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Acetylcholine Induces Mesenchymal Stem Cell Migration via Ca^{2+} /PKC/ERK1/2 Signal Pathway

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Abstract

Acetylcholine (ACh) plays an important role in neural and non-neural function, but its role in mesenchymal stem cell (MSC) migration remains to be determined. In the present study, we have found that ACh induces MSC migration via muscarinic acetylcholine receptors (mAChRs). Among several mAChRs, MSCs express mAChR subtype 1 (m1AChR). ACh induces MSC migration via interaction with mAChR1. MEK1/2 inhibitor PD98059 blocks ERK1/2 phosphorylation while partially inhibiting the ACh-induced MSC migration. InsP3Rs inhibitor 2-APB that inhibits MAPK/ERK phosphorylation completely blocks ACh-mediated MSC migration. Interestingly, intracellular Ca^{2+} ATPase specific inhibitor thapsigargin also completely blocks ACh-induced MSC migration through the depletion of intracellular Ca^{2+} storage. PKC α or PKC β inhibitor or their siRNAs only partially inhibit ACh-induced MSC migration, but PKC- ζ siRNA completely inhibits ACh-induced MSC migration via blocking ERK1/2 phosphorylation. These results indicate that ACh induces MSC migration via Ca^{2+} , PKC and ERK1/2 signal pathways.

Keywords

Mesenchymal stem cell; Acetylcholine; Migration; Protein kinase C; Ras mitogen-activated protein kinase; Calcium; Muscarinic receptors; ERK1/2

Introduction

Mesenchymal stem cells (MSCs) are able to self-renew and differentiate into a variety of cell types including adipocytes, chondrocytes, myocytes, osteoblasts, etc. [Bobis, et al., 2006]. MSCs are traditionally isolated from bone marrow. However, recent reports have shown that cells with MSC characteristics are also isolated from other tissues, such as cord blood, peripheral blood, skeletal muscle, fetal liver, lung, placenta, synovium, and heart

[Huang et al., 2009, Chong et al., 2011]. Moreover, MSCs and its progenies are involved in tissue or organ formation during embryonic development and adult fracture repair or remodeling [Bruder et al., 1994; De Bari et al., 2010]. MSC proliferation and differentiation are controlled by a coordinated action of several signaling pathways including Wnt [Boland et al., 2004], Notch [De Jong et al., 2004], BMP [Sammons et al., 2004], and others such as acetylcholine (ACh) that has not yet been explored.

ACh is a ubiquitously-expressed signaling molecule and an important neurotransmitter in the nervous system and neuromuscular junctions [Wessler et al., 2008]. Recent studies demonstrate that the cholinergic system also plays a role in regulating the function of non-neural tissues or cells [Schraufstatter et al., 2009]. The cholinergic system is composed of acetylcholine (ACh), acetyltransferase, acetylcholinesterase and acetylcholine receptors (AChRs). These components are found to be expressed in a variety of non-neural cells including hematopoietic stem cells (HSC) [Seroby et al., 2007], embryonic stem cell (ES) [Paraoanu et al., 2007], neural stem cell (NSC) [Cui et al., 2006; Ma et al., 2004], MSCs [Kawano et al., 2002; Hoogduijn et al., 2009; Schraufstatter et al., 2009], and skeletal muscle stem cells (SMSC) [Grassi et al., 2004]. These observations suggest a role of ACh in regulating stem cell function. Indeed, AChRs have been shown to regulate the proliferation and differentiation of NSC [Ma et al., 2004], HSC [Seroby et al., 2007], and SMSC [Grassi et al., 2004]. MSCs express both nicotinic (nAChRs) and muscarinic receptors (mAChRs). ACh regulates apoptosis [Danielyan, et al., 2009; Kim et al., 2008], but not proliferation of MSCs [Hoogduijn et al., 2009]. Importantly, mAChRs appear to be involved in the migration of several different cells [Boss et al., 2005; Chernyavsky et al., 2004]. However, little is known about the role of the cholinergic system in the regulation of MSC migration.

In this study, we found that bone marrow-derived MSCs express mAChR. ACh promoted MSC migration via mAChR, which was mediated by Ca^{2+} -dependent ERK1/2 and PKC signaling pathways.

Methods

Isolation, culture, and multi-differentiation of MSC

BMMSC was purified from male Sprague Dawley rats (40~60 g) by flushing the cavity of femurs and tibias with basal Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium. BM cells were seeded into 150-mm dishes and cultured in DMEM supplemented with 15% fetal calf serum (FCS) and antibiotics. Non-adherent hematopoietic cells were removed during routine fresh medium replacement. The adherent and spindle-shaped MSC were expanded and cultured for no more than 3–5 passages/generations before treatment. For osteogenic differentiation, MSC (70% confluent) from passage two were used. The medium was replaced by osteogenic medium with 10^{-7} M dexamethasone, 0.2 mM ascorbic acid, and 10 mM β -glycerophosphate (Sigma). After culturing in osteogenic medium for 15 days, cell colonies displayed bone-like nodular aggregates of matrix mineralization. Von Kossa staining for calcium enabled visualization of mineral deposition. For adipogenic differentiation, the culture medium was replaced by adipogenic medium with 1 μM dexamethasone, 0.2 mM metacel, 0.5 mM isobutyl methylxanthine (IBMX) and 10 $\mu\text{g/ml}$

insulin (Sigma). The medium was replaced every 3 days for 21 days. Fat cells were visualized by oil red staining [Tang et al., 2009a].

FACS analysis

MSC were washed twice and incubated with following antibodies at 4°C for 40 min: fluorescein isothiocyanate (FITC), m1AChR, CD90, CD29, CD34 and CD45 (Supplemental Data 1). Matching isotype antibodies served as controls. Cells were analyzed by flow cytometry using a Coulter Epics XL-MCL™ flow cytometer (Beckman Coulter, Fullerton, CA, USA). Each analysis included 50,000 events.

Cell proliferation assay

Growth studies *in vitro* were conducted using a [³H]-thymidine incorporation assay. MSC were placed in 96-well plates in DMEM with 15% FCS and allowed to adhere overnight. Subconfluent conditions were chosen to allow detection for maximal growth. The medium was changed to DMEM with 2% FBS to induce quiescence for 24 h [Hoogduijn et al., 2009]. Cells were then treated with or without ACh (1×10^{-5} – 10^{-9} M) in medium containing 15% FCS. Cells were pulsed with 1 µCi per ml [³H]-thymidine and incubated for 3 hours. After trypsin treatment, cells were harvested by centrifugation and treated with 5% trichloroacetic acid (TCA) at 4°C for 30 minutes. The TCA-insoluble fraction was resuspended in 0.1% SDS in 200 mM NaOH. The samples, after addition of 5 ml Optifluor (Packard Instruments, Downers Grove, IL), were counted for radioactivity by a liquid scintillation counter (Tricarb 2900 TR, Packard Instruments).

Cell migration assay

MSC were collected and seeded in the top well of a transwell insert (Millipore, Billerica, MA) at a density of 2×10^5 cells/well in 200 µl of 15% FCS-contained DMEM. DMEM (600 µl) with 15% FCS containing ACh (1×10^{-5} – 10^{-9} M) was added to the bottom wells of the transwell plates (pore size, 8 µm). 15% FCS-DMEM was used as a random migration control. For the inhibition experiments, MSC were preincubated with mAChR antagonist atropine, calcium channel blocking agent verapamil (Sigma), MEK1/2 inhibitor PD98059 [Forte et al., 2006], phospholipase C (PLC) inhibitor U73122 (Bioscience, Silverdale, WA) [Petit et al., 2005], Ryanodine receptor inhibitor Ryandoin, Ins (1, 4, 5) P (3) receptor inhibitor 2-APB, Ca²⁺ pump inhibitor thapsigargin (Alexis biochemicals, San Diego, CA) [Kawano et al., 2002; Kim et al., 2009], PKC inhibitor staurosporine (Alexis biochemicals, San Diego, CA) [Jiménez et al., 2005], or PKCα/PKCβ1 inhibitor Gö-6976 (Merk, Darmstadt, Germany) [Kasenda et al., 2008] for 30 min before seeding. MSC were then cultured at 37°C in a humidified atmosphere of 5% CO₂ for 12 h. Transwell inserts were then removed and migration activity was evaluated by the mean number of cells migrating to the bottom wells of 5 high-power fields (200×) per chamber as observed by phase contrast microscopy. The migration index was calculated to express stimulated migration using the following equation: Migration index=Stimulated migration/Random migration. Each assay was carried out in triplicate wells [Tang et al., 2009b].

Scrape migration assay

Scrape migration assays were performed by following the protocol of the CytoSelect™ 24-well Wound Healing Assay Kit (Cell Biolabs, Inc. San Diego, CA). For optimal cell dispersion, add 250 µL of cell suspension to either side of the open ends at the top of the insert. Cells were cultured for 24 h, and the inserts were then removed to create a wound field with a defined gap of 0.9 mm for measuring the migratory rates of cells. Migratory cells were able to extend protrusions and ultimately invade and close the wound field. For the inhibition experiment, MSC were preincubated with mAChR antagonist atropine (50 mM) for 30 min before seeding. MSC were then cultured at 37°C in a humidified atmosphere of 5% CO₂ for 12 h. The migration of cells across the wound was evaluated by phase-contrast microscopy. The percent of closure was measured according to the manufacturer's recommendation from the CytoSelect™. Percent Closure (%) = Migrated Cell Surface Area/Total Surface Area x 100. Total Surface Area = 0.9 mm x length. Migrated Cell Surface Area = length of cell migration (mm) x 2 x length [Ridley et al., 2003]

In vitro knockdown of PKC in MSC using small interfering RNA (siRNA)

The siRNA targeting PKCα, PKCβ and PKCζ were obtained from Santa Cruz Biotechnology. To target mRNA of PKCα, PKCβ or PKCζ, MSCs were planted into 6-well plates. siRNA transfection was performed using siRNA transfection reagent (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's recommendation. After 6 hours of transfection, the culture medium containing 15% FCS was added. The assays were carried out 48 hours post-transfection.

Western blot analysis

Quiescent cultures of MSC were incubated at 37°C with vehicle or agents and then lysed in ice-cold RIPA buffer containing protease inhibitors. 50 µg of proteins were resolved in 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA). After being blocked with 5% nonfat milk, the membrane was incubated with primary antibody (1:1000 of dilution) for 90 min followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. The primary antibodies used were: mouse anti-rat phospho-ERK1/2 (pERK1/2, 1:1000, Cell Signaling Technology, Boston, MA), rabbit anti-goat ERK1/2(tERK1/2, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-rat phospho-PKCα (pPKCα, 1:1000, Santa Cruz), and mouse anti-rat PKCα (tPKCα, 1:1000, Santa Cruz). The secondary antibodies were goat anti-rabbit IgG and rabbit anti-mouse IgG (Santa Cruz). Protein expression was visualized by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ) and measured by densitometry [Tang et al., 2009b].

RNA preparation and RT-PCR

Total RNA was extracted from MSC using TRIzol Reagent (Invitrogen, Carlsbad, CA) by following the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Moloney Murine Leukemia Virus reverse transcriptase (Fermentas Life Science, Ontario, Canada) and oligo (dT) primers according to the

manufacturer's instructions. The primer sequences are shown in Supplemental Data 2. PCR was performed at 95°C for 4 min, followed by 2–33 cycles at 94°C for 45 s, 56–58°C for 40 s, and 72°C for 40 s. The constitutively expressed gene GAPDH was used as an internal control for cDNA input. 20 µl of each PCR product was run in 1.5% agarose gels.

Immunostaining

Cultured MSC were fixed in 4% paraformaldehyde. After a brief wash in PBS, the cells were incubated in a blocking buffer (PBS containing 1% FCS and 0.1% Triton X-100) at room temperature for 1 h. Incubations with antibodies (diluted 1:250 in blocking buffer) were carried out at 4°C overnight for primary antibodies (goat anti-rat m1AChR, 1:150, Santa Cruz), and room temperature for 2 h for secondary antibodies (FITC-conjugated anti-goat IgG, Santa Cruz). The nucleus was stained with DAPI (50 µg/ml).

Data analysis

Data are presented as mean ± SD. Statistical significance between two groups was determined by paired or unpaired Student's *t*-test. Results for more than two experimental groups were evaluated by one-way ANOVA to specify differences between groups. *P* < 0.05 was considered significantly different.

Result

Characterization of MSC

To characterize the phenotype of MSC, the expression of MSC surface markers were analyzed by FACS. Although MSC did not express CD34 and CD45, more than 90% of MSC isolated expressed CD29 and CD90 (Fig 1A–1D). The ability of MSC to differentiate into osteocytes and adipocytes was tested in all cultures from various donors. When cultured in osteogenic medium for 15 days, MSC were positive for Kossa staining, indicating a mineral deposition. The MSC were also able to differentiate into adipocytes as evidenced by the accumulation of lipid vacuoles after cultivation in adipogenic medium (Fig 1E–1F).

Expression of mAChR in MSC

To determine if MSC express mAChR, the mRNA levels of five mAChR subtypes in the MSCs were determined by RT-PCR. We found that m1AChR was highly expressed in MSC; m4AChR expression was very weak, but the m2, m3 and m5 subtypes were not detected (Fig 2A). Immunofluorescent staining showed that m1AChR protein was expressed in both cytoplasm and membrane of MSCs (Fig 2B). Interestingly, only 32% of MSCs isolated from bone-marrow expressed m1AChR as shown by FACS analyses (Fig 2C), suggesting that m1AChR can only mediate the migration of a portion of MSCs. Ach-induced migration of other MSCs is likely to be mediated by nAChR (Schraufstatter et al., 2009). In addition to m1AChR, MSCs also expressed L-type Ca²⁺ channel, InsP3R1, InsP3R2, and RYR1 (Fig 2D–2E).

ACh did not induce MSC proliferation

[³H] thymidine incorporation assay was used to evaluate DNA synthesis as a measure of *in vitro* cell proliferation of ACh-induced MSC. We found that the [³H] thymidine

incorporation rate was not affected by the treatments with different dosage of ACh, suggesting that ACh had no effect on MSC growth (Fig 3A–B).

ACh induced MSC migration via the mAChR

By using Trans-well migration assay, we found that ACh induced a dose-dependent increase in MSC migration. 1×10^{-6} M of ACh stimulated a maximal migration rate (Fig 3C). ACh also stimulated MSC migration in a time-dependent manner. 1×10^{-6} M of ACh stimulated the maximal migration after 24 h of treatment (Fig 3D). ACh-induced migration was blocked by atropine, a selective muscarinic antagonist (Fig 3E), suggesting that mAChR is essential for ACh-induced MSC migration. To further determine the ACh effect on MSC migration, scrape migration assays were performed. Indeed, ACh significantly induced MSC migration in time and dose-dependent matters (Fig 4A and 4B, and Supplemental Figures 1 and 2). This effect was also blocked by atropine (Fig 4C and Supplemental Figure 3), confirming that ACh induced MSC migration via mAChR.

ERK is involved in ACh-induced MSC migration via the mAChR

The main extracellular signal-regulated kinases of MAPKs consist of ERK1/2, p38 MAPK and JNK [Ma et al., 2004]. ERK1/2 pathways have been reported to play a role in ACh-induced keratinocyte directional migration [Chernyavsky et al., 2005]. To determine if ERK1/2 is a downstream signaling pathway of ACh-stimulated muscarinic receptors, we detected ERK1/2 activation in MSC and found that 1×10^{-6} M of ACh significantly increased ERK1/2 phosphorylation. Both mAChR antagonist atropine and MEK1/2-selective inhibitor PD98059 abolished the effect of ACh (Fig 5A), suggesting that ERK1/2 is involved in ACh-mAChR activity. Indeed, blocking ERK1/2 activation by PD98059 inhibited ACh-induced MSC migration (Fig 5B).

PKC is involved in ACh-induced MSC migration via the mAChR

PKC is involved in the migration of various cells [Dempsey et al., 2000]. To determine if PKC is a downstream signaling molecule of ACh-stimulated muscarinic receptors, we first determined if ACh induces PKC α activation. As shown in Fig 6A and 6B, ACh induced PKC α phosphorylation in MSC. However, ACh-induced PKC α phosphorylation was blocked by Atropine as well as PKC α /PKC β selective inhibitor (Fig 6A and 6B). Importantly, ACh-induced MSC migration was also blocked by Gö-6976 or staurosporine, a PKC-non selective inhibitor (Fig 6A and 6B). Moreover, blockade of PKC α , PKC β , or PKC ζ by individual specific siRNAs inhibited ACh-induced MSC migration. Notably, PKC ζ -siRNA appeared to have a significantly greater effect than PKC α or PKC β siRNA, suggesting that PKC ζ signaling pathway may play a major role in mediating ACh-mediated MSC migration.

Role of calcium in ACh-induced MSC migration

Calcium has been shown as an important regulator of cell migration [Agell et al., 2002]. Extracellular Ca²⁺ also stimulated MSC migration (Fig 7A). ACh appeared to enhance MSC migration in the presence of Ca²⁺ (Fig 7A). ACh-induced migration was partially inhibited

by verapamil, a L-type and α_1 calcium channels inhibitor, suggesting that ACh-induced MSC migration was associated with extracellular Ca^{2+} .

To determine if Ca^{2+} release from intracellular stores plays a role in ACh-induced MSC migration, we used a Ca^{2+} -ATPase blocker Thapsigargin to treat MSC. We found that Thapsigargin blocked MSC migration (Fig 7B). Thapsigargin irreversibly inhibits Ca^{2+} -ATPase, eliciting an increase in intracellular Ca^{2+} and depletion of the intracellular calcium pools that is likely involved in the control of MSC migration. These data suggest that endoplasmic reticulum Ca^{2+} stores are important for MSC migration. There are two types of endoplasmic reticulum Ca^{2+} release channels, inositol 1, 4, 5-trisphosphate receptors (InsP3Rs) and ryanodine receptors (RyRs). As shown in Fig 2D, InsP3Rs are abundantly present in MSC. To determine whether ACh stimulated MSC migration by signaling through the InsP3Rs upstream phospholipase C (PLC) pathway, we utilized PLC- β and PLC- γ inhibitor U-73122 to treat ACh-induced MSC. We found that U-73122 significantly inhibited ACh-induced MSC migration (Fig 7B), indicating a PLC dependence.

To explore further the role of InsP3Rs, the effect of IP₃Rs inhibitor 2-APB on MSC migration was determined. We found that ACh-induced MSC migration was completely inhibited by 2-APB. These data demonstrate that ACh-induced MSC migration requires endoplasmic reticulum Ca^{2+} release through IP₃Rs (Fig 7B).

MSC endoplasmic reticulum contains another Ca^{2+} release channel namely RyRs, which are activated by cytoplasmic Ca^{2+} . Ryanodine, selective inhibitor of Ca^{2+} release from endoplasmic reticulum RyRs, failed to prevent MSC migration (Fig 7B), suggesting that ACh-induced MSC migration is not associated with Ca^{2+} release mediated by RyRs.

Role of calcium in ACh-induced PKC and ERK1/2 phosphorylation

Since calcium, ERK1/2 and PKC were all involved in MSC migration; we sought to determine if Ca^{2+} release from intracellular stores is important for ACh-induced PKC and ERK1/2 activation in MSC. We found that ryanodine receptor inhibitor Ryanodine partially affected phosphorylation of ERK1/2 and PKC. InsP3Rs blocker 2-APB completely decreased the phosphorylation levels of ERK1/2 and PKC in ACh-induced MSC (Fig 7C–7D). These data suggest that Ca^{2+} release mediated by InsP3Rs are essential for ACh-induced PKC and ERK1/2 phosphorylation.

Role of PKC in ACh-induced ERK1/2 phosphorylation

PKC-dependent activation of ERK1/2 in response to mAChR stimulation has been observed in various cells [Agell et al., 2002; Jiménez et al., 2002; Jiménez et al., 2005]. To determine if the link between PKC and ERK1/2 pathways is involved in ACh signaling, MSC were pretreated with PKC inhibitor staurosporine or PKC α /PKC β inhibitor Gö-6976 for 30 min. As shown in Fig 8A and 8B, staurosporine or Gö-6976 completely blocked ACh-induced ERK1/2 phosphorylation evoked by ACh. To confirm PKC effect on ERK1/2 phosphorylation, PKC α , PKC β , or PKC ζ expression were blocked by individual siRNAs. As shown in Fig 8C–8H, siRNA knockdown of each PKC isoforms significantly blocked ACh-induced ERK1/2 phosphorylation. Consistent with the effect of PKC ζ on MSC migration (Fig 6D), PKC ζ appeared to have a greater effect on the ERK1/2 phosphorylation than

PKC α or PKC β . PKC ζ These results demonstrate that ACh-induced ERK1/2 activation is mediated by PKC ζ signaling pathway.

Discussion

Although ACh is a neurotransmitter, it has autocrine functions in diverse cell types. ACh has been shown to regulate neuronal differentiation during early development [Wessler, et al. 2008]. In addition to neuronal tissues, mAChRs and nAChRs regulates a wide variety of physiological responses including apoptosis, proliferation, differentiation and migration of muscle cells [Fernandes et al., 1998], NSCs [Resende et al., 2009], blood cells and HSCs [Seroby et al., 2007] and keratinocytes [Chernyavsky et al., 2005]. In this study, we found that ACh activated Ca²⁺ release, ERK1/2 and PKC signaling via interaction with mAChR, leading to MSC migration.

m2AChR mRNA expression has been reported in MSCs previously [Hoogduijn et al. 2009; Kawano, et al. 2002]. In this report, we have found that MSCs express m1AChR at both the mRNA and protein level, consistent with a previous study [Danielyan, et al. 2009]. In response to ACh stimulation in the presence of calcium, MSCs showed an enhanced migratory capacity, which was partially inhibited by membrane calcium channel blocker verapamil, but completely blocked by InsP3Rs inhibitor 2-APB or intracellular Ca²⁺ pump inhibitor thapsigargin. Not surprisingly, RyRs inhibitor ryanodine only partially inhibited the ACh-MSC migration because RyRs are not the major functional Ca²⁺ channel in MSCs. Nevertheless, our data demonstrate that Ca²⁺ signal is essential for ACh-induced MSC migration. Moreover, we found that ACh-induced transient increase of cytosolic [Ca²⁺] in MSCs is mediated by the activation of phospholipase C and production of IP3 (Supplemental Data, Fig S4), which is consistent with previous finding in other cells [Patergnani, et al. 2011; Volpicelli, et al. 2004].

MAPK/ERK1/2 is involved in SDF-1 α - and TNF- α -induced MSC migration [Li et al., 2009; Ryu et al., 2010; Fu et al., 2009]. mAChRs has been found to promote smooth muscle and keratinocyte cell migration through activation of PI3-kinase and its downstream targets, protein kinase B (PKB)/Akt and MAPK/ERK1/2 [Chernyavsky et al., 2005; Yamboliev et al., 2000]. Our studies showed that MEK1/2 inhibitor blocked ACh-induced MSC migration by attenuating ERK1/2 phosphorylation, suggesting that ACh-induced MSC migration is mediated by MAPK/ERK1/2 signaling. Importantly, 2-APB and thapsigargin completely blocked MSC migration while attenuating Erk1/2 phosphorylation, indicating that ERK1/2 activation in ACh-induced MSC migration is Ca²⁺-dependent.

ERK1/2 activation in ACh-induced MSC migration appears to be mediated by PKC. Upon ACh binding, m1AChR activates PKC through diacylglycerol (DAG), which is activated by PLC [Resende et al., 2009; Dempsey et al., 2000; Ma et al., 2004]. MAPK/ERK1/2 can be activated through PKC-dependent and independent mechanisms [Resende, et al., 2009]. We found that MAPK/ERK1/2 activation was partially blocked by the inhibitors of PKC α and PKC β or their siRNAs, but completely blocked by PKC ζ siRNA or high dosage of a relatively non-selective protein kinase inhibitor staurosporine, suggesting that ACh-mediated MAPK/ERK1/2 activation is PKC-, especially PKC ζ -dependent. Functionally,

siRNA knockdown of PKC α or PKC β or inhibition of its activity partially while knockdown of PKC ζ completely blocked ACh-induced MSC migration, suggesting that ACh-induced MSC migration is regulated primarily by PKC-dependent ERK1/2 pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1. MSC phenotype characterization

Flow cytometric analysis showed that MSC expressed CD34 (0.74%) (A), CD45 (0.19%) (B), CD90 (92%) (B), and CD29 (91%) (D). (E-F) MSC differentiation in special culture conditions *in vitro*. (E) Osteocyte differentiation as shown by von Kossa staining. (F) Adipogenic differentiation as shown by Oil-O-Red staining.

Fig 2. mAChR expression in MSC

(A) m1AChR mRNA expression in MSC was detected by RT-PCR. (B) m1AChR expression was detected by immunostaining of MSC. (C) Flow cytometric analysis of m1AChR expression in MSCs. (D) L-Ca²⁺ channel, N-Ca²⁺ channel, and InsP3Rs expression in MSC was detected by RT-PCR. (E) RYRs expression in MSC was detected by RT-PCR.

Fig 3. The effect of ACh on MSC proliferation and migration

(A, B) ACh had no effect on MSC proliferation, * $p > 0.05$ vs Ctrl. (C) ACh induced MSC migration in a dose-dependent manner, * $p < 0.05$ vs Ctrl. (D) ACh induced MSC migration in a time-dependent manner, * $p < 0.05$ vs Ctrl in 12 h, # $p < 0.05$ vs Ctrl in 24 h. (E). ACh-induced MSC migration was inhibited by atropine, a mAChR blocker, # $p < 0.05$ vs ACh (10^{-6} M).

Fig 4. MSC migration determined by wound healing assay

(A) 2×10^5 cells were seeded into 24-well plates coated with 10 mg/ml fibronectin. Monolayer cell wounds were produced by CytoSelect™ insert. 10^{-5} ~ 10^{-9} M of ACh was added to 15% FCS-containing DMEM. MSC migration was photographed at the times indicated. * $p < 0.05$ vs ACh (0, 10^{-7} , 10^{-8} , or, 10^{-9} M). # $p < 0.05$ vs ACh (10^{-6} M). (B) MSCs were treated with ACh (10^{-6} M) for 0, 6, 12, and 24 h, and scrape wound healing assays were performed as described in A. * $p < 0.05$ vs Ctrl in 6 h, & $p < 0.05$ vs Ctrl in 12 h, # $p < 0.05$ vs Ctrl in 24 h. (C) MSCs were treated with ACh (10^{-6} M) with or without atropine treatment (10^{-4} M) for 12 h. Scrape wound healing assays were performed. * $p < 0.05$. vs. ACh (10^{-6} M).

Fig 5. ACh promoted MSC migration via activation of ERK1/2

(A) ACh Activated ERK1/2 via mAChR. Upper panel: western blot detection of ERK1/2 phosphorylation (pERK1/2) and ERK1/2 expression. α -tubulin served as an internal control. Lower panel: Quantification of phosphorylated ERK1/2. *, # $P < 0.05$ vs ACh (10^{-6} M) (n=5). (B) ERK1/2 is essential for ACh-induced MSC migration. * $P < 0.05$ vs ACh (10^{-6} M), n=5.

Fig 6. ACh promoted MSC migration via activation of PKC signaling

(A) ACh Activated PKC α via mAChR. PKC phosphorylation and expression were determined by western blot. α -tubulin served as an internal control. (B) Quantification of phosphorylated PKC α . *,# $P < 0.05$ vs ACh (10^{-6} M), $n=5$. (C) PKC α /PKC β selective inhibitor Gö-6976 (6 nM) blocked ACh-induced MSC migration. *,# $P < 0.05$ vs ACh, $n=5$. (D) siRNA blockade of PKC α , PKC β , or PKC ζ inhibited ACh-induced MSC migration. *,#, & $P < 0.05$ vs ACh (10^{-6} M), $n=5$.

Fig 7. Role of Calcium in ACh-induced MSC migration and PKC and ERK1/2 phosphorylation (A) Extracellular Ca²⁺ in ACh-induced MSC migration, **P*<0.05 vs PBS; #*P*<0.05 vs PBS +ACh. (B) ACh-induced MSC migration required Ca²⁺ release mediated by InsP3Rs. **P*>0.05 vs ACh (10⁻⁶ M); #, &, \$, @*P*<0.05 vs ACh (10⁻⁶ M). (C–D) Role of Calcium in ACh-induced PKC and ERK1/2 phosphorylation. Upper panel: phosphorylated and total PKC (C) or ERK1/2 (D) was detected by western blot. α-tubulin served as an internal control. Lower panel: Quantification of phosphorylated PKC or ERK1/2. *, #*P*<0.05 vs ACh (10⁻⁶ M), n=5.

Fig 8. Role of PKC in ACh-induced ERK1/2 phosphorylation

(A–D) Western blot of ERK1/2 phosphorylation (pERK1/2) and expression (tERK1/2), α-tubulin served as an internal control. *, #, & $P < 0.05$ vs ACh (10^{-6} M). in each panel, $n = 5$.