

## Effect of Protein Kinase C on Glucose-Mediated Insulin Secretion in HIT-T15 Cells

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### ABSTRACT

**Objective** To clarify the regulation of protein kinase C on glucose-mediated insulin secretion.

**Main outcome measures** We examined the effect of protein kinase C on the cytosolic free  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) and the activity of  $Ca^{2+}$ -activated  $K^+$  channels ( $K_{Ca}$ -channel) in the insulinoma cell line, HIT-T15.

**Results** Glucose at a concentration of 10 mmol/L increased the secretion of insulin. This increase was partly inhibited by 1 nmol/L staurosporine, a protein kinase C inhibitor. Staurosporine (1 nmol/L) also attenuated the glucose-induced elevations in  $[Ca^{2+}]_i$ . On the contrary, glibenclamide (100 nmol/L) specifically blocked ATP-sensitive  $K^+$  channels, and increased both  $[Ca^{2+}]_i$  and insulin secretion, but staurosporine had no effect on them. Patch clamp studies showed that 10 mmol/L glucose almost completely blocked  $K_{Ca}$  channel activity, an effect that was reversed by 1 nmol/L staurosporine. Phorbol 12-myristate 13-acetate (1  $\mu$ mol/L), a protein kinase C activator, also decreased  $K_{Ca}$  channel activity.

**Conclusions** These results indicate that the activation of protein kinase C is involved in the glucose-induced release of insulin by modulating  $K^+$  channel function in HIT-T15 cells.

### INTRODUCTION

The physiological secretagogue, glucose, causes insulin to be released by pancreatic beta cells through a complex mechanism. This process involves the closure of ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels [1], which are directly blocked by intracellular ATP [2], membrane depolarization [3], the opening of voltage-gated L-type  $Ca^{2+}$  channels, and a subsequent increase in the cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) [4]. Thus, the closure of  $K_{ATP}$  channels is regarded as a prerequisite for the glucose-induced insulin release. Sulfonylureas, used in the treatment of diabetes mellitus, have been shown to specifically block the  $K_{ATP}$  channel [5], thus inducing cell membrane depolarization and insulin secretion. The opposite has been found for the hyperglycemic drug diazoxide, a compound which opens  $K_{ATP}$  channels, hyperpolarizes the beta cells and inhibits the glucose-induced release of insulin in rat islets [6].

Several reports have shown that glucose can cause the beta-cells to release insulin without altering the cell's membrane potential or  $[Ca^{2+}]_i$  level [7-9]. In addition to the block of the  $K_{ATP}$  channels, insulin release is regulated by a variety of intracellular processes, including changes in intracellular levels of cyclic nucleotides and phosphoinositide turnover. The latter process is mediated by the activation of phospholipase C, resulting in the generation of inositol 1, 4, 5 trisphosphate and diacylglycerol [10]. Whereas inositol 1, 4, 5 trisphosphate releases intracellularly bound

Ca<sup>2+</sup> [11], diacylglycerol exerts its effects through the activation of protein kinase C (PKC) [12]. The activity of this enzyme has been established in both rat [13] and mouse [14] pancreatic islets as well as in insulin-producing tumor cells [15, 16]. In HIT-T15 cells, Deeney et al. [17] reported that glucose caused translocation of PKC from the cytoplasm to the plasma membrane. Several types of ion channels have also been shown to be modulated by the activation of PKC [18]. For example, phorbol esters and synthetic diacylglycerol, which activate PKC, reportedly block K<sup>+</sup> currents [19]. In addition to K<sub>ATP</sub> channels, several other K<sup>+</sup> channels were characterized in cultured pancreatic islet cells [20]. However, it is unclear how the individual K<sup>+</sup> channel currents are affected by PKC in pancreatic beta cells. Therefore, we attempted to examine the effects of PKC on glucose-induced insulin secretion via K<sub>Ca</sub> channels by using a PKC inhibitor, staurosporine, in insulinoma HIT-T15 cells.

## MATERIALS AND METHODS

### Cell Culture

The insulin secreting cell line, HIT-T15 (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan), was cultured at 37 °C in Ham's F12-K medium (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin (all obtained from GIBCO, Grand Island, USA) in an atmosphere of 5% CO<sub>2</sub>. The medium was changed twice a week and the cell line was passaged once a week.

### Measurements of Insulin Secretion

HIT cells were plated into 24-well tissue culture dishes (1 to 10 x 10<sup>5</sup> cells/well). Prior to any analysis, the plates were rinsed with Tyrode's solution. This solution contained 128 mmol/L NaCl, 2.68 mmol/L KCl, 1.8 mmol/L CaCl<sub>2</sub>, 1.64 mmol/L MgCl<sub>2</sub> and 10

mmol/L MOPS (3-[N-morpholinol] propane-sulfonic acid), pH 7.2. Subsequently, the plates were incubated with test reagents diluted in the same buffer for 60 min. The samples were then collected and frozen at -20 °C. Insulin was measured by radioimmunoassay with commercially available kits according to the manufacturer's specifications (Waco, Tokyo, Japan).

### Measurement of Intracellular Ca<sup>2+</sup>

The [Ca<sup>2+</sup>]<sub>i</sub> levels were studied by using the fluorescent dye fura-2AM (Waco, Tokyo, Japan). Cells were cultured on glass coverslips three days before the experiments. At the time of analysis, these cells were immersed in Tyrode's solution containing 4 μmol/L fura-2AM for 30 min at 37 °C. The cells were then transferred to a small incubation bath (0.5 mL) that had been mounted on a microscope stage. The bath temperature was maintained at 37 °C while being perfused with buffer at a rate of 0.8 mL/min during the experimental period. Any fluorescence emitted was measured in the HIT cells on the coverslips by using a fluorescence spectromicroscope (excitation, 340/380 nm, emission, 510 nm). The results were expressed as a ratio of the fluorescence recorded at 340 nm and 380 nm.

### Electrophysiologic Measurements

Membrane currents were recorded in the "cell-attached" and "inside-out" configurations by employing a patch-clamp amplifier as described by Hamill et al. [21]. The bath solution for the cell-attached mode contained 140 mmol/L KCl, 10 mmol/L MOPS-K, and 1 mmol/L CaCl<sub>2</sub>. The pipette solution was the same as the cell-attached solution except for the Ca<sup>2+</sup> concentration (CaCl<sub>2</sub> 10<sup>-7</sup> mol/L). Soft glass pipettes, prepared in an electrode puller (PP-83, Narishige, Tokyo, Japan), were used after being coated with Sylgard. The electrical resistance of the patch pipette was 5 to 7 MΩ for single channel recording. Experiments were conducted at a solution

temperature between 35 and 37 °C. Data were stored in a PCM recorder (model PCM-501ES, Sony Co., Tokyo, Japan) with a low pass filter (3 KHz). The pClamp Ver 6.0 software (Axon Instruments Inc., Foster City, USA) was used to analyze the data on single channel currents. The open probability (NPo) was determined from current amplitude histograms and was calculated by using the following equation:

$$NPo = \frac{\sum_{n=0}^N (n \cdot P_n)}{N}$$

where N is the number of channels in the patch and P<sub>n</sub> is the integrated channel opening.

### STATISTICAL ANALYSIS

Data are expressed as mean±SD. Differences among data sets were evaluated by the Student's t-test. A level of P<0.05 was accepted as statistically significant.

### RESULTS

Table 1 shows the effect of glucose on insulin secretion in cultured HIT cells. Glucose (3, 10, 30 mmol/L) caused insulin secretion in a concentration-dependent manner. To determine whether the effects of glucose on insulin secretion involved a PKC, we studied the effect of a PKC inhibitor, staurosporine, on insulin release. The glucose-induced secretion of insulin (at the concentration of 10

mmol/L: 455±182 pmol/L/h, n=8) was significantly attenuated in the presence of 1 nmol/L staurosporine (286±34 pmol/L/h, n=6, P<0.05 vs. 10 mmol/L glucose). In addition, phorbol 12-myristate 13-acetate (PMA: 1 µmol/L), an activator of PKC, alone significantly increased insulin secretion.

As shown in Table 2, glibenclamide stimulated insulin secretion in a concentration-dependent manner between 10-1,000 nmol/L. Unlike glucose, staurosporine did not attenuate the glibenclamide-induced insulin secretion when stimulated with 100 nmol/L glibenclamide.

Glucose and glibenclamide increased the [Ca<sup>2+</sup>]<sub>i</sub> level indicated by 340/380 ratio in a concentration-dependent manner (Figure 1). The 340/380 ratio was 0.928±0.199 in the resting conditions and the peak elevation by 10 mmol/L glucose was 1.307±0.222 (P<0.001, n=8). To clarify whether the decrease in insulin secretion caused by staurosporine was related to changes in the [Ca<sup>2+</sup>]<sub>i</sub>, we tested the effect of staurosporine on glucose-induced [Ca<sup>2+</sup>]<sub>i</sub> increases. Staurosporine (1 nmol/L) significantly reduced the level of intracellular calcium in the presence of glucose, (340/380 ratio; 1.307±0.222 to 1.123±0.180, P<0.05, n=8, Figure 2A) and the time point was 16 min. PMA alone also increased the [Ca<sup>2+</sup>]<sub>i</sub> (1.036±0.122 to 1.186±0.185, P<0.001, n=11) and the time point was 3 min (Figure 2B).

**Table 1.** Effect of staurosporine on insulin secretion induced by glucose.

	Insulin release (pmol/L/h)
Control	129±16
Glucose (3 mmol/L)	268±95 <sup>a</sup>
Glucose (10 mmol/L)	455±182 <sup>ac</sup>
Glucose (30 mmol/L)	497±254 <sup>b</sup>
Glucose (10 mmol/L) + Staurosporine (1 nmol/L)	286±34 <sup>c</sup>
Phorbol 12-myristate 13-acetate (PMA 1 µmol/L)	280±157 <sup>b</sup>

Results are shown as mean values ± SD

<sup>a</sup> P<0.01 vs. Control

<sup>b</sup> P<0.05 vs. Control

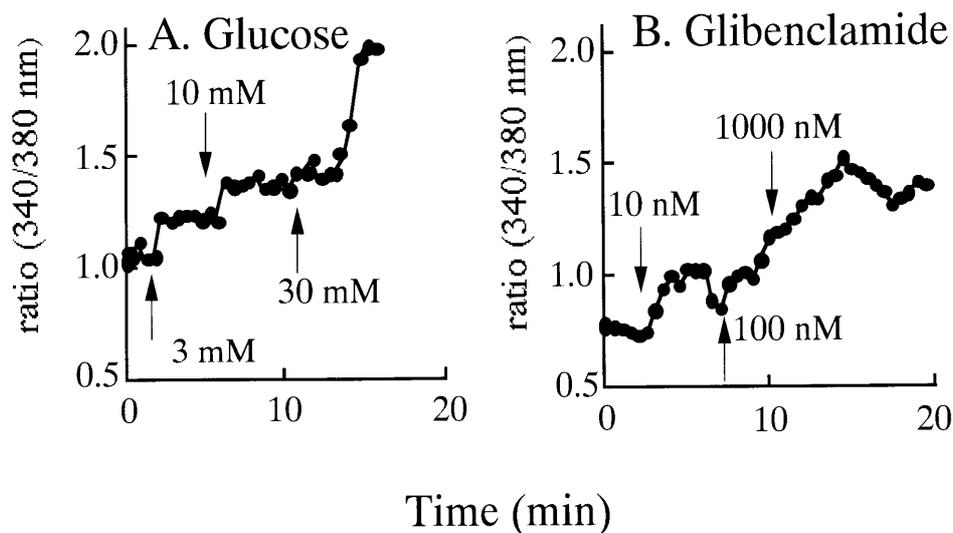
<sup>c</sup> P<0.05 Glucose (10 mmol/L) vs. Glucose (10 mmol/L) + Staurosporine (1 nmol/L)

**Table 2.** Effect of staurosporine on insulin secretion induced by glibenclamide.

	Insulin release (pmol/L/h)
Control	129±16
Glibenclamide (10 nmol/L)	554±173 <sup>a</sup>
Glibenclamide (100 nmol/L)	650±268 <sup>a</sup>
Glibenclamide (1000 nmol/L)	662±192 <sup>a</sup>
Glibenclamide (100 nmol/L) + Staurosporine (1 nmol/L)	606±220

Results are shown as mean values ± SD

<sup>a</sup> P<0.001 vs. Control



**Figure 1.** The effects of glucose and glibenclamide on cytosolic Ca<sup>2+</sup> in HIT cells. **A.** Glucose (3, 10, 30 mmol/L) and **B.** Glibenclamide (10, 100, 1,000 nmol/L) were perfused. The results are presented as the 340/380 nm wavelength ratio of fluorescence.

Glibenclamide at a concentration of 100 nmol/L increased [Ca<sup>2+</sup>]<sub>i</sub> from a control level of 0.968±0.229 to a peak level of 1.407±0.137 (P<0.05, n=5), but staurosporine had no effect on the glibenclamide-induced [Ca<sup>2+</sup>]<sub>i</sub> increase (1.305±0.187, n=5, Figure 2C). These values were obtained after 9 min application of drugs.

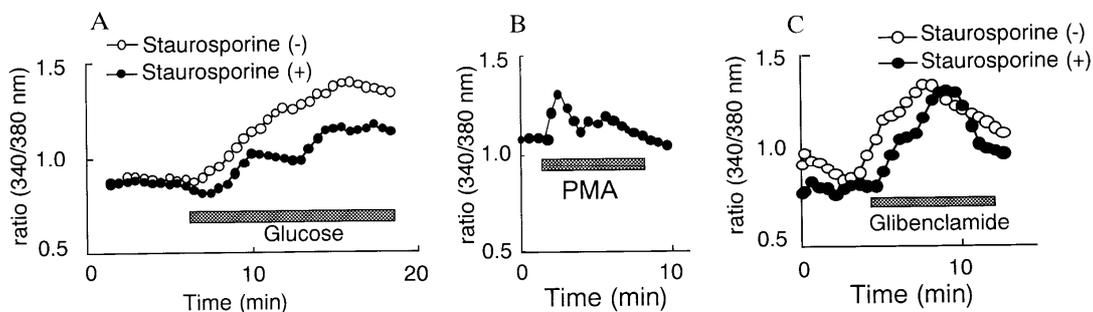
To investigate whether the decrease in insulin secretion and [Ca<sup>2+</sup>]<sub>i</sub> levels caused by staurosporine were related to the activity of the K<sup>+</sup> channels, we studied single channel K<sup>+</sup> currents using patch clamp technique. Figure 3A shows the effect of glibenclamide on K<sub>ATP</sub> (channels that had a conductance of 51±13 pS), (n=9, Figure 3A) and K<sub>Ca</sub> channels (223±35 pS), (n=7, Figure 3B). Glibenclamide

(100 nmol/L) blocked K<sub>ATP</sub> channel (Figure 3A), but had no effect of K<sub>Ca</sub> channel (Figure 3B). The open probability of the K<sub>ATP</sub> channel was decreased from 0.154±0.088 to 0.014±0.025 (P<0.05, n=6). Figure 3C shows the effect of 10 mmol/L glucose on K<sup>+</sup> channel currents in the cell-attached configurations, using symmetrical 150 mmol/L K<sup>+</sup>, without glucose and at a membrane potential of +40 mV. Glucose blocked the K<sub>ATP</sub> channel and K<sub>Ca</sub> channel almost completely (Figure 3C).

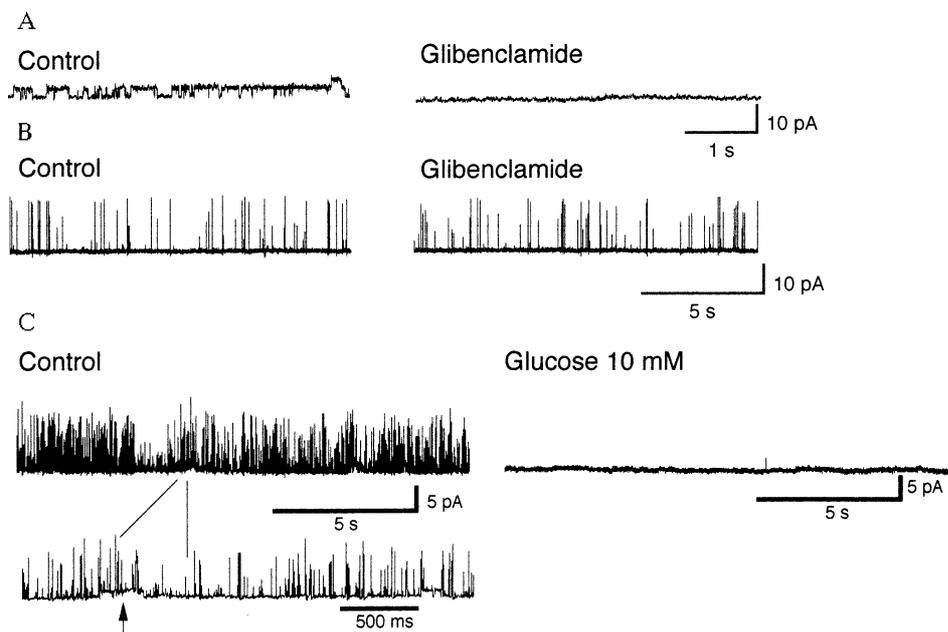
To clarify whether the glucose-induced decrease in K<sub>Ca</sub> channel activity was mediated by PKC, we treated the cells with staurosporine (Figures 4 and 5). To increase K<sub>Ca</sub> channel activity, 1 μmol/L of A23187, a

calcium ionophore, was used before the application of glucose. Glucose significantly suppressed the  $K_{Ca}$  channel activity (Po:  $0.221 \pm 0.224$  to  $0.014 \pm 0.023$ ,  $P < 0.05$ ,  $n = 7$ , Figure 4C). This effect was significantly

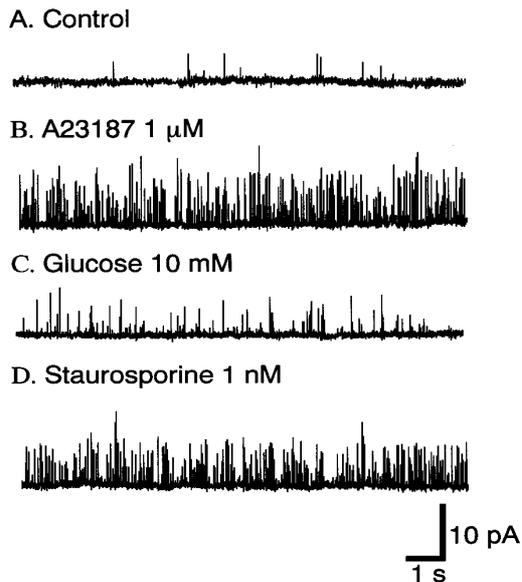
reversed by 1 nmol/L staurosporine ( $0.014 \pm 0.023$  to  $0.195 \pm 0.178$ ,  $P < 0.05$ ,  $n = 7$ , Figure 4D).



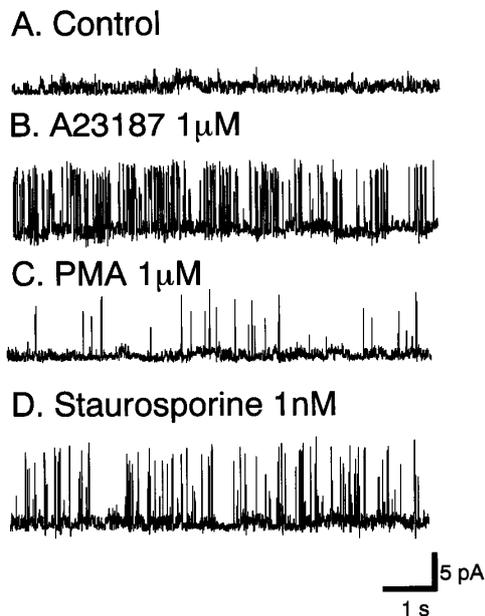
**Figure 2.** **A.** Effect of staurosporine on glucose-induced  $[Ca^{2+}]_i$  increase in HIT-T15 cells. Note that staurosporine partially suppressed increase in  $[Ca^{2+}]_i$  by glucose. **B.** Effect of phorbol 12-myristate 13-acetate (PMA) on  $[Ca^{2+}]_i$  in fura-2 loaded HIT cells. There was a slight increase in  $[Ca^{2+}]_i$  by PMA. **C.** Effect of staurosporine on glibenclamide induced on  $[Ca^{2+}]_i$  in fura-2 loaded HIT cells. The increase in glibenclamide-induced  $[Ca^{2+}]_i$ , and staurosporine and glibenclamide-induced  $[Ca^{2+}]_i$  increase was almost at the same level.



**Figure 3.** **A.** Effect of glibenclamide on  $K_{ATP}$  and  $K_{Ca}$  channels. Control.  $K_{ATP}$  channels were recorded at a membrane potential of  $-50$  mV. After application of glibenclamide (100 nmol/L). Glibenclamide completely blocked  $K_{ATP}$  channels. **B.** Control. The  $K_{Ca}$  channels were recorded at a membrane potential of  $50$  mV. After application of glibenclamide (100 nmol/L). The activities of  $K_{Ca}$  channels were not altered by application of glibenclamide (100 nmol/L). **C.** Effects of 10 mmol/L glucose on ATP-sensitive  $K^+$  ( $K_{ATP}$ ) and  $Ca^{2+}$ -dependent  $K^+$  ( $K_{Ca}$ ) channel. Both  $K_{ATP}$  (small conductance and long opening: arrow) and  $K_{Ca}$  (large conductance and spiky opening) channels were seen before application of glucose. The membrane potential was  $50$  mV. 10 mmol/L glucose blocked both  $K_{ATP}$  and  $K_{Ca}$  channels almost completely.



**Figure 4.** Inhibitory effects of glucose on  $K_{Ca}$  channels. In this experiment, the membrane potential was maintained at 40 mV. In control, infrequent  $K_{Ca}$  channel activity was seen. The application of 1 μmol/L A23187 activated the  $K_{Ca}$  channels by increasing  $[Ca^{2+}]_i$ . The activated  $K_{Ca}$  channels were blocked by 10 mmol/L glucose and reactivated by 1 nmol/L staurosporine.



**Figure 5.** Inhibitory effects of PKC blockade on  $K_{Ca}$  channels. The membrane potential was maintained at 40 mV.  $K_{Ca}$  channels were activated by 1 μmol/L A23187 and 1 μmol/L PMA significantly blocked these channel activities. The  $K_{Ca}$  channels were reactivated by adding 1 nmol/L staurosporine to the same cell.

To test whether a PKC activator could mimic the effects of glucose on  $K_{Ca}$  channels, we tested the effect of PMA on  $K_{Ca}$  channels (Figure 5). PMA significantly reduced  $K_{Ca}$  channel activity ( $0.261 \pm 0.243$  to  $0.027 \pm 0.024$ ,  $P < 0.05$ ,  $n = 7$ ). This inhibition was also reversed by the application of 1 nmol/L staurosporine ( $0.027 \pm 0.024$  to  $0.147 \pm 0.122$ ,  $P < 0.05$ ,  $n = 7$ , Figure 5D).

## DISCUSSION

The purpose of the present study was to clarify the significance of PKC in the secretion of insulin induced by glucose. Staurosporine, a PKC inhibitor, attenuated such secretion in insulinoma HIT cells. In patch clamp studies, glucose blocked both the  $K_{Ca}$  and the  $K_{ATP}$  channels. This blockade appeared to be mediated by PKC, since staurosporine reversed the effect and PMA mimicked it. Staurosporine also attenuated the increase in  $[Ca^{2+}]_i$  levels caused by glucose. Thus, PKC contributed to the glucose-induced secretion of insulin at least partly by blocking the  $K_{Ca}$  channels.

PKC is a calcium- and phospholipid-dependent enzyme that is activated by the endogenous second messenger, diacylglycerol [22]. A variety of approaches have been used to investigate the role of the activation of PKC in stimulated secretion. The activation of PKC by such phorbol esters as 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulates the secretion of insulin in the absence or presence of basal levels of glucose (2.8 mmol/L) in rat islet cells [23, 24] and insulinoma cells [16]. In our present study, staurosporine partially suppressed the secretion of insulin by glucose, thus suggesting the involvement of PKC.

Although PKC has been implicated in secretory responses to glucose, its involvement in the regulation of nutrient-induced insulin secretion is controversial. In isolated rat islet of Langerhans, which had

been pretreated with PMA for 20-24 h to deplete cells of PKC activity, Hii et al. [25] concluded that PKC activation was not essential to the glucose-induced secretion of insulin. However, in HIT cells, Hughes et al. [26] found that PKC depletion blocked the acetylcholine-induced insulin release as well as decreased the levels of insulin released in response to glucose. In our studies, the glucose-induced insulin secretion was assayed not under the PKC depleted condition but under the acute inhibition of PKC. In this condition, staurosporine caused a slight decrease in glucose-induced insulin secretion compared with that seen in cells stimulated by glucose alone. An increased  $[Ca^{2+}]_i$  concentration is considered to be the essential event that initiates glucose-induced insulin secretion, and the activation of PKC reportedly increases  $[Ca^{2+}]_i$  levels. Our study also showed that the inhibition of PKC by staurosporine slightly decreased the  $[Ca^{2+}]_i$  levels. Therefore, the decrease in glucose-induced insulin secretion was due to the diminution of  $[Ca^{2+}]_i$  levels caused by the inhibition of PKC. In contrast with these data, staurosporine had no effect on either glibenclamide-induced insulin secretion or  $[Ca^{2+}]_i$  increase.

Resting HIT-T15 cells show two types of  $K^+$  channels, ATP-sensitive  $K^+$  channels ( $K_{ATP}$ ) and  $Ca^{2+}$ - and voltage-activated  $K^+$  channels ( $K_{Ca}$ ) as in normal pancreatic beta-cells.  $K_{ATP}$  channels have an important role in insulin secretion and  $K_{Ca}$  channels also play an important role in beta-cell membrane repolarization after  $Ca^{2+}$  influx via the voltage-gated  $Ca^{2+}$  channels. In our experiment, glibenclamide blocked  $K_{ATP}$  channels selectively, leading to the increase of  $[Ca^{2+}]_i$  and insulin secretion. It indicates that glibenclamide-induced insulin secretion and  $[Ca^{2+}]_i$  increase come exclusively from the blocking of  $K_{ATP}$  channels.

Phorbol ester, an activator of PKC, has previously been shown to block  $K^+$  currents in several cell types [19]. The ensuing activation of PKC due to an increase in the level of

diacylglycerol leads to the closure of  $K_{ATP}$  channels. In RINm5F cells, it was also reported that PMA reduces  $K_{ATP}$  channel activity, leading to membrane depolarization and an increase in  $[Ca^{2+}]_i$  levels [27]. HIT-T15 cells have the secretory properties of normal islets and respond to nutrient secretagogues such as glucose. Eddkestone et al. demonstrated that glucose depolarizes HIT cells by closing  $K_{ATP}$  channels [28]. Although HIT-T15 cells have been reported to decrease glucose-induced insulin secretion as passage increases, it has both  $K_{ATP}$  and  $K_{Ca}$  channels and they have properties very similar to normal beta cells. Thus, the HIT cells appeared to be a valid model for the investigation. The present study focused on  $K_{Ca}$  channels, so we did not study  $K_{ATP}$  channels extensively. Our data showed that in the cell-attached patch configurations, glucose and PMA inhibited the  $K_{Ca}$  channels that were activated by increasing the  $[Ca^{2+}]_i$  levels by treatment with A23187 (Figures 4 and 5). The PKC inhibitor staurosporine significantly activated the  $K_{Ca}$  channels blocked either by glucose (Figure 4) or PMA (Figure 5). These results indicate that the activation of PKC inhibits  $K_{Ca}$  channels, probably via phosphorylation of the channel protein.

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**Key words** Potassium Channels; Protein Kinase C; Staurosporine

**Abbreviations**  $K_{ATP}$ : ATP-sensitive  $K^+$ ; PKC: protein kinase C; PMA: phorbol 12-myristate 13-acetate

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