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# Calcium influx blocks the skeletal muscle acetylcholine receptor α-subunit gene in vivo

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**Abstract**

The transcriptional activity of the acetylcholine receptor α-subunit gene was measured in denervated chick skeletal muscle in response to calcium-active drugs, using a ribonuclease protection version of the conventional run-off assay The L-channel agonist (−)Bay-K6844 and the calcium ionophore A23187 mimicked, and the intracellular chelator BAPTA and the calcium channel blockers D600 and nifedipine blocked, the effect of electrostimulation These results suggest that influx of extracellular calcium is an integral component of the membrane depolarization–receptor gene inactivation cascade

*Key words* Skeletal muscle, Acetylcholine receptor, Regulation, Calcium, Excitation–transcription coupling, *Gallus domesticus*

## 1. Introduction

Adult myofibers harbor the ε isoform of the nicotinic acetylcholine receptor (AChR) at the endplate, but revert to the extrajunctional expression of the embryonic γ-type receptor upon denervation. Conversely, reinnervation or electrical stimulation of denervated 'supersensitive' fibers rapidly leads to the restitution of the adult phenotype [1] Since depolarization triggers contraction, it has been suspected that elements of the excitation–contraction coupling (ECC) pathway may be shared by the signaling pathway that communicates information on membrane activity to the nucleus. In particular, calcium, which functions as a cytosolic messenger in ECC, has been postulated to serve as a shut-off factor in AChR regulation [2,3] Evidence for the involvement of calcium has come from experiments with cultured cells; there, calcium effectors change the number of surface receptors and affect levels of AChR subunit messages (for reviews see [4,5]). It is not known to what extent message levels reflect gene activity and how reliably in vitro models mimic in vivo processes We have therefore begun to measure gene activity in skeletal muscle in vivo Taking advantage of the ease of preparing active nuclei from skeletal muscle of newborn chicks [6], we have previously identified activation of a protein kinase C in the nucleus as a component of the pathway coupling membrane activity to receptor gene suppression [7] We now report on the effect of calcium drugs in vivo, our findings indicate that calcium entering through the depolarized plasma membrane rapidly inactivates AChR gene transcription.

## 2. Materials and methods

### 2 1 *Animal experimentation*

Sciatic nerve section was performed on 4-day-old Leghorn chicks (Hall's Brothers Hatchery, North Brookfield MA) as described previously [5] Forty hours after sciatic nerve section, 20 μg in 20 μl of 20% dimethyl sulfoxide in ethanol (v/v) of each reagent were injected intramuscularly, drugs were from Biomol (Plymouth Landing PA) except for A23187 (Sigma, St Louis MO) and BAPTA acetoxymethyl ester (BAPTA-AM, a product of Molecular Probes, Eugene, OR) Denervated muscle refers to the leg musculature between knee and ankle 40 h after denervation, at which time AChR genes are maximally active [6] For the stimulation experiments, the protocol of Lømo and Westgaard [1] was adopted Briefly, the denervated leg musculature was stimulated for periods of 30 min in 100-Hz trains, 2 s in duration, and applied once every minute At the desired time, animals were sacrificed, and the leg musculature was processed immediately for subcellular fractionation and isolation of nuclei All animal experimentation utilized protocols approved by the Institutional Animal Care and Use Committee

### 2 2 *Gene activity analysis*

Transcript elongation was carried out with $10^7$ freshly prepared or liquid nitrogen-stored nuclei, using 50 μCi [$^{32}$P]UTP, as described [8] Nascent transcripts were purified through a Sephadex G-50 spun column, followed by TCA and ethanol precipitations Riboprobes were prepared as follows The 399-bp *Hin*dIII–*Eco*RI genomic fragment of the chicken AChR α-subunit gene, containing exon 7 and flanking intron sequences [9], was cloned into the Bluescript plasmid pSK− in order to generate riboprobe (protected region 400 nt) The 3′ end of the chicken MLC cDNA was removed by cutting MLC1f with SstI, an antisense probe was prepared by linearizing the plasmid and transcribing with SP6 (protected region 180 nt) For hybridization, aliquots containing a constant amount of radiolabel ($10^6$ cpm) were incubated with 0 1 μg of antisense RNA probes at 52°C, RNase A digestion and

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*Abbreviations* AChR, acetylcholine receptor, ECC, excitation–contraction coupling


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electrophoretic analysis followed the procedure of Melton et al [10] Results were quantified using a Beta scanner (Ambis, San Diego CA) visualized by autoradiography Solution hybridization followed by RNase protection analysis is very sensitive and permits comparison of the activity of the gene in question with that of any other gene(s) under identical conditions, provided the probes are of distinct size

## 3. Results and discussion

Experiments were performed with denervated chick leg muscle in situ Forty hours after section of the sciatic nerve, when AChR genes are transcribed at maximal rates [6], the denervated muscle was subjected to electrostimulation and/or intramuscular drug injection, nuclei were isolated, and gene activity analysed If calcium mediates depolarization-AChR gene repression coupling, raising the calcium concentration in the cytosol without electrical stimulation should also inactivate receptor genes We therefore tested the effect of A23187, a calcium-specific ionophore that would be expected to dissipate calcium gradients and raise cytosolic calcium, on the activity of the AChR α-subunit gene As can be seen in Fig 1, the ionophore reduced receptor α-subunit gene

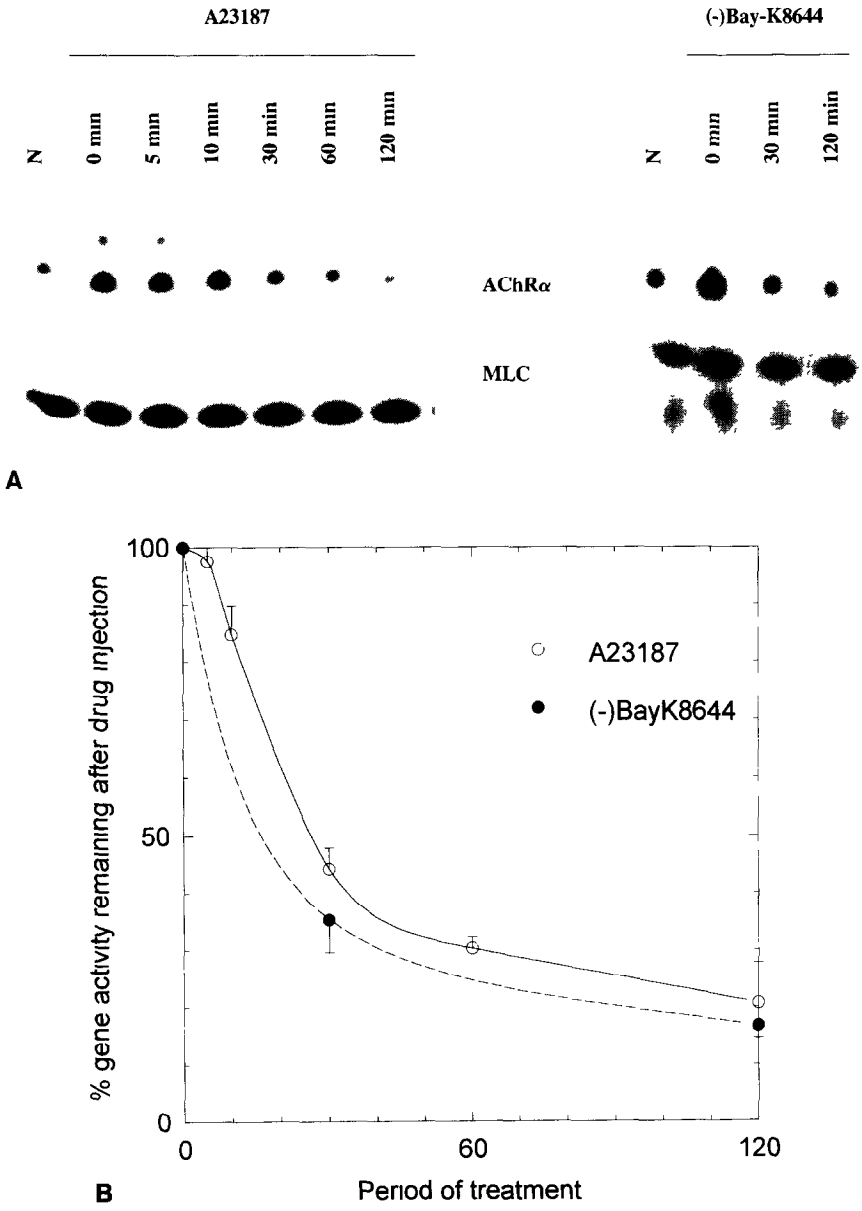


Fig 1 The calcium ionophore A23187 and the calcium channel activator (−)Bay-K8644 inactivate the AChR α-subunit gene in denervated muscle Chicks received injections of A23187 or (−)Bay-K8644 (20 μg in 20% DMSO in ethanol (v/v)) into the denervated musculature After the indicated periods of time, animals were sacrificed, nuclei isolated, and gene activity determined by transcript elongation analysis Results shown were obtained with probes specific for the AChR α-subunit and MLC genes (A) Autoradiograph of ribonuclease protection analysis of the α-subunit gene The top protected band (400 nt) reflects the strength of the α-subunit gene, and the bottom one (180 nt) reveals activity of the MLC gene, N, innervated muscle, no treatment (B) Quantitative analysis Each data point represents the mean of 3 to 7 independent measurements Results were normalized to the transcriptional activity of the α-subunit gene in nuclei from 40-h denervated muscle

activity by approximately 20% within 10 min of the addition of the drug, and to the control (innervated) level within one hour This is most readily explained as a consequence of calcium influx into the myofiber and suggests that the rise in intracellular calcium that accompanies membrane depolarization is sufficient for the effects seen at the gene level Calcium ions may mediate depolarization-gene inactivation coupling as a result of ion influx through voltage-gated channels We therefore tested the effect of (−)Bay-K8644, an activator of the L-type dihydropyridine-sensitive calcium channel Upon addition of the drug, the receptor α-subunit gene returned to control (innervated) levels within 30 min, suggesting that calcium entry or an event downstream of it activates excitation-AChR gene suppression coupling. Because expression of myofibrillar proteins is not drastically affected by denervation and electrostimulation, the myosin light chain (MLC) gene was chosen as a reference gene Its transcriptional activity fell by 30% over a 2-hour period This more moderate effect may arise from a limited similarity in the regulatory regions of the two genes, the enhancers of both the AChR α-subunit [11,12] and MLC [13] genes comprise E boxes which might be activated by the same or related HLH proteins

The calcium hypothesis also predicts that inhibition of either the influx or the accumulation of calcium should disrupt the signaling pathway We therefore tested the effect of calcium blockers The administration of drugs, such as nifedipine and D600, which are known to inhibit the L-type calcium channel, completely blocked the effect of electrical stimulation on receptor gene activity in denervated chick skeletal muscle (Fig 2), but showed no effect on α-subunit gene transcription rate in the absence of depolarization This indicates that in contrast to ECC which in skeletal muscle functions in the absence of extracellular calcium, influx of calcium through the plasma membrane is required for depolarization to affect gene activity Cytosolic calcium can be lowered with membrane-permeant chelators. We treated denervated chick skeletal muscle with BAPTA-AM, a potent cell permeant calcium chelator, prior to electrical stimulation As can be seen from Fig 2, BAPTA-AM abolishes the electrostimulation response of the AChR α-subunit gene, suggesting that cytosolic calcium plays a role in the signaling cascade Treatment with BAPTA-AM did not affect receptor gene activity in the absence of electrical stimulation We have previously shown that a calcium-requiring nuclear PKC mediates depolarization-receptor gene inactivation coupling [7], it is possible therefore that the BAPTA-AM treatment results directly in the inactivation of this calcium-dependent kinase

In skeletal muscle, calcium acts as an indispensible link in ECC, by binding to calmodulin which acts as a regulatory subunit of phosphorylase kinase [14] it also initiates the glucose mobilization required for enhanced mechanical work From a regulatory point of view it makes sense that calcium should trigger the contractile apparatus as well as inform the metabolic machinery of the activity of the muscle fiber Similarly it is reasonable to postulate that the newly innervated and active myofiber utilize calcium signals to notify the genome that the γ-isoform of AChR expressed prior to innervation is no longer required Evidence for the effect of manipulations of the cytosolic calcium levels on AChR levels in cultured muscle cells has been available for years [15–21] More recent investigations have revealed that AChR subunit

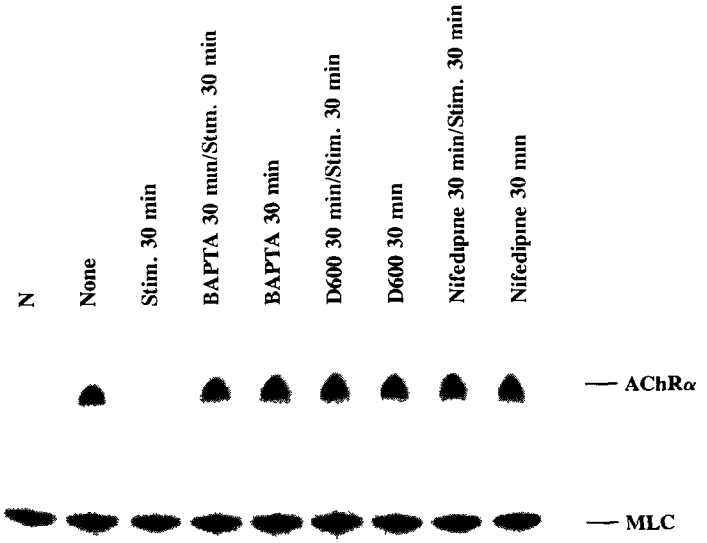


Fig 2 Treatment with calcium channel blockers or an intracellular calcium chelator abolishes the effect of depolarization Chicks received, into the denervated muscle, injections of nifedipine, D600, or BAPTA acetoxymethyl ester (20 μg in 20% DMSO in ethanol (v/v)), after 30 min, the treated musculature of some of the animals was electrically stimulated for another half hour Parallel experiments were performed on denervated chicks with either only 30 min electrical stimulation or only 30 min exposure to drug Nuclei were then isolated, and gene activity was assayed

mRNA levels are similarly affected [22,23]; these latter observations suggested that calcium control may be exerted at the transcriptional level. In addition, we have observed that calcium influx inactivates AChR genes in cultured C2C12 cells (Huang, Flucher and Schmidt, unpublished) The present work firmly establishes calcium as an inhibitor of receptor gene transcription in vivo as well A further dissection of the signaling pathway, focused on the intracellular calcium sensing mechanism, is currently underway

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