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Store-operated calcium entry induced by activation of Gq-coupled alpha1B adrenergic receptor in human osteoblast

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ABSTRACT

Recent studies have revealed that the sympathetic nervous system is involved in bone metabolism. We previously reported that noradrenaline (NA) suppressed K^+ currents via Gi/o protein-coupled alpha$_{1B}$-adrenergic receptor (α_{1B}-AR) in human osteoblast SaM-1 cells. Additionally, it has been demonstrated that the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) was increased by NA via α_{1B}-AR. In this study, we investigated the signal pathway of NA-induced $[Ca^{2+}]_i$ elevation by using Ca^{2+} fluorescence imaging in SaM-1 cells. NA-induced $[Ca^{2+}]_i$ elevation was suppressed by pretreatment with a PLC inhibitor, U73122. This suggested that the $[Ca^{2+}]_i$ elevation was mediated by Gq protein-coupled α_{1B}-AR. On the other hand, NA-induced $[Ca^{2+}]_i$ elevation was completely abolished in Ca^{2+}-free solution, which suggested that Ca^{2+} influx is the predominant pathway of NA-induced $[Ca^{2+}]_i$ elevation. Although the inhibition of K^+ channel by NA caused membrane depolarization, the $[Ca^{2+}]_i$ elevation was not affected by voltage-dependent Ca^{2+} channel blockers, nifedipine and mibefradil. Meanwhile, NA-induced $[Ca^{2+}]_i$ elevation was abolished following activation of store-operated Ca^{2+} channel by thapsigargin. Additionally, the $[Ca^{2+}]_i$ elevation was suppressed by store-operated channel inhibitors, 2-APB, flufenamate, $GdCl_3$ and $LaCl_3$. These results suggest that Ca^{2+} influx through store-operated Ca^{2+} channels plays a critical role in the signal transduction pathway of Gq protein-coupled α_{1B}-AR in human osteoblasts.

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1. Introduction

Bones are constantly remodeled throughout life. Bone homeostasis is maintained by a balance between the activities of bone-forming osteoblasts and bone-resorbing osteoclasts. In recent years, many studies have demonstrated that the sympathetic nervous system is involved in bone metabolism [1–5]. Osteoporosis can be induced by continuously high sympathetic tone, which is recovered from by using β-adrenergic receptor (β-AR) blocker [6,7]. Previous studies, including ours, showed that mRNAs of α- and β-ARs were expressed in human osteoblasts [1,8,9]. Although a number of studies have suggested that up-regulation of osteoclastogenesis and osteoclastic activity via β-AR caused enhancement of bone resorption [3,10,11], the physiological role of α-ARs in bone metabolism has been less well studied.

We previously reported that noradrenaline (NA) increased cell proliferation by suppressing K^+ channels via Gi/o-coupled α_{1B}-AR in human osteoblast SaM-1 cells. On the other hand, application of NA also increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via Gq protein-coupled α_{1B}-AR [12]. In general, NA-induced $[Ca^{2+}]_i$ elevation is mediated by Ca^{2+} release from endoplasmic reticulum via the Gq/phosphoinositide-phospholipase C (Gq/PI-PLC) pathway. However, recent studies have demonstrated that Ca^{2+} influx through Ca^{2+}-permeable channels and Na^+/Ca^{2+} exchanger is involved in α_1-AR-mediated $[Ca^{2+}]_i$ elevation in several tissues [13–19]. The molecular component of Ca^{2+} influx and its importance in Ca^{2+} signaling differ among tissues.

In this study, we investigated the signal transduction pathway of NA-induced $[Ca^{2+}]_i$ elevation in human osteoblast SaM-1 cells. We observed that α_1-AR-mediated $[Ca^{2+}]_i$ elevation was suppressed not only by a PLC inhibitor, U73122, but also by removing extracellular Ca^{2+}. Interestingly, the response to NA was completely abolished in Ca^{2+}-free extracellular solution. This suggested that Ca^{2+} influx plays a predominant role in α_1-AR-mediated Ca^{2+} signaling. Additionally, NA-induced $[Ca^{2+}]_i$ elevation was inhibited by pretreatment with either thapsigargin or store-operated Ca^{2+} channel inhibitors. These results suggested that activation of Gq

Abbreviations: 2-APB, 2-aminoethyl diphenylborate; AR, adrenergic receptor; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; MG-63, human osteosarcoma-derived osteoblast-like cell line; NA, noradrenaline; PDL, population doubling level; PI-PLC, phosphoinositide-phospholipase C; PLC, phospholipase C; SaM-1, human periosteum-derived osteoblastic cells.

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protein-coupled-α_1-AR induces $[Ca^{2+}]_i$ elevation mainly via store-operated Ca^{2+} channels in human osteoblasts.

2. Materials and methods

2.1. Cell culture

The human osteoblasts used in this study, SaM-1 cells, were provided by Dr. Koshihara, who prepared them with informed consent from an explant of ulnar periosteum tissue from a 20-year-old male patient who underwent curative surgery [20]. These cells have a mitotic lifespan of 34 population doubling levels (PDLs), and we used them at a PDL of 22–24 for our experiments. We confirmed that the cells were capable of calcifying at this level [21]. The cells were cultured in alpha-modified minimum essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Moregate Biotech, Bulimba, Australia) and 60 μg/ml kanamycin at 37 °C in 95% humidified air containing 5% CO_2. The growth media were renewed every 2 days. For optical measurements of $[Ca^{2+}]_i$, they were seeded on a glass cover slip 1–2 days before the experiments.

2.2. Optical measurements of $[Ca^{2+}]_i$

We used Cal-520 AM, a highly sensitive Ca^{2+} fluorescent dye, for optical measurements of $[Ca^{2+}]_i$. SaM-1 cells were loaded with Cal-520 AM (2.5 μM) for 30 min and washed three times with extracellular solution, which contained 124 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 14 mM D-glucose and 10 mM HEPES (pH adjusted to 7.4 with NaOH), just before use. Then, the glass cover slip was transferred to a superfusion chamber on the stage of a confocal laser scanning microscope (LSM710, Carl Zeiss, Hallbergmoos, Germany). Cells were superfused with extracellular solution at a rate of 2 ml/min. The fluorescence was recorded every 2 s at room temperature at an excitation wavelength of 488 nm and the data were analyzed using ZEN 2009 software (Carl Zeiss). Stock solutions of drugs were prepared and diluted 1000-fold into extracellular solution just before use. Unless otherwise noted, drugs were bath-applied and fluorescence was recorded from the cells that showed a response to repeated application of NA.

2.3. Chemicals

L-Noradrenaline, prazosin, an α_1-AR selective antagonist, U73122, a PLC inhibitor, nifedipine, an L-type voltage-dependent Ca^{2+} channel blocker, mibefradil, a T-type voltage-dependent Ca^{2+} channel blocker, 2-aminoethyl diphenylborate (2-APB), flufenamate, $GdCl_3$ and $LaCl_3$ were purchased from Sigma Aldrich (St. Louis, MO, USA). KB-R-7943, a Na^+/Ca^{2+} exchanger reverse mode inhibitor, was purchased from Tocris Biosciences (Bristol, UK). Thapsigargin was purchased from Wako (Osaka, Japan). Cal-520 AM was purchased from COSMO BIO (Tokyo, Japan). Cal-520 AM, U73122, nifedipine, KB-R-7943, thapsigargin, 2-APB and flufenamate were dissolved in dimethyl sulfoxide. All other chemicals used were of reagent grade.

2.4. Statistical analysis

All data are expressed as mean ± SEM. In the optical measurements of $[Ca^{2+}]_i$, fluorescence intensity recorded from each cell was used for analysis. The data were recorded from more than 3 independent experiments. The comparison of NA-induced $[Ca^{2+}]_i$ elevation before and after drug treatment was carried out with the paired *t*-test. For multiple comparisons, the two-tailed *t*-test combined with Bonferroni's correction following one-way analysis of variance was used. Differences with p values <0.05 were considered significant.

3. Results

3.1. Involvement of Ca^{2+} influx in NA-induced $[Ca^{2+}]_i$ elevation

Consistent with previous studies, bath application of NA dose-dependently increased $[Ca^{2+}]_i$ and the response was significantly inhibited by prazosin and a PLC inhibitor, U73122 (Fig. 1A–C). To examine whether Ca^{2+} influx was involved in the NA-induced $[Ca^{2+}]_i$ elevation, we used Ca^{2+}-free extracellular solution, which contained 5 mM EGTA instead of 2 mM $CaCl_2$. In the Ca^{2+}-free extracellular solution, NA had no effect on Ca^{2+} fluorescence (Fig. 1D). Additionally, we examined the effects of NA on $[Ca^{2+}]_i$ elevation induced by switching perfusate from Ca^{2+}-free solution to normal solution. Pretreatment with NA significantly increased the Ca^{2+} influx from extracellular fluid (Fig. 1E).

3.2. Elucidation of Ca^{2+}-influx pathway

Previous studies have demonstrated that activation of α_1-AR can induce Ca^{2+} influx via several kinds of pathway, including voltage-dependent Ca^{2+} channel, reverse mode of Na^+/Ca^{2+} exchanger, store-operated Ca^{2+} channel and receptor-operated Ca^{2+} channel in several tissues [13–19].

First, we examined the involvement of voltage-dependent Ca^{2+} channels. The expression of L-type and T-type voltage-dependent Ca^{2+} channel families was previously reported in osteoblasts [22,23]. However, neither nifedipine, an L-type voltage-dependent Ca^{2+} channel blocker, nor mibefradil, a T-type voltage-dependent Ca^{2+} channel blocker, inhibited NA-induced $[Ca^{2+}]_i$ elevation (Fig. 2A, B and D).

In general, Na^+/Ca^{2+} exchanger plays an important role in Ca^{2+} homeostasis by pumping Ca^{2+} out of the cytosol. On the other hand, it was suggested that local accumulation of Na^+ drove Na^+/Ca^{2+} exchanger in reverse mode, and the mechanism was involved in α_1-AR-mediated $[Ca^{2+}]_i$ elevation [16]. However, Na^+/Ca^{2+} exchanger inhibitor, KB-R-7943, did not suppress, but rather enhanced NA-induced $[Ca^{2+}]_i$ elevation in SaM-1 cells (Fig. 2C and D).

Next, we examined the involvement of store-operated and receptor-operated Ca^{2+} channels. Passive depletion of endoplasmic reticulum Ca^{2+} store by a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, activated store-operated Ca^{2+} channels. Bath application of NA had no effect on Ca^{2+} fluorescence following treatment with thapsigargin (Fig. 3A). This result suggested that NA-induced Ca^{2+} influx was mediated through store-operated Ca^{2+} channels, but not through receptor-operated Ca^{2+} channels. Additionally, we examined the effects of store-operated channel inhibitors, 2-APB, flufenamate, $GdCl_3$ and $LaCl_3$, on NA-induced $[Ca^{2+}]_i$ elevation. In the presence of any of these inhibitors, NA-induced $[Ca^{2+}]_i$ elevation was significantly suppressed (Fig. 3B–E).

4. Discussion

Our previous study suggested that α_{1B}-AR can be coupled to both Gq-protein and Gi/o-protein, and NA increased $[Ca^{2+}]_i$ via the Gq/PI-PLC pathway and also inhibited K^+ current via the Gi/o/G$\beta\gamma$ pathway in human osteoblast SaM-1 cells [12,24]. In this study, NA-induced $[Ca^{2+}]_i$ elevation was significantly suppressed by a PLC inhibitor, U73122. This result is in agreement with our conventional understanding. On the other hand, NA-induced $[Ca^{2+}]_i$ elevation was completely abolished in Ca^{2+}-free extracellular fluid. Additionally, pretreatment with NA significantly

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