Antisense oligonucleotides against ‘cardiac’ and ‘skeletal’ DHP-receptors reveal a dual role for the ‘skeletal’ isoform in EC coupling of skeletal muscle cells in primary culture

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SUMMARY

Two dihydropyridine receptor mRNA isoforms (cardiac and skeletal) are expressed in rat skeletal muscle cells in primary culture. The progressive changes in excitation-contraction coupling mode from dual mode (‘skeletal’ and ‘cardiac’) to predominant ‘skeletal’ one during in vitro myogenesis are thought to be linked to the developmental changes in the relative expression of the two types of molecular entity previously observed in this preparation. In order to test this hypothesis, myotube cultures (5- to 7-day-old) were treated with antisense phosphorothioated oligodeoxynucleotides against cardiac or skeletal \(\alpha_1\) subunit of L-type calcium channel. The oligodeoxynucleotide uptake by cells was checked by means of imaging of fluorescent oligodeoxynucleotide derivatives within the cells. Optimum concentration used (10 \(\mu\)M in the extracellular medium) and incubation time (70 hours) were empirically determined. Antisense directed against the cardiac type led to a 54\% decrease in the averaged L-type calcium current peak density at \(-10\) mV. The same type of experiment was performed with antisense against the skeletal isoform and led to a same order of inhibition (46\%). This result clearly shows that the two isoforms can work as a calcium channel. Conversely, analysis of the shape of \(T-V\) (relative contractile amplitude versus membrane potential) curves shows that the treatment with ‘skeletal’ antisense depressed the contractile response in the medium membrane potential range whereas treatment with ‘cardiac’ antisense had no effect. This and other results taken together suggest that the skeletal isoform of dihydropyridine receptor is involved in both ‘cardiac’ and ‘skeletal’ types of EC coupling mechanisms at work in early stages of myotubes in vitro development. The type of coupling probably depends on the proximity of the skeletal dihydropyridine receptor and the ryanodine receptor.

Key words: Skeletal muscle cell, Myogenesis, Excitation-contraction coupling, Dihydropyridine receptor, Antisense oligodeoxynucleotide

INTRODUCTION

The term excitation-contraction (EC) coupling is used to describe the coupling between sarcolemmal depolarisation and sarcoplasmic reticulum (SR) calcium release, leading to activation of contraction. Two main molecular components are involved in EC coupling: the L-type calcium channel (Beam et al., 1986; Cognard et al., 1986), also known as dihydropyridine receptor (DHPR), localised in transverse tubules of mature skeletal muscle, and the ryanodine receptor (RyR) which acts as a calcium release channel in the sarcoplasmic reticulum membrane. Two mechanisms have been proposed to explain the nature of the coupling. A molecular interaction between the DHPRs and RyRs is thought to be involved in the first mechanism, named ‘skeletal’ type EC coupling since it is predominant in mature skeletal muscle (for review, see Melzer et al., 1995). According to this model, influx of calcium through the L-type calcium channel is not necessary for calcium release. The DHPR responds to transverse tubule depolarisation by sensing the voltage variation and inducing calcium release from the SR via a direct activation of the RyR. Conversely, in the second mechanism, predominant in cardiac muscle, the so-called ‘cardiac’ type EC coupling, SR calcium release cannot be activated by sarcolemmal depolarisation per se, and requires calcium influx through DHP-sensitive calcium channel (Callewaert, 1992).

In skeletal muscle, during the first stages of in vitro development, a calcium current-dependent component of contraction is present and consequently EC coupling displays both skeletal and cardiac features (Cognard et al., 1992). Nevertheless, the cardiac relative part declines with time in culture becoming undiscernible in well-developed cells (Cognard et al., 1992).

Skeletal and cardiac isoforms of DHPR (\(\alpha_1\) subunit of L-type calcium channel) are issued from two genes (Tanabe et al., 1987; Mikami et al., 1989) and both isoforms are present...
and differentially expressed during in vitro myogenesis (Chaudhari and Beam, 1993; Bulteau et al., 1997). The main differences in primary structure between the two isoforms reside in the large putative cytoplasmic regions, i.e. the amino- and carboxy-terminal regions and the regions linking repeats I and II and repeats II and III. The latter appears to be critical for determining the skeletal- or cardiac-like nature of EC coupling (Tanabe et al., 1990). The progressive changes observed in EC coupling mode from dual mode (‘skeletal’ and ‘cardiac’) to a predominant ‘skeletal’ one during in vitro myogenesis have led to the idea that these changes could be linked to the developmental ones in the relative expression of the two isoforms (Bulteau et al., 1997).

In the present study, the ‘antisense strategy’ was used. Antisense oligodeoxynucleotides (AS-ODNs) are complementary to a specific mRNA sequence, and the RNA-DNA duplexes so formed can block translation and/or processing of the targeted mRNAs. These duplexes are expected to inhibit protein synthesis by different mechanisms: they can impede translocation of the mRNA along the ribosome, prevent splicing by hybridising between introns and exons, or activate ribonuclease H-dependent degradation of mRNA (for review, see Pilowsky et al., 1994; Phillips and Gyrurko, 1997). Since AS-ODNs are expected to block, or at least attenuate, protein synthesis, specific oligos which selectively interfere with the expression of cardiac or skeletal α1 subunit isoforms were used to test the above hypothesis. Calcium current and contraction were recorded on cells treated with these AS-ODNs, to investigate the effect of the differential inhibition of α1 subunit isoforms expression. The data led us to reinterpret the former results and to propose a new view in which the transitory coexistence of two EC coupling mechanisms during in vitro development of skeletal muscle cells in primary culture is due to a dual role of the skeletal isoform and not to the presence of the two isoforms of the DHPR.

### MATERIALS AND METHODS

#### Cell culture

Primary cultures of mammalian skeletal muscle cells were initiated from satellite cells obtained by trypsinisation of muscle pieces of 1- to 3-day-old rats hindlimbs (for details see Cognard et al., 1993). Cells were maintained for two days in growth medium, consisting of HamF12 (Gibco BRL, Life Technologies, Cergy Pontoise, France), supplemented with 5% heat-inactivated horse serum and 1% antibiotics. This medium exchange operation was used as time zero to age cells in culture. For current and contraction recordings, antisense or scrambled ODNs were introduced into the culture medium on day 2 at different concentrations. In order to use cells suitable for electrophysiological recordings (see Cognard et al., 1993), colchicine (30 nM; Sigma), was added when elongated myotubes appeared (around day 2) to favour the formation of rounded cells (‘myoballs’). Patch-clamp experiments and mechanical recording were carried out on 5- to 7-day-old myoballs.

### Oligodeoxynucleotides

The ODNs used were chemically modified at the terminal 3′ phosphate with phosphorothioate linkages to block exonucleolytic degradation and to increase cellular uptake. Two antisense phosphorothioated ODNs (Eurogentec Bel, Belgium), and their controls were used (Fig. 1). The skeletal AS-ODN was specific for the rat skeletal isoform of α1 subunit since it was chosen in the II-III cytoplasmic loop of the corresponding rat cDNA partly cloned (see Bulteau et al., 1997). This part of the molecule shows a high diversity degree between cardiac and skeletal isoforms. In the same way, there was no homology between the cardiac and skeletal domain N termini, where the cardiac oligo was chosen. For cytofluorescence analysis, the same phosphorothioated anti-α1S ODN, conjugated to fluorescein isothiocyanate (FITC), was used (Eurogentec).

### Cytofluorescence imaging

Two days after fusion, myotubes were treated in the dark with the FITC-conjugated antisense ODNs, at a final concentration of 10 μM. After 5 hours of ODNs incubation, cells were washed twice in a standard saline solution (Earle medium) and fluorescent cells were imaged with a laser scanning confocal microscope (ACAS 570, Meridian Instruments, Okemos, Michigan, USA), every hour, for 19 hours. The fluorochrome was excited with the blue line (488 nm) of an Argon laser. The ODNs used were chemically modified at the terminal 3′ phosphate with phosphorothioate linkages to block exonucleolytic degradation and to increase cellular uptake. Two antisense phosphorothioated ODNs (Eurogentec Bel, Belgium), and their controls were used (Fig. 1). The skeletal AS-ODN was specific for the rat skeletal isoform of α1 subunit since it was chosen in the II-III cytoplasmic loop of the corresponding rat cDNA partly cloned (see Bulteau et al., 1997). This part of the molecule shows a high diversity degree between cardiac and skeletal isoforms. In the same way, there was no homology between the cardiac and skeletal domain N termini, where the cardiac oligo was chosen. For cytofluorescence analysis, the same phosphorothioated anti-α1S ODN, conjugated to fluorescein isothiocyanate (FITC), was used (Eurogentec).

![Fig. 1. Locations and sequences of antisense oligodeoxynucleotides.](image)

(A) Antisense location on DHPR. The skeletal antisense (sk) was designed to complement 15 nt in the II-III cytoplasmic loop of α1 subunit. The cardiac antisense (card) complemented 15 nt at the NH2 terminus. (B) Oligodeoxynucleotide sequences of antisense and scrambled oligos targeted to DHPR mRNA. ‘Skeletal’ is a part of the original rat skeletal muscle DHPR cDNA sequence corresponding to bases 2,015 to 2,029 of the rabbit one (Tanabe et al., 1987). Cardiac corresponds to the rabbit cardiac DHPR cDNA sequence (Mikami et al., 1989) running from base 293 to 307. Sk. antisense and card. antisense are the antisense phosphorothioated oligos targeted against these regions of skeletal and cardiac α1 subunits, respectively. Missense are the respective control oligos corresponding to the normal sequences shown just above them, in which four bases (in italic) are mesapparied.
Excitation-contraction coupling and antisense

Current recording
Voltage-clamp experiments were performed at room temperature (20±2°C) in the whole-cell configuration of the patch-clamp technique. Pipettes (2-5 mΩ) were connected to the head stage of a patch-clamp amplifier (RK300, Biologic, Grenoble, France), driven by a PC compatible microcomputer equipped with a Labmaster A/D conversion board (Scientific solutions, Solon, USA). Membrane voltage clamping, data acquisition, and analysis were performed by means of a software package (pClamp, Axon Instruments, Foster City, USA).

Mechanical recording
Contractile responses were recorded simultaneously with membrane currents (Rivet et al., 1989) by means of a photomultiplier tube (IP28, Hamamatsu, Japan) mounted on an auxiliary light-path exit of the inverted microscope. This device measured the changes in light transmission resulting from cell deformation during contraction. In spite of limitations of this semi-quantitative method, this technique remains the only one to estimate the contractile activity of cultured adherent cells. For this reason relative contractions were not calibrated, but expressed in an arbitrary unit that corresponded to the electrical potential at the exit of the current/voltage converter following the IP28 tube.

Solutions and experimental procedures
The control Earle medium, for cytofluorescence analysis contained: 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 10 mM Hepes, 5.6 mM glucose; pH adjusted at 7.4 with NaOH.

For patch-clamp experiments, the culture medium was exchanged for a saline bath Na⁺- and K⁺-free solution to suppress Na⁺ and K⁺ currents. This solution contained: 135 mM TEACl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 10 mM Hepes, 5.6 mM glucose; pH adjusted to 7.4 with Tris base. The pipette contained: 145 mM CsCl, 1 mM MgCl₂, 1 mM EGTA, 5×10⁻³ mM CaCl₂, 10 mM Hepes, 5.6 mM glucose; pH adjusted to 7.2 with Tris base. When used, the Ca²⁺ current inhibitor cobalt was added to the external solution at the desired final concentration from a stock solution through a home-made superfusion system. Currents and contraction were elicited by a series of increasing depolarising steps, 500 milliseconds in duration, from a holding potential (HP) of −90 mV. Before recordings, series resistances (around 10 MΩ) were 80% compensated with the built-in device of the amplifier. Data were corrected for leakage currents from a linear extrapolation of membrane current magnitudes (assumed to be ohmic) for small depolarisations which did not induce any dynamic currents. The current amplitude was measured as the difference between peak amplitude of inward current and the background current level before the test pulse. For the plotting of current/voltage (I/V) curves, current amplitudes were then expressed as current densities (pA/pF) calculated by ratioing the amplitude over the membrane capacity. This latter was estimated from the capacitive transient elicited by a 10 mV depolarising step from a HP value of −90 mV.

RESULTS
Before experiments on current and contraction, control experiments to check ODN entry and distribution, survival and differentiation of muscle cells in the presence of ODNs were performed, and the optimum ODN incubation parameters determined.

Time course of entry and distribution of ODNs in myotubes
Internalisation of fluorescent labelled AS-ODNs by myotubes was checked by cytofluorescence (Fig. 2). After a 5 hour exposure to AS-ODNs (Fig. 2A), a great fluorescence was visualised within the cells, demonstrating that the modified ODNs were stable, in the culture medium employed, for at least 5 hours, and that the uptake of ODNs was effective after this delay. A punctuate fluorescent pattern, characteristic of localisation in the endosomal compartment, was first visible in the subplasmalemmal cytoplasmic domain. At this stage, fluorescence was never detected in the cell nucleus (Fig. 2A). Then fluorescence, organised in streaks, migrates within the cytoplasm and appears to be perinuclearly localised (Fig. 2B). After a 10-11 hour ODN exposure, the labelling is homogeneous throughout the entire cell (Fig. 2C). The nuclei are no longer visible. Until 19 hours after ODN treatment, fluorescence was always visible (Fig. 2D).

Survival and differentiation of myotubes and myoballs in the presence of ODNs
Primary cultures of skeletal muscle were incubated in parallel...
with no ODNs, AS-ODNs or missense (NS) ODNs, when myoblasts begin to fuse into myotubes (generally two days after exchanging the culture medium). The micrographs in Fig. 3 allow morphological comparison between control cells and cells treated with $10^{-3}$ M of NS- or AS-ODNs. In the three groups, the differentiation into well-formed myotubes (Fig. 3A,B,C: 24 hours of ODN incubation) takes place, then into myoballs (Fig. 3D,E,F: 70 hours of ODN incubation) after adding colchicine in the culture medium. The same development was observed for all ODN concentrations tested and for all incubation times. These micrographs demonstrate that myotubes, in the presence of ODNs, are able to normally differentiate and to form myoballs.

Selection of antisense ODN concentration and incubation time

In order to determine the most effective concentration and incubation time of AS-ODNs, cells were treated in parallel with four ODN concentrations (Fig. 4A) and for three incubation times (Fig. 4B). ODNs were added to the culture medium at 2 μM, 5 μM, 10 μM and 20 μM, and cells were first incubated for 70 hours. Ionic currents were recorded on 5-day-old myoballs. Bargraphs show that for a depolarising pulse to $-10$ mV (Fig. 4A), only two ODN concentrations resulted in current density decreases (47% and 51% in cells treated with 10 μM and 20 μM AS-ODNs, respectively). These results show that the antisense effect on current density is dose-dependent. In parallel, cells were incubated with 10 μM of AS-ODNs for 28 hours, 70 hours, and 144 hours (Fig. 4B), in order to determine the optimum incubation time. For a test pulse to $-10$ mV, only the 70 hour incubation time led to a significant decrease of the current density (47%, versus 31% for a 144 hour incubation).

In a general manner, higher concentrations or long time incubations led to side or reverse effects. For example (data not shown), for the highest concentration tested (20 μM), the reversal potential is shifted by at least 10 mV toward positive potentials (from +50 mV to approximately +65 mV). This is probably due to a non-specific antisense effect at this

**Fig. 3.** Comparison of morphological aspect of developing skeletal muscle cells in primary culture, incubated or not with ODNs. Micrographs were obtained by standard photonic transmission (phase contrast) microscopy. They display 3 day-old myotubes (up) and 5 day-old myoballs (down) without any treatment (A and D) or incubated since 24 hours and 70 hours with NS- (B and E) or AS-ODNs (C and F). Bar, 30 μm.

**Fig. 4.** Effect of different AS-ODNs concentrations and incubation times on current densities. Calcium currents were recorded in 5- to 7-day-old myoballs. Bar graphs show mean ± s.e.m. of calcium current density from several different cells. (A) Cells were incubated 70 hours with different AS-ODNs concentrations or with NS-ODNs (NS), or with no oligos (control). (B) Cells were incubated with 10 μM of AS-ODNs for different incubation times. ns: not significantly different from control cells, $P<0.01$; *significantly different, $P<0.01$; $n$=number of tested cells, Student unpaired two-tailed $t$-test. Test pulse to $-10$ mV.
Table 1. Time-to-peak and time constants of inactivation of ICaL in control, NS treated cells, and in cells incubated with skeletal or cardiac AS-ODNs

<table>
<thead>
<tr>
<th>Cells</th>
<th>Time-to-peak (ms)</th>
<th>( \tau_{\text{inac}} ) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59±6</td>
<td>ns</td>
</tr>
<tr>
<td>(n=24)</td>
<td></td>
<td>(n=23)</td>
</tr>
<tr>
<td>NS</td>
<td>59±2</td>
<td>ns</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td>(n=14)</td>
</tr>
<tr>
<td>Skeletal AS</td>
<td>67±10</td>
<td>ns</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td>(n=11)</td>
</tr>
<tr>
<td>Cardiac AS</td>
<td>80±5</td>
<td>ns</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td>(n=14)</td>
</tr>
</tbody>
</table>

Values are means±s.e.m. \( \tau_{\text{inac}} \) are obtained from mono-exponential fit of calcium current decay.

*Significantly different from the NS-cells value with \( P<0.01 \); ns: non significantly different (\( P>0.05 \)), one-way ANOVA test and Newman-Keuls post test. HP=−90 mV. Test pulse to +10 mV.

Effect of AS-ODNs on calcium current

Currents were recorded on 5- to 6-day-old myoballs where the two types of EC coupling coexist (Cognard et al., 1992). Fig. 5A shows examples of inward calcium current traces elicited by depolarising pulses from −90 mV to −10 mV. The shape of current is similar in the three types of cell culture conditions: in the presence of 10 \( \mu \)M of skeletal (left) or cardiac (middle left) AS-ODNs, or in the presence of NS-ODNs (middle right), or with no oligo (right). The time-course of calcium currents displayed slow activation and inactivation phases that characterise skeletal L-type calcium current. Comparison of time-to-peak and time constants of inactivation (Table 1) shows that there are weak changes but no significant difference between control cells and cells incubated with skeletal AS-ODNs. Conversely, the inhibition of cardiac \( \alpha_1 \) synthesis in the presence of cardiac AS-ODNs revealed a higher inactivation time constant and an increased time-to-peak. Further, the inward current is partly inhibited in cells treated with AS-ODNs (skeletal or cardiac), as compared to control cells. No significant decrease of current amplitude is observed between cells incubated with NS-oligos and control (without any treatment) cells. The I/V curves (Fig. 5B) indicate a potential for maximum L-type calcium peak current amplitude around 0 mV (NS), or +10 mV (AS and control). Incubation of cells with AS-ODNs against isoforms of \( \alpha_1 \) subunit leads to a decrease in current densities, particularly at −10 mV and 0 mV and, to a lesser extent, at +10 mV. The corresponding inhibition values are provided in Table 2.

Effect of AS-ODNs on contraction

Contraction was simultaneously recorded with current. Fig. 6A shows examples of current (a) and contraction (b) traces obtained in the absence (squares) and in the presence (circles) of cobalt for the depolarising pulses from −90 mV to potential indicated below each panel. In control medium, the corresponding T/V curve (Fig. 6B) exhibits a mechanical threshold at −30 mV, a maximum peak amplitude at −10 mV and a decrease for larger depolarising pulses. The addition of 5 mM cobalt to the bath led to a full blockade of the calcium current (Fig. 6Aa). This calcium current inhibition, clearly observed at −10 mV (central panel), was accompanied, at least for the medium potentials (−30 and −10 mV), by a parallel block of an outward current previously studied (Constantin et al., 1993). This blocking effect on ICa was also accompanied by a reduction in the mechanical response amplitude (Fig. 6Ab and B) with a maximum contractile inhibition observed at medium depolarisations as shown by the corresponding inhibition values provided by Table 3 and the subtraction of the T/V curve obtained with cobalt to the one obtained without cobalt (Fig. 6Cc). In spite of a possible shift of the T/V curve induced by the high concentration of cobalt (see Discussion), this demonstrates the existence of a current-dependent component of contraction (Fig. 6Cc) as previously described in the presence of 1.5 mM cadmium, another calcium current inhibitor (Rivet et al., 1989; Cognard et al., 1992). Since skeletal and cardiac \( \alpha_1 \) subunits of the L-type calcium channel are present in these cultured cells (Bulteau et al., 1997), it can

Table 2. Calcium current densities measured in myoballs incubated with 10 μM of skeletal or cardiac AS-ODNs, with NS-ODNs, or with no oligos

<table>
<thead>
<tr>
<th>Pulse to (mV):</th>
<th>Cells (n)</th>
<th>Current ± s.e.m. (pA/pF)</th>
<th>Inhibition (%)</th>
<th>Current ± s.e.m. (pA/pF)</th>
<th>Inhibition (%)</th>
<th>Current ± s.e.m. (pA/pF)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−10</td>
<td>Skeletal AS</td>
<td>15</td>
<td>−1.36±0.20</td>
<td>46*</td>
<td>−2.60±0.29</td>
<td>41*</td>
<td>−3.11±0.29</td>
</tr>
<tr>
<td></td>
<td>Cardiac AS</td>
<td>16</td>
<td>−1.16±0.25</td>
<td>54*</td>
<td>−2.93±0.50</td>
<td>33</td>
<td>−3.45±0.43</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>14</td>
<td>−2.51±0.45</td>
<td>−</td>
<td>−4.35±0.49</td>
<td>−</td>
<td>−4.20±0.42</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>25</td>
<td>−2.57±0.39</td>
<td>2</td>
<td>−3.78±0.36</td>
<td>13</td>
<td>−4.50±0.30</td>
</tr>
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</table>

Values of inhibition correspond to the decrease percentage of the current densities obtained in the presence of the different AS-ODNs (skeletal AS and cardiac AS) or control cells as compared to the ones obtained in the NS-ODNs incubated cells.

*Significantly different from NS-cell values, \( P<0.05 \); ns: non significantly different (\( P>0.05 \)), one-way ANOVA test and the Newman-Keuls post test. HP=−90 mV.

‡The minus sign corresponds to an increase.
be supposed that the skeletal isoform led to a coupling mechanism independent of calcium entry ('skeletal'-type EC coupling) whereas the cardiac one led to the calcium current-dependent part of the contraction ('cardiac'-type EC coupling). Consequently, this latter is mainly observed at medium depolarising pulses for which the calcium current is strongly activated.

The specific inhibition of either skeletal or cardiac α1 subunit with AS-oligos could allow us to check this hypothesis. But technical limits (relative contraction amplitude measurements and not absolute ones) preclude direct comparison of the contractions recorded in different cells. So investigations of contraction with oligo treatment require normalising contractile amplitudes. A normalisation potential of +50 mV, a potential at which the ‘cardiac’-type EC coupling is thought to be very weak, was chosen. Then the T/V curve obtained in the presence of ‘cardiac’ oligos (Fig. 7A, filled triangles), can be compared with the control one (filled squares) and the curve resulting from the subtraction of the curves (Fig. 7B, open triangles) analysed. Surprisingly, no significant effect of the ‘cardiac’ AS-ODNs can be observed (Table 4). By contrast, the T/V curve obtained in the presence of ‘skeletal’ oligos (Fig. 7A, filled circles) and the curve resulting from the subtraction (Fig. 7B, open circles) clearly exhibit an inhibition of the contractile response (Table 4). This is quite different from the expected result: in the hypothesis in which inhibition of the skeletal isoform expression led to a decrease of the ‘skeletal’-type EC coupling (that is to a predominant inhibiting effect at high potentials, in particular at +50 mV), the T/V amplitude curve, after normalisation, should increase (dashed arrow, Fig. 7A), in the range of medium potentials. For example, the T/V curve of the calcium current-dependent component (open squares, in Fig. 6B) normalised in this way and plotted again in Fig. 7A, open squares, dashed line, appeared over the control one. Clearly the reverse effect (black arrow, Fig. 7A) is obtained.

Table 3. Contractile activity recorded in control cells in presence and in absence of cobalt

<table>
<thead>
<tr>
<th>Pulse to (mV):</th>
<th>10</th>
<th>10</th>
<th>30</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contraction</strong></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td><strong>Inhibition</strong></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>1.00±0.02</td>
<td>0.98±0.03</td>
<td>0.88±0.03</td>
<td>0.85±0.03</td>
</tr>
<tr>
<td><strong>Cobalt</strong></td>
<td>0.18±0.08</td>
<td>82*</td>
<td>0.69±0.06</td>
<td>30*</td>
</tr>
</tbody>
</table>

Relative contraction is expressed as mean values normalised to the maximum value obtained in absence of cobalt. Values of inhibition are the decrease percentage between the contraction obtained in cells in the presence and in the absence of cobalt.

*Significantly different from control values, \( P<0.01 \).

**Significantly different, \( P<0.05 \). Student unpaired two-tailed \( t \)-test. HP=−90 mV.
DISCUSSION

This study demonstrates the possibility to inhibit (or at least to modulate), in skeletal muscle cells, calcium channel function by action of AS-ODNs targeted against the α1 subunit of L-type calcium channel expression.

The first main result concerns the use of AS-ODNs per se. Imaging data show that the uptake of oligos by skeletal muscle cells is possible and that their location within the cell varies with time. Perinuclear and nuclear locations were observed after 10 hours, but a cytoplasmic distribution remained at this time. Such a perinuclear distribution of oligos appears to be in agreement with previous observations (Wyrob et al., 1996), where only traces of scattered fluorescence within and around the cell nucleus were showed. However, in the latter study, oligonucleotides were used at low concentrations (no more than 2.5 μM). Other studies have shown a nuclear distribution of AS-ODNs. In those studies, higher concentrations of oligos (Whang et al., 1992) similar to those of our work were used, or oligonucleotides were introduced directly into the cell cytoplasm by means of microinjection (Fakler et al., 1994), HVJ-liposomes (Morishita et al., 1993), or other cationic liposomes (Galderisi et al., 1996). The locations observed in our study could result from different modes of action of AS-oligos, since a perinuclear distribution can indicate a translational arrest, after mRNA is transported from the nucleus to the cytoplasm, whereas the AS-oligos localised in the nucleus may enable the protein synthesis inhibition by other mechanisms (for instance, RNase H activation). As for the remaining cytoplasmic location of AS-ODNs after 10 hours of incubation, it can be hypothesised that it may result either from a higher tendency of phosphorothioates to non-specific binding to cellular components, or from their increased resistance to cellular nucleases. The use of different

Fig. 6. Contractile activity elicited in 5- to 7-day-old myoballs by electrical stimulation. (A) Examples of current (a) and contraction (b), elicited by depolarising pulses from a holding potential of −90 mV to −30 mV (left), −10 mV (middle), and +50 mV (right), in a control cell in the absence (squares) or in the presence (circles) of 5 mM cobalt. (B) Mean (± s.e.m.) values of relative contraction normalised to the maximum contraction value in control and plotted against membrane potential. Contractions were elicited in several control cells in response to various depolarising pulses from a holding potential of −90 mV to test values from −50 mV to +50 mV (20 mV increments), in the absence (squares) or in the presence (circles) of 5 mM cobalt. n indicates the total number of cells tested. The curve in C corresponds to the subtraction of the T/V curve recorded in the presence of cobalt to the T/V one in the absence of cobalt.
concentrations and incubation times of AS-ODNs shows that their inhibitory effect is dose- and time-dependent with, for very long time of incubation (144 hours), or for high concentration (20 µM), an increase of the peak amplitude or a shift of the reversal potential, respectively. Biro et al. (1993) reported a stimulatory effect on cell proliferation, which may be attributed to a mitogenic effect of nucleotides and nucleosides resulting from the degradation of the oligos. Here, maybe nucleotides activate genes, leading to a stimulation of calcium channel translation or to a positive modulation of channel activity. For a 144 hour incubation or a 20 µM concentration, the degradation should be high, and the non-specific effects due to nucleotides could compete with the intact oligo’s inhibitory effects. So it is necessary, before doing experiments, to determine the optimum experimental conditions concerning the use of AS-ODNs. Moreover, as a whole inhibition of the protein synthesis is difficult to obtain, the results must be cautiously interpreted. These features point out the limits of the ‘antisense strategy’, but nevertheless, this method remains the only one which allows us to specifically inhibit gene expression.

The second point resulting from this work is the inhibition of Ca²⁺ current density with AS-ODNs against either skeletal or cardiac α1 subunit, which demonstrates that both isoforms work as an L-type Ca²⁺ channel. This conclusion is confirmed by the slight slowing down of calcium current kinetics observed in cells incubated with cardiac AS-ODNs. The partial inhibition of a ‘cardiac’ component with faster kinetics can probably account for the slight modifications observed in time-to-peak and time constant of inactivation of the remaining current; as in skeletal muscle fibres or in cultured cells, L-type Ca²⁺ current activates and inactivates more slowly (Donaldson and Beam, 1983; Cognard et al., 1986; Beam et al., 1986) than in cardiac cells (Bean, 1989). Nevertheless, the Ca²⁺ current, which persists after treatment of cells with skeletal AS-ODNs, does not actually present cardiac L-type Ca²⁺ current characteristics (for instance as regards the time-to-peak) and our results also disagree with transfection experiments performed in dysgenic cells. When dysgenic myotubes, which lack normal L-type calcium current (Beam et al., 1986) because of the protein mutation (Chaudhari, 1992), were transfected with α1C, they showed a fast activated current, whereas transfection with α1S leads to a slow activation (Tanabe et al., 1991). In spite of the fact that α1 subunit alone can act as a Ca²⁺ channel and possesses sites for activation kinetics modulation (Tanabe et al., 1991; Nakai et al., 1994; Wang et al., 1995) the role of the ancillary subunits, and particularly β subunit, in the regulation of channel activity has been demonstrated. As dysgenic myotubes ‘naturally’ lack normal α1 subunit, it can be hypothesized that expression of α1C in these cells leads to a β2 subunit (cardiac isoform) endogenous expression, which could partly contribute to the fast kinetics observed. Moreover many studies concerning the role of the other subunits, and particularly the β subunit, in the regulation of the channel activity, have been performed in heterologous expression systems and it is difficult to extrapolate the results in native tissues. The effect of the β1 (skeletal type) subunit on the activation rates varies, depending on the expression system. Strube et al. (1996) have reported in cells devoid of the β1 subunit (β1-null myotubes), that activation of the Ca²⁺ current was faster than in normal myotubes. This result was consistent with the observation, in HEK cells, that β1 slowed down the kinetics of Ca²⁺ current produced by α1C (Perez-Garcia et al., 1995). In other expression systems, opposite results were observed: β1 accelerated the kinetics of α1C subunit in oocytes (Wei et al., 1991) and of α1S in L cells (Varadi et al., 1991; Lory et al., 1992). Therefore it can be supposed that in our study association of the native β1 subunit with α1C results in the slight slowing down of the ‘cardiac-type’ Ca²⁺ current

**Table 4. Contractile activity recorded in myoballs incubated with 10 µM of skeletal or cardiac AS-ODNs, and in control cells**

<table>
<thead>
<tr>
<th>Cells (n)</th>
<th>Contraction (± s.e.m.)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal AS 13</td>
<td>0.7±0.07</td>
<td>41&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cardiac AS 24</td>
<td>1.08±0.02</td>
<td>9</td>
</tr>
<tr>
<td>Control 15</td>
<td>1.18±0.03</td>
<td>ns</td>
</tr>
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Contraction is elicited by a depolarising pulse from a HP of –90 mV to –10 mV. Relative contraction values are normalised to the value at +50 mV. Values of inhibition are the decrease percentage between the normalised contraction in control cells and in cells incubated with skeletal or cardiac AS. *Significantly different from control values, P<0.01; ns: non significantly different (P≥0.05), one-way ANOVA test and the Newman-Keuls post test.
Membrane depolarisation, have been previously shown (Rivet et al., 1989; Cognard et al., 1992) by blocking $I_{\text{Ca}}$ with cadmium. In the present work the dual control of contractile activity was confirmed using cobalt (Fig. 6), another divalent inorganic blocker of $I_{\text{Ca}}$. The high concentration of divalent cations (5 mM Co\textsuperscript{2+} and 1.8 mM Ca\textsuperscript{2+}) probably induced a shift of the T/V curve through a ‘charges screening’ effect. Nevertheless this curve shift could not totally explain the observed difference of the curves shapes with or without Co\textsuperscript{2+} and particularly the decrease of the contractile response for high depolarisation values in control medium (Fig. 6B) more readily due to the progressive reduction of calcium current with the increasing potentials as previously shown with cadmium (Rivet et al., 1989).

The absence of effect on contractile response of AS-ODNs on contractile activity and the role which can be attributed to the two different $\alpha 1$ isoforms in EC coupling mechanisms.

In these cells developing in primary culture, the coexistence of two components of contractile response, a calcium current-dependent one and a component more directly controlled by membrane depolarisation, have been previously shown (Rivet et al., 1989; Cognard et al., 1992) by blocking $I_{\text{Ca}}$ with cadmium. In the present work the dual control of contractile activity was confirmed using cobalt (Fig. 6), another divalent inorganic blocker of $I_{\text{Ca}}$. The high concentration of divalent cations (5 mM Co\textsuperscript{2+} and 1.8 mM Ca\textsuperscript{2+}) probably induced a shift of the T/V curve through a ‘charges screening’ effect. Nevertheless this curve shift could not totally explain the observed difference of the curves shapes with or without Co\textsuperscript{2+} and particularly the decrease of the contractile response for high depolarisation values in control medium (Fig. 6B) more readily due to the progressive reduction of calcium current with the increasing potentials as previously shown with cadmium (Rivet et al., 1989).

The absence of effect on contractile response of AS-ODNs against $\alpha 1\text{C}$ while the AS-ODNs against $\alpha 1\text{S}$ alter the contractile response characteristics (changes in the shape of the T/V curve), ruled out the possibility of the involvement of cardiac isoform in EC coupling mechanism and the former hypothesis (Bulteau et al., 1997) used to explain the presence of the calcium current-dependent component of contraction observed in young myotubes in primary cultures (Rivet et al., 1989; Cognard et al., 1992). In this hypothesis the coexistence of the two types (‘cardiac’ and ‘skeletal’) of EC coupling was thought to be due to the presence of the two isoforms of the $\alpha 1$ subunit: the cardiac isoform works by means of a calcium current through the pore of the $\alpha 1$ subunit triggering a secondary calcium release from the RyR of sarcoplasmic reticulum membrane (CICR mechanism) whereas the role in the EC coupling of the skeletal isoform doesn’t require a calcium influx because of the involvement of a more direct molecular interaction between the $\alpha 1$ subunit and the RyR. As the inhibition of cardiac isoform expression by cardiac AS-ODNs failed to alter the contractile response, it can be suggested that the skeletal isoform must be also involved in the ‘cardiac’ type EC coupling in order to explain the calcium current-dependent (cobalt or cadmium sensitive) component of contraction previously reported during the early phases of in vitro myogenesis (primary culture). Thus, the skeletal DHPR must be a calcium channel molecular entity working both through a calcium entry (‘cardiac’ coupling) and as a voltage-sensing molecule more directly interacting with the RyR (‘skeletal’ coupling), to trigger in either case a secondary release of calcium ions from SR.

Is this hypothesis in agreement with data on ultrastructural organisation of DHPR, RyR and the different membrane elements of the muscle cells developing in culture? Immunolabeling of the T-tubule system in in vitro myotubes has shown that ultrastructural organisation was the same as in mature skeletal muscle (Flucher et al., 1991, 1992). Tetrads, which are clusters of four DHPRs (Block et al., 1988; Takekura et al., 1994), are initially targeted to the surface membrane, before being localised in the T-tubules, at TT-SR junctions. Morphological studies have shown that each tetrad was in close apposition with one foot, which represents the large cytoplasmic domain of the SR calcium release channel or RyR (Inui et al., 1987; Lai et al., 1988). This is in agreement with a specific interaction and direct coupling, characteristic of skeletal EC coupling. In cardiac muscle, tetrads are absent (Sun et al., 1995), which is in accordance with a less direct communication between the DHPRs and RyRs in cardiac EC coupling. In our view (see diagram in Fig. 8), during the first stages of in vitro myogenesis, some skeletal DHPRs might be organised into tetrads and establish tight association with RyRs, acting as ‘voltage-sensor’ to trigger directly the calcium release whereas others, unlinked to RyRs, might work as calcium channels involved in the CICR mechanism. The organisation of skeletal DHPRs into tetrads could increase during in vitro myogenesis,

**Fig. 8.** Schematic model of developmental changes in interaction between the two molecular components involved in EC coupling of skeletal muscle cells developing in primary culture. The diagram represents the functional and spatial links between the DHPR cardiac or skeletal isoform, in the plasma or transverse tubules (TT) membrane, and the RyRs of the sarcoplasmic reticulum in young (left) and older (right) myotubes. In the right panel, coupling is almost exclusively provided by a direct molecular link between skeletal DHPRs and RyRs whereas in early myotubes coupling is provided both through a direct coupling and a CICR mechanism due to a less tight spatial link between the two proteins. In addition, the cardiac to skeletal isoforms ratio decreases in older myotubes (right) because of the progressive reduction of the number of cardiac type isoforms expressed. In either case only the skeletal DHPRs are involved in EC coupling mechanisms in spite of involvement of the two isoforms in L-type calcium current.
since the direct coupling becomes the main mechanism in late
myotubes (Cognard et al., 1992). As for the partial inhibition of
calcium current by both the ‘cardiac’ and ‘skeletal’ AS-ODNs,
it can be supposed that other cardiac or skeletal DHPRs are
located in the TT membrane or surface membrane but far from
the SR membrane, which precludes any link with the RyR.
In this view, the role of unlinked DHPRs remains unknown.

This work was supported by CNRS/Université de Poitiers UMR 6558.
Françoise Mazin is gratefully acknowledged for expert
technical assistance in cell culture.

REFERENCES

mice eliminates the slow calcium current in skeletal muscle cells. Nature
320, 1690-170.
Physiol. 51, 367-384.
antisense oligodeoxynucleotides targeting c-myc mRNA on smooth muscle
Structural evidence for direct interaction between the molecular
components of the transverse tubular/sarcoplasmic reticulum junction in
Bulteau, L., Cogné, M., Cognard, C. and Raymond G. (1997). Reversal of
the relative expression of cardiac and skeletal alpha1 subunit isoforms of L-
type calcium channel during in vitro myogenesis. Pflügers Arch. 433, 376-
378.
Chaudhari, N. and Beam, K. (1994). Different types of Ca 2+
Sci. USA 83, 517-521.
Cognard, C., Rivet-Bastide, M. and Raymond G. (1986). Different types of Ca 2+
105, 507-515.
Cognard, C. and Raymond G. (1987). Reduced Ca 2+ current, charge movement and absence of Ca 2+
transients in skeletal muscle deficient in dihydropyridine receptor β1
Sun, X.-H., Protasi, F., Takahashi, M., Takeshima, H., Ferguson, D. G. and
129, 659-671.
Takekura, H., Bennett, L., Tanabe, T., Beam, K. G. and Franzini-
myotubes by dihydropyridine receptor cDNA. Biophysical J. 67, 793-803.
Tanabe, T., Takeshima, M., Mikami, A., Flockerzi, V., Takahashi, H.,
Primary structure of the receptor for cardiac channel blockers from skeletal
Regions of the skeletal muscle dihydropyridine receptor critical for
dihydropyridine receptor is critical in determining cardiac
Acceleration of activation and inactivation by the β subunit of the skeletal
calcium channel. Nature 352, 159-162.
oligo(deoxynucleotides to G, protein α-subunit sequence accelerate
Wyroba, E., Pawlowska, Z., Kobylanska, A., Puska, E., Maszewskia, M.,
oligo(deoxynucleotide antisense to type-1 plasminogen activator inhibitor
mRNA in endothelial cells: A three-dimensional reconstruction by confocal