Eur J Med Res (2006) 11: 93-96

Calcium Antagonist Isradipine-induced Calcium Influx through Nonselective Cation Channels in Human Gingival Fibroblasts

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Abstract
Isradipine raises the cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) in human gingival fibroblasts by enhancing Ca^{2+} influx through the plasma membrane. To research the pathways through which Ca^{2+} enters the cells, we examined the interactive effects of isradipine and blockers or enhancers of nonselective cation channels (NSCCs) and Na^+/Ca^{2+} exchangers (NCXs). Normal human gingival fibroblast Gin-1 cells were used. The $[Ca^{2+}]i$ was measured with the Ca^{2+}-sensitive fluorescent dye fura-2/AM. Changes in the fluorescence intensity of fura-2 in the cells were recorded with a video-imaging analysis system. Ca^{2+} antagonists (nifedipine, verapamil, and diltiazem in the concentration range of 1 to 20 μM) other than isradipine also raised the $[Ca^{2+}]i$. All of the NSCC inhibitors (SK&F 96365, $GdCl_3$, $HgCl_2$, and flufenamic acid), but none of the NCX inhibitors (KB-R 7943 and benzamil), significantly decreased the $[Ca^{2+}]i$ raised by isradipine (3 μM). Neither the Na^+ ionophore monensin nor Na^+/K^+ ATPase inhibitor ouabain had any significant effect on the isradipine-induced $[Ca^{2+}]i$ rise. Taken together, our data indicate that Ca^{2+} entry through the NSCCs is involved in the isradipine-induced $[Ca^{2+}]i$ rise. The results obtained here play an important role in the development of drugs for etiologic therapy of gingival overgrowth.

Key words: Isradipine; NSCC; calcium influx; gingival fibroblast

Introduction

Calcium (Ca^{2+}) antagonists are well known to cause gingival overgrowth as a side effect. Isradipine is one of dihydropirydine-derivative calcium antagonist, which also causes proliferation of gingival fibroblasts (Hattori et al. 2004). However, it unexpectedly raises the cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) in a concentration-dependent manner in human gingival fibroblasts (Hattori and Wang 2005). From a very recent investigation on the origin of elevated Ca^{2+}, we obtained results indicating that isradipine releases Ca^{2+} from intracellular Ca^{2+} stores and also enhances the Ca^{2+} influx from the extracellular environment through the plasma membrane (Hattori and Wang 2005). Although isradipine is a Ca^{2+} antagonist, it raises the $[Ca^{2+}]i$. From this fact, voltage-dependent Ca^{2+} channels must be excluded as a possible contributor to the elevated $[Ca^{2+}]i$ although Gin-1 cells possess the voltage-dependent Ca^{2+} channels (Hattori and Wang 2005). Nonselective cation channels (NSCCs) and Na^+/Ca^{2+} exchangers (NCXs) play important roles in the intake of Ca^{2+} by cells in various tissues. For example, the former perform this function in arterial (Raingo et al. 2004) and gastric smooth muscles (So and Kim 2003); and the latter in heart (Shpak et al. 2004) and astrocytes (Lenart et al. 2004). In the present study, in order to research the pathways through which Ca^{2+} enters the cells, we examined the interactions between isradipine and the blockers or enhancers of NSCCs and NCXs. The results obtained suggest that isradipine causes the Ca^{2+} influx through the NSCCs.

Materials and Methods

Normal human gingival fibroblast Gin-1 cells obtained from Dainippon Pharmaceutic Co. Ltd. (Japan) were used in our experiments. They were cultured for 7–10 days in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum. The cells ($5 \times 10^3/cm^2$) were plated on poly-L-lysine-coated cover slips adhered to a flexiperm chamber (Heraeus GmbH, Göttingen, Germany).

$[Ca^{2+}]i$ was measured with the Ca^{2+}-sensitive fluorescent dye fura-2/AM (5 μM). The cells were kept in a solution consisting of 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 20 mM HEPES-NaOH (pH 7.4). Changes in the fluorescence intensity of fura-2 in the cells were recorded with a video-imaging analysis system (Furusawa Labo Appliance, Kawagoe, Japan).

Drugs were added to the perfusate at appropriate concentrations. The solvent used to dissolved isradipine was dimethyl sulfoxide, and its final concentration was 1 % in each case.

We adopted the peak concentrations of $[Ca^{2+}]i$ induced by a 1-min application of isradipine as the effect of the drug. Each value of the data was presented as the mean value ± the standard error of the mean and the number (N) of cells examined. Statistical analysis of the data was performed by using 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.

Isradipine was a generous gift from Novartis Pharma (Basel, Switzerland). KB-R 7943 came from Tocris (Bristol, UK). Fura-2/AM was obtained from Dojindo Laboratories (Kumamoto, Japan). Tissue culture reagents were purchased from Gibco BRL (Rockville, USA). All other chemicals were from Nacalai Tesque (Kyoto, Japan).

Details of the methods were described before (Hattori and Maehashi 1999a).

Results

We examined the effects of 3 kinds of Ca^{2+} antagonists (dihydropyridine-, phenylalkylamine-, and benzothiazepine-derivatives) on the $[Ca^{2+}]i$ in Gin-1 cells to ensure that Ca^{2+} antagonists other than isradipine also elevate the $[Ca^{2+}]i$. Fig. 1 shows the elevation of $[Ca^{2+}]i$ induced by the Ca^{2+} antagonists in the concentration range of 1 to 20 μM. All kinds of Ca^{2+} antagonists (nifedipine, verapamil, and diltiazem, respectively) raised the $[Ca^{2+}]i$ nearly in a concentration-dependent manner.

Fig. 2 illustrates effects of NSCC inhibitors on the isradipine-induced rise in $[Ca^{2+}]i$. All of the inhibitors tested, i.e., SK&F 96365 (10 μM), $GdCl_3$ (50 μM), $HgCl_2$ (0.1 μM) and flufenamic acid (50 μM), significantly decreased the $[Ca^{2+}]i$ raised by isradipine (3 μM) to approx. half of the control values. Each concentration of the NSCC inhibitors was determined since it significantly inhibits NSCC (Kawanabe et al. 2001). A trypan blue dye exclusion test was performed to rule out the possibility of the cytotoxicity to Gin-1 cells.

The effects of NCX inhibitors on the $[Ca^{2+}]i$ are shown in Fig. 3. Although both mean values obtained with KB-R 7943 (3 μM, Iwamoto 2004) and benzamil (200 μM, Fisher et al. 2002) showed a slight reduction in the $[Ca^{2+}]i$ raised by isradipine (3 μM), they were not statistically significantly different from the control values.

Fig. 4 illustrates the effects of monensin and ouabain on the $[Ca^{2+}]i$. Neither monensin (10 μM), a Na^+ ionophore, nor ouabain (10 μM), a Na^+/K^+ ATPase inhibitor, had any significant increasing effect on the $[Ca^{2+}]i$ raised by isradipine (3 μM). We supposed that if NCXs participate in the isradipine-induced $[Ca^{2+}]i$ rise, both monensin (Fisher et al. 2002) and ouabain (Dong et al. 2004) would have enhanced the isradipine-induced $[Ca^{2+}]i$ rise by activating the NCXs; however, they did not so.

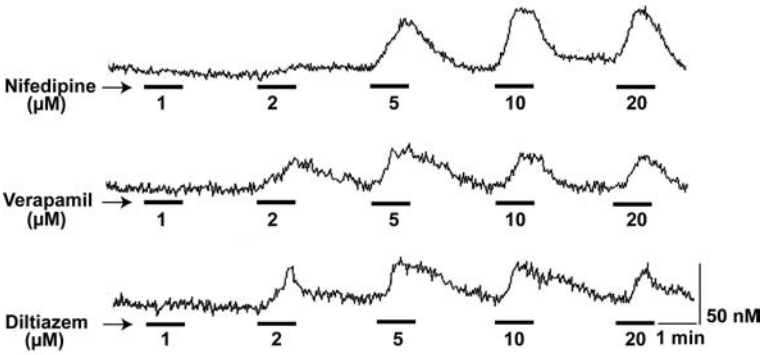

Fig. 1. Effects of 3 kinds (dihydropyridine-, phenylalkylamine-, and benzothiazepine-derivatives) of Ca^{2+} antagonists in the concentration range of 1 to 20 μM on the $[Ca^{2+}]i$ in Gin-1 cells. All of the Ca^{2+} antagonists (nifedipine, verapamil, and diltiazem, respectively) other than isradipine also raised the $[Ca^{2+}]i$.

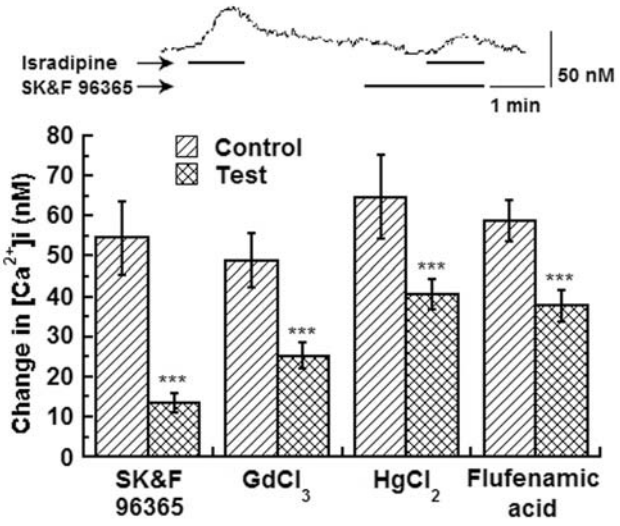

Fig. 2. Effects of NSCC inhibitors on the $[Ca^{2+}]i$. All of the inhibitors, i.e., SK&F 96365 (10 μM), $GdCl_3$ (50 μM), $HgCl_2$ (0.1 μM), and flufenamic acid (50 μM) significantly decreased the $[Ca^{2+}]i$ raised by isradipine (3 μM). ***$p < 0.005$. N = 14 – 17.

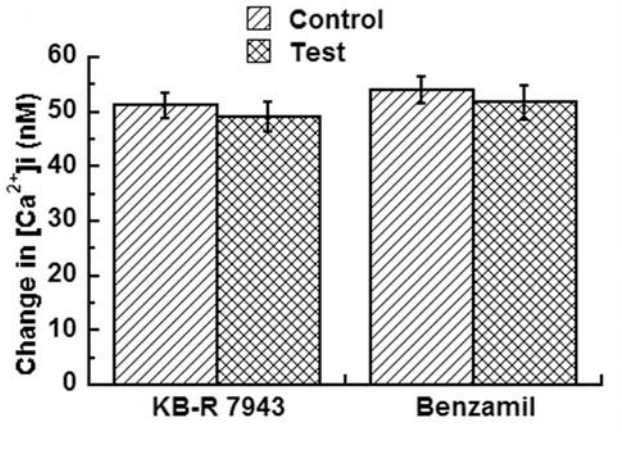

Fig. 3. Influences of NCX inhibitors on the $[Ca^{2+}]i$. Neither KB-R 7943 (3 μM) nor benzamil (200 μM) reduced the $[Ca^{2+}]i$ raised by isradipine (3 μM). N = 15 and 17.

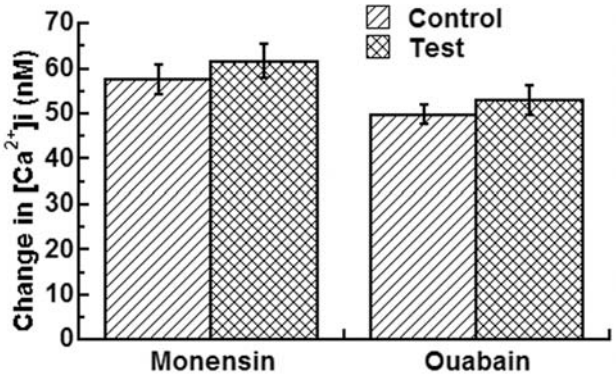

Fig. 4. Effects of monensin and ouabain on the $[Ca^{2+}]i$. Neither monensin (10 μM) nor ouabain (10 μM) had any significant effect on the isradipine (3 μM)-induced $[Ca^{2+}]i$ rise. N = 23 and 32.

Discussion

We examined whether Ca^{2+} entry through NSCCs is involved in the isradipine-induced $[Ca^{2+}]i$ rise in gingival fibroblasts. Dihydropyridine-derivative Ca^{2+} antagonists generally inhibit Ca^{2+} influx in both excitable, e.g., PC12 cells (Hattori and Maehashi 1999b) and non-excitable cells, e.g., MC3T3-E1 cells (Hattori et al. 2001). However, isradipine (a dihydropyridine-derivative Ca^{2+} antagonist) enhances, but not inhibits the Ca^{2+} entry in gingival fibroblasts (Hattori and Wang 2005). Not only isradipine but also other Ca^{2+} antagonists (nifedipine, verapamil, and diltiazem) raised the $[Ca^{2+}]i$ (Fig. 1). This fact suggests that Ca^{2+} antagonists do not act on Ca^{2+} channels but instead other Ca^{2+} pathways in gingival fibroblasts. Thus, in this study, we investigated the influence of blockers or enhancers of the NSCCs and NCXs on isradipine-enhanced Ca^{2+} influx. All of the NSCC blockers (SK&F 96365, $GdCl_3$, $HgCl_2$, and flufenamic acid) reduced the $[Ca^{2+}]i$ raised by isradipine (Fig. 2), indicating that Ca^{2+} passes through the NSCCs. By using endothelin-1, a powerful activator of NSCCs, Kawanabe et al. (2001) observed that although Ca^{2+} influx through NSCCs played an important part in endothelin-1-induced mitogenesis in cultured rat thoracic aorta smooth muscle cells, A7r5 cells, nifedipine, another dihydropyridine-derivative Ca^{2+} antagonist, had only a minor role. They did not claim any excitatory effect of nifedipine on NSCCs. This result, different from ours, might be ascribed to some difference between the materials. In addition, since SK&F 96365, a NSCC blocker selective for NSCC-2, decreased the $[Ca^{2+}]i$, NSCC-2 among the NSCCs (Miwa et al. 1999) may be operative in gingival fibroblasts.

On the other hand, neither of the NCX blockers tested had any effect on the isradipine-induced rise in $[Ca^{2+}]i$ (Fig. 3). Furthermore, monensin and ouabain, which ought to enhance the Ca^{2+} influx via NCXs, did not alter the $[Ca^{2+}]i$ (Fig. 4). This result suggests that NCXs are not involved in the Ca^{2+} rise induced by isradipine. Lee et al. (2001) observed that application of dichlorobenzamil, another NCX inhibitor, and KB-R 7943, a selective reverse-mode NCX inhibitor, completely abolished the Ca^{2+} oscillations induced by phenylephrine in the rabbit inferior vena cava. Thus, they concluded that NCXs generated the Ca^{2+} oscillations. If NCXs are involved in isradipine-induced $[Ca^{2+}]i$ rise, similar results should have been obtained in our study. However, our results were opposite to theirs. This fact indicates that NCXs are not involved in the isradipine-induced $[Ca^{2+}]i$ rise. Moreover, monensin and ouabain also had no increasing effect on the isradipine-induced $[Ca^{2+}]i$ rise, confirming that NCXs have no relation to the isradipine-induced $[Ca^{2+}]i$ rise.

Taken together, our data allow us to conclude that Ca^{2+} entry through NSCCs is involved in the isradipine-induced $[Ca^{2+}]i$ rise in gingival fibroblasts. Furthermore, we speculate the possibility that this isradipine-induced $[Ca^{2+}]i$ rise participates in the proliferation of gingival fibroblasts (Hattori et al., 2004). The results obtained here play an important role in the development of drugs for etiologic therapy of gingival overgrowth (Hattori and Wang 2004).

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Received: November 17, 2005 / Accepted: February 15, 2006

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