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Hydrocortisone Influences Voltage-Dependent L-Type Calcium Channels in Cultured Human Skeletal Muscle

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The glucocorticoid hydrocortisone (HC), applied for up to 2 weeks to either aneurally or innervated cultured human muscle, produced 2-fold increase of the number of dihydropyridine ([^{3}H]PN200-110) binding sites. The K^+-induced, nifedipine-inhibited Ca^{2+} uptake was increased 40%. The effect of HC was concentration- and time-dependent. [^{3}H]PN200-110 affinity for its receptor was not affected by HC treatment. HC did not exert significant influence on the total amount of protein, CK activity, and the number of myotubes. These results indicate that voltage-dependent L-type Ca^{2+} channel expression in human muscle is regulated by glucocorticoid.

Key words: voltage-dependent Ca^{2+} channels, glucocorticoid, human muscle cultures, PN200-110, Ca^{2+} uptake

INTRODUCTION

Among all tissues, skeletal muscle exhibits the highest density of voltage-dependent L-type Ca^{2+} channels. In muscle, the L-type Ca^{2+} channels are located in the T-tubular system and are inhibitable by dihydropyridines. They first appear during fusion of myoblasts into myotubes and they increase dramatically during the postnatal development of the skeletal muscle (Renaud et al., 1989). The increased number of Ca^{2+} channels is accompanied by a decrease of dihydropyridine affinity for their receptor (Renaud et al., 1989). L-type Ca^{2+} channels play a key role in excitation-contraction coupling as voltage sensors (Tanabe et al., 1987). As calcium transporters, voltage-dependent Ca^{2+} channels induce a) the stabilization of acetylcholine receptors (AChRs) in the postsynaptic membrane (Rotzler et al., 1991) through calcium-dependent phosphorylation reactions (Caroni et al., 1993), and b) the accumulation of acetylcholinesterase (AChE) in the synaptic cleft (Decker and Berman, 1990). Voltage-dependent Ca^{2+} channels are regulated by a variety of factors. β-adrenergic agonists (Schmid et al., 1985), insulin (Desnuelle et al., 1987), and thyroid hormone (Brodie and Sampson, 1990) enhance Ca^{2+} channel expression in skeletal muscle, whereas FGF and TGFβ1 inhibit their expression (Shih et al., 1990).

Glucocorticoids (GCs) exert a profound influence on skeletal muscle and neuromuscular transmission (Wilson et al., 1974). They cause increased accumulation of nonjunctional AChRs on aneurally cultured human muscle (Askanas et al., 1986). On cultured human muscle that has been innervated for 4 weeks by rat spinal cord neurons, they increase accumulation of junctional AChRs (Braun et al., 1993) and AChE (Askanas et al., 1992).

We now report that hydrocortisone influences the development of L-type Ca^{2+} channels in aneurally and innervated cultured human muscle.

MATERIALS AND METHODS

Muscle Cultures

Human muscle cultures were established from satellite cells of portions of diagnostic muscle biopsies of patients considered free of muscle disease after diagnostic studies were performed, according to our standard technique (Askanas and Engel, 1975) with the current modifications (Askanas and Gallez-Hawkins, 1985). (Biopsies were obtained after informed consent.)

Aneural muscle cultures were established from dissociated cells plated in collagen-coated 35-mm petri dishes (1.5×10^5 cells per dish) and grown, unless specified otherwise, in an "aneural medium" composed of F14 medium (Gibco, Gaithersburg, MD) supplemented with 2 mM glutamine (Gibco), 10 μg/ml insulin

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(Sigma, St. Louis, MO), 10 ng/ml EGF, 12.5 ng/ml FGF (Collaborative Research, Bedford, MA), 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), and an antibiotic-antimycotic mixture (Gibco) (Askanas and Gallez-Hawkins, 1985). When the serum-free medium was used, cells were first grown for up to 10 days in the "aneural medium," and then, at the time when HC treatment was initiated, switched to the serum-free medium (Pegolo et al., 1990).

Human muscle-rat spinal cord cocultures were established as described previously (Askanas et al., 1987). In brief, immediately after myoblast fusion, explants of whole transverse slices of 13-day-old rat embryo spinal cords with dorsal root ganglia attached were placed on the muscle monolayer, 4–5 explants per 35-mm petri dish. Innervated cultures were maintained in F14 medium supplemented with 10% FBS, 10 μg/ml insulin and antibiotic-antimycotic mixture. Four weeks after innervation, FBS was decreased to 5%. At that time, innervated muscle fibers located in proximity to the spinal cord explants were virtually continuously contracting, they were cross-striated, and they had positive patches of AChE staining corresponding strictly to AChR clusters at the neuromuscular junction (Kobayashi et al., 1987). As described previously (Martinuzzi et al., 1986, 1988), innervated fibers were morphologically and spatially distinct from the noninnervated fibers and could easily be dissected out. Figure 1 illustrates human muscle cultured under the various conditions described above.

Treatment with HC (2×10^{-5}–10^{-12} M) (Sigma) of aneural cultures was initiated after myoblast fusion was completed (7–10 days after initiation of a culture), and continued for up to 10 days. Treatment of innervated cultures was initiated after 4 weeks of muscle-spinal cord coculture and continued for up to 2 weeks.

Binding Assays

Dihydropyridine binding sites were assayed using (+)-[*methyl*-^{3}H]PN200-110 (New England Nuclear [NEN], Boston, MA) according to the method of Desnuelle et al. (1987) with slight modifications.

When aneural cultures were studied, cells from 3 entire culture dishes were prepared. When using cocultures, innervated and noninnervated muscle fibers from 5–8 culture dishes were carefully dissected out as described previously (Martinuzzi et al., 1986, 1988) and separately harvested. Cultures were washed 3 times in Dulbecco's phosphate-buffered saline, without calcium and magnesium, and containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). Cells were centrifuged in the same buffer, and pellets were stored at −80°C until use. Upon use, cell homogenates were prepared by sonication of the pellets.

Quantitative measurement of the total number of [^{3}H]PN200-110 binding sites was performed on aliquots of cell homogenates (0.1–0.2 mg protein). Aliquots were incubated for 1 hr at 25°C in 1 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM PMSF, with 2 nM [^{3}H]PN200-110, in the presence (nonspecific binding) or absence (total binding) of 10^{-5} M unlabeled nifedipine (Sigma). After incubation, 400-μl aliquots were filtered in duplicate under vacuum on Whatman GF/B filters (Whatman, Maidstone, England) and washed twice with 10 ml of the Tris buffer. Radioactivity retained by the filters was measured using Biofluor (NEN) in a Beckman LS 6000SC scintillation spectrometer. Specific activity was calculated by subtracting counts obtained after blocking from total counts, and expressed as femtomoles of bound radioactivity.

For saturation-isotherm experiments, aliquots of cell homogenate (0.1–0.2 mg protein) were incubated, as with quantitative measurements, with 0.05–3.2 nM [^{3}H]PN200-110 in the presence or absence of nifedipine.

All procedures were done under dim light because of the light-sensitivity of the dihydropyridine derivatives.

Calcium Flux Experiments

Calcium uptake studies were performed on aneural cultures according to the method of Hayashi et al. (1991) with some modifications. Duplicates of culture dishes were washed 3 times with 1.5 ml of buffer A (10 mM Hepes-Tris, pH 7.4 containing 35 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM choline chloride, and 5 mM glucose). The dishes were then preincubated for 20 min at 37°C in the same buffer in the presence or absence of 10^{-5} M nifedipine. To initiate $^{45}Ca^{2+}$ uptake, the preincubation solution was replaced by 1 ml of the same buffer but containing 1.5 μCi $^{45}Ca^{2+}$ (NEN) with either 5 mM or 40 mM KCl (when KCl was 40 mM, choline chloride was reduced accordingly) in the presence (specific Ca^{2+} uptake) or absence (total Ca^{2+} uptake) of nifedipine. After 2 hr incubation at 37°C with $^{45}Ca^{2+}$, culture dishes were washed 3 times in less then 15 sec with 2 ml of ice-cold buffer A devoid of calcium and containing 1 mM $LaCl_3$. The cells were solubilized with 2 ml of 0.1 N NaOH for 2 hr. After solubilization, 1.8-ml aliquots were neutralized and their radioactivity was counted in 15 ml of Aquassure scintillation solution (NEN). Specific radioactivity incorporated by the cells was determined by subtracting counts obtained after blocking from total counts, and expressed as nanomoles of Ca^{2+} incorporated per dish or per mg of protein. All experiments were performed under dim light.

Protein and Total Creatine Kinase (CK) Determination

Protein content of the samples was measured according to the method of Bradford (1976). CK activity of

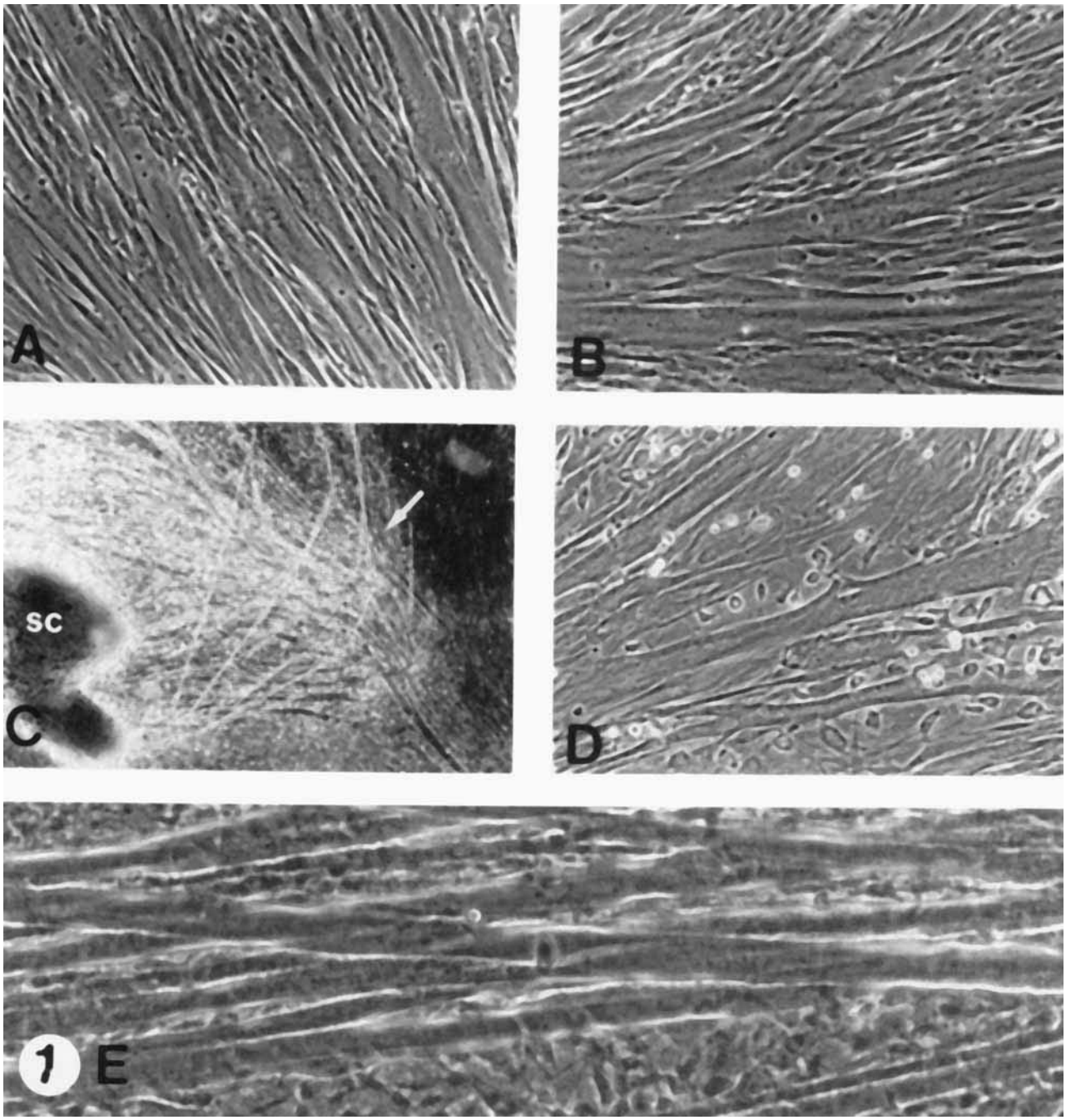

Fig. 1. Phase-contrast and darkfield microscopy of living cultured human muscle. Muscle cultured in aneural medium (**A**) and in serum-free medium (**B**). C,D,E: Cultured human muscle innervated by fetal rat spinal cord neurons for 7 weeks. **C**: Darkfield low-power microscopy of muscle-spinal cord cocultures. Single arrow points toward a group of densely packed muscle fibers, located close to a spinal cord explant (sc). **D**: Non-innervated, non-contracting not cross-striated human muscle fibers, located away from the innervated area.**E**: Innervated contracting, well-differentiated muscle fibers. Magnifications: ×120 (A,B,D); ×25 (C); ×275 (E).

TABLE I. Effect of HC on Human Muscle Cultured Aneurally in Different Culture Media†

Culture media		Ca^{2+} channels (fmol/dish)	CK (mU/dish)	Proteins (mg/dish)	Number of myotubes/field
Aneural media	Control	8.1 ± 1.1	29.6 ± 16.4	0.080 ± 0.014	20.0 ± 5.8
	HC	13.8 ± 3.1*	23.1 ± 10.2, ns	0.114 ± 0.021, ns	17.5 ± 5.2, ns
SFM	Control	8.4 ± 2.0	105.5 ± 58.5	0.112 ± 0.018	ND
	HC	16.7 ± 4.0*	97.4 ± 56.0, ns	0.130 ± 0.016, ns	ND
F14/FBS	Control	6.1 ± 0.9	52.8 ± 28.8	0.138 ± 0.021	17.5 ± 3.2
	HC	17.0 ± 5.9*	72.3 ± 15.0, ns	0.201 ± 0.039, ns	18.9 ± 7.2, ns

†Treatment with HC (2×10^{-5} M) continued for 7 days in cultures grown in aneural medium and SFM, and for 10 days for F14/FBS. Data are mean ± SEM of 5 experiments. ND, not determined; ns, not significant.
*$P < 0.05$.

the cell homogenates was measured spectrophotometrically using a CK-NAC-activated kit (Sigma).

Number of Myotubes

Myotubes were counted in aneural cultures on duplicate dishes (5 fields per dish were randomly chosen), using an inverted phase contrast microscope and a 20× objective lens (Olympus, Tokyo, Japan).

Statistical Analysis

All data were analyzed using a paired Student's *t*-test. A total of 25 experiments (750 cultures) was performed. Since no statistical difference in the amplitude of the effect of HC on Ca^{2+} channels between the various culture conditions (aneural medium, serum-free medium, F14/10% FBS) was found, data were combined for analysis when appropriate.

RESULTS

Effect of HC on Aneurally Cultured Human Muscle

As shown in Table I, treatment with HC induced a 2-fold increase of the number of dihydropyridine binding sites. This effect was not dependent on media used. No significant effect of HC on total creatine kinase, protein amount, or number of myotubes was found (Table I).

The effect of HC was dependent on the concentration of the hormone and the duration of treatment (Fig. 2A,B).

Because here as well as in previous studies (Braun et al., 1993; Askanas et al., 1992) 2×10^{-5} M of HC induced the strongest effect, this dose was used for the studies below.

$^{45}Ca^{2+}$ uptake experiments performed on aneurally cultured human muscle showed that the L-type Ca^{2+} channel-specific Ca^{2+} flux was increased 40% by treatment with 2×10^{-5} M HC (Fig. 3), but the affinity of Ca^{2+} channels of aneurally cultured muscle fibers was not modified by HC treatment. K_D of control and HC-treated cultures were 0.12 ± 0.01 nM and 0.17 ± 0.03 nM, respectively, as indicated by saturation-isotherm experiments (3 experiments) (Fig. 4).

Effect of HC on Innervated Human Muscle

Table II illustrates that 2 weeks of treatment with HC (2×10^{-5} M) increased the expression of voltage-dependent Ca^{2+} channels on innervated muscle fibers 2-fold. In noninnervated muscle fibers which are present in the same culture dish as innervated muscle fibers, but located a considerable distance from spinal cord explants and not contacted by spinal cord neurites (Martinuzzi et al., 1986, 1988) (Fig. 1), the expression of voltage-dependent Ca^{2+} channels was also increased 2-fold by HC treatment (Table II). Moreover, as reported previously (Sarkozi et al., 1992), untreated innervated-contracting muscle fibers had a much higher number of dihydropyridine binding sites as compared to untreated aneurally-cultured muscle fibers.

The amount of creatine kinase per mg of protein was not affected by HC treatment (Table II), but as in previous reports (Martinuzzi et al., 1986, 1988), innervated cultured muscle fibers had higher levels of creatine kinase activity than their noninnervated counterparts and aneurally-cultured muscle.

DISCUSSION

This study demonstrates that HC enhances, in a dose- and time-dependent fashion, the total number of [^{3}H]PN200-110 binding sites, in both aneurally cultured and innervated muscle fibers. This increase in aneurally-cultured human muscle occurred without modification of the affinity of the receptor for the specific ligand [^{3}H]PN200-110.

The K^+-induced, nifedipine-inhibited Ca^{2+} uptake was also increased by HC. 1,4-dihydropyridine-sensitive and K^+-induced Ca^{2+} uptake was demonstrated previously in cultured embryonic chick muscle and was related to Ca^{2+} flux through voltage-dependent L-type Ca^{2+} channels (Schmid et al., 1984). As in the chick model, we found in cultured human muscle cells that 40

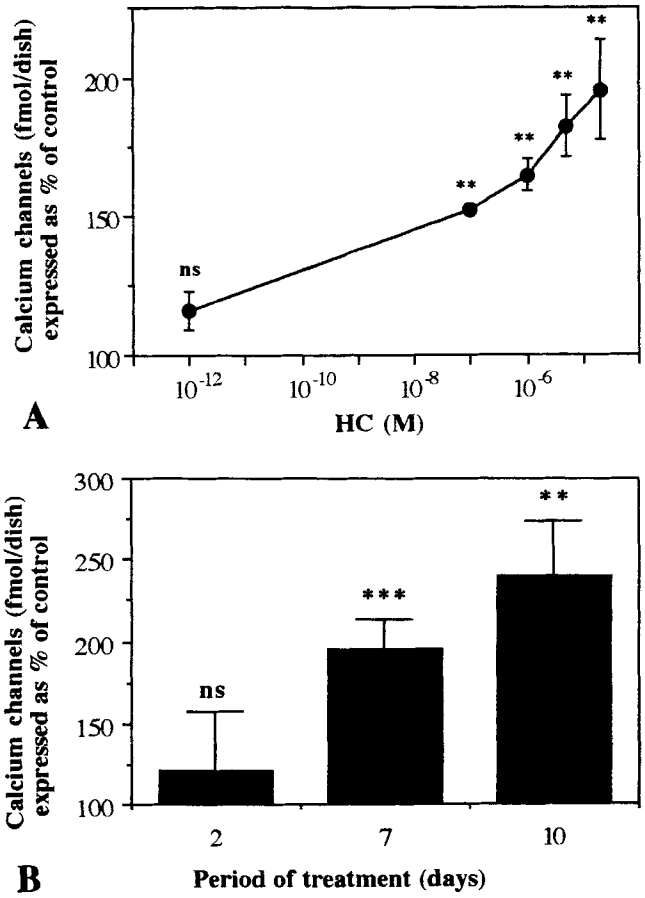

Fig. 2. Influence of HC concentration and duration of treatment on the number of Ca^{2+} channels of aneurally cultured human muscle. **A:** Effect-concentration relationship. Treatment with various concentrations of HC (from 10^{-12} M to 2×10^{-5} M) continued for 7 days. Results were gathered from 9 experiments using either aneural medium or SFM. Data are mean ± SEM. SEM values are indicated by vertical bars if they extend beyond the symbols. ns, not significant; $^{**}P < 0.01$. **B:** Influence of time on HC effect. Concentration of HC used was 2×10^{-5} M. Cultures were grown in either aneural medium or SFM. Data were gathered from 9 experiments. Bars are mean ± SEM. ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$. During the period of treatment, the number of Ca^{2+} channels/dish was relatively stable (7.7 ± 3.3 fmol/dish 2 days after treatment with HC was initiated, and 9.4 ± 2.5 fmol/dish 8 days later).

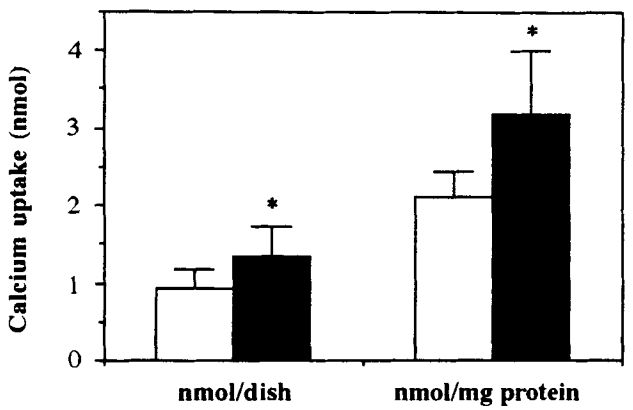

Fig. 3. Effect of HC on Ca^{2+} uptake. Ca^{2+} uptake by aneurally cultured control and HC-treated human muscle. □, control cultures; ■, cultures treated with HC, 2×10^{-5} M for 7 days. $^{*}P < 0.05$ (n = 4 experiments). Ca^{2+} uptake was increased by 36.8 ± 3% when expressed as nmol/dish, and 42.8 ± 13.7% when expressed as nmol/mg protein.

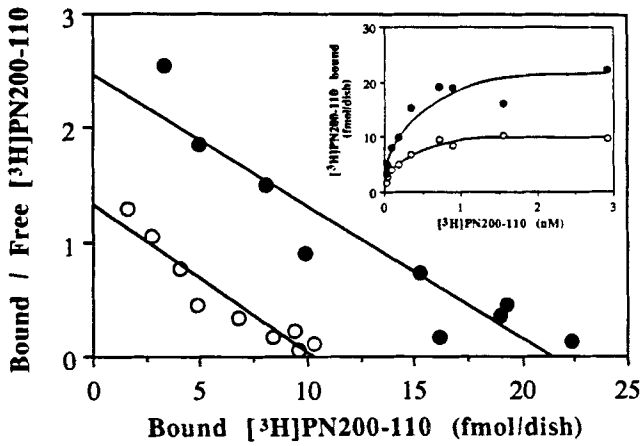

Fig. 4. Effect of HC on [^{3}H]PN200-110 binding to aneurally cultured human muscle. Scatchard plot of the data leading in this typical example to K_D = 0.13 nM and B_{max} = 10.2 fmol/dish in control cultures, and K_D = 0.14 nM and B_{max} = 21.5 fmol/dish in HC-treated cultures. **Inset:** Equilibrium binding of (+)-[*methyl*-^{3}H]PN200-110 to crude homogenates of aneurally cultured human muscle. ○, control culture; ●, culture treated with HC 10^{-5}M for 7 days.

mM K^+ caused change in muscle membrane potential (−53 ± 6 mV to −29 ± 4 mV (n = 10) (Kobayashi and Askanas, unpublished observations; Desnuelle et al., 1987), and caused muscle to accumulate $^{45}Ca^{2+}$, demonstrating depolarization-induced calcium uptake. The K^+-induced calcium uptake was not due to other voltage-dependent channel such as AChR, since specific blockers did not modify the amount of $^{45}Ca^{2+}$ incorporated by the cells (data not shown). Nor was it due to Na^+/Ca^{2+} exchange, since the same $^{45}Ca^{2+}$ accumulation could be demonstrated when NaCl had been replaced by choline in the incubation buffer. The $^{45}Ca^{2+}$ uptake was also inhibited by 2 μM nitrendipine (Desnuelle et al., 1987). Thus, the calcium flux we measured reflects L-type Ca^{2+} channel activity.

The effect of HC on voltage-dependent Ca^{2+} channels was specific, since other developmental muscle markers (i.e., creatine kinase activity, number of myo-

TABLE II. Effect of HC on Ca^{2+} Channel Expression in Nerve-Muscle Cocultures†

	Innervated fibers		Noninnervated fibers	
	Ca^{2+} channels (fmol/mg proteins)	CK (mU/mg proteins)	Ca^{2+} channels (fmol/mg proteins)	CK (mU/mg proteins)
Control	222 ± 12	1242 ± 188	88 ± 17	476 ± 130
HC	387 ± 53*	1390 ± 222, ns	164 ± 31*	537 ± 120, ns

†Treatment with HC (2×10^{-5} M) continued for 2 weeks. Data are mean ± SEM of 5 experiments. ns, not significant.
*$P < 0.05$.

tubes, and amounts of protein) were not influenced by HC treatment. The influence of HC did not depend on the presence of serum, growth factors and innervation, since both innervated and contracting muscle fibers, which are more mature than aneural cultures (Martinuzzi et al., 1986, 1988; Saito et al., 1990), and noninnervated and noncontracting muscle fibers present in the cocultures (Askanas et al., 1987), responded to HC treatment in the same way.

We do not know how HC stimulates L-type Ca^{2+} channel activity. This effect may be due to an enhancement of membrane insertion and/or stabilization of the L-type Ca^{2+} channels or its α1 subunit, by an unknown direct or indirect mechanism. It is also unknown whether the Ca^{2+} channel is enhanced or if the number of channels is modified. Since the affinity of voltage-dependent Ca^{2+} channels is not modified by HC treatment, a rise in the intracellular level of cyclic AMP, which has been found previously to increase Ca^{2+} channel expression and simultaneously to decrease its affinity, is probably not involved in the HC effect described here. Even though an increased organization of the T-tubules by HC is a plausible explanation, this mechanism is not likely, since a) HC treatment does not seem to influence the number and ultrastructural organization of the T-tubules in our culture system (Askanas, unpublished observation), and b) a similar effect of GCs on L-type Ca^{2+} channel expression has been found in T-tubule-free cells, namely, in a smooth cell line (Hayashi et al., 1991) and in rat brain synaptosomes (Sze and Iqbal, 1991). However, it is possible that the HC-induced increase of both specific sites and specific $^{45}Ca^{2+}$ uptake might reflect an increase of the number of L-type Ca^{2+} channels per T-tubule. Electrophysiological studies should complete our understanding of the phenomenon.

The fact that both aneural and innervated and noninnervated muscle fibers are able to respond to HC treatment indicates that innervation is not required for its effect on Ca^{2+} channels to take place. Similarly, it has been shown by others that appearance of L-type Ca^{2+} channels on skeletal muscle cells is not dependent on innervation (Moody-Corbett and Virgo, 1991), and their presence is insensitive to denervation (Renaud et al., 1989; Shih et al., 1990).

It is known that GCs exert a wide variety of effects on pre- and postsynaptic components of the neuromuscular junctions and other synapses (reviewed in Braun et al., 1993). We have previously demonstrated that GCs increase the number of AChRs in aneural (Askanas et al., 1986) and innervated (Braun et al., 1993) cultured human muscle. Moreover, insulin and β-adrenergic agonists also influence the expression of both acetylcholine receptors (Askanas et al., 1985; Blosser, 1983) and L-type Ca^{2+} channels (Desnuelle et al., 1987; Schmid et al., 1985). Thus, the expression or activity of both AChRs and voltage-dependent Ca^{2+} channels in cultured muscle may be regulated by GC in parallel ways. A functional relationship between AChRs and voltage-dependent Ca^{2+} channels was previously suggested (Desnuelle et al., 1987). Recently, Rotzler et al. (1991) showed that stimulation of Ca^{2+} channels promotes AChR stabilization in the postsynaptic membrane. However, we do not know presently whether the effect of GCs on AChRs is exerted through their influence on L-type Ca^{2+} channels.

L-type Ca^{2+} channels play an important role in excitation-contraction coupling and have been shown to be causatively involved in muscle pathology (Chaudhari, 1992). Therefore, tissue culture of human muscle should provide an excellent model for studying molecular mechanisms underlying the influence of drugs, hormones, and various factors on voltage-dependent Ca^{2+} channels in normal and diseased muscle.

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