Abstract: The effect of cyclopiazonic acid (CPA) on changes in the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_i]) evoked by bradykinin (BK), histamine (HIST) and thapsigargin (TG) was investigated in human gingival fibroblasts. CPA itself dose-dependently stimulated [Ca\(^{2+}\)_i] responses in both the absence and presence of extracellular Ca\(^{2+}\). Pretreatment with CPA (< 5 µM) enhanced the [Ca\(^{2+}\)_i] responses evoked by 5 nM BK and 1 mM HIST. However, CPA-pretreatment depressed the [Ca\(^{2+}\)_i] response evoked by 1 µM TG in a dose-dependent manner. Moreover, CPA accelerated the Ca\(^{2+}\) influx caused by 5 nM BK and 1mM HIST, but did not alter that caused by 1 µM TG. These results indicate that CPA discharges intracellular Ca\(^{2+}\) stores, resulting in their depletion, and enhances Ca\(^{2+}\) influx across the plasma membrane. (J. Oral Sci. 45, 139-144, 2003)

Key words: cyclopiazonic acid; bradykinin; histamine; thapsigargin; intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_i]); human gingival fibroblast.

Introduction

Cyclopiazonic acid (CPA), a mycotoxin derived from the *Aspergillus* and *Penicillium* genera, has been reported to inhibit the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum (SR) of striated muscle (1-3), smooth muscle (4-8) and cardiac muscle (9), the endoplasmic reticulum of lymphocytes (10), and aortic endothelial cells (11). CPA has also been reported to stimulate Ca\(^{2+}\) influx through non-specific cation channels in endothelial cells (12,13), in an inositol 1,4,5-trisphosphate (IP\(_3\)) -independent manner in cultured bovine pulmonary arterial endothelial cells (14) and in urinary bladder smooth muscle (15). However, the effect of CPA on the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_i]) in gingival fibroblasts has not been elucidated.

In the course of a study to clarify the effects of calcium channel blockers such as nifedipine on gingival overgrowth, we demonstrated that fibroblasts originating from nifedipine responders (nifedipine responder, NIFr) tended to exhibit a higher cell proliferation rate and increased synthesis of DNA and collagen than those from nifedipine non-responders (nifedipine non-responder, NIFn) in the presence of 1 µM nifedipine (16). Furthermore, gingival fibroblasts originating from NIFrs showed a greater cytosolic calcium response to bradykinin (BK), thrombin, prostaglandins E\(_2\) and F\(_{2\alpha}\) and platelet-derived growth factor, but not to histamine (HIST) and bombecin, than ones originating from NIFn (17). We also demonstrated that tenidap caused discharge of intracellular Ca\(^{2+}\) stores and inhibited Ca\(^{2+}\) influx in cultured human gingival fibroblasts (18). Tenidap also inhibited DNA and collagen synthesis, depressed cell proliferation and lowered the intracellular pH in these cells (19). From these recent data, we concluded that tenidap might be useful in preventing gingival overgrowth caused by calcium channel blockers, especially nifedipine.

Since CPA has quite similar properties to tenidap, it was thought worthwhile to investigate its effects on human gingival fibroblasts.

The objectives of the present study were therefore to...
determine how CPA affects the \([\text{Ca}^{2+}]_i\) responses to BK, HIST and thapsigargin (TG) in cultured human gingival fibroblasts.

**Materials and Methods**

**Chemicals and Reagents**

CPA, BK, ionomycin, ethylene glycol-bis (-amino-ethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and TG were purchased from Sigma Chemical Co., MO, USA. Fura-2/acetoxymethyl ester (Fura-2/AM) and N,N,N′,N′-tetrakis-(2-pyridilmethyl)-ethylenediamine (TPEN) were purchased from Dojin Laboratories, Japan. All the chemicals and reagents used in tissue culturing were purchased from Invitrogen, Carlsbad, CA, USA.

**Cells**

The cell culture was prepared according to a previously reported method (16,20). Briefly, cultures of fibroblast-like cells were established from a gingival specimen obtained from a NIFr patient (a 47 year-old woman who had been treated with nifedipine 40 mg/day for 2 years 8 months) during the clearance of her remaining teeth. The use of the gingival samples was approved by the Committee on Studies Involving Human Beings of Nihon University School of Dentistry at Matsudo (No. EC99-001). Fibroblasts were obtained by trypsinization of the primary outgrowth of cells using a previously described procedure (16,18,21). After establishment, the cells were maintained in DMEM-10 medium (Dulbecco’s modified Eagle’s medium [DMEM] supplemented with 10% fetal calf serum [FCS], streptomycin 100 µg/ml, penicillin G 200 U/ml and amphotericin B 0.2 µg/ml), and routinely passaged using 0.25% trypsin and 0.02% ethylenediamine-N, N′, N″, N‴-tetraacetic acid (EDTA) in Dulbecco’s phosphate-buffered saline (DPBS). The homogeneity of the fibroblasts was determined by flow cytometry (FACS Vantage, Nippon Becton Dickinson, Japan). The fibroblasts used in the experiments exhibited logarithmic-phase proliferation between the 5th and the 8th passage.

**Determination of \([\text{Ca}^{2+}]_i\)**

The determination of \([\text{Ca}^{2+}]_i\) was performed in essentially the same manner as reported previously (17,18,20). The fibroblasts were harvested by treatment with 0.25% trypsin and 0.02% EDTA in DPBS, and routinely passedaged using 0.25% trypsin and 0.02% ethylenediamine-N, N′, N″, N‴-tetraacetic acid (EDTA) in Dulbecco’s phosphate-buffered saline (DPBS). The homogeneity of the fibroblasts was determined by flow cytometry (FACS Vantage, Nippon Becton Dickinson, Japan). The fibroblasts used in the experiments exhibited logarithmic-phase proliferation between the 5th and the 8th passage.

**Results**

**Homogeneity of the fibroblasts**

The cell strain used in the present experiment was subjected to flow cytometry to ensure homogeneity in appearance, size (scatter forward, SCF), surface characteristics (FCS) and cytoplasmic features (scatter side, SSC).

**Increase in the intracellular \([\text{Ca}^{2+}]_i\) response to BK, HIST and TG after CPA-pretreatment**

The dose-response curve for CPA is shown in Figure 1. In both the presence and absence of 1.5 mM extracellular \(\text{Ca}^{2+}\), CPA evoked a \([\text{Ca}^{2+}]_i\) response in a dose-dependent manner. The \([\text{Ca}^{2+}]_i\) response was greater in the presence of extracellular \(\text{Ca}^{2+}\) than in its absence, indicating that CPA causes \(\text{Ca}^{2+}\) influx through the plasma membrane.

The effects of pretreatment with CPA for 2 min on the \([\text{Ca}^{2+}]_i\) responses evoked by BK (5 nM), HIST (1 mM) and TG (1 µM) are shown in Figures 2a, 2b and 2c, respectively. In the presence of 1.5 mM of extracellular \(\text{Ca}^{2+}\), the \([\text{Ca}^{2+}]_i\) response evoked by 5 nM BK was increased by pretreatment with CPA (< 5 µM) in a dose-dependent manner (Fig. 2a). In the absence of extracellular \(\text{Ca}^{2+}\), CPA pretreatment did not alter the BK-induced \([\text{Ca}^{2+}]_i\) response. The same trend was seen with the \([\text{Ca}^{2+}]_i\) response evoked by HIST (1 mM) (Fig. 2b). However, CPA-pretreatment dose-dependently depressed the \([\text{Ca}^{2+}]_i\) response evoked by TG (1 µM) (Fig. 2c).

**Effect of CPA on \(\text{Ca}^{2+}\) influx after BK-, HIST- or TG-pretreatment**

The effect of CPA on \(\text{Ca}^{2+}\) influx across the plasma membrane after depletion of intracellular \(\text{Ca}^{2+}\) stores by...
5 nM BK and 1 mM HIST in the absence of extracellular Ca\(^{2+}\) is summarized in Figures 3a and 3b, respectively. Ca\(^{2+}\) influx at 0 and 5 min after the addition of CPA was increased in line with the CPA dose by both BK (Fig. 3a) and HIST (Fig. 3b) pretreatment. However, Ca\(^{2+}\) influx was CPA dose-dependently depressed by TG pretreatment (Fig. 3c). The peak [Ca\(^{2+}\)] response is summarized in Table 1.

**Discussion**

As previously reported by others (1-7,10), CPA depleted cytoplasmic Ca\(^{2+}\) stores in gingival fibroblasts. Thus, the [Ca\(^{2+}\)] was increased CPA dose-dependently in the absence of extracellular Ca\(^{2+}\) (Fig. 1). The difference in [Ca\(^{2+}\)] values between experiments carried out in the presence and absence of extracellular Ca\(^{2+}\) indicates that CPA induced the influx of Ca\(^{2+}\) through the plasma membrane. In the presence of 5 nM BK or 1 mM HIST plus extracellular Ca\(^{2+}\), the highest [Ca\(^{2+}\)] response and Ca\(^{2+}\) influx was observed at 5 \(\mu\)M CPA (Figs. 2a and 2b). However, in the presence of 1 \(\mu\)M TG plus extracellular Ca\(^{2+}\), the [Ca\(^{2+}\)]
Fig. 3 Effects of CPA on Ca\textsuperscript{2+} influx after pretreatment with various stimulants (BK, HIST or TG) for 2 min. Pretreatment with either 5 nM BK, 1 mM HIST or 1 \(\mu\)M TG was carried out for 2 min in the absence of extracellular Ca\textsuperscript{2+} in order to deplete intracellular stores. This was followed by the addition of Ca\textsuperscript{2+} (final concentration of 1.5 mM) to the extracellular solution at 0 and 5 min after the addition of CPA (1, 5 or 50 \(\mu\)M). (a) [Ca\textsuperscript{2+}]i response evoked by BK (5 nM). (b) [Ca\textsuperscript{2+}]i response evoked by HIST (1 mM). (c) [Ca\textsuperscript{2+}]i response evoked by TG (1 \(\mu\)M).
response and Ca\textsuperscript{2+} influx decreased in line with the CPA dose (Fig. 2c). This indicates that the effect of CPA is the same as that of TG (i.e., inhibition of Ca\textsuperscript{2+}-ATPase), but not completely the same as those of BK and HIST (which produce IP\textsubscript{3}, thereby resulting in the depletion of Ca\textsuperscript{2+} stores). The Ca\textsuperscript{2+} influx at 0 and 5 min after the addition of CPA was dose-dependently accelerated by CPA when 5 nM BK or 1 mM HIST was used for pretreatment (Figs. 3a and 3b). However, pretreatment with 1 µM TG did not produce a CPA dose-dependent alteration in Ca\textsuperscript{2+} influx (Fig. 3c). This also indicates that CPA possess the same function as TG.

In our previous study, we demonstrated that tenidap depleted intracellular Ca\textsuperscript{2+} stores and decreased Ca\textsuperscript{2+} influx in gingival fibroblasts (18,19). Since CPA has the same effect as tenidap in depleting intracellular Ca\textsuperscript{2+} stores, we were interested to clarify whether it shared the same ability to inhibit Ca\textsuperscript{2+} influx through the plasma membrane. We found that CPA depletes intracellular Ca\textsuperscript{2+} stores by inhibiting Ca\textsuperscript{2+}-ATPase, and that it enhances Ca\textsuperscript{2+} influx; these properties are exactly the same as those of TG. However, CPA did not inhibit Ca\textsuperscript{2+} influx through the plasma membrane. Therefore, unlike tenidap, CPA might not be suitable for depressing gingival overgrowth (19).

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### Table 1 Effects of CPA on Ca\textsuperscript{2+} influx: Cells were subjected to various pretreatments before the addition of 1.5 mM Ca\textsuperscript{2+} to the extracellular solution. All values represent the [Ca\textsuperscript{2+}]\textsubscript{i} (nM) after the addition of Ca\textsuperscript{2+}.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>0 min</th>
<th>5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>49.0</td>
<td>35.8</td>
</tr>
<tr>
<td>1 µM CPA</td>
<td>72.5</td>
<td>57.5</td>
</tr>
<tr>
<td>5 µM CPA</td>
<td>131.5</td>
<td>87.8</td>
</tr>
<tr>
<td>50 µM CPA</td>
<td>216.0</td>
<td>212.8</td>
</tr>
<tr>
<td>5 nM BK + 1 µM CPA</td>
<td>133.0</td>
<td>99.0</td>
</tr>
<tr>
<td>+ 5 µM CPA</td>
<td>163.0</td>
<td>154.0</td>
</tr>
<tr>
<td>+ 50 µM CPA</td>
<td>358.0</td>
<td>231.0</td>
</tr>
<tr>
<td>1 mM HIST + 1 µM CPA</td>
<td>129.0</td>
<td>108.0</td>
</tr>
<tr>
<td>+ 5 µM CPA</td>
<td>144.0</td>
<td>141.0</td>
</tr>
<tr>
<td>+ 50 µM CPA</td>
<td>340.0</td>
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<tr>
<td>1 µM TG + 1 µM CPA</td>
<td>246.5</td>
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<tr>
<td>+ 5 µM CPA</td>
<td>258.0</td>
<td>356.0</td>
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<tr>
<td>+ 50 µM CPA</td>
<td>193.5</td>
<td>203.5</td>
</tr>
</tbody>
</table>

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