ORIGINAL ARTICLE

[6]-Shogaol Induces Ca^{2+} Signals by Activating the TRPV1 Channels in the Rat Insulinoma INS-1E Cells

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ABSTRACT

Context [6]-shogaol is a vanilloid compound present in steamed ginger (*Zingiber officinale*), a commonly used spice. Pancreatic beta-cells respond to nutrients like glucose, amino acids and fatty acids, by an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), which mediates diverse cellular processes in these cells. Some vanilloid compounds activate the transient receptor potential vanilloid receptor type 1 (TRPV1) channel. **Objective** We investigated whether [6]-shogaol could trigger Ca^{2+} signals in the beta-cell. **Methods** $[Ca^{2+}]_i$ was measured from single INS-1E cells by microscope-based fluorometry using fura-2 as the Ca^{2+} indicator. **Results** In fura-2 loaded single rat insulinoma INS-1E cells, a widely used model of beta-cell, [6]-shogaol increased $[Ca^{2+}]_i$ in a concentration-dependent manner. $[Ca^{2+}]_i$ increase by [6]-shogaol was completely blocked when Ca^{2+} was omitted from the extracellular medium. Capsazepine, an inhibitor of the TRPV1 ion channel completely inhibited the [6]-shogaol-induced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ increase obtained by 1 μM [6]-shogaol was greater than that obtained by 10 mM glucose. Moreover, a sub-stimulatory concentration of [6]-shogaol (300 nM), significantly enhanced the glucose-induced $[Ca^{2+}]_i$ increase in these cells. **Conclusion** We conclude that [6]-shogaol induces Ca^{2+} signals in the beta-cell by activating the TRPV1 channels, and it sensitizes the beta-cells to stimulation by glucose.

INTRODUCTION

An increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a critical signalling event that enables the pancreatic beta-cells function as "fuel sensors" [1]. These cells sense the concentration of nutrients, e.g. glucose, amino acids and fatty acids, derived from foods. The ATP-sensitive potassium channel (K_{ATP}), which senses an increase in the cytoplasmic [ATP]/[ADP] ratio, plays an important role in this process. Closure of the K_{ATP} channels leads to membrane depolarization, opening of the voltage-gated Ca^{2+} channels, and consequent increase in the $[Ca^{2+}]_i$, which mediates diverse cellular processes [2]. Apart from the nutrients, many incretin hormones and neurotransmitters also activate beta-cells by triggering the Ca^{2+}- or the cAMP-dependent signalling pathways.

Although spices are common ingredients of food, it is not known whether active ingredients derived from spices can trigger Ca^{2+} signals in the beta-cells. It is now known that several ion channels belonging to the transient receptor potential (TRP) super-family can be activated by chemicals derived from spices [3]. Transient receptor potential channel vanilloid type 1 (TRPV1) is a Ca^{2+} permeable channel that can be activated by vanilloid compounds present in many spices [4, 5]. We have shown that the insulin secreting INS-1E cells have functional TRPV1 channels [6]. [6]-shogaol is a vanilloid compound present in ginger, which is the rhizome of the plant *Zingiber officinale* Roscoe [7]. In this report, we have investigated the effects of [6]-shogaol on $[Ca^{2+}]_i$ in the pancreatic beta-cells.

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Abbreviations $[Ca^{2+}]_i$: cytoplasmic free Ca^{2+} concentration; K_{ATP}: ATP-sensitive potassium channels; TRP: transient receptor potential; TRPV1: transient receptor potential vanilloid type 1

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MATERIALS AND METHODS

Cell Culture and Chemicals

We used a highly differentiated clone of rat insulinoma cells (S5 cells) derived from INS-1E cells. The cells were cultured in a medium

composed of RPMI-1640, supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 500 μM 2-mercaptoethanol, 2.5 % fetal bovine serum, 50 I.U./mL penicillin, and 50 μg/mL streptomycin. Cells were cultured in a humidified incubator in 5% CO_2. The medium was changed every other day and the cells were passaged by mild trypsinization once a week. For measurement of $[Ca^{2+}]_i$, cells were cultured on glass coverslips. In single cell experiments, only cells that were round, without processes, and thus looked like differentiated beta-cells were chosen.

Fura-2 acetoxymethyl ester (98% pure) was from Invitrogen (Lidingö, Sweden). Capsazepine was from Sigma-Aldrich (Stockholm, Sweden). [6]-shogaol (1-4-hydroxy-3-methoxyphenyl-4-decen-3-one) was from ChromaDex, Inc. (Irvine, CA, USA). Cell culture materials were from Invitrogen.

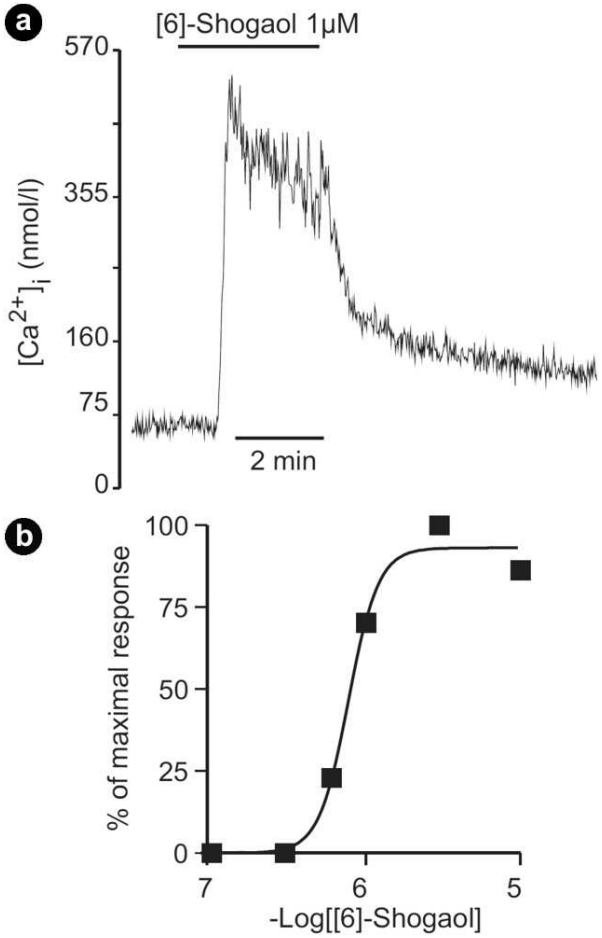

Figure 1. [6]-shogaol increased $[Ca^{2+}]_i$ in a dose-dependent manner. Effect of [6]-shogaol on $[Ca^{2+}]_i$ in the INS-1E cells was measured by microfluorometry, using fura-2 as the Ca^{2+} indicator. **a.** The trace shows $[Ca^{2+}]_i$ increase by [6]-shogaol (1 μM). The trace is representative of at least three experiments. **b.** Concentration-response curve for [6]-shogaol-induced $[Ca^{2+}]_i$ increase. Maximal increase of $[Ca^{2+}]_i$ was obtained by 3 μM [6]-shogaol. Each data point represents mean of at least three experiments.

Measurement of $[Ca^{2+}]_i$ by Microscope-Based Fluorometry

Cells cultured on glass coverslips were incubated in RPMI 1640 medium containing 0.1% bovine serum albumin, and 1 μM fura-2 acetoxymethyl ester for 35 min in a humidified incubator. The coverslips were then left for another 10 min at room temperature in a physiological solution containing (in mM) 140 NaCl, 3.6 KCl, 0.5 NaH_2PO_4, 0.5 $MgSO_4$, 1.5 $CaCl_2$, 10 HEPES, 3 glucose, and 0.1% bovine serum albumin (pH 7.4). Nominally Ca^{2+}-free buffer was prepared by omitting Ca^{2+} from the solution and adding EGTA (0.5 mM). Coverslips were then mounted as the bottom of an open perfusion chamber on the stage of an inverted microscope (Olympus CK40, Stockholm, Sweden). The temperature within the chamber was maintained at 37°C. Single cells isolated optically by means of a diaphragm, were studied by using a 40×1.3 numerical aperture oil immersion objective (40× UV apochromatic). Cells were excited alternately at 340 and 380 nm wavelengths selected by a monochromator (DeltaRam, Photon Technology International (PTI), Inc., Birmingham, NJ, USA). The emitted light selected by a 510 nm filter was monitored by a photomultiplier tube detector. The emissions at the excitation wavelength of 340 nm ($F340$) and that of 380 nm ($F380$) were used to calculate the fluorescence ratio ($R340/380$). The excitation wavelengths were alternated to obtain one ratio data point per second. The fluorescence system was controlled by the Felix software (Version 1.42b, Photon Technology International (PTI), Inc., Birmingham, NJ, USA). The background fluorescence was measured, and subtracted from the traces. The $[Ca^{2+}]_i$ was calculated from $R340/380$ as described before [8]. R_{max} and R_{min} were determined by using thin films of external standards containing fura-2 free acid and 2M sucrose. The Kd for Ca^{2+}-fura-2 was taken as 225 nM.

STATISTICS

Data are shown as mean values and standard error of mean (SEM). Student's t-test (unpaired) was used for testing statistical significance and two-tailed $P<0.05$ was accepted as statistically significant. Data were analyzed by means of the SigmaPlot package (Systat Software Inc., San Jose, CA, USA).

RESULTS

[6]-Shogaol Increased $[Ca^{2+}]_i$ in the INS-1E Cells

[6]-shogaol increased $[Ca^{2+}]_i$ in the INS-1E cells in a dose dependent manner, the estimated EC50 being about 770 nM (Figure 1ab). After switching to the [6]-shogaol-containing solution, there was an initial delay of about 25 seconds because of the delay in the perfusion system. Once [6]-shogaol reached the

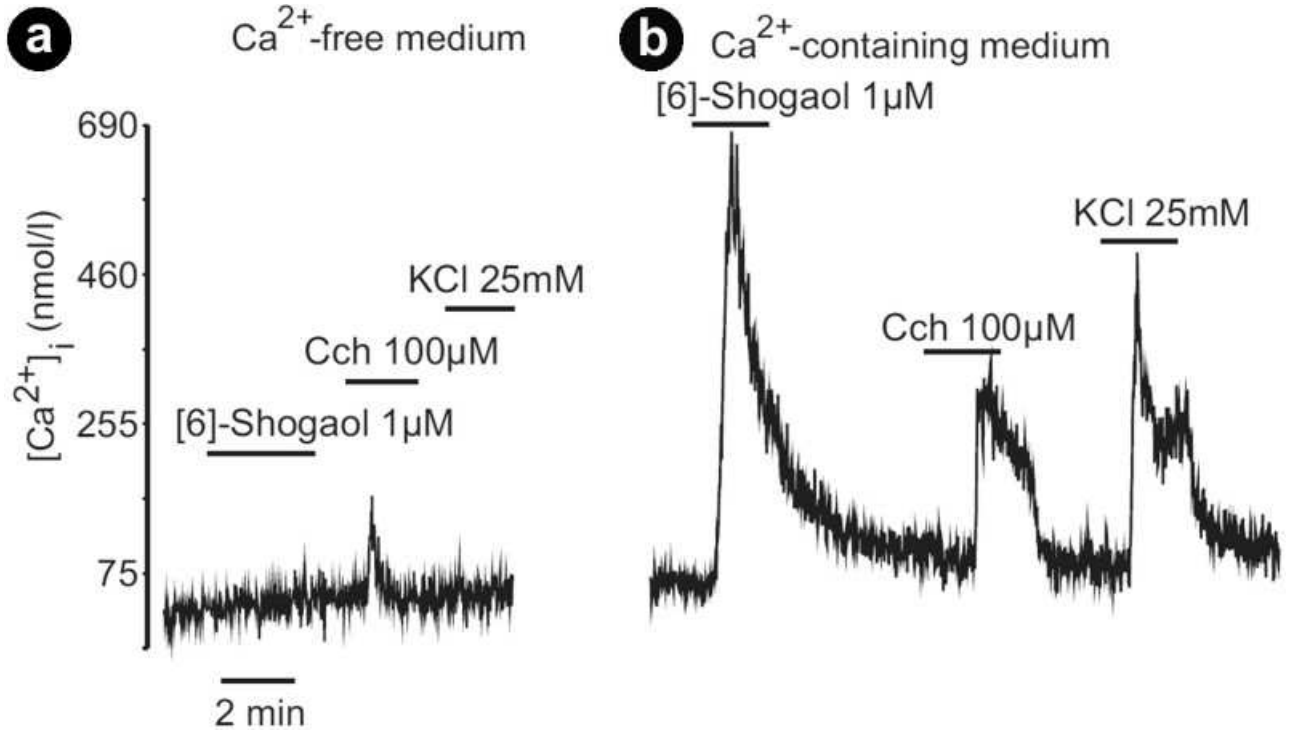

Figure 2. Extracellular Ca^{2+} was essential for [6]-shogaol-induced $[Ca^{2+}]_i$ increase. **a.** INS-1E cells were superfused with nominally Ca^{2+}-free medium. [6]-shogaol (1 μM), carbachol (Cch, 100 μM), and KCl (25 mM) were present in the medium at times indicated by the horizontal bars. Under these conditions, [6]-shogaol failed to increase the $[Ca^{2+}]_i$. **b.** Control experiment, where the extracellular medium contained 1.5 mM Ca^{2+}. Traces A and B are representatives of four independent experiments.

chamber, $[Ca^{2+}]_i$ increased rapidly (within less than 5 sec) to a peak. Two patterns of $[Ca^{2+}]_i$ changes were observed. In some cells, after an increase of $[Ca^{2+}]_i$ to the peak, there was a plateau phase of elevated $[Ca^{2+}]_i$ (Figure 1a). In other cells, the plateau phase of $[Ca^{2+}]_i$ increase was missing (Figure 2b). On wash out of [6]-shogaol, $[Ca^{2+}]_i$ returned completely to the base line. These results demonstrated that [6]-shogaol increased $[Ca^{2+}]_i$ in a dose-dependent, and reversible manner in the INS-1E cells.

Increase of $[Ca^{2+}]_i$ by [6]-Shogaol Was Due to Ca^{2+} Entry Across the Plasma Membrane

To test whether [6]-shogaol increased $[Ca^{2+}]_i$ by inducing Ca^{2+} entry across the plasma membrane, or by releasing Ca^{2+} from the intracellular stores, we stimulated the cells by [6]-shogaol, in the absence of

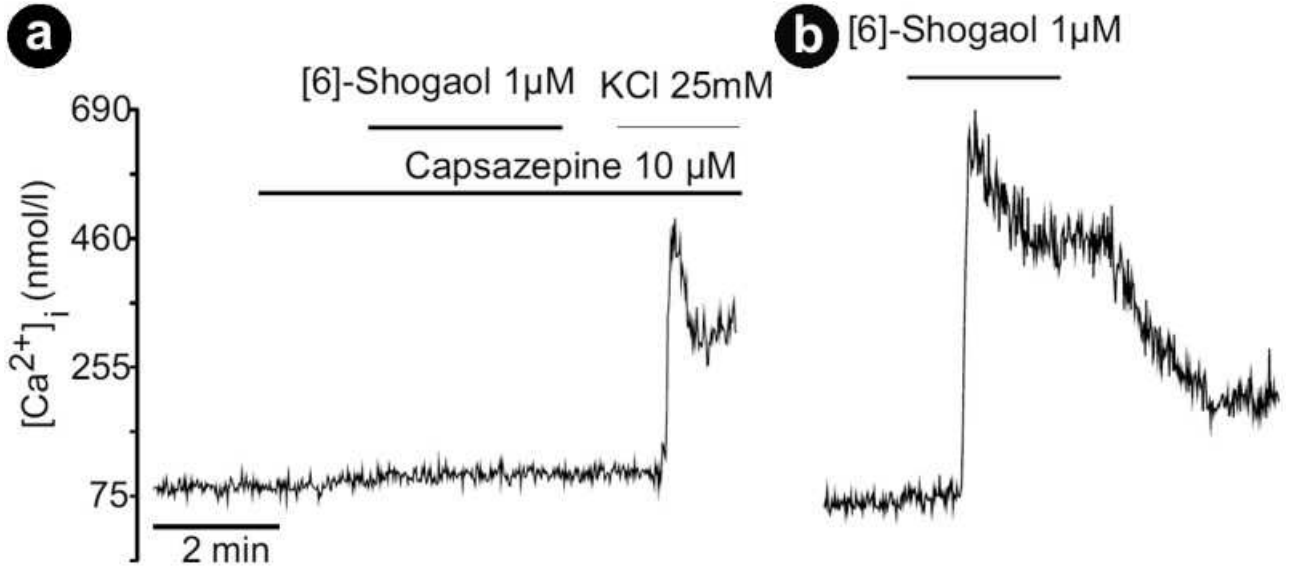

Figure 3. Capsazepine inhibited the $[Ca^{2+}]_i$ increase by [6]-shogaol. Conditions of experiments were as described in the legend to the figure 1. **a.** Capsazepine (10 μM) itself did not increase $[Ca^{2+}]_i$, but it completely inhibited the $[Ca^{2+}]_i$ increase by [6]-shogaol (1 μM). At the end of the experiment KCl (25 mM) was added as a positive control. **b.** Control experiment, where [6]-shogaol was added in the absence of capsazepine. The traces are representatives for three experiments with similar results.

extracellular Ca^{2+}. Under this condition, [6]-shogaol did not increase $[Ca^{2+}]_i$ (Figures 2ab). Carbachol, an agonist of muscarinic receptors, increased $[Ca^{2+}]_i$ indicating that the intracellular stores were not empty when Ca^{2+} was omitted from the extracellular medium (Figure 2a). KCl which depolarizes the plasma membrane and thereby triggers Ca^{2+} entry through the voltage-gated Ca^{2+} channels, did not increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. In control experiments, where [6]-shogaol and KCl was applied in the presence of extracellular Ca^{2+}, large $[Ca^{2+}]_i$ increase was observed (Figure 2b). These results demonstrated that the [6]-shogaol-induced increase of $[Ca^{2+}]_i$ was due to Ca^{2+} entry through the plasma membrane, and not due to the release of Ca^{2+} from the intracellular Ca^{2+} stores.

Capsazepine Inhibited $[Ca^{2+}]_i$-Increase by [6]-Shogaol

To test whether $[Ca^{2+}]_i$ increase by [6]-shogaol was due to the activation of the TRPV1 channels, we used capsazepine, a specific inhibitor of this channel [9]. As shown in Figure 3, capsazepine did not alter $[Ca^{2+}]_i$ by itself, but it completely blocked the $[Ca^{2+}]_i$ increase by [6]-shogaol. $[Ca^{2+}]_i$ increase by KCl was not blocked by capsazepine (Figure 3a). In control experiments, where [6]-shogaol was used without capsazepine, there was a characteristic increase of $[Ca^{2+}]_i$ (Figure 3b). These results indicated that $[Ca^{2+}]_i$ increase by [6]-shogaol was due to the activation of the TRPV1 channel.

[6]-Shogaol Increased Glucose-Induced $[Ca^{2+}]_i$ Response

A low concentration of [6]-shogaol (300 nM) did not increase $[Ca^{2+}]_i$ (Figure 4a). Addition of glucose (10 mM) to the cells increased $[Ca^{2+}]_i$ by 100±30 nM (mean±SEM; n=3) (Figure 4bd). When 10 mM glucose was added in the presence of 300 nM [6]-shogaol, the magnitude of $[Ca^{2+}]_i$ increase was about three-fold more compared to that obtained in the absence of [6]-shogaol (322±62 nM, P=0.036; n=4) (Figure 4cd).

DISCUSSION

An increase in the $[Ca^{2+}]_i$ in the beta-cells is a hallmark of activation of these cells. In our experiments, [6]-shogaol induced a rapid and large increase of $[Ca^{2+}]_i$ in the beta-cells in a dose-dependent manner. This increase of $[Ca^{2+}]_i$ by [6]-shogaol was due to influx of Ca^{2+} across the plasma

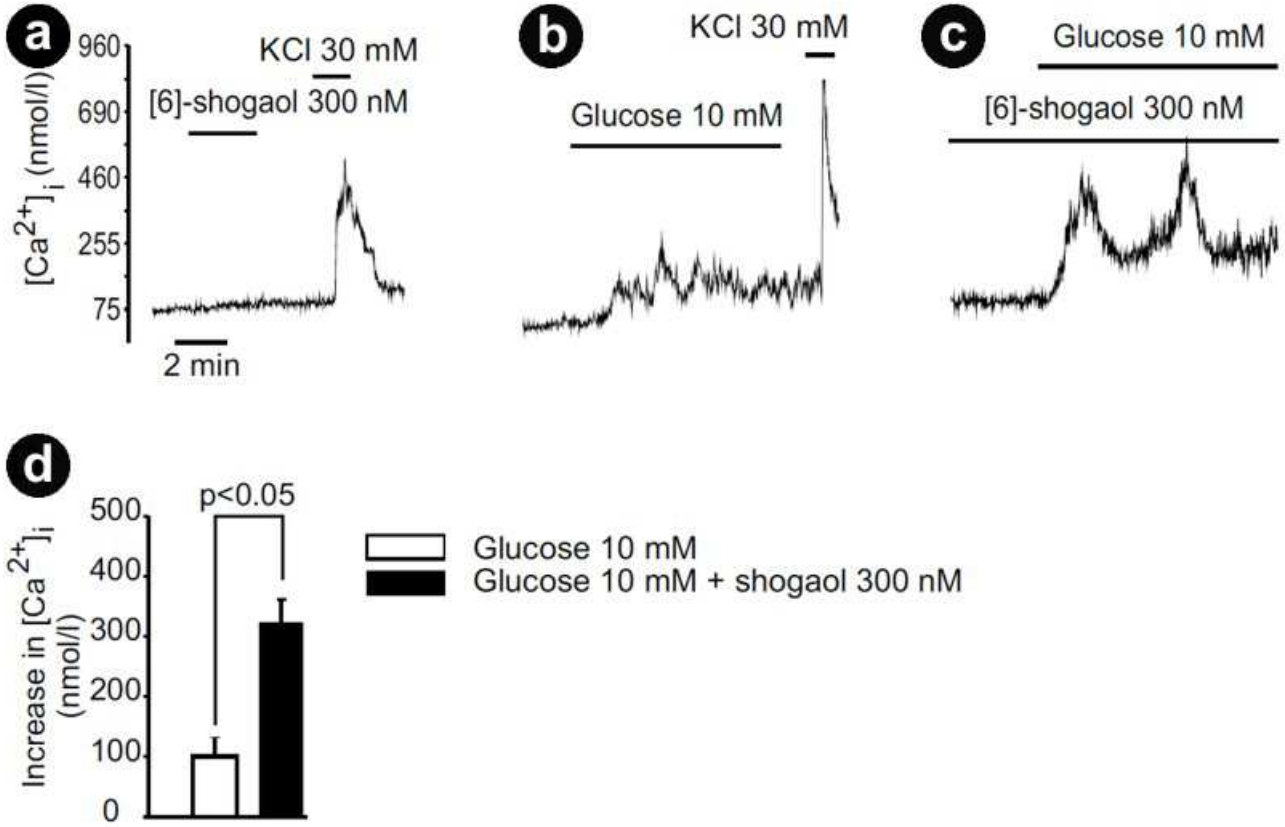

Figure 4. [6]-shogaol sensitized glucose-induced $[Ca^{2+}]_i$ response. $[Ca^{2+}]_i$ was measured from the INS-1E cells loaded with fura-2. **a.** [6]-shogaol (300 nM) did not increase $[Ca^{2+}]_i$ by itself. KCl (25 mM) was added as a positive control, and it increased $[Ca^{2+}]_i$. **b.** Glucose (10 mM) increased $[Ca^{2+}]_i$. **c.** Glucose (10 mM) was added in the presence of 300 nM [6]-shogaol, and this caused a larger increase of $[Ca^{2+}]_i$ (*versus* Figure 4b). **d.** $[Ca^{2+}]_i$ increase by glucose (10 mM) was significantly higher when the sugar was added in the presence of 300 nM [6]-shogaol. The traces are representatives of at least three independent experiments.

membrane, and was not due to any release of Ca^{2+} from the intracellular Ca^{2+} stores. To the best of our knowledge, this is the first demonstration of $[Ca^{2+}]_i$ increase by shogaols in any insulin-secreting cell, and our results are consistent with the $[Ca^{2+}]_i$-increasing effect of shogaols described in other cells that express TRPV1 [10, 11]. $[Ca^{2+}]_i$ increase by [6]-shogaol was completely blocked by capsazepine, a specific blocker of the TRPV1 channel, indicating that the vanilloid compound induced Ca^{2+} entry by activating this channel. These results are consistent with a previous report that pancreatic beta-cells express TRPV1 channels [12]. Furthermore, we demonstrate that [6]-shogaol not only increased $[Ca^{2+}]_i$ by itself, but also sensitized the $[Ca^{2+}]_i$ response of the beta-cells to glucose. We speculate that sub-threshold concentration of [6]-shogaol promotes glucose-induced depolarization of the plasma membrane of the INS-1E cells by providing an inward depolarizing current through the TRPV1 channels. Thus, closure of the K_{ATP} channels by increased ATP/ADP ratio upon metabolism of glucose, together with the inward depolarizing currents provided by the activation of the TRPV1 channels, causes increased depolarization of the plasma membrane potential to the threshold for the activation of the L-type voltage-gated Ca^{2+} channels leading to the increase of $[Ca^{2+}]_i$.

It is well known that nutrients increase $[Ca^{2+}]_i$ in the beta-cells by increasing the cytoplasmic ATP/ADP ratio leading to the closure of the K_{ATP} channels, membrane depolarization and opening of the voltage-gated Ca^{2+} channels [2]. Commonly used drugs for the treatment of type 2 diabetes, e.g. the sulphonylurea compounds, also work by blocking the K_{ATP} channels, and thereby increasing the $[Ca^{2+}]_i$ [2]. Our results show that high concentrations of [6]-shogaol can bypass this well known chain of events, and instead, increases $[Ca^{2+}]_i$ by a distinct mechanism. Our results illustrate that certain chemicals derived from spices may induce Ca^{2+} signals in the beta-cells by directly acting on ion channels, other than the K_{ATP} channels. The physiological relevance of our data obtained from the INS-1E cells is unclear since primary human beta-cells apparently do not express functional TRPV1 channels [6]. Nevertheless, the INS-1E cells can be used as a model for the study of the native TRPV1 channels in a mammalian cell line.

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Conflict of interest notification None

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