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A Possible Role of Kainate Receptors in C2C12 Skeletal Myogenic Cells

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Ca^{2+} influx appears to be important for triggering myoblast fusion. It remains, however, unclear how Ca^{2+} influx rises prior to myoblast fusion. Recently, several studies suggested that NMDA receptors may be involved in Ca^{2+} mobilization of muscle, and that Ca^{2+} influx is mediated by NMDA receptors in C2C12 myoblasts. Here, we report that other types of ionotropic glutamate receptors, non-NMDA receptors (AMPA and KA receptors), are also involved in Ca^{2+} influx in myoblasts. To explore which subtypes of non-NMDA receptors are expressed in C2C12 myogenic cells, RT-PCR was performed, and the results revealed that KA receptor subunits were expressed in both myoblasts and myotubes. However, AMPA receptor was not detected in myoblasts but expressed in myotubes. Using a Ca^{2+} imaging system, Ca^{2+} influx mediated by these receptors was directly measured in a single myoblast cell. Intracellular Ca^{2+} level was increased by KA, but not by AMPA. These results were consistent with RT-PCR data. In addition, KA-induced intracellular Ca^{2+} increase was completely suppressed by treatment of nifedifine, a L-type Ca^{2+} channel blocker. Furthermore, KA stimulated myoblast fusion in a dose-dependent manner. CNQX inhibited not only KA-induced myoblast fusion but also spontaneous myoblast fusion. Therefore, these results suggest that KA receptors are involved in intracellular Ca^{2+} increase in myoblasts and then may play an important role in myoblast fusion.

Key Words: KA receptors, AMPA receptors, Myoblast fusion, Ca^{2+} influx

INTRODUCTION

There is considerable evidence that glutamate is a principal neurotransmitter mediating fast excitatory synapse transmission in the vertebrate central nervous system (Foster & Fagg, 1984). The family of glutamate-gated ion channels includes receptors activated by *N*-methyl-D-aspartate (NMDA), kainic acid (KA), and α-amino-3-hydroyl-5-methyl-4-isoxazole propionic acid (AMPA) (for review, see Petralia et al, 2000). Metabotropic glutamate receptors are linked to the second messenger system via GTP-binding protein and participate in the generation of slow synaptic responses and modulation of neuronal excitability (Schoepp & Johnson, 1989). A large body of evidence indicates that most of these glutamate receptors are localized in both postsynaptic and presynaptic neurons.

Recently, some evidences showed that functional glutamate receptors (GluRs) are not restricted only to neuron, but also expressed in non-neuronal cells. Among glial cells, macroglial cell types, astrocytes, and oligodendrocytes are known to express various types of GluRs (for review, see Steinhuser & Gallo, 1996), and microglial cells have also been demonstrated to express GluRs (Noda et al, 2000). However, the presence of glutamate receptors in neuromuscular junction (NMJ) has not well been defined. Previous studies on glutamate receptors in NMJ have mainly been focused on the role of these receptors, which are present in presynaptic motoneuron (Schramm et al, 1997; Cairns et al, 1998).

It is well known that NMDA receptors consist of NR1 and NR2A-2D, and that natural NMDA receptors occur only when NR1 subunit is in hetero-oligomeric configurations with NR2A-2D (Sun et al, 2000). Recently, Luck *et al.* (2000) demonstrated co-localization of nitric oxide synthase I (NOSI) and NR1 at the NMJ in rat and mouse skeletal muscle. This result suggests that functional NMDA receptors could be expressed in skeletal muscle. In fact, we observed that C2C12 myogenic cells express both NR1 and NR2D subunits, whose channels are involved in Ca^{2+} influx in myoblasts (unpublished data). Although NMDA receptors seem to be functional in skeletal muscle, it has not yet been determined whether non-NMDA receptors functionally exist in myogenic cells. In this study, we showed that KA receptors are also expressed in C2C12 myoblasts and involved in Ca^{2+} influx in myoblasts.

METHODS

Cell culture

C2C12 cell, a mouse skeletal myogenic cell line, was

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ABBREVIATIONS: NMDA, N-methyl-D-aspartate; KA, Kainic acid; AMPA, a-amino-3-hydroyl-5-methyl-4-isoxazole propionic acid; GluR, glutamate receptor; NMJ, neuromuscular junction; NR, NMDA receptor.

obtained from the American Type Culture Collection. Cultures were plated at a density of 3×10^4 cells/ml in growth medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution) and cultured at 37°C for 2 days. Differentiation from myoblasts to myotubes was induced by changing the growth medium with differentiation medium (Dulbecco's modified Eagle's medium with 2% horse serum and 1% antibiotic-antimycotic solution). When needed, drugs were added at the time of medium change.

To determine the extent of myoblast fusion, the cells were fixed with 1 % (v/v) glutaraldehyde for 30 min, stained with 0.1% hematoxylin for 30 min, and observed under a microscope at 200X magnification. Cells were considered fused only if there was clear cytoplasmic continuity and at least three nuclei were present. Each data point represents the means of the counts from more than 30 randomly chosen fields.

RT-PCR analysis

RT-PCR was performed as described previously (Stefani et al, 1998) with slight modification. Briefly, cellular mRNA was extracted using TRIZOL (Life Technologies), according to the protocol provided by the manufacturer. One microgram of total RNAs was reverse transcribed using Super Script II RT (Life Technologies) in the presence of random hexamer (50 pmole) and dNTP (1 mM) at 42°C for 1 hr. The DNA from RT of RNA in C2C12 cells was subjected to PCR to detect the expression of glutamate receptor RNAs.

Amplification was performed on a thermal cycler (GeneAmp 2400; PerkinElmer, Turku, Finland) using DNA polymerase, premix *Taq* (TaKaRa, Tokyo, Japan) under the following cycle condition: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min (repeated 40 cycles). After PCR amplification, aliquot (10 μl) of reaction product was analyzed with agarose gel (2%) electrophoresis and stained with ethidium bromide. The primers used for the amplification of AMPA receptor subunits and KA receptor subunit were: a sense primer 5'-TGGGCCTTCACCTTGATCATCA-3' and an antisense primer 5'-TCGTACCACCATTTGTTTTTCA-3' (for AMPA receptor 1-4 subunit), a sense primer 5'-TGGGCCTTCACCTTGATCATCA-3' and an antisense primer 5'-CTGTGGTCCTCCTCCTTGGG-3' (for KA1-KA2 receptor). Negative controls for contamination from extraneous and genomic DNA were employed. To ensure that genomic DNA did not contribute to the PCR products, the cells were processed in the normal manner, except that the reverse transcriptase was omitted and contamination from extraneous sources was checked by replacing the cellular template with water. Both controls were consistently negative in these experiments.

Ca^{2+} imaging

Intracellular Ca^{2+} concentration was analyzed by Fluo-3 AM fluorescence dye detection method under a conventional inverted microscope equipped for epi-fluorescence and transmitted illumination (IX-70, Universal infinity system; Olympus America, Inc., Melville, NY). Fluorescence images were acquired using a 40X objective lens. The excitation light source was a 75 W xenon arc lamp (Ludl Erectronic Product Ltd., NY, USA) coupled to excitation filter wheels. The emission filter wheel was coupled to the output port of the microscope and then to the camera. Images were captured using a slow scan, liquid nitrogen-cooled CCD (charge-coupled device) camera with a back-thinned, back illuminated imaging chip (Quantix, Photometrics, Ltd., Tucson, AZ). The digital image output of the camera was 512×512 pixels with 16 bits resolution. For these studies, all images were collected using constant integration times. Optical excitation for Fluo-3 AM was accomplished using the 488 nm wavelength of xenon light. The emitted fluorescence was passed through a 535 nm emission filter before it reached CCD camera. The fluorescence images were acquired in a slow mode (a frame/5 sec). The fluorescence data from a single cell were stored, processed in MetaFluor (Universal Imaging, West Chester, PA), and later exported to the EXCEL graphic program.

For Ca^{2+} imaging, cells were plated onto the gellatin-coated glass coverslips at a density of 3×10^4 cells/ml. After 2 days, the cells were washed twice with normal bath solution. The cells were loaded with 5 μM Fluo-3 AM by incubation for 60 min at room temperature. Fluo-3 AM solution was then removed by rinsing twice with normal bath solution, and the cells were kept in normal bath solution for 10 min at room temperature to complete ester-hydrolysis. Since Fluo-3 AM is a single-wavelength chromophore, the fluorescence was a function of the $[Ca^{2+}]_i$ as well as the dye concentration. However, for a fixed region, the change of fluorescence directly reflects the change of $[Ca^{2+}]_i$. Therefore, the data were obtained by averaging raw intensities of fluorescence change of at least four individual myoblasts, and then displayed as the ratio of average fluorescence change. All experiments were repeated at least three times.

Solutions and materials

Solutions used were as follows. Normal bath solution: 140 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes) (pH 7.4, adjusted with NaOH). Ca^{2+}-free solution: 140 mM NaCl, 5.0 mM KCl, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM Hepes (pH 7.4, adjusted with NaOH). L-glutamate, AMPA, Kainic acid, MK801, and CNQX were purchased from Research Biochemical Inc.. Culture dishes were purchased from Corning, and other culture reagents were obtained from Gibco BRL.

RESULTS

Expression of non-NMDA receptor subunits in C2C12 cells

To examine whether non-NMDA receptors are expressed in C2C12 myogenic cells, we examined expression of AMPA and KA receptors using a RT-PCR method. Although KA receptor was detected in both myoblasts (2 days after plating) and myotubes (8 days after plating), AMPA receptors were expressed in only fully matured myotubes (Fig. 1A). The primer set for the detection of AMPA receptor subunit (Glu1-Glu4) cDNA is common to GluR1-GluR4 mRNA, and all four AMPA receptor subunits are coamplified (Noda et al, 2000). The size of amplified products is 749 bp for GluR1, GluR2, and GluR4, and 755 bp for

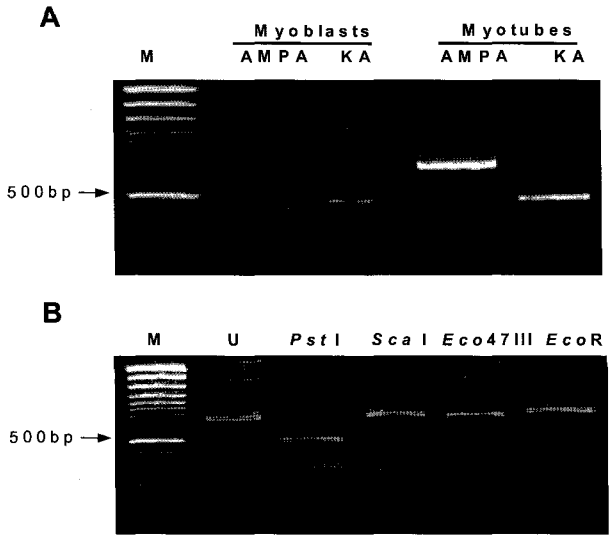

Fig. 1. Expression of non-NMDA receptor subunits in C2C12 cells. (A) RT-PCR was performed on mRNA from C2C12 cells (2 days and 8 days after plating). To determine the subunits of AMPA, cDNAs for GuR1-GluR4 subunits were simultaneously PCR- amplified using primers common to all four subunits. For PCR analysis of KA receptor mRNAs, KA1-KA2 specific primer sets were used. (B) Separate fractions of AMPA PCR products were then treated with subunit-specific restriction enzyme for GluR1 (Pst I), GluR2 (Sca I), GluR3 (Eco47III), GluR4 (EcoRI), or without treatment (U).

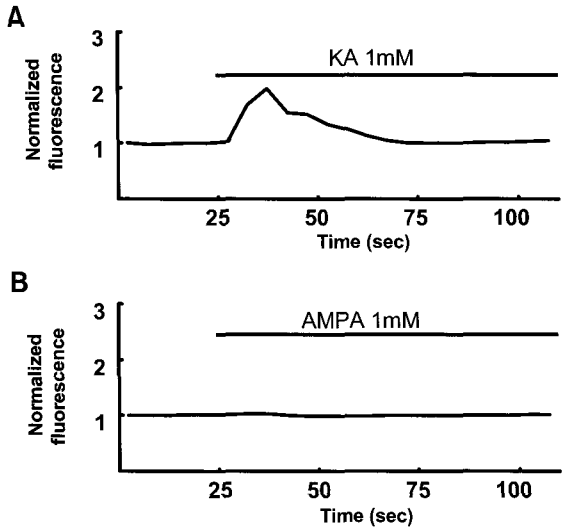

Fig. 2. Effect of AMPA and KA on intracellular Ca^{2+} levels. Intracellular Ca^{2+} levels were estimated using Fluo-3 AM fluorescence dye. The vertical axis represents relative fluorescence intensity normalized to that of basal level. Changes in intracellular Ca^{2+} level were monitored in the presence of 1 mM KA (A) or 1 mM AMPA (B).

GluR3, and they appear as a single band on electrophoresis gel. Therefore, to determine the presence of specific GluR1-GluR4 mRNA, the amplified PCR products were cut with subunit-specific restriction enzymes. Aliquot (10 μl) of the PCR product was digested with *Pst*I (GluR1), *Sca*I (GluR2), *Eco*47III (GluR3), and *Eco*RI (GluR4) at 37°C for 2 hr. The

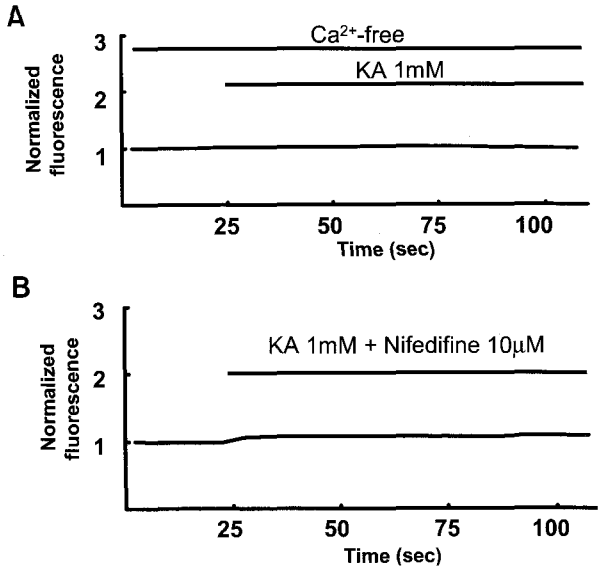

Fig. 3. Effects of Ca^{2+} -free solution or Nifedifine on KA induced Ca^{2+} increase. Effect of 1 mM KA on intracellular Ca^{2+} levels was monitored under Ca^{2+} -free bath solution (A) and co-treatment of Nifedifine (B).

enzyme-cleaved products were then analyzed with gel electrophoresis. The predicted restriction fragments are 275 and 474 bp for GluR1, 692 and 57 bp for GluR2, 354 and 401 bp for GluR3, and 412 and 337 for GluR4. Using the subunit-specific restriction enzyme reactions, we confirmed that only AMPA R1 (GluR1) m RNA was expressed in fully matured C2C12 cells (Fig. 1B, lane 3). These results indicate that functional KA receptors may exist in C2C12 myoblasts, suggesting that these ionotropic channels are involved in Ca^{2+} mobilization of myoblasts.

KA receptor is involved in Ca^{2+} influx in myoblasts

Since KA1-KA2 mRNA was expressed in myoblasts, we next examined whether KA was able to increase intracellular Ca^{2+} level using Fluo-3 AM. As shown in Fig. 2A, 1mM KA markedly increased intracellular Ca^{2+} level in a physiological solution, containing 2.5 mM Ca^{2+} ion (see Materials and Methods). The intracellular Ca^{2+} level was increased transiently and then decreased. On the other hand, AMPA was unable to elevate intracellular Ca^{2+} level (Fig. 2B). This result was consistent with the above RT-PCR data, which showed that AMPA receptor was not expressed in myoblasts (see Fig. 1A). The KA-induced intracellular Ca^{2+} increase showed a dose-dependent manner up to 5 mM (data not shown). These results clearly showed that KA receptors, but not AMPA receptors, are involved in intracellular Ca^{2+} increase in C2C12 myoblasts.

To clarify the source of Ca^{2+} ion, the cells were first kept in a Ca^{2+}-free solution, and then the effect of KA on intracellular Ca^{2+} level was examined. As shown in Fig. 3A, 1 mM KA was unable to increase intracellular Ca^{2+} level (Fig. 3A), showing that KA-induced intracellular Ca^{2+} increase was originated from extracellular fluids. It is well known that KA receptors are more permeable to Na^{+} or K^{+} rather than Ca^{2+}, and involved in depolarization of membrane potential. Therefore, KA could trigger Ca^{2+}

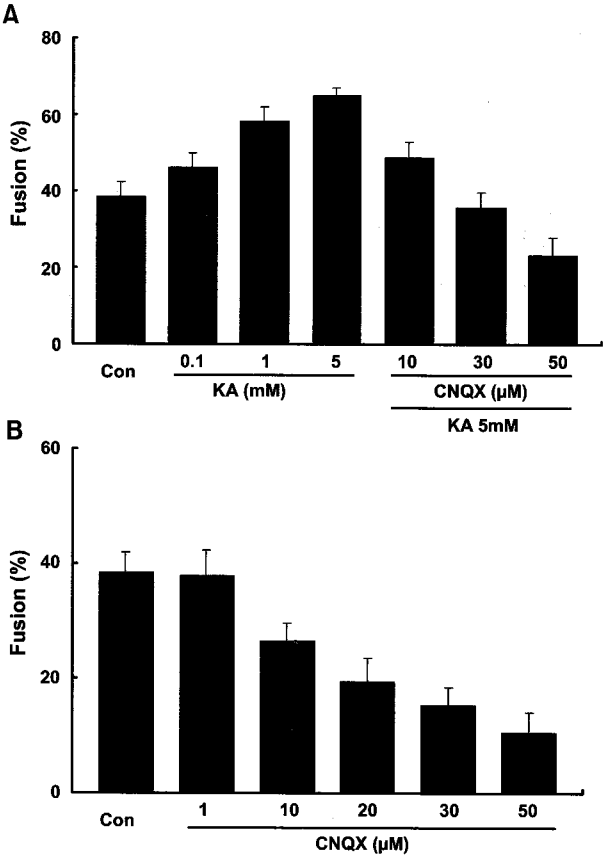

Fig. 4. Effects of KA or CNQX on myoblast fusion. C2C12 myoblasts were treated with indicated amounts of the drugs 2 days after plating, and the extent of fusion was assessed after 8 days. Each bar represents mean±SE of three different cultures. (A) Effect of KA on myoblast fusion in the presence and absence of CNQX. (B) Dose-dependent effect of CNQX on spontaneous myoblast fusion.

influx mediated by voltage dependant Ca^{2+} channels (Burnashev et al. 1996). As shown in Fig. 3B, the KA-induced intracellular Ca^{2+} increase disappeared by treatment of nifedifine, a L-type Ca^{2+} channel blocker. These data suggest that KA-induced Ca^{2+} influx was mediated by L-type Ca^{2+} channels.

Effect of KA on the myoblast fusion

The myoblast fusion is known to be a Ca^{2+} dependent process (Shainberg, et al, 1971b; David, et al, 1981; Entwistle, et al, 1988a). Since KA increased intracellular Ca^{2+} level in myoblasts, we next examined the effect of KA on myoblast fusion. Fig. 4A shows that KA promoted myoblast fusion in a dose-dependent manner with a maximal effect at 5 mM. And the KA-stimulated myoblast fusion was suppressed by CNQX, a non-NMDA receptor blocker. In addition, CNQX also completely blocked spontaneous myoblast fusion in a dose-dependent manner (Fig. 4B). These results strongly suggest that KA receptors are involved in Ca^{2+} influx and then trigger myoblast fusion.

DISCUSSION

Increase of intracellular Ca^{2+} concentration is essential for the cell fusion in myoblasts (David et al, 1981). This Ca^{2+} increase seems to be a prerequisite for myoblast fusion, since a partial buffering of intracellular Ca^{2+} can reduce myotube formation. Several studies showed that this essential cytosolic Ca^{2+} increase is involved in the machinery which provides signals for myoblast fusion and/or in the control of the machinery necessary for the fusion of adjacent membranes (Choi et al, 1992; Kwak et al, 1993; Lee et al, 1994). However, the mechanism of how Ca^{2+} influx rises prior to myoblast fusion remains unclear.

Several studies suggested that Ca^{2+}-permeable channels, such as stretch-activated channels (SACs) and nicotinic acetylcholine receptors (nAChRs), are involved in myoblast Ca^{2+} influx (Cognard et al, 1993; Shin et al, 1996; Park et al, 2002). Besides of these channels, NMDA receptors seem to be also involved in myoblast Ca^{2+} influx, since recent data suggested the expression of NMDA receptors in skeletal muscle. In rat and mouse skeletal muscles, the NMDA receptor subunit R1 protein was identified in NMJs (Grozdanovic & Gossrau, 1998), and NMDAR1 and the acetylcholine receptor (AChR) were found to be linked to nitric oxide synthase in C2C12 skeletal myotubes (Luck et al, 2000). Previously, we also detected expression of NR1 and NR2D in both C2C12 myoblasts and myotubes (unpublished data).

There still remins an unanswered question of how motoneurons are able to release glutamate as a neurotransmitter at NMJs. N-acetylaspartylglutamate, a dipeptide localized in putative glutamatergic neurons in brain, is found in mammalian motoneurons (Ory-Lavollee et al, 1987). This dipeptide is cleaved to yield extracellular glutamate and N-acetylasparate by the N-acetylated alpha-liked acidic dipeptidase (NAALADase) (Slusher et al, 1990), which is also detected at the NMJs (Berger et al, 1995). Based on these results, it is possible that glutamate might be released by motoneurons as a neurotransmitter.

Based on these studies, we presently examined whether myoblasts could also express non-NMDA receptors as a new pathway for Ca^{2+} influx, and showed for the first time that KA receptors, non-NMDA receptors, also are expressed in myoblasts and involved in Ca^{2+} influx, a prerequisite for myoblast fusion. C2C12 myoblasts expressed KA1-KA2 mRNA, and myotubes also expressed both KA1-KA2 / AMPAR1 (GluR1) mRNA (Fig. 1). In addition, KA provoked Ca^{2+} influx through L-type Ca^{2+} channels in C2C12 myoblasts (Fig. 2), implying that KA receptors are candidates for another pathway for Ca^{2+} influx. Furthermore, KA promoted myoblast fusion, and these fusion-promoting effects were abolished by CNQX, a inhibitor of non-NMDA receptors (Fig. 4). The spontaneous myoblast fusion was also prevented with CNQX. Although the function of KA and AMPA receptors in myotube was not examined, these findings nevertheless suggest that the effect of KA on myoblast fusion was mediated by KA receptors, since the RT-PCR analysis showed that AMPA receptor was not expressed in myoblasts.

In conclusion, the present study strongly indicates that KA receptor is a new route for Ca^{2+} influx during myoblast fusion.

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