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Short communication

Elevation of cytosolic calcium level triggers calcium antagonist-induced gingival overgrowth

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Abstract

Calcium (Ca^{2+}) antagonists induce gingival overgrowth as a side effect but the pathogenic mechanism is still unknown. The Ca^{2+}-channel activator Bay K 8644 elevates intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and enhances the cell proliferation of gingival fibroblasts in a dose-dependent manner. Verapamil, an L-type Ca^{2+}-channel blocker, also elevates $[Ca^{2+}]i$ in gingival fibroblasts, but it has no effect on other fibroblasts such as those of the lung, skin, and muscle. Moreover, verapamil enhances the proliferation of fibroblasts of the gingiva but has no effect on the proliferation of those of other tissues. These findings confirm that $[Ca^{2+}]i$ elevation induces the proliferation of gingival fibroblasts.

Keywords: Calcium antagonist; Gingival overgrowth; Gingival fibroblast; Calcium ion

1. Introduction

It is recognized that calcium (Ca^{2+}) antagonists cause gingival overgrowth as a side effect of their primary action (Varnfield and Botha, 2000), but the pathogenic mechanism remains unknown. Kataoka et al. (2001) reported the hypothesis that decreased collagen degradation due to reduced phagocytosis is closely associated with increased type-I collagen accumulation in nifedipine-treated rat gingiva. Munaron (2002) claimed that elevation of cytosolic Ca^{2+} has a key role in the mechanism underlying Ca^{2+}-dependent cell proliferation. We recently found that the dihydropyridine derivative isradipine enhances the proliferation of gingival fibroblasts (Hattori et al., 2004) and raises intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) (Hattori and Wang, 2005) by increasing Ca^{2+} influx through the plasma membrane and releasing Ca^{2+} from intracellular Ca^{2+} storage sites (Hattori and Wang, 2006). We proposed that elevation of $[Ca^{2+}]i$ is closely involved in this process, from the results that phospholipase C induced Ca^{2+} release from the Ca^{2+} storage sites through the production of inositol 1,4,5-triphosphate (Hattori et al., 2005). The aim of the study we present here was to confirm that elevation of $[Ca^{2+}]i$ triggers Ca^{2+} antagonist-induced gingival overgrowth.

2. Materials and methods

2.1. $[Ca^{2+}]i$ measurement

Because $[Ca^{2+}]i$ is closely related to cell proliferation, $[Ca^{2+}]i$ was measured using the Ca^{2+}-sensitive fluorescent dye fura-2/AM (Dojindo Laboratories, Kumamoto, Japan). The cells were stored in a solution of 135-mM NaCl, 5-mM KCl, 1-mM $CaCl_2$, 1-mM $MgCl_2$, 10-mM glucose and 20-mM HEPES-NaOH (pH 7.4), and loaded with the dye (5 μM) by incubation for 45 min at 37 °C. The fura-2/AM was then washed out 5 times and the cells were incubated in medium without fura-2/AM for 15 min at 37° as a post-incubation. The excitation light was provided by a xenon lamp through a 340- or 360-nm filter. The wavelength of emission for analysis was 510 nm. Changes in the intensity of fluorescence of fura-2 in the cells were recorded with a video-imaging analysis system (FC-400, Furusawa Lab Appliance, Kawagoe, Japan). Fura-2 fluorescence in terms of $[Ca^{2+}]i$ was calibrated from the ratio of 340/360-nm excitation fluorescence values. The standard calibration curve was obtained using a Calcium Calibration Buffer Kit I (Molecular Probes, Eugene, OR, USA).

A bath temperature controller (DTC-100A; DIA Medical Systems, Kunitachi, Japan) was used to keep the cells at 32 °C during fluorescence measurements to minimize leakage of fura-

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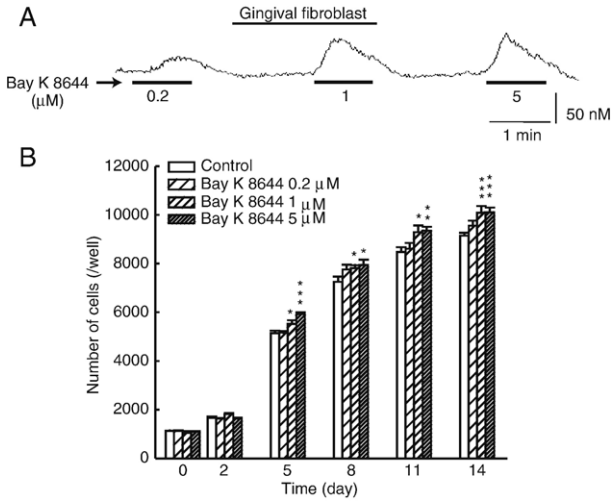

Fig. 1. Elevation of $[Ca^{2+}]i$ and enhancement of proliferation by Bay K 8644 in gingival fibroblasts. A: Incubation of Bay K 8644 with gingival fibroblasts leads to a dose-dependent increase in $[Ca^{2+}]i$. B: Incubation with Bay K 8644 leads to a dose-dependent increase in gingival fibroblast cell number. $*P<0.05$,$**P<0.01$, $***P<0.005$, $n=8$ for each time column.

2 (Takemura et al., 1991). The cells were soaked in a flexiperm chamber containing 0.5 ml of saline and perfused at 6.0 ml/min with a tubing pump system (Masterflex 7524-10; Cole-Parmer Instrument Company, Barrington, IL, USA). The drugs were added to the perfusate at appropriate concentrations. To reduce statistical deviation of the data, the results were obtained only from cells containing $[Ca^{2+}]i$ in the range of 50–200 nM under normal conditions.

Normal human gingival fibroblast Gin-1 cells obtained from Dainippon Pharmaceutic (Osaka, Japan) were used. Other fibroblasts such as those from human lung (TIG-1-20), human muscle (TIG-2M-30) and human skin (TIG-103) were purchased from Health Science Research Resources Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (Medium 41; Dainippon Pharmaceutic) supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The medium also contained antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Sigma, St Louis, MO, USA) and was changed at intervals of two or three days. The medium containing Bay K 8644 or verapamil was used in the test groups.

2.2. Fibroblast proliferation

Cell proliferation was examined using water-soluble tetrazolium-1 (WST-1), which is supplied in a commercially available assay kit (Dojindo Laboratories). The cells were cultured in 96-well microculture plates. Briefly, at selected times during the culture period, WST-1 (10 μl) was added to each well and the plate was then incubated at 37 °C for 2 h. The optical density was read using a microplate reader (Model 550; BioRad Laboratories, Hercules, CA, USA) at 450 nm as a test wavelength and 655 nm as a reference according to the manufacturer's instructions. Assays of both the control and the test groups were performed in quadruplicate. Cell numbers were calculated by reference to WST-1 values obtained beforehand for a known number of viable Gin-1 cells.

2.3. Chemicals

Verapamil hydrochloride was obtained from Nakalai Tesque (Kyoto, Japan). Bay K 8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2′-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester) was purchased from Calbiochem (CA, USA).

2.4. Statistical analysis

The data are presented as the mean ± standard error of the mean (S.E.M.) and the number of wells (n). Statistical analysis of the data was performed using Student's simple t-test for cell counts. Differences between mean values were considered significant if the probability of error (P) was less than 0.05.

3. Results

The effects of $[Ca^{2+}]i$ elevation on gingival fibroblasts were examined using the Ca^{2+} channel activator Bay K 8644, which raises $[Ca^{2+}]i$ in a dose-dependent manner (Fig. 1A). Our results

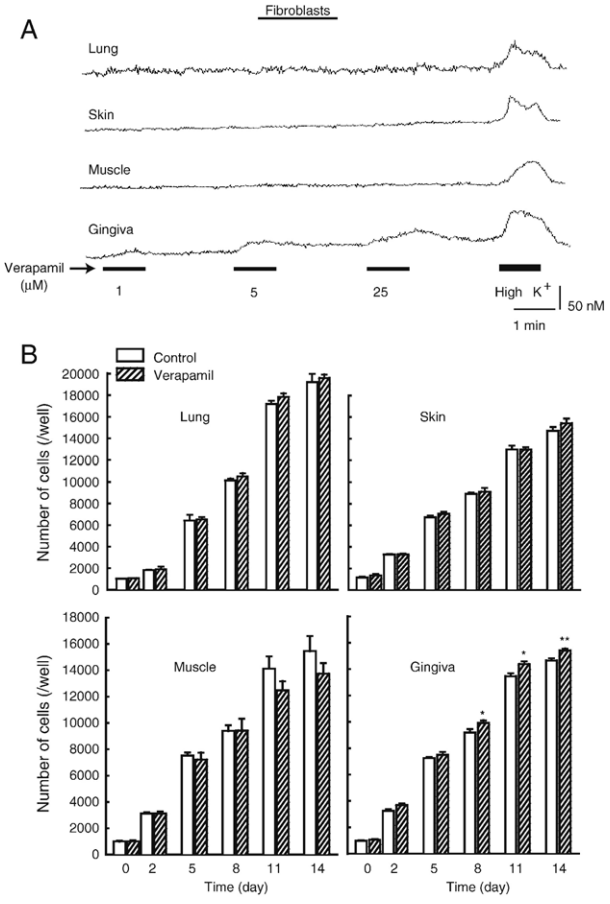

Fig. 2. The effect of verapamil on $[Ca^{2+}]i$ and proliferation of different fibroblast types. A: Our results show that verapamil has no effect on the $[Ca^{2+}]i$ of lung, skin or muscle fibroblasts but it causes a dose-dependent increase of $[Ca^{2+}]i$ in gingival fibroblasts. B: Verapamil (10 μM) was added to the fibroblast samples daily and the cell counts recorded over a 14-day period. The results show that verapamil had no effect on the proliferation of fibroblasts other than those of gingiva, for which it caused a significant increase compared with control after eight days of treatment. $*P<0.05$, $**P<0.01$, $n=8$ for each time column.

show that, upon addition of Bay K 8644, proliferation of gingival fibroblasts is also increased in a dose-dependent manner (Fig. 1B).

The effects of Bay K 8644 on other fibroblast types (lung, muscle, and skin) were also examined for comparison. Interestingly, concentrations of 0.2–5-μM Bay K 8644 caused dose-dependent elevation of $[Ca^{2+}]i$ but had no effect on cell proliferation (data not shown).

To investigate the effect of Ca^{2+} antagonist-induced $[Ca^{2+}]i$ elevation, the response of fibroblasts from lung, skin, and muscle were compared with that of gingival fibroblasts to the Ca^{2+} antagonist verapamil. Verapamil dose-dependently elevated the $[Ca^{2+}]i$ in gingival fibroblasts but had no effect on the other fibroblasts (Fig. 2A). The viability of all of the fibroblasts was confirmed by the observation that high K^+ saline raised the $[Ca^{2+}]i$ of all fibroblast types.

The effects of verapamil on the proliferation were then tested. Cell counts of lung, skin, and muscle fibroblasts incubated with verapamil were not altered compared with those of the controls over the 14-day period of the experiment (Fig. 2B). However, upon addition of the drug (10 μM) on the 8th day, the level of proliferation of gingival fibroblasts was significantly higher than that of the control (Fig. 2B). The proliferation of the gingival fibroblasts remained at this elevated level thereafter (Fig. 2B).

4. Discussion

The aim of this study was to confirm that Ca^{2+} ions, which are required for cell proliferation (Rodrigues et al., 2007), are also closely related to gingival overgrowth. We have shown that, in gingival fibroblasts, the Ca^{2+}-channel blocker verapamil and the Ca^{2+}-channel activator Bay K 8644 both increase $[Ca^{2+}]i$ in a dose-dependent manner (Figs. 1A and 2A). In addition, both enhance gingival fibroblast proliferation (Figs. 1B and 2B). Importantly, however, verapamil has no effect on the $[Ca^{2+}]i$ or proliferation of lung, skin, or muscle fibroblasts (Fig. 2B). Thus, our results indicate that $[Ca^{2+}]i$ elevation is crucial for the proliferation of gingival fibroblasts. Kataoka et al. (2001) claimed the pathogenic mechanism of Ca^{2+} antagonist-induced gingival overgrowth, but they did not describe why it does not occur in tissues other than gingiva. Munaron (2002) and Munaron et al. (2004), although they did not investigate the detailed mechanism of Ca^{2+} antagonist-induced gingival overgrowth, reported that intracellular Ca^{2+} signals control cell proliferation in fibroblasts, which supports the results of our study.

The effects of Ca^{2+} antagonists on the $[Ca^{2+}]i$ of fibroblasts other than those of the gingiva have not been previously investigated. Our results show that, although the $[Ca^{2+}]i$ and proliferation of lung, skin, and muscle fibroblasts responded to high K^+ saline, they were not affected by verapamil. This result agrees with the fact that Ca^{2+} antagonists do not cause overgrowth in tissues other than the gingiva.

In light of our results, we propose that a novel type of receptor exists that is responsible for $[Ca^{2+}]i$ elevation and overgrowth in gingival fibroblasts, and we speculate that these receptors are stimulated by Ca^{2+} antagonists and transmit signals to both non-selective cation channels (McGehee et al., 1997) and Ca^{2+} stores (Remy et al., 2007) to elicit $[Ca^{2+}]i$ elevation and, therefore, increased cell proliferation. At present, we are working to identify these proposed receptors in gingival fibroblasts.

In conclusion, we confirm that elevation of $[Ca^{2+}]i$ triggers Ca^{2+} antagonist-induced gingival overgrowth. Thus, we advocate the "Ca^{2+} trigger theory" as the explanation for the pathogenic mechanism by which Ca^{2+} antagonists cause gingival overgrowth. That is, Ca^{2+} antagonists elevate the $[Ca^{2+}]i$ (Hattori and Wang, 2005), which cause the exocytosis of basic fibroblast growth factors (bFGF). This factor then, combines with bFGF receptors to activate tyrosine kinase, which causes the cell proliferation by activating Ras protein, MAP kinase, and *c-fos* genes (Hattori et al., 2005).

Increased $[Ca^{2+}]i$ and gingival overgrowth is not only induced by Ca^{2+} antagonists (Hattori and Wang, 2006) but also by the antiepileptic phenytoin (Modéer et al., 1991) and the immunosuppressant cyclosporin A (Bullon et al., 2007; Varnfield and Botha, 2000). Gingival overgrowth induced by these drugs may be explained by the Ca^{2+} trigger theory.

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