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Induction of normal ultrastructure by CGRP treatment in dysgenic myotubes

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The calcitonin gene-related peptide (CGRP) restores an apparant normal ultrastructure in *mdg/mdg* muscle cells in vitro, including a normal triadic organization which is known to be essential for excitation-contraction (E-C) coupling. However, neither slow L-type Ca^{2+} channel activity nor E-C coupling, which are absent in *mdg/mdg* muscle, were re-established. These observations suggest a potential role of CGRP (and also of cAMP as the intracellular messenger) in the morphological development of the muscle fiber.

Muscle; Calcitonin gene-related peptide; Excitation-contraction coupling; Ca^{2+} channel; Triad; Muscular dysgenesis

1. INTRODUCTION

mdg/mdg mice suffer from a skeletal muscle disease [1–3] characterized by an immature ultrastructural organization of the muscle [1,4–6], a drastic decrease of the level of 1,4-dihydropyridine receptor [6] and of the L-type Ca^{2+} channel activity [7,8] and, as a result, a lack of contractile activity [6–8]. Co-culture of diseased mouse cells with spinal cord cells from normal mice was shown to restore several features of the normal ultrastructure and excitation–contraction (E-C) coupling [9]. The calcitonin gene-related peptide (CGRP) coexists with acetylcholine in spinal cord motoneurons [10] and affects both the biosynthesis [11–13] and the biophysical properties of the nicotinic acetylcholine receptor. The purpose of this work is to describe the spectacular properties of the action of CGRP in dysgenic myotubes.

2. MATERIALS AND METHODS

Primary cultures of skeletal muscles from newborn normal and mutant mice were prepared as previously described [5]. In treated dishes, CGRP was added at the final concentration of 10^{-7} M after the 6th day in culture. Culture dishes were fixed 1 h in 2% paraformaldehyde in 0.1 M phosphate, pH 7.0 at room temperature, immersed for 15 min in a PBS solution containing 0.1 M glycine and washed in PBS for 15 min. The cultures were then incubated overnight at 4°C in a mouse IgM anti-α-actinin antibody (Amersham) diluted in 3% BSA in PBS. The incubation with an IgG-anti-IgM antibody was followed by the incubation of a FITC-conjugated second antibody (Eurobio). Photomicrographs were taken with a Zeiss photomicroscope equipped for observation with fluorescein and phase optics.

For electron microscopy, cultures were fixed for 2 h in 2.5% glutaraldehyde 0.5% tannic acid in 0.1 M phosphate buffer at pH 7.4 at 4°C, followed by 0.6% glutaraldehyde 0.5% tannic acid in the same buffer overnight. Post-fixation was performed in 2% osmotic acid in 0.1 M phosphate buffer for 1 h at 4°C. Then the cultures were dehydrated in graded alcohol and embedded in Epon resin. Ultra-thin sections of about 65 nm were stained with a saturated solution of uranyl acetate in 50% acetone followed by staining with 0.2% lead citrate for 4 min. The observations were made with a Philips EM 410 electron microscope (accelerating voltage 80 kV, objective aperture 20 μm).

The membrane currents were recorded with the whole-cell variant of the patch-clamp technique [14]. The pipette solution contained (in mM): 140 CsCl, 5 EGTA, 4 $MgCl_2$, 3 ATP; this solution was buffered at pH 7.3 with 10 mM Hepes/CsOH. The external solution contained (in mM): 140 TEA, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 glucose; this solution was buffered at pH 7.4 with 10 mM TEAOH. Tetrodotoxin (5 μM) was added to the external solution to prevent any contamination with Na^+ currents. Patch pipettes (2–5 MΩ) were connected to the head stage of the recording apparatus (RK300, Bio-Logic, Grenoble, France). The mechanical activity was recorded simultaneously with the electrical activity using a 128 × 128 photodiode array matrix associated with an image analyzer giving an image rate analysis up to 200/s.

3. RESULTS AND DISCUSSION

All results presented in this work have been obtained with myotubes grown in vitro [4,5] from mutant *mdg/mdg* mice. Their phenotypically normal littermates will be referred to as *+/mdg?* since +/+ and *+/mdg?* mice are indistinguishable. Control *+/mdg?* myotubes spontaneously contract and display a regular

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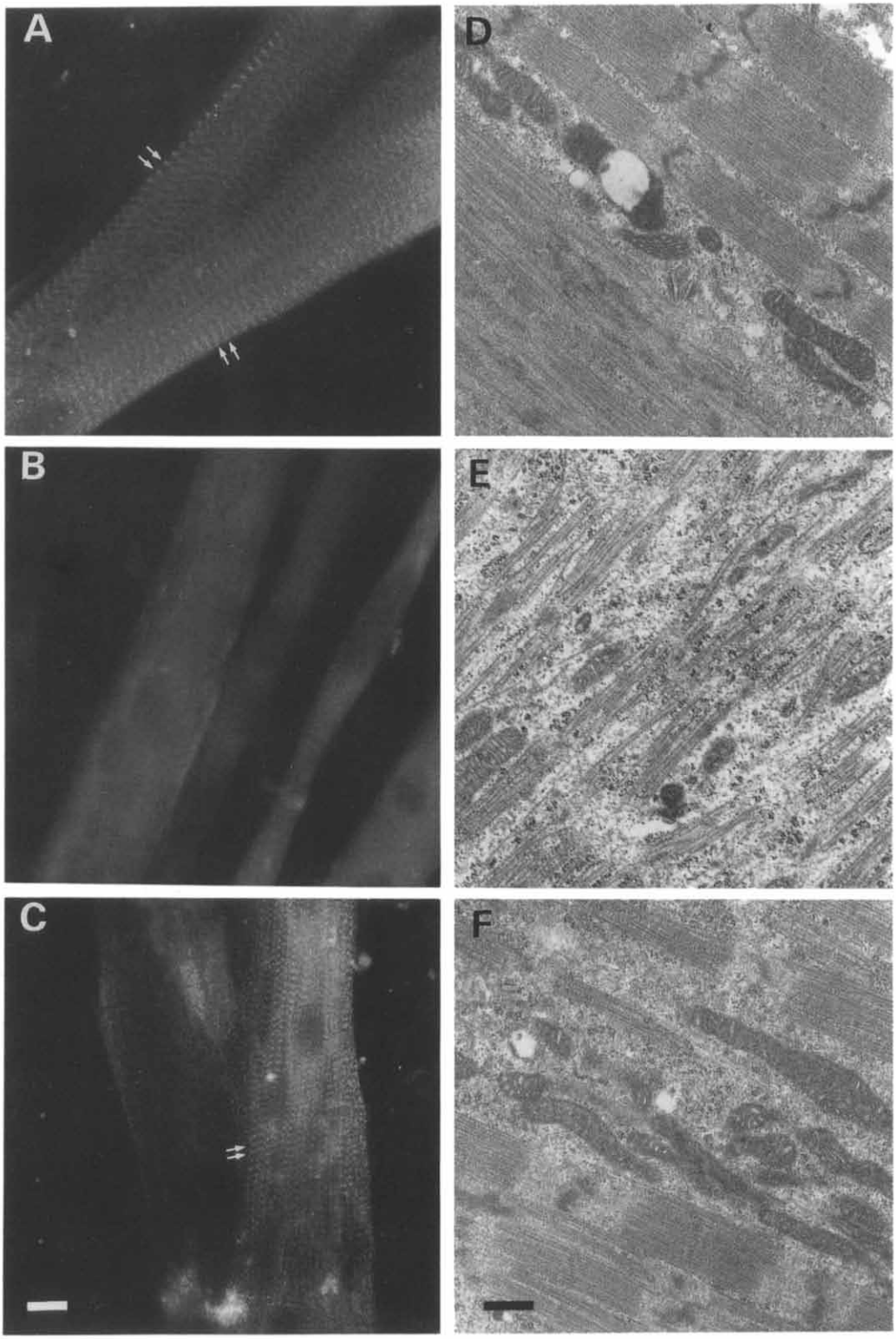

Fig. 1. Morphological reorganization of the internal structure of CGRP-treated *mdg/mdg* myotubes. (A–C) Immunodetection of α-actinin as a marker of Z line organization (scale bar = 10 μm). (A) In normal contracting myotubes, the transverse sarcomeric organization was observed all along the fibers by α-actinin labelling. (B) In *mdg/mdg* myotubes, the α-actinin labelling appeared diffusely distributed. (C) After CGRP treatment, the α-actinin labelling in *mdg/mdg* myotubes demonstrated areas with distinct sarcomeric organization. The myotube on the left is out of focus and it is not representative. (D–F) Electron microscopy; myofibrillar and sarcomeric organization of myotubes corresponding to those shown in Fig. A–C, respectively.

sarcomeric organization and Z striations. *mdg/mdg* myotubes: (i) never normally contract; (ii) lack a normal sarcomeric organization; (iii) display an absence of basal lamina [15]; and (iv) have an abnormal triadic structure [6,9]. *mdg/mdg* myotubes in culture were chronically exposed to 0.1 μM CGRP for 6–7 days. After this period of time, major changes in their ultrastructure were observed when compared to untreated mutant myotubes. The distribution pattern of α-actinin, an important component of the Z line [16], was used as an index of the organization of the myofibrillar network. Fig. 1A–C shows the distribution of α-actinin (using a polyclonal antibody) in 12-day-old *+/mdg?*, *mdg/mdg*, and CGRP-treated *mdg/mdg* myotubes. α-Actinin is not absent in *mdg/mdg* myotubes (Fig. 1B) but the well-organized striated pattern and the well-defined Z lines observed in normal myotubes (Fig. 1A) are absent. In contrast, extensive areas of quasi-normal sarcomeric organization are observed (Fig. 1C) in CGRP-treated *mdg/mdg* myotubes. These observations were confirmed at the ultrastructural level by classical electron microscopy (Fig. 1D–F). In control myotubes, myofibrillar and sarcomeric organization are already widespread (Fig. 1D), although not systematic, as generally observed in late fetal muscle. In *mdg/mdg* myotubes, little or no fibrillar or sarcomeric organization is visible (Fig. 1E) although some aberrantly deposited Z material is sometimes observed, as already reported [17]. In CGRP-treated *mdg/mdg* myotubes, clear reorganization has occurred in extended areas in good agreement with the light microscopy observations and is typically illustrated by Fig. 1F. Histograms comparing the organization of α-actinin in *+/mdg*, *mdg/mdg*, and CGRP-treated *mdg/mdg* myotubes are presented in Fig. 2. Most of the CGRP-treated myotubes, i.e. more than 80%, have gained a normal sarcomeric organization. In addition to its effects on the sarcomeric structure, CGRP treatment elicits the accumulation of a well-defined basal lamina in *mdg/mdg* myotubes (Fig. 3).

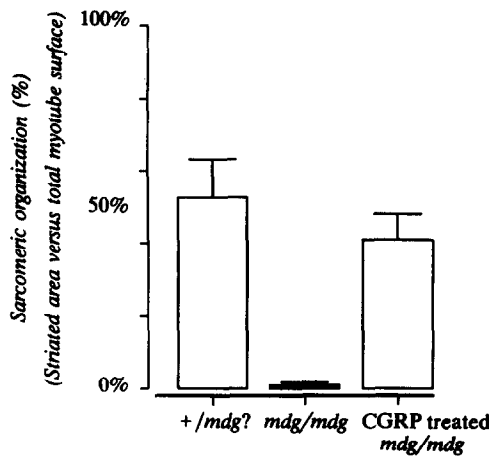

Fig. 2. Histogram representation of sarcomeric organization in normal control myotubes and untreated and CGRP-treated mutant myotubes. In untreated mutant myotubes, there was essentially no sarcomeric organization. After a few days (6–7) in the presence of CGRP, mutant myotubes exhibit an increase of organized area which becomes intermediate between that of normal and untreated mutant myotubes. Micrograph fields were chosen at random, in each experiment, and comparable numbers of fields were analyzed under each condition. The computer image analysis was performed on a TITN Samba system (Grenoble, France) using a simple software based on direct manual determination of organized vs inorganized areas displayed on screen.

A major defect of *mdg/mdg* myotubes, which is probably a critical factor for the absence of E-C coupling in mutant muscle, is the absence of normal triads [6,9] with no spaced densities (junctional 'feet') between T-tubules and the sarcoplasmic reticulum. Upon CGRP exposure, most of the triadic junctions acquire regularly spaced densities (Fig. 3). Out of a total of 36 triads observed in an optimal longitudinal orientation, and in 4 separate experiments, 25 had normal features with regularly spaced densities. In normal myotubes almost 100% of the triads observed possessed regularly spaced densities. The total number of triads per volume unit did not seem to be significantly different in normal and CGRP-treated *mdg/mdg* myotubes, suggesting a normal overall density of differentiated normal sarcoplasmic reticulum-T-tubule (SR-TT) junctions.

Since CGRP had such a remarkable organizing effect on the ultrastructure of *mdg/mdg* myotubes, one could have expected that the physiological defects of the diseased muscle, i.e. the lack of L-type Ca^{2+} channel activity associated with a drastic decrease of the level of 1,4-dihydropyridine receptor and the lack of E-C coupling, might have also been restored by the peptide treatment. Voltage-clamp results presented in Fig. 4 show that exposure to CGRP does not restore the slow L-type Ca^{2+} current activity which is observed in normal muscle and which is virtually absent in non-treated *mdg/mdg* muscle [7,8]. In previous experiments in which mutant myotubes were co-cultured with spinal cord cells [9], the restoration of a normal morphogenesis of the triads in innervated *mdg/mdg* myotubes was accompanied by the appearance of L-type Ca^{2+} currents and the restoration of contraction. In CGRP-treated myotubes, triads were normally differentiated but slow L-type Ca^{2+} currents were still absent. In addition, contractile activity was observed in none of the mutant myotubes tested after exposure to CGRP. Chronic treatment with this peptide was therefore clearly without effect on both L-type Ca^{2+} channel activity and contraction. Nevertheless, modifications of the electrophysiological properties of *mdg/mdg* myotubes were observed after CGRP treatment. CGRP treatment led to a spontaneous bursting activity which was not observed in untreated *mdg/mdg* myotubes (Fig. 5A,B). This spontaneous firing was systematically of a higher frequency than that of *+/mdg?* myotubes in sister cultures (Fig. 5B,C).

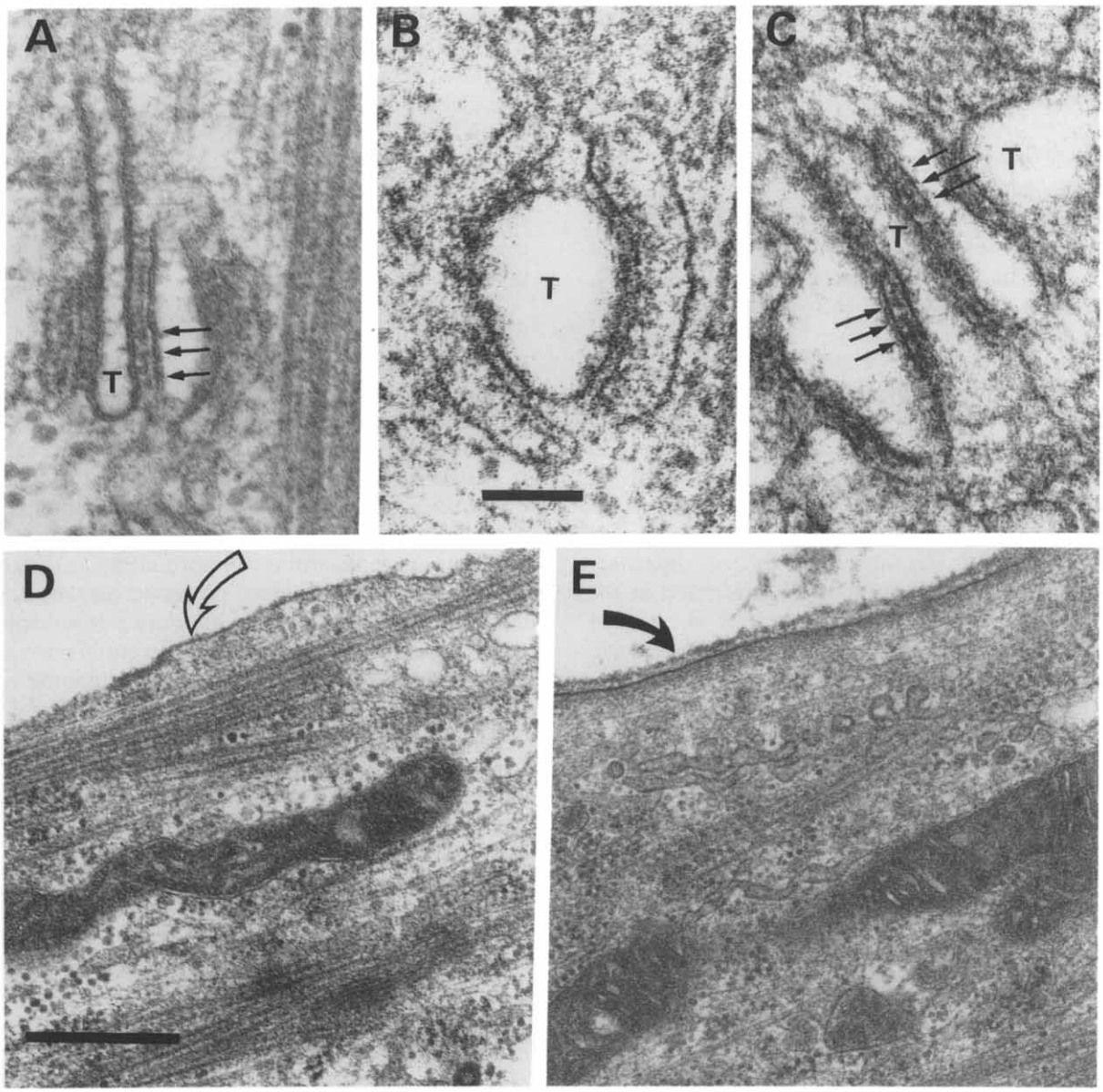

Fig. 3. Ultrastructural analysis of triadic junctions in the normal, mutant and CGRP-treated mutant myotubes. (A) In normal contracting myotubes, triads frequently present well-defined regularly spaced densities (× 130150, see scale bar in B). (B) In *mdg/mdg* myotubes, triads are rare and spaced densities are not observed between T-tubule and sarcoplasmic reticulum membranes, although unorganized material is frequently present (× 130150, scale bar = 0.1 μm). (C) After several days in the presence of CGRP, triads in *mdg/mdg* myotubes acquire a normal morphology with regularly spaced densities (× 130150, scale bar as in B). (D) Organized basal lamina is not observed in *mdg/mdg* myotubes (× 42750, scale bar = 0.5 μm). (E) A well-defined basal lamina is present on the surface of CGRP-treated mutant myotubes (× 42750, scale bar as in D).

The second messenger of CGRP action in muscle has previously been shown to be cAMP [18]. Similarly to CGRP, chronic treatment of *mdg/mdg* myotubes with dibutyryl cAMP (final concentration of 1 μM) induced the differentiation of 87% of the observed triads, as well as a sarcomeric organization and a basal lamina accumulation (not shown). Spontaneous electrical activity was also observed in *mdg/mdg* myotubes treated with dibutyryl cAMP (Fig. 5D). Treatment with cholera toxin (2 ng/ml), which is a well-known activator of cAMP production, also mimicked the effects described above for CGRP although to a lesser extent (not shown).

The triad is thought to be a key structural element for E-C coupling in skeletal muscle. During muscle ontogenesis, E-C coupling does not occur as long as triads are not formed [19]. Also, removal of the triad structure by detubulation of myotubes by glycerol treatment eliminates E-C coupling. The voltage-sensitive L-type Ca^{2+} channel, although it is situated in T-tubules, does not seem to be the essential element for contraction [19–22]. Elimination of the L-type Ca^{2+} channel activi-

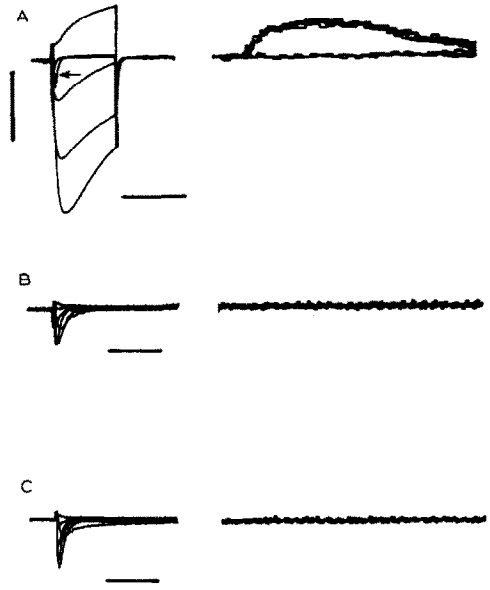

Fig. 4. Voltage-clamp analysis of Ca^{2+} currents and the associated contractions in normal (A), *mdg/mdg* (B), and CGRP-treated *mdg/mdg* (C) myotubes. Left traces: superimposed Ca^{2+} currents associated with step depolarizations to −50, −30, −10, +10, +30, +50 mV from a holding potential of −90 mV. Right traces: the associated contractions in arbitrary units. In normal myotubes (A), the presence of two distinct Ca^{2+} currents, a fast activating and inactivating current (arrow) with a low membrane potential threshold (−50 mV) (I_{fast}) and a slow activating current with a higher threshold (−20 mV) (I_{slow}). Note the absence of I_{slow} and of the contractions but the presence of I_{fast} in *mdg/mdg* (B) and CGRP-treated *mdg/mdg* (C) myotubes. Horizontal bars = 500 ms (A) or 200 ms (B, C). Vertical bar = 1 nA.

ty by Ca^{2+} removal, blockade with some Ca^{2+} channel blockers or simply the culture conditions [21,22], does not eliminate E-C coupling. Situations in which L-type Ca^{2+} channels are functional but triads are not formed, do not lead to contraction [22]. The present work shows that triad formation induced by CGRP in *mdg/mdg* myotubes is not sufficient by itself to obtain muscle contractile activity.

The α_1 subunit of the 1,4-dihydropyridine (DHP) receptor seems to serve both as an L-type Ca^{2+} channel and as a voltage sensor [19,21]. In its voltage sensing function, the DHP receptor transmits the depolarization of the T-tubule membrane (by a mechanism which is still unknown but which does not involve transmembrane Ca^{2+} translocation for the external medium) to ryanodine-sensitive Ca^{2+} channels located in SR thereby releasing Ca^{2+} from internal stores for contraction [23–25]. The DHP receptor is clearly an essential element of E-C coupling which probably remains absent, in insufficient amounts, or is not functional in CGRP-treated mutant myotubes. This suggests that normal levels of voltage sensor are not necessary for the morphogenesis of muscle ultrastructure and, particularly, for triad formation.

Tanabe et al. [26] have recently published data in-

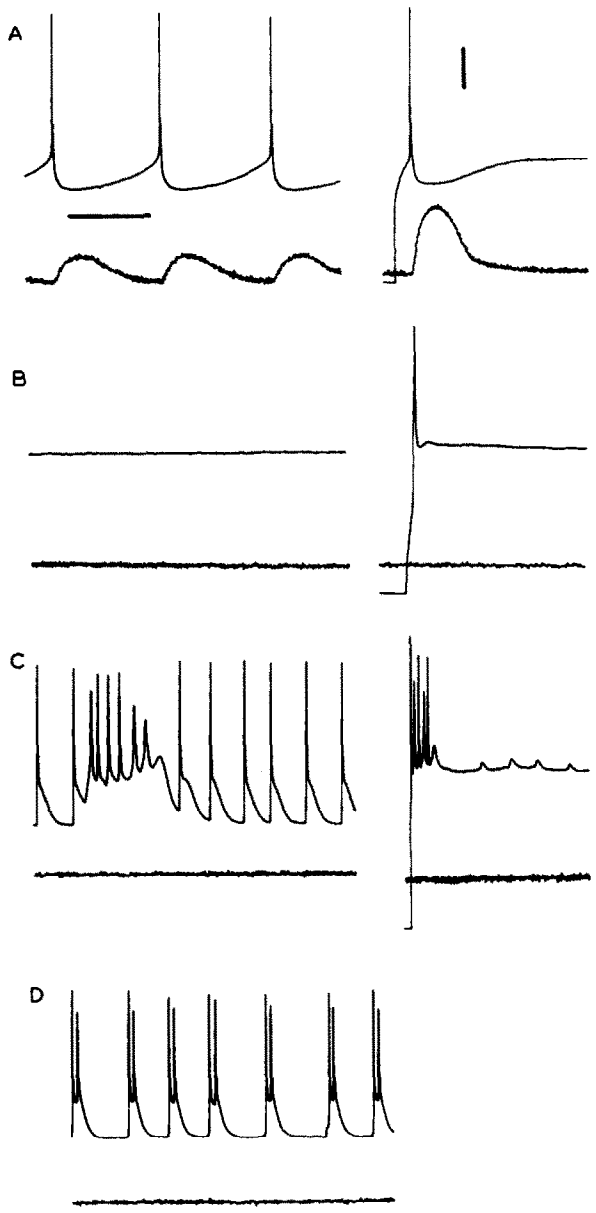

Fig. 5. Spontaneous and evoked action potential by anodal break stimulation (upper traces) and contractions (lower traces) in arbitrary units in normal myotubes (A), *mdg/mdg* myotubes (B), and *mdg/mdg* myotubes treated chronically with CGRP (0.1 μM) (C). (A) In normal myotubes, spontaneous action potentials (left traces) or evoked action potentials (right traces) induced contractions and were followed by a long-lasting after-hyperpolarization (ahp). (B) *mdg/mdg* myotubes were often quiescent (left trace). Evoked action potentials were not followed by an ahp and did not produce contractions (right trace). (C) Spontaneous or evoked pattern of burst activity in *mdg/mdg* myotubes treated by CGRP. No contractile activity was associated with the action potentials. (D) Spontaneous bursting electrical activity (upper trace) without mechanical activity (lower trace) induced by chronic application of dibutyryl cAMP. Horizontal bar in A = 500 ms; vertical bar in A = 20 mV. Methods: The external Earle medium contained (in mM): 140 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose. This solution was buffered at pH 7.4 with 10 mM Hepes/KOH. Intracellular recordings were made using conventional microelectrodes filled with 3 M KCl, with resistances of 20–50 MΩ. The microelectrode was connected to an electrometer amplifier (WPI.M707) allowing simultaneous current injection and voltage recording through the same electrode.

dicating that the genome of dysgenic mice lacks a functional copy of the normal gene for the DHP receptor. A mutation of the DHP receptor gene would explain that CGRP does not restore L-type Ca^{2+} channel activity and E-C coupling.

The *mdg/mdg* muscle has been an important system for demonstrating that the α_1 subunit of the DHP receptor is the essential functional subunit for L-type Ca^{2+} channel activity [26,27]. The present work shows in addition that this diseased muscle system is of particular interest for studying the role of muscle morphogenesis and the conditions for maturation of the structures involved in E-C coupling. It is tempting to speculate that CGRP may be one of the trophic factors necessary for the maturation, stabilization and/or maintenance of normal cell/cell interactions in the neuromuscular system, although no direct evidence has yet been gathered for such a role.

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