of ^{3}H-Ryanodine to cells homogenates of RCMH exposed 10–12 days to a differentiating culture media (stage 3). Curve fitting gave the following values for $K_d = 2.95$ nM and $B_{max} = 0.73$ pmol mg^{-1} protein. The ^{3}H-Ryanodine binding to cells homogenates of RCMH exposed to a non-differentiating culture media (stage 1) is also shown (open circles); curve was fitted to values for $K_d = 14.5$ and $B_{max} = 0.1$ pmol mg^{-1} protein. (B) ^{3}H-InsP_3 binding was measured as described in Materials and methods; open circles represent binding to non-differentiated cells (stage 1) and the solid line shows fitting to the following parameters: $B_{max} = 3.9$ pmoles mg^{-1} protein and a $K_d = 100$ nM. Solid circles represent binding to differentiated cells (stage 3) and the broken line shows fitting to a $K_d = 100$ nM and $B_{max} = 4.6$ pmoles mg^{-1} protein.

Undifferentiated cells in stage 1 (open circles), revealed a very low density (less than 0.1 pmole mg^{-1} protein) of receptors for ^{3}H-ryanodine.

Calcium release from intracellular compartments after membrane depolarization is a marker for skeletal muscle function. We studied calcium induced fluorescence changes (Fig. 8) in fluo-3 loaded cells in stage 1 upon addition of saline containing 40 mM K^+ (replacing Na^+). A clear increase in fluorescence is seen after 2 s (Fig. 8). Fluorescence slowly returns towards basal values during the next several seconds. As both control and high K^+ saline contained EGTA, the effect could not be attributed to external Ca^{2+} (0.6 nM free external calcium was estimated in the experimental conditions used). Membrane potential was measured in these cells in normal saline and was found to be -32 ± 6 mV ($n = 10$); it was reduced to -14 ± 3 mV ($n = 12$) for cells incubated in solutions containing 40 mM K^+.

Discussion

The main findings for this model cell line can be summarized as follows; (1) this is the first human muscle cell line in permanent culture that, after more than 200 passages and while growing in standard culture media is able to express a variety of muscle cell markers; (2) upon differentiation of this cell line, expression of several ion channels was enhanced; and (3) differential expression of channels and in particular that of two types of intracellular calcium release channels make this cell line a good model to study the different mechanisms involved in calcium release.

Table 2 summarizes data on ion currents at the different stages of differentiation for RCMH cells. Cells in stage 1 did not express ion currents. Potassium channels (and eventually T-type calcium channels) are the first to be expressed in stage 2 of differentiation. Several types of K^+ channels have been described in muscle cell lines, among them inward rectifier and ATP dependent K^+ channels (Kubo, 1991) as well as Ca^{2+} activated K^+ channels (Hamann *et al.*, 1994). Although we cannot discard the presence of Ca^{2+} activated channels, our results suggest that K^+ channels first expressed in RCMH cells are of the delayed rectifier type.

Voltage-dependent sodium channels, expressed as TsTX-γ toxin receptors are present in stages where no sodium current was seen; it is unlikely that channels could have been lost due to intracellular perfusion through the patch pipette, since they were not recorded as single channels using the cell attached patch technique (Caviedes *et al.*, 1992). On the other hand, these receptors appear not to be intracellular since they are detected by binding to intact cells (Table 1). This evidence suggests the

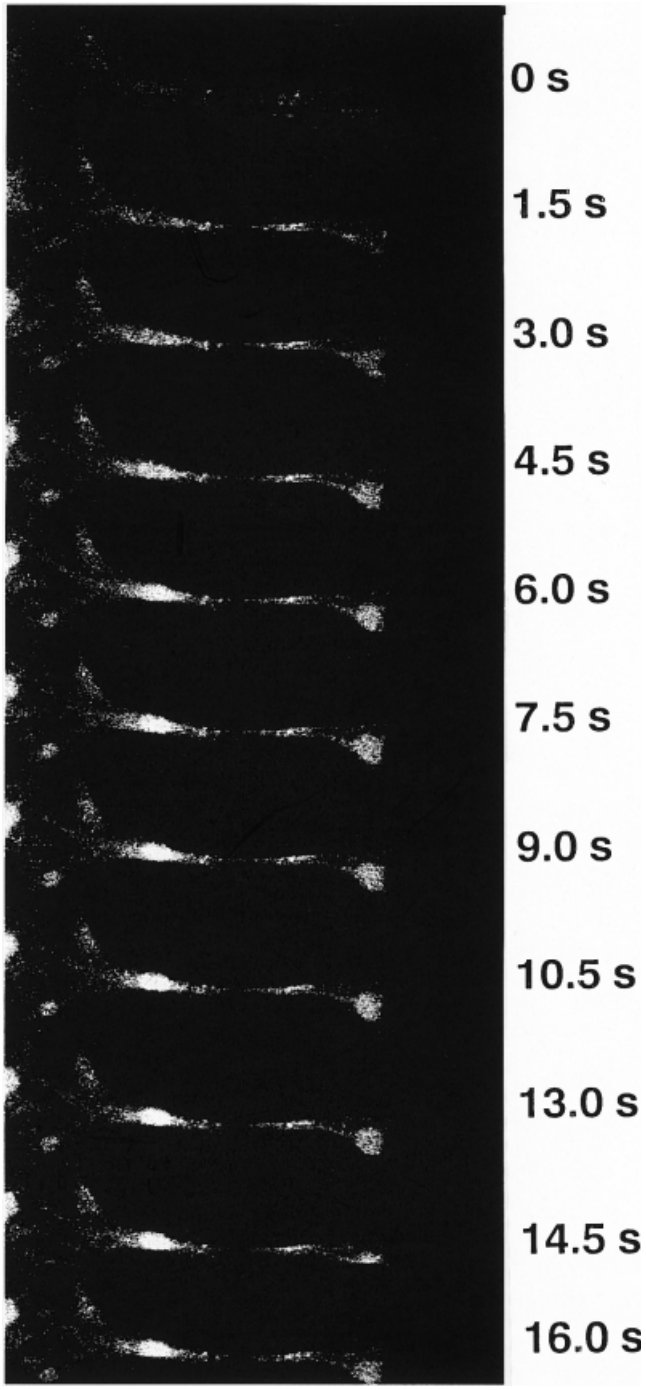

Fig. 8. Time sequence of confocal images of fluo-3 fluorescence from a cell incubated in a calcium free medium (in mM: 145 NaCl, 5 KCl, 1$MgCl_2$, 15 Na-HEPES, 5.6 glucose, 1 Na-EGTA, pH 7.4). At time 1 s, perfusion with 40 mM K^+ (replacing Na^+) in the incubation chamber began. Notice the fast spread of fluorescence throughout the cell and the slower rise in fluorescence around the nuclei. The total cell length was 47 μm.

existence of 'immature' or 'silent' channels in these cells (Frelin *et al*., 1984). Cell differentiation induced by serum deprivation plus addition of hormones and nutrients to the incubation media resulted in a moderate increase of TsTX-γ toxin receptors (Table 1), these results correlated with the appearance of a fast inactivating sodium current, partly abolished by TTX (Fig. 4) found in stage 3. We can conclude that functional Na^+ channels are expressed only in differentiated RCMH cells. Besides the existence of functional and 'silent' sodium channels in developing muscle, previous studies have demonstrated the presence of subtypes of sodium channels with different affinities for TTX or STX (Frelin *et al*., 1983, 1984; Sherman *et al*., 1983; Gonoi *et al*., 1985). The criteria used have included binding of TTX derivatives, TTX sensitivity of sodium fluxes measured by a combination of veratridine and ATX II or veratridine and scorpion toxin (Haimovich *et al*., 1986) and voltage clamp studies (Hamann *et al*., 1994). However, the *in vitro* studies with developing muscle have left the relationship between channel subtypes detected with these methods and receptors detected with toxin binding assays during early muscle differentiation unresolved. Our results confirmed the presence of both functional and non-functional channels in RCMH cells and we could also distinguish between currents with high and low sensitivity to TTX such as those reported in the L6 muscle cell line (Haimovich *et al*., 1986), in proliferating human muscle satellite cells (Hamann *et al*., 1994) or in denervated muscles (Lupa & Caldwell, 1994). We see a single type of receptor for TsTX-γ in these cells; whether this toxin recognizes one or the two types of sodium channel is not yet evident. The possibility that the current seen in the presence of TTX could correspond to either a transient calcium current or to a sodium current passing through calcium channels seems unlikely, since the voltage dependence for both types of transient currents is different (Figs 4 and 5) and the calcium concentration used in the experiments illustrated in Figure 4 (50 μM) is low enough to avoid a significant calcium current and high enough to keep calcium selectivity of channels intact (Almers *et al*., 1984).

More than one type of calcium current was seen in RCMH cells; although transient, T-type currents were prominent only in some cells in stage 2 of differentiation. These currents are not apparent in the presence of potassium in the patch pipette (Fig. 3; Table 2) since they are probably masked by the much larger outward potassium currents. Both the kinetics and the voltage dependence of the calcium currents are roughly similar to those reported for skeletal muscle cells in primary culture (Rivet *et al*., 1992; Cognard *et al*., 1993) and to a current expressed transiently during development of embryoid bodies, a model for myogenesis (Rohwedel *et al*., 1994). These currents differ significantly from those seen in

a cardiac cell line immortalized in the same way (Caviedes *et al*., 1993). The presence in stage 3 of net inward currents with no apparent inactivation, using caesium in the pipette, suggest the expression of L-type calcium currents; the observed activity is consistent with an important increase in dihydropyridine receptors in this stage (from 0.5 to 2 pmol mg^{-1} protein). As mentioned above, no ion currents are evident in stage 1, despite the presence of a basal level of dihydropyridine receptors, implying that these receptors are non-functional as Ca^{2+} channels. There are two possible explanations for these results; first, it is possible that dihydropyridine receptors in stage 1 represent 'immature' channels, lacking some functionally critical component and second, as these receptors have been postulated to function both as calcium channels and as voltage sensors in excitation–contraction coupling (Ríos & Pizarro, 1991), it is possible that the receptors expressed at early stages of differentiation do not function as calcium channels but as voltage sensors. RCMH cells are capable of releasing calcium from intracellular stores after plasma membrane depolarization, thus implying that a voltage sensor must indeed be active in these cells.

We have found that the ryanodine receptor is expressed at very low levels in proliferating undifferentiated cells (Table 2), and increases up to sevenfold upon differentiation. This is consistent with the data of Marks and colleagues (1989), where the ryanodine receptor mRNA was not observed in undifferentiated dividing BC_3H1 cells but was detected in cells after the onset of differentiation and with that of Airey and colleagues (1991) who found a similar pattern of expression for the ryanodine receptor in the BC_3H1 and C2C12 murine cell lines. Similar results have been reported for rat myotubes in primary culture (Kyselovic *et al*., 1994), suggesting a general pattern of late expression of ryanodine receptors during development.

The presence of $InsP_3$ receptors in skeletal muscle cells has been hard to document; low-affinity receptors have been reported in frog skeletal muscle SR membranes (Rojas & Hidalgo, 1990) but their quantification has not been possible. Cultured skeletal muscle on the other hand, clearly shows a single type of $InsP_3$ binding site both in rat embryo primary culture (Jaimovich *et al*., 1995) and in RCMH cells (Fig. 7B). The precise role for $InsP_3$ receptors in skeletal muscle is yet to be determined; nevertheless, our results suggest that $InsP_3$ may be involved in calcium release induced by membrane depolarization before ryanodine receptors are fully expressed. The calcium release signal seen upon potassium depolarization in undifferentiated cells suggest that such cells are capable both of sensing membrane potential and of transducing the signal to calcium release.

On the other hand, expression of functional $InsP_3$ receptors may be developmentally important for skeletal muscle; functions other than excitation–contraction coupling such as gene expression and various enzyme activities may be critically dependent on intracellular calcium. A detailed study of calcium release during muscle development may help to elucidate such a role.

Acknowledgements

We thank Drs E. Rojas and M. Pincon-Raymond for critical reading of early versions of this manuscript. This research was financed by the Muscular Dystrophy Association, FONDECYT 1931089 and DTI. Thanks are also due to Conselho Nacinal de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenaçâo de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

References

AIREY, J. A., BARING M. D. & SUTKO, J. L. (1991) *Develop. Biol.* **148**, 365–74.

ALMERS, W., McCLESKEY, E. W. & PALADE, T. D. (1984) *J. Physiol.* **3563**, 565–83.

ARISPE, N., JAIMOVICH, E., LIBERONA, J. L. & ROJAS, E. (1988) *Pflügers Archiv.* **411**, 1–7.

BARHANIN, J., PAURON, D., LOMBET, A., NORMAN, R., VIJVERBERG, H., GIGLIO, J. & LAZDUNSKI, M. (1983) *EMBO J.* **2**, 915–20.

CAVIEDES, R., LIBERONA, J. L., HIDALGO, J., TASCÓN, S., SALAS, K. & JAIMOVICH, E. (1992) *Biochim. Biophys, Acta* **1134**, 247–55.

CAVIEDES, P., OLIVARES, E., SALAS, K., CAVIEDES, R. & JAIMOVICH, E. (1993) *J. Mol. Cell Cardiol.* **25**, 829–45.

COGNARD, C., CONSTANTIN, B., RIVET-BASTIDE, M., IMBERT, N., BESSE, C. & RAYMOND, G. (1993) *Development* **117**; 1153–61.

FRELIN, C., VIGNE, P. & LAZDUNSKI, M. (1983) *J. Biol. Chem.* **258**, 7256–9.

FRELIN, C., VIJVERBERG, H., ROMEY, G., VIGNE, P. & LAZDUNSKI, M. (1984) *Pflügers Arch.* **402** 121–8.

GONOI, T & HASEGAWA, S. (1988) *J. Physiol.* **401**, 617–37.

GONOI, T., SHERMAN, S. J. & CATTERALL, W. A. (1985) *J. Neurosci.* **5**, 2559–64.

HAIMOVICH, B., TANAKA, J. & BARCHI, R. L. (1986) *J. Neurochem.* **47**, 1148–53.

HAMANN, M., WIDMER, H., BAROFFIO, A., AUBRY, J-P., KRAUSE, R. M., KAELIN, A. & BADER, C. R. (1994) *J. Physiol.* **475**, 305–17.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANNN, B. & SIGWORTH, F. J. (1981) *Pflügers Arch.* **391**, 85–100.

HILLE, B. (1984) *Ionic Channels of Excitable Membranes.* Sunderland, MA: Sinauer Press.

HUNTER, W. A. & GREENWOOD, F. C. (1962) *Nature* **194**, 495–6.

JAIMOVICH, E., DONOSO, P., LIBERONA, J. L. & HIDALGO, C. (1986) *Biochim. Biophys. Acta* **855**, 89–98.

JAIMOVICH, E., LIBERONA, J. L., PÉREZ, T., HIDALGO, J. &

CAVIEDES, R. (1995) Calcium waves and IP_3 receptors in cultured skeletal muscle. *Biophys. J.* **68,** A391.

KIDOKORO, Y. (1975) *J. Physiol.* **244,** 145–59.

KUBO, Y. (1991) *J. Physiol.* **442,** 743–59.

KYSELOVIC, J., LEDDY, J. J., RAY, A., WIGLE, J. & TUANA, B. S. (1994) *J. Biol. Chem.* **269,** 21770–7.

LESAGE, F., ATTALI, B., LAZDUNSKI, M. & BARHANIN, J. (1992) *FEBS* **310,** 162–6.

LUPA, M. T. & CALDWELL, J. H. (1994) *J. Cell Biol.* **124,** 139–47.

MARKS, A. R., TEMPST, P., HWANG, K. S., TAUBMAN, M. B. INUI, M., CHADWICK, C., FLEISCHER, S. & NADAL-GINARD, B. (1989). *Proc. Natl Acad. Sci. USA* **86,** 8683–7.

MISHINA, M., TAKAI, T., IMOTO, K., NODA, M., TAKASHASHI, T. & NUMA, S. (1986) *Nature* **321,** 406–11.

NEW, H. V. & MUDGE, A. W. (1986) *Nature* **323,** 809–11.

NORMAN, R. I., SCHMID, A., LOMBET, A., BARHANIN, J. & LAZDUNSKI, M. (1983) *Proc. Natl Acad Sci. USA* **80,** 4164–8.

OLSON, E. N., STERNBERG, E., HU, J-S. & WILCOX, C. (1986) *J. Cell Biol.* **103,** 1799–805.

OROZCO, C. B., SMITH, S. A., EPSTEIN, C. J. & RAPOPORT, S. J. (1987) *Dev. Brain Res.* **32,** 111–22.

RÍOS, E. & PIZARRO, G. (1991) *Physiological Reviews*, **71,** 849–908.

RIVET, M., COGNARD, C., IMBERT, N., RIDEAU, Y., DUPORT, G. & RAYMOND, G. (1992) *Neuroscience Letters* **138,** 97–102.

ROHWEDEL, J., MALTSEV, V., BOBER, E., ARNOLD, H. H., HESCHELER, J. & WOBUS, A. M. (1994) *Development Biol.* **164** 87–101.

ROJAS, C & HIDALGO, C. (1990) *Biophys. J.* **57,** 342a.

ROMEY, G., GARCIA, L., DIMITRIADOU, V., PINCON-RAYMOND, M., RIEGER, F. & LAZDUNSKI, M. (1989) *Proc. Natl Acad. Sci. USA* **86,** 2933–7.

SAMPAIO, S. V., LAURE, C. J., GIGLIO, J. R. (1983) *Toxicon.* **21,** 265–77.

SHERMAN, S. J., LAWRENCE, J. C., MESSNER, D., JACOBY & CATTERALL, W. A. (1983) *J. Biol. Chem.* **258,** 2488–95.

SHIMAHARA, T. & BOURNAUD, R. (1991) *Cell Calcium* **12,** 727–33.

WEISS, R. E. & HORN, R. (1986) *Science* **233,** 361–4.