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Induction of Na^+/K^+-ATPase activity by long-term stimulation of nicotinic acetylcholine receptors in C2C12 myotubes

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1 To investigate the role of long-term stimulation of nicotinic acetylcholine receptors (AChRs) on the regulation of membrane potential, non-contracting C2C12 myotubes were stimulated for 1–4 days with carbachol (10 μM) and membrane potentials were measured by the intracellular microelectrode technique after washing out of the drug.

2 The membrane potential (− 45.7 mV) gradually increased by 10.1 mV to − 55.8 mV during 4 days treatment, which was caused by enhanced electrogenic Na^+/K^+-pumping.

3 The concentration-dependent enhancement of Na^+/K^+-ATPase activity in long-term carbachol-treated myotubes (4 days, EC_{50} = 5.3 μM) was prevented by co-treatment with the competitive nicotinic AChR antagonist, pancuronium but not by the muscarinic antagonist, atropine.

4 Enhanced Na^+/K^+-ATPase activity still developed in carbachol-stimulated myotubes during co-treatment (4 days) with the nicotinic AChR-channel blocker, chlorpromazine (1 μM). Membrane depolarization as such, obtained by incubation in high K^+ medium (40 mM, 4 days) did not enhance Na^+/K^+-ATPase activity.

5 Non-treated myotubes possessed a high-affinity ouabain binding site (K_d = 119 nM) in association with the low Na^+/K^+-pumping activity. Long-term stimulation of myotubes (4 days) with carbachol or with a combination of carbachol and chlorpromazine was accompanied by the development of an additional low-affinity ouabain binding site (K_d = 13 μM).

6 Binding of monoclonal antibodies directed against either α_1- or α_2-subunit of Na^+/K^+-ATPase were both increased in myotubes treated with carbachol (4 days).

7 These results support the concept that nicotinic AChRs regulate Na^+/K^+-ATPase activity, independent of the functionality of the receptor-operated ion-channel.

Keywords: Na,K transporting ATPase; nicotinic acetylcholine receptor; C2C12 myotubes

Introduction

Long-term interruption of neuromuscular transmission by drugs or toxins induces denervation-like changes in skeletal muscle (Chang *et al.*, 1975; Simpson, 1977). Thus, long-term administration of competitive blockers of the nicotinic acetylcholine receptor (AChR) is associated with the development of extrajunctional nicotinic AChRs and a decrease in membrane potential (Berg & Hall, 1975; Pestronk *et al.*, 1980). The development of these properties is thought to be related to the cessation of muscle contraction, for they are almost completely counteracted by direct stimulation (Drachman & Witzke, 1972). However, the disruption of stimulation of nicotinic AChRs might also contribute to the effects observed on long-term administration of competitive blockers. Indeed, some studies suggest a trophic role for ACh. For instance, it has been found that drugs interfering only with nerve impulse-dependent ACh release induce less extrajunctional nicotinic AChRs than agents that block spontaneous ACh release as well (Pestronk *et al.*, 1976; Mathers & Thesleff, 1978). Moreover, a possible role for nicotinic AChRs is strongly supported by a study demonstrating denervation-like changes in the skeletal muscle of animals that had been chronically treated with low concentrations of the nicotinic AChR antagonist (+)-tubocurarine not interfering with muscular contraction (Hogue *et al.*, 1992). To examine the role of nicotinic AChRs in the regulation of skeletal muscle properties, we studied the effect of long-term stimulation of nicotinic AChRs on the membrane potential of non-innervated, non-contracting C2C12 myotubes.

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Methods

Cell culture

C2C12 cells, a murine myoblast cell line (Yaffee & Saxel, 1977) were obtained from the American Tissue Type Collection, Rockville, U.S.A. Cells were cultured, for most experiments on glass cover slips, in 9.6 cm^2 plastic wells at 37°C in Dulbecco's modified essential medium, 7 mM $NaHCO_3$ and 10 mM HEPES (DMEM) supplemented with 10% foetal calf serum. When cells reached 80% confluence, medium was changed to DMEM supplemented with 5% horse serum (HS). Low Na^+ (23 mM) medium was obtained by diluting DMEM with medium of the following composition (mM): glucaminechloride 125, KCl 6, $CaCl_2$ 1.2, $MgCl_2$ 2.5, NaH_2PO_4 1.2, glucose 11, HEPES 10, HS 5% (pH 7.4). High K^+ (60 mM) medium was obtained by mixing DMEM with medium of the following composition (mM): KCl 131, $CaCl_2$ 1.2, $MgCl_2$ 2.5, NaH_2PO_4 1.2, glucose 11, HEPES 10, HS 5% (pH 7.4). Control medium was obtained by appropriately diluting DMEM with medium of the following composition (mM): NaCl 125, KCl 6, $CaCl_2$ 1.2, $MgCl_2$ 2.5, NaH_2PO_4 1.2, glucose 11, HEPES 10 (pH 7.4). Myotubes were used 7 days after initiating myoblast fusion. Spontaneous contractions of the myotubes were not observed.

Electrophysiology

Membrane potential was measured by microelectrodes filled with 1 M KCl as described previously (Henning *et al.*, 1992). Experiments were performed following a 15 min wash at 25°C in a 1 ml bath superfused at a rate of 1.5 ml min^{-1} with the following solution (mM): NaCl 125, KCl 6, $CaCl_2$ 1.2,

$MgCl_2$ 2.5, NaH_2PO_4 1.2, glucose 11, HEPES 10 (pH 7.4). In the low Na^+ solution (23 mM), NaCl (102 mM) was replaced with glucaminechloride. Drugs used were applied by changing the superfusing fluid with buffer containing the required drug concentration.

[^{3}H]-ouabain binding

The number of [^{3}H]-ouabain binding sites was determined by measuring the specific binding of [^{3}H]-ouabain at several ouabain concentrations (20 nM–1 μM). Total binding at 20°C was assessed in buffer of the following composition (mM): NaCl 150, $MgCl_2$ 4, HEPES 10 (pH 7.4). Non-specific binding was determined in a buffer of the following composition (mM): KCl 150, $MgCl_2$ 4, HEPES 10 (pH 7.4). Cells were successively washed three times in the buffer, incubated for 30 min in 1 ml buffer containing the final ouabain concentration including [^{3}H]-ouabain (0.5–3.0 μCi.ml^{-1}) and washed four times with ice-cold buffer. A final wash was performed with additional 2% ethanol. Thereupon, cells were solubilized in 1 ml NaOH (1 M) and radioactivity was measured by liquid scintillation counting. Data were subjected to kinetic modelling (Munson & Rodbard, 1980). A two-site binding model was preferred over a one-site binding model if a significant reduction in the residual sum of squares of the fitted curve was obtained (F test, $P<0.05$).

Analysis of Na^+/K^+-ATPase antibody binding

Two monoclonal antibodies were used (courtesy of Dr K.J. Sweadner). McK1 is a mouse IgG_1 antibody raised against rat kidney Na^+/K^+-ATPase, which cross-reacts with the α_1-subunit of both native and denatured mouse Na^+/K^+-ATPase (Felsenfeld & Sweadner, 1988). McB2 is a mouse IgG_1 antibody raised against rat axolemma Na^+/K^+-ATPase and recognizes the α_2 subunit of the native and denatured enzyme (Urayama *et al.*, 1989). Staining procedure consisted of an initial wash (30 min) of cells grown on monolayers on glass coverslips, followed by fixation for 30 min in periodate-lysine-paraformaldehyde, an overnight wash in 0.02 M potassium phosphate buffered saline (KPBS, pH 7.4) and treatment with Triton X-100 (0.3%) and H_2O_2 (10%) in KPBS for three periods of 10 min. After washing with KPBS containing 0.3% Triton X-100 (KPBS-T), the monolayers were incubated with McK1 (dilution 1:24) or McB2 (1:10) for 60 h. Secondary antibodies (RAM/IgG (H + L), 1:400, Nordic Immunological Lab, Tilburg, the Netherlands) were introduced for 2 h after washing in KPBS-T. After washing, cells were subsequently incubated with rabbit peroxidase-anti-peroxidase (1:800, Nordic) in a solution containing 0.05% 3,3-diaminobenzidine (DAB), 2.5% nickel ammonium sulphate (NAS), 0.04% ammonium chloride and 0.004% H_2O_2. Staining intensity was determined after washing in KPBS-T by scanning the absorbance of the cells at 497 nm (100 pixels at 10 μm distance in an area of 200 × 200 μm) using a Leitz orthoplan microscope with 10 × objective and equipped with a Leitz MPV compact photometer with scanning stage control unit as described by Wolters *et al.* (1984). Coverslips were scanned at 20 randomly chosen areas. Data were corrected for non-specific absorbance of the cells and are presented as mean ± s.e.mean of the absorbance. Control coverslips with treated and non-treated myotubes that were processed without incubation with the primary or secondary antibody did not stain.

Results

Membrane potential: acute effects of carbachol

In acute experiments, the non-hydrolysable AChR agonist, carbachol (1–30 μM) evoked a sustained and reproducible depolarization of the cells (Figure 1a). The amplitude of the depolarization was dependent on the agonist concentration, reaching a maximum at 30 μM (43.4 ± 2.2 mV; $n = 6$). This action of carbachol was inhibited by the competitive nicotinic AChR antagonist, pancuronium (Figures 1b,d), but not by the muscarinic receptor antagonist, atropine (1 μM; not shown). Under low Na^+ conditions (23 mM), the depolarization evoked by carbachol (30 μM) was greatly reduced, being 9.2 ± 1.0 mV ($n = 6$). Chlorpromazine is known to block the nicotinic AChR-coupled ion-channel use-dependently (Changeux, 1990). In fact, carbachol (10 μM) evoked only an initial depolarization in the presence of chlorpromazine (1 μM; Figure 1c).

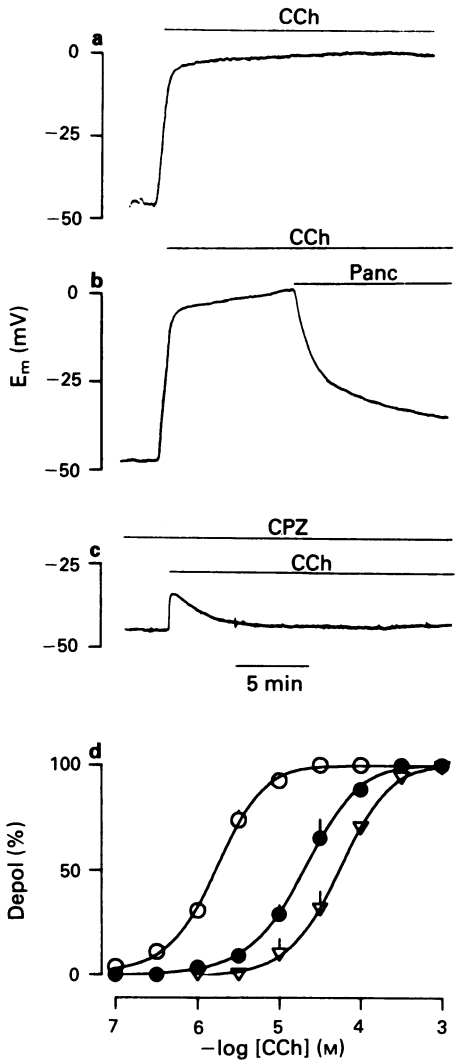

Figure 1 The acute effect of carbachol (CCh) on membrane potential (E_m) of C2C12 myotubes under different experimental conditions. (a) Recording of the sustained depolarization evoked by carbachol (10 μM; horizontal bar); (b) the antagonistic effect of pancuronium (Panc, 1 μM; horizontal bar); (c) use-dependent block by chlorpromazine (CPZ, 1 μM); (d) concentration-effect curve of carbachol (○; $n = 6$) and in the presence of pancuronium (0.3 μM, ●; $n = 4$) and (1 μM, ▽; $n = 4$). Data (± s.e.mean) are expressed as the percentage of the maximal depolarization (43.4 ± 2.2 mV; $n = 6$).

Membrane potential: long-term effects of carbachol

The effect of chronic stimulation of the nicotinic AChRs was studied by treatment of the C2C12 myotubes for several days with carbachol and assessed by measuring their membrane potential in the absence of the agonist (≥ 15 min). Non-treated myotubes possessed a relative small membrane potential of about −46 mV and inhibition of Na^+/K^+-pumping by ouabain (200 μM) resulted in a minor reduction of their

Table 1 The effect of long-term stimulation of nicotinic acetylcholine receptors (AChRs) on membrane potential of C2C12 myotubes

	Incubation	*After washing*			
Treatment	E_m† (mV)	E_m (mV)	E_m (Ouab) (mV)	V_{el} (mV)	n
None	− 46.2 ± 1.5	− 45.7 ± 0.6	− 43.5 ± 0.8	− 1.2 ± 1.0	30
Carbachol	− 8.4 ± 2.2	− 55.8 ± 1.3*	− 45.4 ± 1.4	− 10.4 ± 1.9*	12
Carbachol/CPZ	− 54.6 ± 2.9	− 52.9 ± 1.3*	− 44.1 ± 1.3	− 8.8 ± 1.8*	12
High K^+	− 13.7 ± 3.3	− 42.6 ± 1.3	− 44.6 ± 0.5	− 2.0 ± 1.4	12

Cells had been incubated for 4 days in the presence of carbachol (10 μM), the combination of carbachol (10 μM) and chlorpromazine (CPZ; 1 μM) or high K^+ (40 mM) medium; membrane potential was measured by the intracellular microelectrode technique. E_m† represents the membrane potential still in the presence of drugs or high K^+ ($n = 4$). After washing, the membrane potential was determined in the absence (E_m) and in the presence of ouabain (200 μM; E_m (Ouab)) to calculate electrogenic Na^+/K^+-pumping (V_{el}). Values are expressed as mean ± s.e.mean of n experiments.
*Significantly different from non-treated cells, $P < 0.01$.

membrane potential (1.2 mV; Table 1; Figure 2a). Myotubes incubated for 4 days with carbachol (10 μM) maintained their depolarization in the presence of the agonist (Table 1). However, after removing carbachol (10 μM), the myotubes showed a pronounced increase in membrane potential of about − 10 mV compared to non-treated cells (Table 1; Figure 2a,b). Inhibition of Na^+/K^+-ATPase in carbachol-treated myotubes by ouabain (200 μM) caused a significantly higher reduction in membrane potential than in non-treated cells (Figure 2a,b; Table 1). The membrane potential of non-treated and carbachol-treated cells reached comparable values in the presence of ouabain (Figure 2a,b; Table 1). The enhanced Na^+/K^+-ATPase activity induced by carbachol (10 μM) developed gradually during the 4 days treatment and declined slowly after withdrawal of the nicotinic AChR agonist (Figure 2c,d). Carbachol treatment for 4 days produced a concentration-dependent increase in electrogenic Na^+/K^+-pumping ($EC_{50} = 5.3$ μM; Figure 2e), at similar concentrations to those observed in acute experiments (Figure 1d).

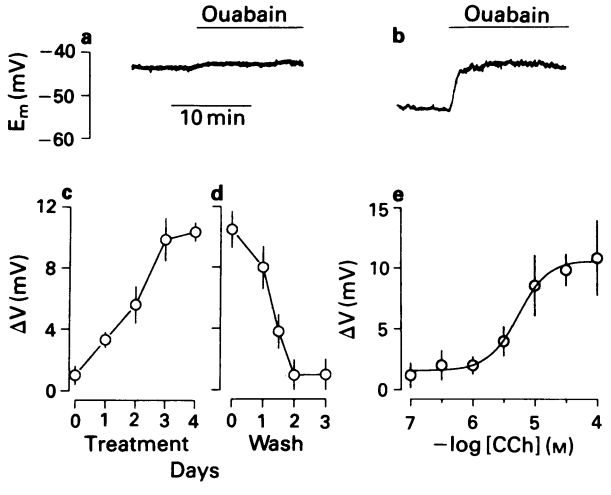

Figure 2 The effect of 4 days treatment of the cells with carbachol (10 μM) on membrane potential and electrogenic Na^+/K^+-pumping in C2C12 myotubes, measured in the absence of carbachol. (a) The membrane potential of non-treated cells showed a small reduction upon inhibition of electrogenic pumping with ouabain (200 μM; horizontal bar); (b) treated cells showed a pronounced decrease in membrane potential in the presence of ouabain (200 μM); (c) the development of electrogenic pumping in time during carbachol treatment; (d) the decline of electrogenic pumping in time after washing 4 days carbachol treated cells; (e) concentration-effect curve of the amount of electrogenic pumping after 4 days of treatment with various concentrations of carbachol (0.1–100 μM). Values (± s.e. mean) are expressed as the difference between membrane potential in the absence and presence of ouabain (200 μM; $n \geq 12$).

To assure that the development of enhanced Na^+/K^+-ATPase activity was mediated by nicotinic AChRs, myotubes were treated with carbachol (4 days) in the presence of identical concentrations of selective antagonists. Myotubes simultaneously treated with carbachol (10 μM) and the nicotinic AChR antagonist, pancuronium (1 μM) possessed the same membrane potential (− 46.8 ± 1.7 mV; $n = 10$) as non-treated or pancuronium-treated myotubes. Pancuronium-treated myotubes showed the same membrane potential as non-treated cells (− 45.0 ± 2.9; $n = 6$). In contrast, the muscarinic antagonist, atropine (1 μM) did not prevent the increase in Na^+/K^+-pumping of myotubes as observed in cells treated for 4 days with carbachol (10 μM). A similar increase in membrane potential (10.1 ± 2.0 mV; $n = 12$) as in carbachol-treated myotubes was observed in the presence of ouabain (200 μM).

The development of enhanced Na^+/K^+-ATPase activity in long-term carbachol-stimulated myotubes might be due to the Na^+-influx passing the receptor coupled ion-channel. This was investigated in myotubes treated for 4 days with carbachol and chlorpromazine, inhibiting the nicotinic AChR coupled Na^+-influx (Figure 1c). In the presence of chlorpromazine, the carbachol-stimulated nicotinic AChR-channel was completely blocked, as demonstrated by the observation that the membrane potential of cells, treated for 4 days with carbachol (10 μM) and chlorpromazine (1 μM), still in the presence of both drugs (− 54.6 mV; Table 1), did not change after withdrawal of carbachol (− 51.6 ± 1.2 mV; $n = 8$). After removing both drugs, myotubes treated with a combination of carbachol (10 μM) and chlorpromazine (1 μM) showed an increase in membrane potential comparable to that of long-term carbachol-treated cells (Table 1). Long-term treatment of the myotubes with chlorpromazine alone did not change the membrane potential (− 44.0 ± 2.4 mV; $n = 10$) compared to non-treated cells. Inhibition of the Na^+/K^+-ATPase by ouabain (200 μM) of cells treated with carbachol (10 μM) and chlorpromazine (1 μM), caused a pronounced increase in membrane potential, as found in myotubes treated with carbachol alone (Table 1).

To investigate whether the development of Na^+/K^+-ATPase activity was related to the maintained depolarization of carbachol-treated myotubes, the effect of high K^+ medium (40 mM; 4 days) was investigated. At the end of the incubation period, myotubes showed a depolarization of 32.5 mV still in the presence of high K^+ (40 mM) (Table 1). Changing to normal conditions after the high K^+ treatment produced a normal membrane potential as observed in non-treated cells, not significantly affected by ouabain (200 μM; Table 1).

Na^+/K^+-ATPase up-regulation

Ouabain binding The nature of the development of enhanced Na^+/K^+-ATPase activity in carbachol-treated myotubes was investigated by determination of [^{3}H]-ouabain binding in the myotubes. Non-treated myotubes possessed a high-affinity site (Figure 3a, Table 2). In myotubes treated with carbachol (10 μM) or a combination of carbachol and chlorpromazine

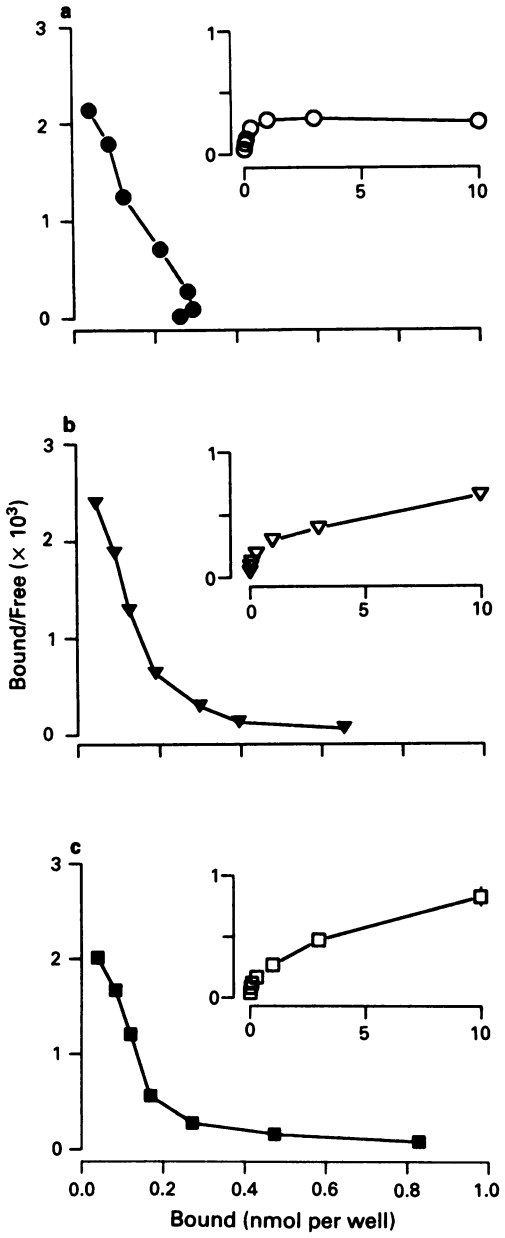

Figure 3 The effect of carbachol treatment of cells on ouabain binding represented by Scatchard plots and saturation binding curves (inserts). (a) A single high-affinity binding site was found in non-treated cells; (b) an additional low-affinity binding site was analysed after 4 days treatment with carbachol (10 μM); (c) a high-affinity as well as a low-affinity binding site was also observed after 4 days treatment with carbachol (10 μM) and chlorpromazine (1 μM). Inserts: abscissae represent ouabain concentration (μM), ordinates represent specific binding (nM); data are expressed as mean ± s.e.mean. Binding parameters are provided in Table 2.

Table 3 The effect of treatment (4 days) with carbachol (10 μM) on Na^+/K^+-ATPase-antibody binding in C2C12 myotubes

	Absorbance (× 100)	
	Control	*Carbachol*
McK1	22.1 ± 1.5	32.2 ± 1.1*
McB2	22.9 ± 1.7	32.2 ± 1.4*

Values are expressed as mean ± s.e.mean of the absorbance of 497 nm in areas of 0.04 mm^2. Data are pooled across 20 series of 100 measurements each.
*$P < 0.01$.

(1 μM), both possessing increased electrogenic Na^+/K^+-pumping, the ouabain binding was characterized by two sites ($P < 0.005$). Besides the high-affinity site (nM range) also found in non-treated myotubes, an additional low-affinity site (μM range) was present (Figure 3b,c; Table 2). The appearance of the low-affinity site accounted for a 4–5 fold increase in the total number of ouabain binding sites in myotubes treated with carbachol (10 μM) or a combination of carbachol and chlorpromazine (1 μM)-treated myotubes (Table 2).

Antibody binding To obtain a more specific characterization of the increase in Na^+/K^+-ATPase activity of carbachol-treated myotubes, binding of two monoclonal antibodies was studied, i.e. McK1 (Felsenfeld & Sweadner, 1988) and McB2 (Urayama *et al.*, 1989) directed against α_1- or α_2-subunits of Na^+/K^+-ATPase, respectively. Both antibodies stained the myotubes intracellularly and at the plasma membrane (not shown). Incubation of the myotubes for 4 days with carbachol (10 μM) caused an increase in staining intensity of about 45% for both antibodies compared to non-treated myotubes (Table 3).

Discussion

The results presented here show that long-term treatment of C2C12 myotubes with carbachol, measured in the absence of the agonist, induced a concentration-dependent increase in membrane potential of C2C12 myotubes, caused by an increased Na^+/K^+-ATPase activity, accompanied by an increased binding of ouabain and Na^+/K^+-ATPase directed antibodies. The carbachol-induced increase in Na^+/K^+-ATPase activity was abolished in the presence of pancuronium, but not by atropine at an equal concentration. Therefore, pancuronium showed a greater potency in antagonizing the carbachol-induced response than atropine, demonstrating that the carbachol-induced increase in Na^+/K^+-ATPase activity was mediated by nicotinic rather than muscarinic AChRs (Buckett *et al.*, 1968). It is unlikely that nicotinic AChR-mediated enhancement of Na^+/K^+-ATPase activity is caused by the depolarization of the myotubes, as long-term incubation in high K^+ medium failed to induce an excess Na^+/K^+-ATPase activity. Intracellular Na^+ might represent the mechanism responsible for the induction of

Table 2 The effect of long-term stimulation of nicotinic acetylcholine receptors (AChRs) on ouabain binding in C2C12 myotubes

Treatment	n	K_{d1} (nM)	K_{d2} (μM)	B_{max1} (fmol per well)	B_{max2} (fmol per well)
None	5	119 ± 11	–	296 ± 10	–
Carbachol	5	79 ± 14	13 ± 5*	227 ± 27	983 ± 35
Carbachol/CPZ	3	56 ± 8	10 ± 2*	160 ± 14	1330 ± 125

Cells had been incubated for 4 days in the presence of carbachol (10 μM) or the combination of carbachol (10 μM) and chlorpromazine (CPZ; 1 μM). [^{3}H]-ouabain binding was measured after extensive washing of the drugs and is represented by the affinity (K_{d1}, K_{d2}) and the number of associated binding sites (B_{max1}, B_{max2}). Values are expressed as mean ± s.e.mean of n experiments.
*Represents the preference of a two-site binding model over a one-site model (F-test, $P < 0.001$).

Na^+/K^+-ATPase activity in carbachol-stimulated C2C12 myotubes. In chick myotubes, long-term treatment with veratridine to activate voltage-operated Na^+-channels, up-regulated Na^+/K^+-ATPase by increasing synthesis of the β-subunit of the enzyme for 24 h (Taormino & Fambrough, 1990), followed by a decrease of the Na^+/K^+-ATPase degradation rate (Wolitzky & Fambrough, 1986). However, it seems unlikely that an increase in nicotinic AChR-mediated intracellular Na^+ is responsible for the increase in Na^+/K^+-ATPase activity of carbachol-stimulated C2C12 myotubes, as carbachol-stimulated myotubes still developed enhanced Na^+/K^+-ATPase activity and increased ouabain binding in the presence of the nicotinic AChR ion-channel blocking agent, chlorpromazine. That different mechanisms are involved in the carbachol and veratridine induction of Na^+/K^+-ATPase activity is supported by the time-course of the action of carbachol, being 3–4 fold longer than in veratridine-stimulated myotubes. These results show that the nicotinic AChR is not only a classical ligand-operated ion-channel (Changeux, 1990), but also regulates long-term cellular processes.

The development of enhanced Na^+/K^+-ATPase activity in carbachol-stimulated myotubes was accompanied by an increase of ouabain binding and an enhanced binding of monoclonal antibodies directed against the α_1- and the α_2-subunit of the Na^+/K^+-ATPase. These results suggest that the increase in Na^+/K^+-ATPase activity of nicotinic AChR-stimulated myotubes is due to an increase in the number of Na^+/K^+-ATPase sites. An attempt to study involvement of *de novo* synthesis of Na^+/K^+-ATPase by incubating the carbachol-treated myotubes in the presence of chlorheximide (1 $\mu g\ ml^{-1}$) was obstructed because of detachment of the cells within 48 h. The development of the low-affinity binding site accounted for the increase in total ouabain binding. The existence of multiple ouabain binding sites has been attributed to the expression of different isozymes of the Na^+/K^+-ATPase α-subunit at the plasma membrane (Sweadner, 1989). In contrast, the development of a specific ouabain binding site in carbachol-treated myotubes was accompanied by an equal increase in binding of antibodies directed against the α_1- or α_2-subunit of the Na^+/K^+-ATPase. This contradiction might be explained by the ability of the antibodies to bind to plasma membrane as well as to intracellular Na^+/K^+-ATPase, thus being unable to detect α-subunit specific Na^+/K^+-ATPase at the plasma membrane. Moreover, development of the low-affinity ouabain binding site might be dependent on mechanisms other than regulation of α-subunit incorporation, such as e.g. phosphorylation of Na^+/K^+-ATPase subunits (Vasilets *et al.*, 1990; Chibalin *et al.*, 1992). Nevertheless, although the precise molecular mechanism increasing the Na^+/K^+-ATPase binding in C2C12 cells is poorly understood, our study clearly demonstrates that long-term stimulation of nicotinic AChRs results in an up-regulation of Na^+/K^+-ATPase binding sites.

In view of the observation that the increased Na^+/K^+-ATPase activity in carbachol-treated C2C12 myotubes is independent of ion-transport, nicotinic AChR-mediated activation of a second messenger system should be considered. So far, stimulation of nicotinic AChRs has been shown to activate the phospholipase C (PLC) route, inducing formation of inositol(1,4,5)trisphosphate and mobilization of Ca^{2+} from internal stores in C2C12 myotubes (Grassi *et al.*, 1993). The regulation of *Xenopus* oocyte Na^+/K^+-ATPase by protein kinase C (Vasilets *et al.*, 1990), which is activated by the PLC route, suggests that the contribution of the PLC route in the induction of Na^+/K^+-ATPase activity in long-term nicotinic AChR-stimulated myotubes needs further attention.

Thus, long-term stimulation of nicotinic AChRs induced an adaptive response in C2C12 myotubes, represented by an increase in Na^+/K^+-ATPase activity and a concomitant increase in membrane potential. In this respect, long-term stimulation of nicotinic AChRs of non-contracting C2C12 myotubes serves as a model of innervation of skeletal muscle (Berg & Hall, 1975; Chang *et al.*, 1975; Mathers & Thesleff, 1978; Pestronk *et al.*, 1980). Further studies examining the involvement of nicotinic AChRs in the regulation of other innervation-related properties of skeletal muscle (Pestronk *et al.*, 1980; Rogart & Regan, 1985) are in progress.

In summary, we have shown that long-term activation of nicotinic AChRs in C2C12 myotubes increases the membrane potential by augmentation of electrogenic Na^+/K^+-pumping. This increase in Na^+/K^+-ATPase activity is associated with the appearance of an additional low-affinity [^{3}H]-ouabain binding site and an increase in binding of antibodies specific for α_1- and α_2-subunits of the enzyme. Consequently, this study exposes the apparent ability of ligand-operated nicotinic AChRs to mediate adaptive responses of the cell upon chronic receptor activation.

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