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Acetylcholine Induces Hyperpolarization Mediated by Activation of $K_{(Ca)}$ Channels in Cultured Chick Myoblasts

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Our previous report demonstrated that chick myoblasts are equipped with Ca^{2+}-permeable stretch-activated channels and Ca^{2+}-activated potassium channels (K_{Ca}), and that hyperpolarization-induced by K_{Ca} channels provides driving force for Ca^{2+} influx through the stretch-activated channels into the cells. Here, we showed that acetylcholine (ACh) also hyperpolarized the membrane of cultured chick myoblasts, suggesting that nicotinic acetylcholine receptor (nAChR) may be another pathway for Ca^{2+} influx. Under cell-attatched patch configuration, ACh increased the open probability of K_{Ca} channels from 0.007 to 0.055 only when extracellular Ca^{2+} was present. Nicotine, a nAChR agonist, increased the open probability of K_{Ca} channels from 0.008 to 0.023, whereas muscarine failed to do so. Since the activity of K_{Ca} channel is sensitive to intracellular Ca^{2+} level, nAChR seems to be capable of inducing Ca^{2+} influx. Using the Ca^{2+} imaging analysis, we were able to provide direct evidence that ACh induced Ca^{2+} influx from extracellular solution, which was dramatically increased by valinomycin-mediated hyperpolarization. In addition, ACh hyperpolarized the membrane potential from -12.5 ± 3 to -31.2 ± 5 mV by generating the outward current through K_{Ca} channels. These results suggest that activation of nAChR increases Ca^{2+} influx, which activates K_{Ca} channels, thereby hyperpolarizing the membrane potential in chick myoblasts.

Key Words: Acetylcholine, Nicotinic acetylcholine receptor, K_{Ca} channel, Ca^{2+} influx, Hyperpolarization

INTRODUCTION

During the differentiation of skeletal muscle, myoblasts recognize each other, align along their bipolar axes, and fuse to form multinucleate myotubes. Muscle fibers undergo electrophysiological maturation during the differentiation as evidenced by the maintenance of large resting membrane potential (Fischbach et al, 1971), the generation of action potentials (Spector & Prives, 1977) and the acquisition of functional excitation-contraction coupling (Romey et al, 1989).

Myoblast fusion depends on the entry of external Ca^{2+} (Shainberg et al, 1971). It has been reported that the fusion of chick embryonic skeletal muscle cells can reversibly be suppressed by lowering the external Ca^{2+} concentration (Easton & Reich, 1972; Wakelam, 1985). For instance, D600 and lanthanium, antagonists of voltage-dependent Ca^{2+} channels, prevent the fusion of myoblasts (David et al, 1981; Entwistle et al, 1988). Despite of these findings, several patch-clamp studies failed to detect voltage-dependent Ca^{2+} channels in myoblasts (Schmid et al, 1984; Cognard et al, 1993; Caffrey et al, 1987). It has also been reported that D600 and lanthanium could affect K^+ channels and nAChRs (McDonald et al, 1994; Miledi & Parker, 1980).

It has recently been suggested that, in different species or at different states of development, different mechanisms might induce Ca^{2+} influx during myoblast fusion (Constantin et al, 1996; Park et al, 2002; Bernheim and Bader, 2002). Voltage-dependent T-type Ca^{2+} channels are expressed and involved in Ca^{2+} influx in human proliferating myoblasts (Bernheim and Bader, 2002), and rat myoblasts and chick myoblasts seem to develop different routes for Ca^{2+} influx such as nAChRs and stretch-activated channels (Constantin et al, 1996; Shin et al, 1996; Park et al, 1999).

It has been well known that nAChR mediates depolarization of adult muscle in response to ACh released from motoneurons, and ACh-induced depolarization triggers voltage-dependent Ca^{2+} channels (Hille, 1992). Functional nAChRs are also likely to be involved in myoblast fusion. For instance, the activation of nAChRs modulates the fusion in chick embryonic myoblasts and human myoblasts (Siegelbaum et al, 1984; Cossu et al, 1987; Krause et al, 1995; Entwistle et al, 1988). However, electrophysiological properties of chick myoblasts have been shown to be different from those of myotubes. Chick myoblasts expressed Ca^{2+}-activated potassium channels (K_{Ca}) exclusively and had higher membrane potential (-15 ± 1 mV)

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ABBREVIATIONS: K_{Ca}, Ca^{2+}-activated potassium channels; ACh, acetylcholine; nAChr, nicotinic acetylcholine receptor; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

(Park et al, 1999). Because of different electrophysiological properties, nAChR in myoblasts may have other roles different from that in myotubes. However, the effects of ACh on membrane potential in myoblasts and the mechanism by which nAChR modulates myoblast fusion remain undetermined.

In the present study, we examined the effect of ACh on membrane potential and intracellular Ca^{2+} level of chick myoblasts. We also directly recorded the currents directly activated by the fast application of ACh to chick myoblasts.

METHODS

Myoblast culture

Chick myoblast cultures were prepared according to the method of O'Neill and Stockdale (O'Neil & Stockdale, 1972). Briefly, the breast muscle from 12-day-old chick embryos was dissected out, minced, digested with 0.1% trypsin (Gibco) for 30 min, and dispersed by repeated pipetting. The cells were collected by centrifugation, resuspended in Eagle's minimal essential medium (MEM, Gibco) containing 10% (v/v) horse serum and 10% (v/v) embryo extracts, and plated onto the collagen coated glass cover slips at a density of 1×10^5 cells/ml. To differentiate myoblasts, culture medium was changed 24 hr after plating with MEM containing 10% (v/v) horse serum and 2% (v/v) embryo extracts. After 2 days, cells were used for patch clamp recording and Ca^{2+} imaging analysis.

Electrophysiology and analysis

Patch clamp experiments were performed using the standard method (Hamill et al, 1981). Resistance of patch pipettes was 2~10 MΩ, when filled with the pipette solutions. The seal resistance was in the range of 5-20 GΩ. Ionic currents and the membrane potential were measured with Axopatch 200A amplifier (Axon instruments). Currents were low-pass filtered at 0.5~2 kHz (−3 dB, four-pole bessel) and digitized at 3~5 kHz using an 80486 based personal computer equipped with Digidata 1200 acquisition board (Axon Instruments). For data acquisition and analysis, pClamp software (Axon instruments) was used. Single channel recordings were stored in a digital tape recorder (DTR 1204, Biologic Science Instruments) and analyzed later after digitization. Channel open probability (P_o) was estimated under steady-state conditions of long-lasting periods (10~30 s) from the mean current (I), the maximal number of simultaneously open channels (N), and the single channel current (i); i.e., $P_o=I/Ni$. When necessary, membrane potential was measured in current clamp mode (I=0). Membrane potentials were given for the inner side with respect to the outer side of the membrane. All experiments were done at room temperature (22~25°C). RC-13 recording camber (Warner Instruments Co.) was routinely used. The flow rate of bath application was about 0.7 ml/min. For rapid application of drugs (Fig. 5), we used a high-speed solution-exchange system (HSSE-2, Ala Scientific Instruments Inc.). Drugs were applied within 100 μm of the recorded cell allowing rapid application of agonists.

Solution

Solution compositions were as follows: Normal external Na^+-rich solution; 140 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES, pH 7.4 adjusted with NaOH: External K^+-rich solution; 140 mM K-aspartate, 5 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM N-2-hydroxyethypiperazine-N'-2-ethanesulphonic acid (HEPES), pH 7.4 adjusted with KOH: External Ca^{2+}-free K^+-rich solution; 140 mM K-aspartate, 5 mM KCl, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES, 5 mM ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), pH 7.4 adjusted with KOH: Intracellular solution; 140 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM HEPES, pH 7.2 adjusted with KOH: All reagents were from Sigma Chemical Co.

Ca^{2+} imaging

For Ca^{2+} imaging, cells were washed twice with normal external Na^+-rich solution. Subsequently, they were loaded with 5 μM fluo-3 AM (Molecular Probes, Eugene, OR., USA) for 60 min at room temperature. Then, fluo-3 AM solution was removed by rinsing twice with normal external Na^+-rich solution, and the cells were left at room temperature for 10 min for completion of ester-hydrolysis. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored under confocal microscope (Leica, Germany). The videoscan module (Leica, TCS 4D, Germany) was mounted on an inverted Leica microscope (DM IRB). Optical excitation was accomplished using 488 nm line of an argon laser. The emitted fluorescence was passed through 515 nm primary barrier filter before it reached to the photomultiplier tube. The laser intensity was minimized to prevent the dye bleaching during the course of measurements. During experiments, the fluorescence images were acquired in a slow mode (one frame/5 sec), and the fluorescence intensitiy at defined cells were stored and analyzed with EXCEL graphic program.

RESULTS

ACh induces Ca^{2+} influx in chick myoblasts

Embryonic chick myoblasts are highly enriched with the large-conductance Ca^{2+}-activated potassium (K_{Ca}) channels (Shin et al, 1996). Because open probability of the K_{Ca} channel is sensitive to the $[Ca^{2+}]_i$, the channels can be used as an intracellular Ca^{2+} indicator (Marty, 1981; Latorre et al, 1989). Therefore we measured activities of the K_{Ca} channels at cell-attached patch configuration to examine whether ACh increased intracellular Ca^{2+} concentration in chick myoblasts. The pipette potential was clamped at −40 mV, and external K^+-rich solution was used to zero the potential across the cell membrane. As shown in Fig. 1A, 1 μM ACh increased the open probability of the K_{Ca} channels from 0.007 to 0.055. This result suggests that ACh increases intracellular Ca^{2+} concentration of chick myoblasts.

To determine which type of AChR was involved in ACh-mediated increase of intracellular Ca^{2+} level, we examined effects of nicotine and muscarine on the activity of the K_{Ca} channels. As shown in Fig. 1B and C, nicotine increased the open probability of K_{Ca} channels from 0.008 to 0.023, whereas muscarine failed to do so. It appears that nAChR is involved in the increase of $[Ca^{2+}]_i$ caused by ACh.

Next, we asked a question of whether ACh-mediated Ca^{2+} increase was derived from extracellular solution. ACh was unable to elevate the open probability of the K_{Ca} channels

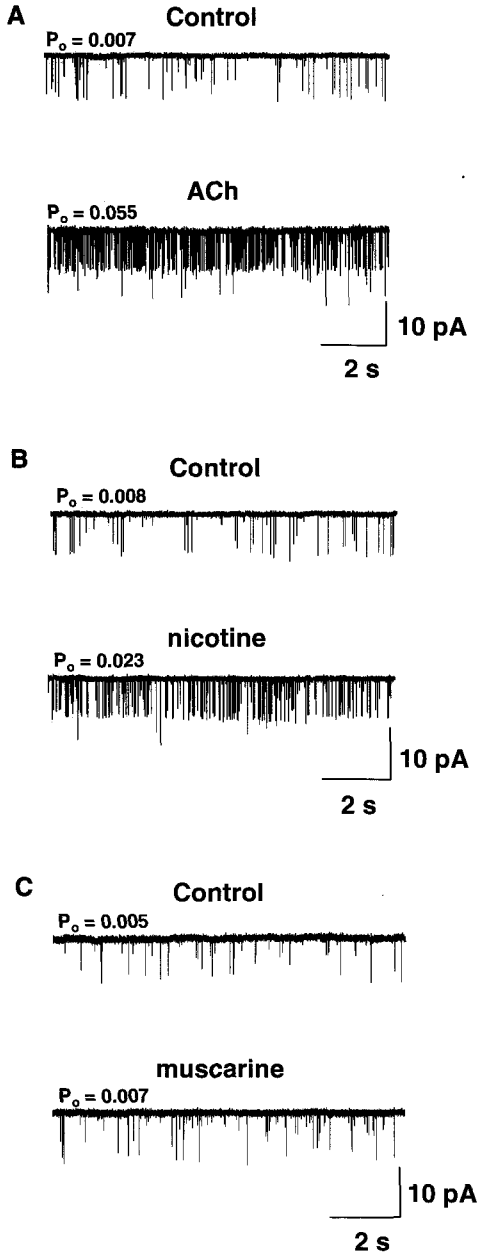

Fig. 1. Effects of acetylcholine receptor (AChR) agonists on the activity of K_{Ca} channels. Currents were recorded from cell-attached patches held at −40 mV pipette potential with the pipette containing normal external 140 mM Na^+-rich solution before and after treatment of 1 μM ACh. External 140 mM K^+-rich solution was used as the bathing solution to zero the cell membrane potential. Pipette solution contained 2.5 mM Ca^{2+} (A). Same experiments were done with nicotine and muscarine. The bath solution was changed to the solution containing 100 μM nicotine (B) or 100 μM muscarine (C). Open probability (P_0) of K_{Ca} channels was determined from the recordings of each experiment. P_o was estimated as described in 'Methods'. All traces were from the same patch.

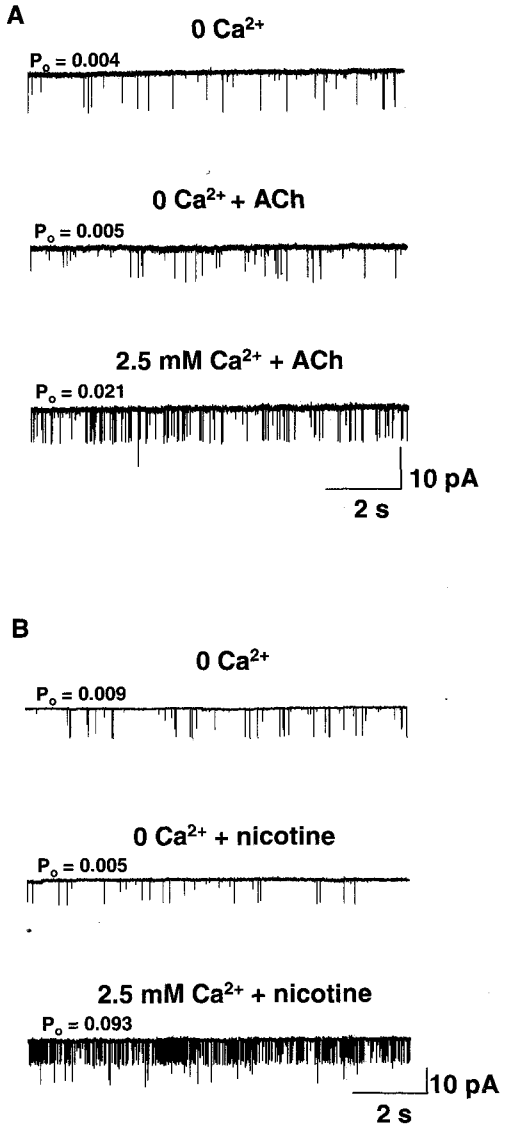

Fig. 2. Effect of extracellular Ca^{2+} on the activation of K_{Ca} channels. Recordings were made at −40 mV using cell-attached patch with the pipette containing normal external 140 mM Na^+-rich solution. External 140 mM K^+-rich solution was used as the bathing solution to zero the cell membrane potential. Activities of K_{Ca} channels were determined by treating the cells with 1 μM ACh (A) or 100 μM nicotine (B) in the absence and presence of 2.5 mM Ca^{2+} in bath solutions.

when the cells were kept in external Ca^{2+}-free K^+-rich solution (Fig. 2A). On the other hand, ACh increased the activities of the K_{Ca} channels in the bath solution containing 2.5 mM Ca^{2+}. Similar results were obtained, when nicotine was used (Fig. 2B). These results suggest that the increase in $[Ca^{2+}]_i$ by ACh was derived from the extracellular solution.

Ca^{2+} influx mediated by nAChRs is potentiated by hyperpolarization

ACh is known to induce depolarization of the membrane potential of myotubes (Hille, 1992). However, the effect of ACh on membrane potential in myoblasts is unknown.

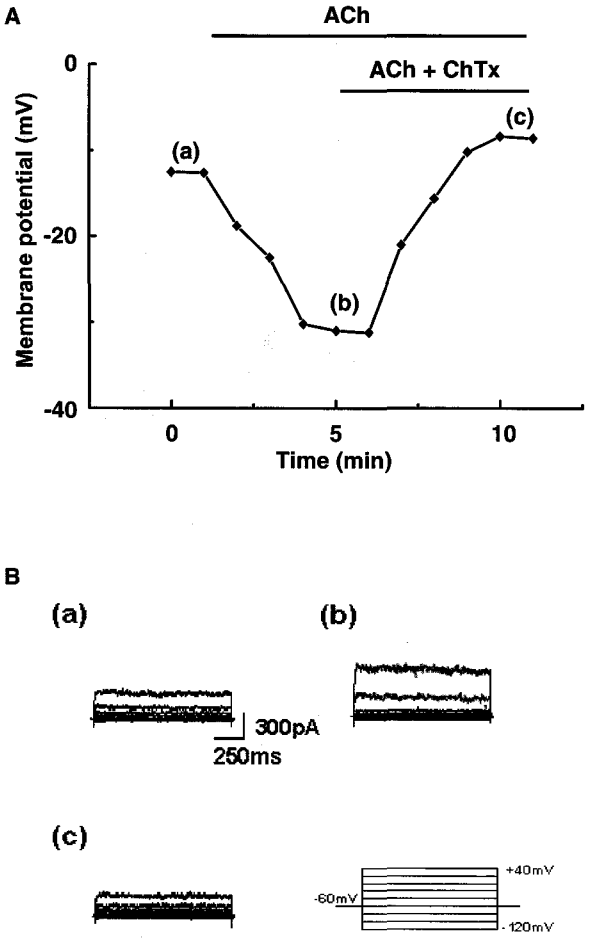

Fig. 3. Effect of ACh and K_{Ca} channel blocker on membrane potential of myoblasts. (A) Membrane potential was measured under control conditions (a), after exposure 1 μM ACh (b), and then after exposure to the drug together with 30 nM charybdotoxin (ChTx) (c). The membrane potential was determined in current clamp mode (I=0). Similar data were obtained by six independent experiments. (B) Whole-cell currents were also measured at the time of the drug treatment by depolarizing steps from a holding potential of -60 mV to the values from -120 mV to $+40$ mV with 20 mV increments. Whole-cell recordings were made with the pipet containing intracellular 140 mM K^+-rich solution, and normal external 140 mM Na^+-rich solution was used.

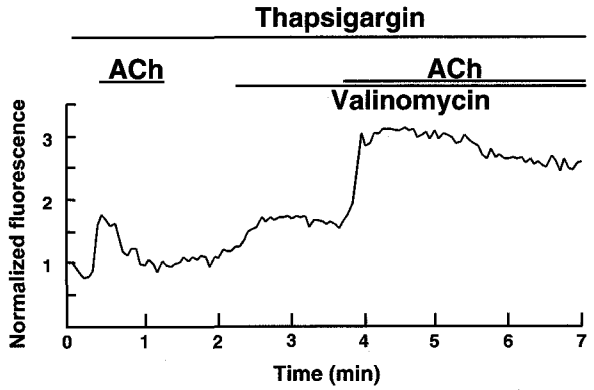

Fig. 4. Effect of hyperpolarization on the ACh-induced Ca^{2+} influx. Intracellular Ca^{2+} levels were estimated by Fluo-3 AM fluorescence dye detection method using confocal microscope. To deplete intracellular Ca^{2+} storage, myoblasts were incubated with 1 μM thapsigargin (Thap) for 30 min prior to the experiments. Changes in the fluorescent intensity were then monitored by treating the cells with 10 μM ACh in the absence and presence of 10 μM valinomycin (Val). Ca^{2+} imaging analysis was performed with normal external 140mM Na^+-rich solution.

Previously, we demonstrated that the K_{Ca} channels are highly expressed and activation of these channels dramatically hyperpolarize the membrane potential in chick myoblasts (Shin et al, 1996; Park et al, 2002). Because ACh increases the activity of the K_{Ca} channels as shown in Fig. 1A, it is quite possible that the potassium outward current increased by ACh may hyperpolarize the membrane potential. We, therefore, performed whole-cell recording to examine the effects of ACh on the resting membrane potential and whole-cell currents of myoblasts. As shown in Fig. 3A, ACh (1 μM) hyperpolarized the membrane potential of chick myoblasts from -12.5 ± 3 to -31.2 ± 5 mV (mean$\pm$SE, n=6), and these effects were completely reversed by 30 nM charybdotoxin, a selective blocker of K_{Ca} channel with large conductance. Whole-cell currents were also measured at the three distinct points by depolarizing voltage steps from a holding potential of -60 mV to the values from -100 to $+40$ mV with 20 mV increments (Fig. 3B). These results strongly suggested that the hyperpolarization of membrane potential and the increased outward currents are likely due to the activation of the K_{Ca} channels. When similar experiments were performed in the Ca^{2+}-free bath solution, ACh was unable to hyperpolarize the membrane potential and activate K_{Ca} channels (data not shown). Therefore, it is likely that Ca^{2+} influx through nAChR increases the activity of the K_{Ca} channels and opening of the K_{Ca} channels, in turn, hyperpolarizes the membrane, thereby providing a bigger electrochemical driving force for Ca^{2+} ions.

ACh can increase $[Ca^{2+}]_i$ in rat myoblasts in extracellular Ca^{2+}-dependent manner (Constantin et al, 1996), and hyperpolarization generated by the increased activity of K_{Ca} channels induces Ca^{2+} influx in chick myoblasts (Shin et al, 1996; Park et al, 1999). Since ACh increased the activity of the K_{Ca} channels mediated by Ca^{2+} influx in chick myoblasts (Fig. 2A), we then examined the relationship between hyperpolarization and ACh-induced Ca^{2+} influx by using Ca^{2+} imaging analysis. In this experiment, to eliminate any possible involvement of Ca^{2+} ions from intracellular storage, internal Ca^{2+} stores were depleted by treatment with thapsigargin, an inhibitor of microsomal Ca^{2+}-ATPase, for 30 min prior to experiments. As shown in Fig. 4, intracellular Ca^{2+} level was transiently increased by treatment of ACh. Because valinomycin, a well known potassium carrier, hyperpolarizes the membrane potential in chick myoblasts (Park et al, 1999), we next treated the cells with this drug in order to induce hyperpolarization. In this case, small increase of Ca^{2+} influx was also observed, similar to our previous result (Park et al, 1999; Park et al, 2002). In addition, Ca^{2+} influx was dramatically magnified, when the myoblats were treated with ACh and valinomycin together. It appears, therefore, that ACh-mediated Ca^{2+} influx can significantly be magnified under the hyperpolarization of membrane potential in chick

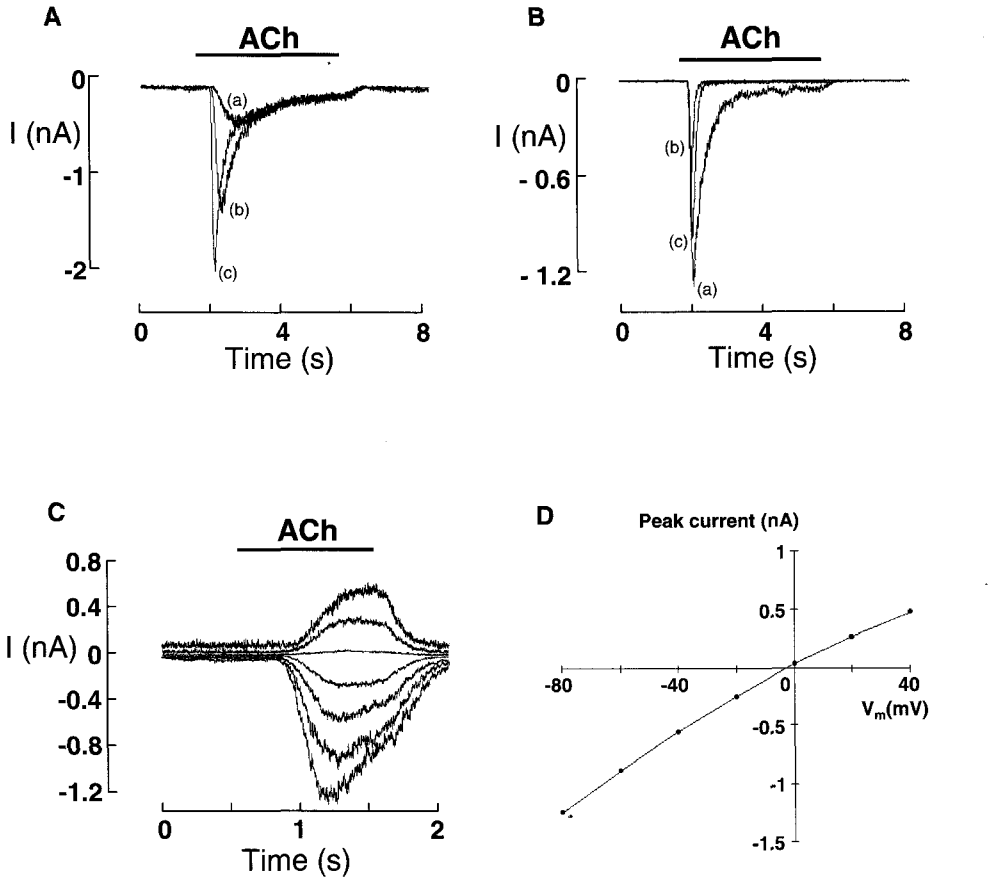

Fig. 5. ACh-induced currents in chick myoblasts. (A) Whole-cell currents were recorded from a chick myoblast held at −70 mV by treating the cell for 4 s with 1 μM (a), 10 μM (b), or 100 μM ACh (c). (B) ACh-activated whole-cell currents were recorded in a myoblast held at −70 mV by exposing the cell to 10 μM ACh. Each currents (a, b, and c) were recorded in control, 5 μM d-tubocurarine and washing conditions, respectively. (C) Whole-cell currents were recorded from a fusion-competent myoblast held at voltages between −80 mV and +40 mV and exposed to 10 μM ACh for 1 s. (D) Peak amplitude of each event in (C) was plotted as a function of membrane potential. Reversal potential was −2.5 mV. Whole-cell recordings were made with the pipet containing intracellular 140 mM K^+-rich solution, and normal external 140 mM Na^+-rich solution was used.

myoblasts.

Presence of nAChR in chick myoblasts

Although the nAChR has been well defined in myotubes (Hille, 1992), the current induced by ACh in chick myoblasts has not yet been identified. AChRs are desensitized within several seconds in the presence of ACh (Arias, 1998). To determine the ACh-induced currents in chick myoblasts, we performed fast application of ACh to whole-cell recordings. When solution exchange rate was measured by junction potential measurement, our system was able to exchange the solution within 1 ms. As seen in Fig. 5A, ACh induced an inward current at negative potentials, and peak amplitude of the inward current was reduced, when the concentration of ACh was lowered. Peak amplitude of the inward current increased with the increase of ACh concentration. The amplitudes of the steady-state currents were, however, similar at higher than 1 μM concentrations of ACh. Fig. 5B shows that *d*-tubocurarine, an antagonist of the nAChR, reduced ACh-activated currents, the currents were recovered when *d*-tubocurarine was removed. Reversal potential of ACh-induced currents was −2.5±3 mV (mean±SE, n=6) (Fig. 5D). ACh induced inward currents at negative potentials and became outward currents at positive potentials. These results indicate that electrical properties of AChRs in chick myoblasts are similar to those shown in myotubes (Siegelbaum et al, 1984; Krause et al, 1995).

DISCUSSION

The entry of Ca^{2+} into myoblasts is known to be pivotal for the cell fusion (Easton & Reich, 1972; Wakelam, 1985). However, little is known about the channels that are responsible for the Ca^{2+} influx. In non-excitable cells, Ca^{2+} influx is generated directly by either gating of receptor-operated channels and non-selective cation channels or indirectly by second messenger-operated channels (Penner

et al, 1988; Oettgen et al, 1985; Meldolesi & Pozzan, 1987; Nathanson, 1988). Ca^{2+} influx in these cells is reduced by depolarization and increased by hyperpolarization. Therefore, it appears possible that membrane hyperpolarization also increases Ca^{2+} influx in chick myoblasts.

It has been proposed that stimulation of voltage-dependent Ca^{2+} channels by membrane depolarization is involved in the Ca^{2+} influx, thereby inducing myoblast fusion (Entwistle et al, 1988). However, the Ca^{2+} currents activated by depolarization have never been detected in myoblasts (Caffrey et al, 1987; Cognard et al, 1993). Recently, nAChR and stretch-activated channels have been suggested as an alternative route for Ca^{2+} influx (Constantin et al, 1996; Shin et al, 1996; Park et al, 2002). It has been found that activation of nAChR is involved in spontaneous myoblast fusion (Cossu et al, 1987; Krause et al, 1995), and that myoblasts synthesize an ACh-like compound that activates the nAChR (Hamann et al, 1995). The present study clearly demonstrates that activation of nAChR increases intracellular Ca^{2+} level. In fact, Constantin et al. (1996) have demonstrated that ACh can increase internal Ca^{2+} level in rat myoblasts in extracellular Ca^{2+}-dependent manner.

It has been known that ACh induces membrane depolarization in adult muscle cell and subsequently increases intracellular Ca^{2+} level by opening voltage-dependent Ca^{2+} channels (Randall et al, 1992). However, myoblasts are unlikely to have voltage-dependent Ca^{2+} channels (Cognard et al, 1993). In addition, myoblasts have depolarized resting membrane potentials, which differs from typical excitable cells; the resting potential in mononucleated myoblasts is rather high ($-10 \sim -15$ mV), compared to that in multinucleated myotubes ($-60 \sim -80$ mV) (Shin et al, 1997; Liu et al, 1998; Park et al, 1999). With different electrical properties of myoblast membrane, it is likely that effects of ACh on membrane potential of myoblasts is different from that of myotubes. Indeed, our results show that ACh gradually hyperpolarizes membrane potential from -12.5 ± 3 mV to -31.2 ± 5 mV (mean$\pm$SE, n=6) by activation of K_{Ca} channels (Fig. 4A). The present study clearly demonstrated that ACh-induced outward currents evoke hyperpolarization of the membrane potential rather than depolarization in chick myoblasts.

There are two opposing hypotheses to explain the role of hyperpolarization in the myoblast fusion: 1) increase in sensitivity to depolarizing signals, and 2) increase in electrical driving force for Ca^{2+}. It has been hypothesized that hyperpolarization may render myoblasts more sensitive to putative depolarizing signals such as ACh (Constantin et al, 1996). However, in the present study, ACh increased intracellular Ca^{2+} and hyperpolarized membrane potential in chick myoblasts. Based on our results, it seems likely that hyperpolarization of membrane potential can increase the magnitude of Ca^{2+} influx by providing the driving force for Ca^{2+} (Tsien & Tsien, 1990; Rich & Rae, 1995). Indeed, the present study clearly demonstrated that valinomycin increased ACh-induced Ca^{2+} influx (see Fig. 4). In our previous study, valinomycin, a potassium carrier, hyperpolarized the membrane potential from -15 to -66 mV in chick myoblasts (Park et al, 1999). This result suggests that hyperpolarization may be responsible for the increase in ACh-induced Ca^{2+} influx.

In fact, voltage-dependent Ca^{2+} channels could not have been detected in chick and rat myoblasts. Cognard et al. (1993) have revealed that dynamic Ca^{2+} responses cannot be observed in rat myoblasts upon superfusion with 100 mM potassium by laser cytofluorimetry. Moreover, Ca^{2+} imaging analysis with 40 mM potassium solution has failed to detect voltage-dependent Ca^{2+} increase in rat (Constantin et al, 1996) and chick myobalsts (Park et al, 2002). Despite these findings, we could not yet exclude the possibility that Ca^{2+} influx may occur through other Ca^{2+} permeable channels such as stretch-activated channels and voltage-dependent Ca^{2+} channels in chick myoblasts. In conclusion, activation of nAChR increases Ca^{2+} influx, which activates the K_{Ca} channels, thereby hyperpolarizing the membrane potential in the chick myoblasts.

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