Manganese Stimulates Ca^{2+} Mobilization in Human Gingival Fibroblasts

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Abstract

In fura-2-loaded human gingival fibroblasts, 1-50 μM MnCl_2 evoked an increase in intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in a dose-dependent manner. This increase was abolished in the absence of extracellular Ca^{2+} . MnCl_2 induced an increase in $[\text{Ca}^{2+}]_i$ provoked prostaglandin E_2 release in human gingival fibroblasts in time- and dose-dependent manners. MnCl_2 failed to evoke prostaglandin E_2 release in the absence of extracellular Ca^{2+} . A protein kinase C activator and a tyrosine kinase inhibitor inhibited the increase in $[\text{Ca}^{2+}]_i$ induced by MnCl_2 . These results suggest that Mn^{2+} evokes Ca^{2+} entry and prostaglandin E_2 release in human gingival fibroblasts, which are regulated by protein kinase C and tyrosine kinase activities.

Introduction

Manganese, an essential trace element, is required for catalytic activity or activation of various enzymes such as pyruvate kinase, pyruvate carboxylase, superoxide dismutase and arginase, and is involved in amino acid, lipid, protein, and carbohydrate metabolism (1). Numerous manganese-deficiency problems in animals and humans have been reported (1). On the other hand, manganese toxicity has also been recognized in animals and humans. In humans, manganese toxicity reportedly induces neuronal damage, reproductive and immune system dysfunction, nephritis, pancreatitis, and hepatic damage (1, 2).

In the field of experimental study, manganese ion (Mn^{2+}) is widely using as a tool for the study of calcium ion (Ca^{2+}) entry into cells because fluorescence Ca^{2+} indicators, such as fura-2, have high affinity for this ion and their fluorescence is quenched by Mn^{2+} (3). In the presence of Mn^{2+} in an extracellular solution, quenching of the intracellular dye fluorescence is induced by an agonist stimulation, attributed to stimulated Mn^{2+} influx, in various kinds of cells such as platelets (3), neutrophils (4), salivary parotid acinar cells (5), and vascular smooth muscle cells (6).

We have previously demonstrated that chemical mediators, such as bradykinin and histamine, and platelet-derived growth factor, a biological mediator for connective tissue cells, provoke an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in human gingival fibroblasts (7-11), which is essential to prostaglandin E_2 release, arachidonic acid release, and potentiation of prostaglandin E_2 synthesis by the cytokine interleukin- 1β (7, 8, 11-13). During the study of Ca^{2+} mobilization, we found that micromolar concentrations of Mn^{2+} induced Ca^{2+} mobilization in fibroblasts. Here we demonstrate Mn^{2+} -induced Ca^{2+} mobilization in human gingival fibroblasts.

Materials and Methods

Materials

Fura-2/AM was purchased from Dojindo Laboratory (Kumamoto, Japan). α -Minimum essential medium (α -MEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from GIBCO BRL Life Technologies (Tokyo, Japan). 12-Tetradecanoyl phorbol-13-acetate (PMA) was purchased from Sigma (St. Louis, MO, USA). Herbinycin A was purchased from Wako Pure Chemical Industries (Osaka, Japan). Prostaglandin E_2 EIA Kit was obtained from Cayman (Ann Arbor, MI, USA).

Cell cultures were grown in α -MEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin. Human gingival fibroblasts were obtained from explants of healthy human gingival connective tissue according to the method previously described (14). When the cells surrounding the tissue explants were confluent, they were subcultured with 0.05% trypsin and 0.02% EDTA in Hanks balanced salt solution and transferred to a tissue culture flask (15).

Determination of the intracellular Ca^{2+} concentration

Confluent human gingival fibroblasts were loaded with 2 μ M fura-2/AM in α -MEM for 30 min at 37 °C and detached from the flask by a trypsin-EDTA solution. Cells were washed by centrifugation at 50 g for 1 min and suspended in α -MEM. Just before $[\text{Ca}^{2+}]_i$ determination, the cell suspension was diluted with 10 volumes of Krebs-Ringer-HEPES solution [120 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 0.96 M NaH_2PO_4 , 0.2% glucose, 0.1% BSA, 10 mM HEPES, pH 7.4, and 1 mM CaCl_2 (Ca^{2+} -containing solution)] and centrifuged at 50 g for 1 min. The cells were resuspended in Krebs-Ringer-HEPES solution for the determination of $[\text{Ca}^{2+}]_i$ (11). In the determination of $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} , Krebs-Ringer-HEPES solution without CaCl_2 (Ca^{2+} -free solution) was used instead of Ca^{2+} -containing solution. When the effect of herbimycin A was examined, fibroblasts were preincubated with 1 μ M herbimycin A for 16 h before loading with fura-2/AM. When the effect of PMA was examined, fura-2-loaded fibroblasts suspended in Krebs-Ringer-HEPES solution were incubated with 100 nM PMA for 10 min before addition of MnCl_2 . The fluorescence of fura-2-loaded human gingival fibroblasts was monitored with a CAF-110 spectrophotometer (Nihon Bunko) with excitation at 340 nm and 380 nm and emission at 500 nm. The cell suspension was continuously stirred using a magnetic stirring rod at 37 °C. The intracellular calcium ion concentration was calculated from the measurement of the ratio of fluorescence intensities (16).

Measurement of prostaglandin E_2

Human gingival fibroblasts were cultured in 24-well culture plates in α -MEM containing 10% fetal calf serum. When the cells were confluent, they were deprived of serum for 18 h. The MnCl_2 was added at various time intervals, then medium was collected and the amount of prostaglandin E_2 was determined by enzyme immunoassay using a commercially available kit (11, 12).

Statistical analysis

All experiments were performed 3-5 times with different cell batches and the results were presented as means $\pm$ SE. Statistical differences were determined using Student's *t* test. *P* values below 0.05 were regarded as statistically significant and are indicated by asterisks (*).

Results

In fura-2-loaded human gingival fibroblasts, the effect of MnCl_2 on $[\text{Ca}^{2+}]_i$ was examined. As Fig. 1 shows, addition of 5 μ M MnCl_2 to fura-2-loaded fibroblasts caused a transient increase in the wavelength ratio (340/380), which was due to a transient increase in fluorescence (500 nm emission) with excitation at 340 nm and a synchronous decrease at 380 nm. Such changes in fluorescence were similar to those provoked by bradykinin and histamine (7-9), indicating that Mn^{2+} induces an increase in $[\text{Ca}^{2+}]_i$. Concentrations of MnCl_2 ranging from 1-50 μ M evoked an increase in $[\text{Ca}^{2+}]_i$ in a dose-dependent manner (Fig. 2). When the cells were suspended in Ca^{2+} -free solution, MnCl_2 failed to induce an increase in $[\text{Ca}^{2+}]_i$ (Fig. 2).

We have previously demonstrated that the increase in $[\text{Ca}^{2+}]_i$ induced by chemical mediators, such as bradykinin and histamine, provokes prostaglandin E_2 release in human gingival fibroblasts (7, 8, 12). Therefore, we examined the effect of MnCl_2 on prostaglandin E_2 release. As Fig. 3 shows, 5 μ M MnCl_2 stimulated prostaglandin E_2 release in a time-dependent manner. The concentrations of MnCl_2 that induced an increase in $[\text{Ca}^{2+}]_i$ provoked prostaglandin E_2 release (Table 1). The prostaglandin E_2

Fig.1. Manganese-evoked calcium ion mobilization in human gingival fibroblasts. Manganese chloride (5 μ M) was added to fura-2-loaded human gingival fibroblasts (indicated by an arrow) after preincubation. Fluorescence (500 nm emission) with excitation at 340 nm and 380 nm and the subsequent ratio (340/380) were recorded. This result is representative of five independent experiments.

Table 1. Dose-dependent effect of manganese on prostaglandin E₂ release in human gingival fibroblasts.

MnCl ₂ concentration (μ M)	Prostaglandin E ₂ (pg/10 ⁵ cells)
0	351.7 $\pm$ 85
1	679.4 $\pm$ 105*
5	1178.7 $\pm$ 159.2*
10	1627.9 $\pm$ 124.8*
50	2731.3 $\pm$ 464.1*

Human gingival fibroblasts were incubated for 10 min without or with varying concentrations of manganese chloride. Prostaglandin E₂ levels released to the medium were determined. *Values are compared to the control (in the absence of manganese chloride).

Fig. 2. Dose-dependent effect of manganese on the increase in $[Ca^{2+}]_i$. Various concentrations of manganese chloride were added to fura-2-loaded human gingival fibroblasts in the presence (closed circle) or absence of extracellular Ca^{2+} (closed square). Peak levels of increase in $[Ca^{2+}]_i$ induced by manganese chloride were plotted. *Values are compared to the control (in the absence of manganese chloride).

Fig. 3. Manganese-induced prostaglandin E₂ release in human gingival fibroblasts. Human gingival fibroblasts were incubated without (squares) or with 5 μ M manganese chloride (circles) in the presence (closed shapes) or absence of extracellular Ca^{2+} (open shapes). Prostaglandin E₂ levels in the medium were determined at the indicated time. *Values are compared to the control (at 0 min).

release induced by 5 μ M $MnCl_2$ was abolished in the absence of extracellular Ca^{2+} (Fig. 3).

Next we examined the effects of protein kinases on the Mn^{2+} -induced increase in $[Ca^{2+}]_i$. As Fig. 4 shows, in the fibroblasts pretreated with 100 nM PMA, a protein kinase C activator, the Mn^{2+} -induced

Fig. 4. Inhibitory effects of a protein kinase C activator and a tyrosine kinase inhibitor on the manganese-induced increase in $[Ca^{2+}]_i$. Manganese chloride ($5 \mu M$) was added to the fura-2-loaded human gingival fibroblasts pretreated without or with $100 \mu M$ PMA, $1 \mu M$ herbimycin A, or $100 \mu M$ PMA/ $1 \mu M$ herbimycin A. The increase in $[Ca^{2+}]_i$ induced by manganese chloride in the absence of the activator or the inhibitor was indicated as 100% (control). *Values are compared to the control.

increase in $[Ca^{2+}]_i$ was reduced to 47% of the control value. In the fibroblasts pretreated with $1 \mu M$ herbimycin A, a tyrosine kinase inhibitor, the increase in $[Ca^{2+}]_i$ induced by Mn^{2+} was also reduced to 32% of the control value. In the fibroblasts pretreated with both $100 nM$ PMA and $1 \mu M$ herbimycin A, the Mn^{2+} -induced increase in $[Ca^{2+}]_i$ was reduced to 5% of the control value, suggesting that protein kinase C and tyrosine kinase are involved in the effect of Mn^{2+} on the increase in $[Ca^{2+}]_i$ in human gingival fibroblasts.

Discussion

We demonstrated here that Mn^{2+} stimulated Ca^{2+} mobilization in human gingival fibroblasts. Ca^{2+} mobilization induced by metal ions has been shown in osteoclasts (17, 18), although the effect of Mn^{2+} is unknown. In hepatocytes, metal ions other than Mn^{2+} have been reported to stimulate Ca^{2+} mobilization (19). On the other hand, metal ions containing Mn^{2+} have been demonstrated to provoke Ca^{2+} mobilization in skin fibroblasts, coronary endothelial cells,

umbilical artery muscle cells, and neuroblastoma cells (20, 21). In such cells, metal ions were thought to evoke Ca^{2+} release from intracellular Ca^{2+} stores because metal ions stimulated the formation of inositol polyphosphates. Heavy metal ions such as cadmium ion (Cd^{2+}) and nickel ion (Ni^{2+}) evoked Ca^{2+} mobilization even in the absence of extracellular Ca^{2+} in human gingival fibroblasts, suggesting that Cd^{2+} and Ni^{2+} stimulate Ca^{2+} release from the intracellular Ca^{2+} stores (22). On the other hand, Mn^{2+} failed to induce Ca^{2+} mobilization in the absence of extracellular Ca^{2+} in human gingival fibroblasts. Therefore, it is likely that Mn^{2+} -induced Ca^{2+} mobilization is due to Ca^{2+} influx from an extracellular site into the cells rather than Ca^{2+} release from the intracellular stores.

Mn^{2+} -induced Ca^{2+} mobilization was inhibited by the protein kinase C activator PMA and the tyrosine kinase inhibitor herbimycin A. This observation suggests that protein phosphorylation regulated by protein kinase C inhibition and tyrosine kinase activation is involved in Mn^{2+} -induced Ca^{2+} mobilization in human gingival fibroblasts. We have previously reported that protein kinase C regulates histamine receptor activation coupled to Ca^{2+} mobilization in human gingival fibroblasts (9). As cation receptors for Cd^{2+} have already been advocated (20), Mn^{2+} receptors appear to exist in human gingival fibroblasts. On the other hand, various kinds of enzymes activated by Mn^{2+} have been demonstrated (1, 23). Therefore, it is probable that Mn^{2+} -induced Ca^{2+} mobilization is caused by the activation of metabolism by Mn^{2+} . We have also demonstrated that tyrosine kinase inhibition activates receptor-activated Ca^{2+} entry in human gingival fibroblasts (10). Because the effect of tyrosine kinase inhibitor on Mn^{2+} -induced Ca^{2+} entry was opposite to that on the receptor-activated Ca^{2+} entry, it is unlikely that Mn^{2+} activates receptor-activated Ca^{2+} channels. Further studies on the mechanism of Mn^{2+} -induced Ca^{2+} mobilization via protein phosphorylation are necessary.

Prostaglandin E_2 is thought to be involved in the pathogenesis of periodontal disease because elevated

levels of prostaglandin E₂ were detected in the inflamed gingival tissues and crevicular fluids and were associated with an increased severity and progression of periodontal lesions and bone loss (24-28). In this paper, Mn²⁺ induced prostaglandin E₂ release via Ca²⁺ mobilization, which had an effect is similar to that of inflammatory chemical mediators in human gingival fibroblasts (7, 8, 12). Therefore, Mn²⁺ appears to be involved in periodontal disease.

In conclusion, Mn²⁺ stimulated Ca²⁺ mobilization and prostaglandin E₂ release in human gingival fibroblasts. The Mn²⁺-induced Ca²⁺ mobilization was inhibited by protein kinase C activation and tyrosine kinase inhibition.

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