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Calcium Entry through Voltage-Dependent L-Type Calcium Channels in Preosteoblastic MC3T3-E1 Cells

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Summary

This study was undertaken to confirm that there are voltage-dependent L-type Ca^{2+} channels in osteoblastic MC3T3-E1 cells and that the type of Ca^{2+} channel involved in the Ca^{2+} entry is the L-type. We investigated the effects of a Ca^{2+}-free saline and Bay K 8644, an L-type Ca^{2+} channel activator, on intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). Although high K^+ (100 mM) alone raised the $[Ca^{2+}]i$, high K^+ did not raise the $[Ca^{2+}]i$ in the absence of extracellular Ca^{2+}. Bay K 8644 (1 μM) augmented the high K^+-induced $[Ca^{2+}]i$ rise. In conclusion, it is confirmed that Ca^{2+} invades through the voltage-dependent L-type Ca^{2+} channels in osteoblastic MC3T3-E1 cells.

Introduction

L-type calcium channels are distributed in both excitatory and non-excitatory organs of the body including muscle, heart, brain[1], lung, kidney, and pancreas[2]. These channels are modulated by dihydropyridine antagonists such as nifedipine, nicardipine, and nitrendipine. Benzodiazepine such as diltiazem and phenylalkylamine such as verapamil also block the channel. Lieberherr[3] observed that the 1,25 $(OH)_2D_3$- and PTH-induced rapid increase in the intracellular calcium concentration ($[Ca^{2+}]i$) in osteoblasts isolated from the parietal bones of neonatal mice is abolished by the prior addition of EGTA, nifedipine, and verapamil and suggested that L-type Ca^{2+} channels are involved in this Ca^{2+} influx. Yamaguchi *et al*.[4] also observed that depolarization-activated Ca^{2+} channels and Ba^{2+} entry through the L-type Ca^{2+} channels in osteoblast-like UMR-106 cells. Furthermore, Chesnoy-Marcharis and Fritsch[5] showed that cells derived from neonatal rat calvaria possess both T- and L-type Ca^{2+} channels. However, they never examined whether or not Bay K 8644, an L-type Ca^{2+} channel activator, enhances the high K^+-induced $[Ca^{2+}]i$ rise and never investigated the effects of a Ca^{2+}-free saline containing EGTA.

Thus, the present study was undertaken to confirm that the voltage-dependent L-type Ca^{2+} channels are involved in the Ca^{2+} entry into MC3T3-E1 cells.

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Materials and Methods

The materials were osteoblastic MC3T3-E1 cells derived from neonatal mouse calvarial bone, which cells were obtained from the Riken Cell Bank (Japan). The cells were cultured in α-MEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and in a fully-humidified atmosphere of 95% air and 5% CO_2 at 37℃. Cells were plated on poly-L-lysine-coated cover slips adhered with flexiperm (Heraeus GmbH, Germany) 3-7 days before the experiments. All cells were subconfluent at the time of the experiments[6]. Since alkaline phosphatase activity, as a characteristic of differentiation, begins to rise about 6 days after the start of the culture[7], the cells used here are considered to be "preosteoblasts" [6].

The $[Ca^{2+}]i$ was measured with the Ca^{2+}-sensitive fluorescent dye fura-2.The cells were kept in a solution consisting of 120 mM-NaCl, 5 mM-KCl, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 10 mM-glucose, 0.1%-bovine serum albumin Fraction V (Gibco, USA), and 10 mM-HEPES-NaOH (pH 7.4)[8]. Changes in the fluorescence intensity of fura-2 in the cells were recorded with a video-imaging analysis system (FC-400, Furusawa Labo Appliance, Japan).

Cells were kept at 32℃ during fluorescence measurements to minimize leakage of fura-2 from the cells[9]. Depolarization-induced Ca^{2+} influx into the cells was produced by exchanging the normal medium for solutions containing high K^+ at the concentration of 100 mM. NaCl was isosmotically replaced with KCl. EGTA (1 mM) was added in a Ca^{2+}-free saline. Drugs were added to the perfusate at appropriate concentrations.

Fura-2 pentapotassium salt for $[Ca^{2+}]i$ calibration and fura-2/AM were obtained from Dojindo Laboratories (Japan). All other chemicals were from Nacalai Tesque (Japan).

Each value of the data represents the mean value ± the standard error of the mean and the number of observations (N). Statistical analyses of the data were performed by the Student's 2-sided paired *t*-test. Differences between mean values were considered significant if the probability of error (p) was less than 0.05.

The detailed methods have been described in our previous paper[10].

Results

In order to examine whether or not MC3T3-E1 cells possess voltage-dependent Ca^{2+} channels and to confirm that extracellular Ca^{2+} invades into the cell, we investigated the effect of depolarization of the cell membrane by elevating the extracellular K^+ concentration and that of a Ca^{2+}-free saline on the high K^+-induced $[Ca^{2+}]i$ rise. Fig.1 illustrates the depression of the high K^+-induced $[Ca^{2+}]i$ rise by the Ca^{2+}-free saline. That is, although high K^+ (100 mM) alone raised the $[Ca^{2+}]i$, high K^+ did not raise the $[Ca^{2+}]i$ in the absence of the extracellular Ca^{2+}, suggesting the existence of the voltage-dependent Ca^{2+} channels in the cell.

Next, the experiment was performed to determine the type of the Ca^{2+} channels participating in the Ca^{2+} influx. Fig.2 illustrates the effect of Bay K 8644, an L-type Ca^{2+} channel activator. Bay K 8644 (1 μM) significantly augmented the high K^+ (100 mM)-induced $[Ca^{2+}]i$ rise, implying that the Ca^{2+} entry through the voltage-dependent L-type Ca^{2+} channels occurs.

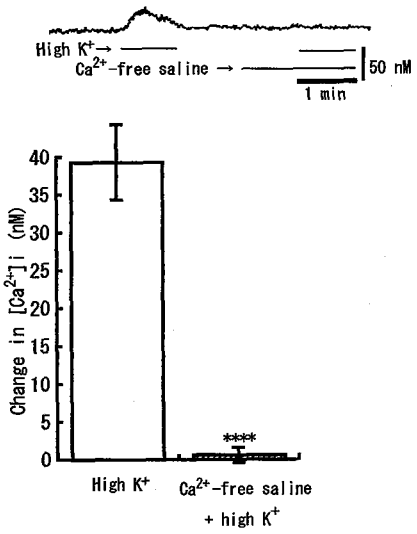

Fig.1 : Depression of high K^+-induced $[Ca^{2+}]i$ rise by a Ca^{2+}-free saline. High K^+ (100mM) did not raise the $[Ca^{2+}]i$ in the absence of extracellular Ca^{2+}.
****: $p<0.001$, N=23.

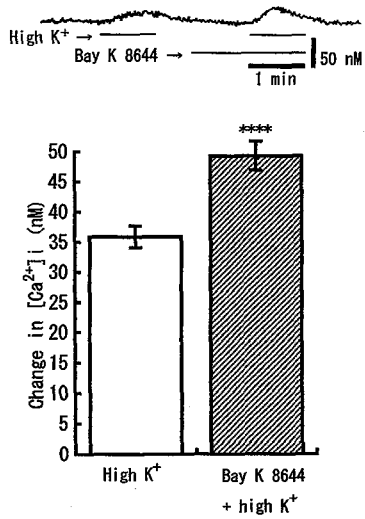

Fig.2 : Effect of Bay K8644 on the high K^+-induced $[Ca^{2+}]i$ rise. Bay K8644 (1 μM) significantly increased the high K^+ (100mM)-induced $[Ca^{2+}]i$ rise.
****: $p<0.001$, N=20.

Discussion

From the results that high K^+ raised the $[Ca^{2+}]i$ and Ca^{2+}-free depressed it (Fig.1), we confirmed the existence of voltage-dependent Ca^{2+} channels in MC3T3-E1 cells. In contrast, Shoda[11] could not observe any high K^+ (50, 75, 100mM)-induced $[Ca^{2+}]i$ rise in MC3T3-E1 cells and concluded that there were no voltage-dependent Ca^{2+} channels in these cells. The cause of the difference from our data might be that electromagnetic stimulation in their case had already depolarized the cell before the application of high K^+ or perhaps the number of experiments was merely too small.

The L-type Ca^{2+} channel agonist (Bay K8644) significantly augmented the high K^+-induced $[Ca^{2+}]i$ rise (Fig.2). This result indicates that the type of the Ca^{2+} channel participating in the high K^+-induced $[Ca^{2+}]i$ rise is the L-type but not the N- or T-type.

Duncan *et al*.[12] proposed that a mechanical strain activated the L-type Ca^{2+} channels in osteoblast-like UMR-106.01 cells. Recently, Meszaros *et al*.[13] and Liu *et al*.[14] reported that the Ca^{2+} influx in osteoblastic cells (ROS 17/2.8) occurs through the L-type voltage sensitive Ca^{2+} channels. These reports support our interpretation.

In rat femoral explant-derived osteoblasts, Gu *et al*.[15] recently observed that the number of cells possessing T-type Ca^{2+} channels decreased more extensively than that of cells possessing L-type ones with increasing days of sub-culture. Our experiments were performed on cells cultured for 3 and over 3 days. Therefore, it is not surprising that we could not find the T-type Ca^{2+} channels, as we used the cells possessing only a small number of T-type ones.

The findings mentioned above lead us to the conclusion that MC3T3-E1 cells possess the voltage-dependent L-type Ca^{2+} channels and that Ca^{2+} invades through the L-type ones under the condition of the membrane depolarization. We speculate that the Ca^{2+} channels may facilitate bone calcifica-

tion, since the rise in the $[Ca^{2+}]i$ is indispensable for accumulation of Ca^{2+} in the matrix vesicle in the osteoblast and their budding them from the cell[16]. It is necessary to examine in detail the participation of the Ca^{2+} channels in the formation of the matrix vesicles themselves.

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抄録：骨芽細胞様細胞 MC3T3-E1 における電位依存性L型カルシウムチャネルからのカルシウムの流入

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電位依存性L型カルシウム（Ca^{2+}）チャネルは興奮性細胞，非興奮性細胞を問わず広く分布している．骨芽細胞におけるCa^{2+}動態は骨形成過程において非常に重要な役割を担っており，細胞外からのCa^{2+}取り込みにはこのチャネルも関与している可能性がある．ここでは材料として骨芽細胞様細胞MC3T3-E1を用いて，この細胞にL型Ca^{2+}チャネルが存在し，それを介してCa^{2+}が流入することを確かめることを目的として実験を行なった．

細胞内Ca^{2+}濃度（$[Ca^{2+}]i$）の測定はCa^{2+}蛍光指示薬のfura-2/AMを細胞内に負荷し,その蛍光強度の変化を画像解析することにより行なった．

高K^+灌流液により $[Ca^{2+}]i$ は上昇したが，細胞外液をCa^{2+}-freeとした条件下では $[Ca^{2+}]i$ 上昇はほぼ完全に消失した．Bay K8644（L型Ca^{2+}チャネル活性化薬）は高K^+による $[Ca^{2+}]i$ の上昇を有意に増大させた．

以上の結果からMC3T3-E1細胞には電位依存性L型Ca^{2+}チャネルが存在し,それを介してCa^{2+}流入の起こることが確かめられた．