Somatostatin and Insulin Secretion Due to Common Mechanisms by a New Hypoglycemic Agent, A-4166, in Perfused Rat Pancreas

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***N*-[(*trans*-4-isopropylcyclohexyl)-carbonyl]-D-phenylalanine (A-4166) is a nonsulfonylurea hypoglycemic agent that decreases blood glucose levels in nondiabetic and diabetic animals. In the present study, we attempted to determine the effect of A-4166 on hormone secretion from the in vitro–perfused rat pancreas and to examine the underlying secretory mechanisms. In the presence of basal glucose (3 mmol/L), A-4166 markedly stimulated insulin and somatostatin release in a concentration-dependent manner over 0.03 to 3 mmol/L. A sulfonylurea, tolbutamide, also stimulated insulin and somatostatin release. A-4166 and tolbutamide elevated the level of glucagon release; however, the change lacked a clear concentration-dependent property. A-4166 at 0.3 mmol/L and tolbutamide at 3 mmol/L exhibited maximal stimulation of insulin release to a similar extent, indicating that A-4166 is one log-order more potent than and as effective as tolbutamide. By contrast, A-4166 stimulated somatostatin release to a threefold greater extent than tolbutamide. A-4166 evoked an increase in the cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat pancreatic β cells. $[Ca^{2+}]_i$ and insulin secretory responses to A-4166 were inhibited by nitrendipine (NTD), a blocker of the L-type Ca^{2+} channel, and by diazoxide (DAZ), an opener of the adenosine triphosphate (ATP)-sensitive K^+ channel. Furthermore, A-4166–stimulated somatostatin release was also inhibited by NTD and by DAZ. The results indicate that A-4166 and tolbutamide stimulate the release of insulin and somatostatin, and that A-4166 is much more effective than tolbutamide in releasing somatostatin, a hormone that attenuates hyperglycemia under certain circumstances. It is concluded that A-4166–induced insulin release is mediated by an increase in $[Ca^{2+}]_i$ in β cells. An inhibition of ATP-sensitive K^+ channels and a consequent activation of L-type Ca^{2+} channels appear to play a key role not only in insulin secretion from β cells, but also in somatostatin secretion from δ cells in response to A-4166.**

SULFONYLUREAS have been widely used to treat patients with non–insulin-dependent diabetes mellitus. However, some patients experience excessive hypoglycemia induced by sulfonylureas[1] or a failure to respond to the agents after long-term therapy.[2,3] Numerous efforts have been undertaken to develop new hypoglycemic agents without these defects.[1]

N-[(*trans*-4-isopropylcyclohexyl)-carbonyl]-D-phenylalanine (A-4166) is a new oral hypoglycemic agent, the chemical structure of which is distinct from any of the known hypoglycemic agents.[4,5] A-4166 exhibits hypoglycemic action in a more rapid and shorter-lasting manner than sulfonylureas. The rapid and short-term hypoglycemic action made it possible to decrease postprandial blood glucose level preferentially without inducing significant delayed hypoglycemia.[6]

The hypoglycemic effect of A-4166 is mainly due to the stimulation of insulin release.[6,7] It was recently shown that A-4166 increases cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat pancreatic β cells, and the $[Ca^{2+}]_i$ increase may be related to insulin release.[8] However, in that study, $[Ca^{2+}]_i$ and insulin release were measured under different experimental conditions. Therefore, a definitive functional link between the β-cell $[Ca^{2+}]_i$ increase and insulin release, as well as further insulinotropic mechanisms of A-4166, remain to be determined. Moreover, little is known about the effect of A-4166 on the release of pancreatic hormones other than insulin. Glucagon, released from islet α cells, is a major physiologic hormone that accounts for the elevation of blood glucose levels and sometimes relates to hyperglycemia in diabetics.[9,10] It has been suggested that somatostatin, a hormone released from islet δ cells, affects carbohydrate metabolism by a paracrine influence on both glucagon and insulin release[11] and also by extrapancreatic effects,[12,13] although a physiological relevance of the paracrine role of somatostatin is still in controversy because a directed microcirculation of β to α to δ cells in islets argues against it.[14] Somatostatin injection has been shown to counteract the progression of hyperglycemia and ketosis in diabetics.[10,15,16] In the present study, we attempted to determine the effect of A-4166, in comparison to a sulfonylurea, tolbutamide, on the release of insulin, glucagon, and somatostatin from isolated perfused rat pancreas, and to investigate the mechanisms for the hormone release. We report here that A-4166, more potently than tolbutamide, stimulates the release of somatostatin and insulin but not glucagon, and that a stimulated Ca^{2+} influx through the L-type Ca^{2+} channel, due to blockade of the adenosine triphosphate (ATP)-sensitive K^+ channel, appears to play a key role in somatostatin secretion from δ cells and in insulin secretion from β cells in response to A-4166.

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MATERIALS AND METHODS

Perfusion of Rat Pancreas

Male Wistar rats (Charles River, Yokohama, Japan) aged 8 to 15 weeks and fed ad libitum were studied following an overnight fast. The animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight). Pancreases were isolated and perfused using the system of Grodsky et al[17] with some modifications. The basal perfusion medium consisted of 140 mmol/L NaCl, 4.0 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.0 mmol/L

$MgCl_2$, and 10 mmol/L HEPES, pH 7.4, supplemented with 4% (wt/vol) dextran and 3 mmol/L glucose without bovine serum albumin (BSA). The flow rate was 2 mL/min. Pancreases were first perfused with the basal medium for 20 minutes, and then with the medium containing stimulus with or without inhibitor. Perfusate was collected into a cooled glass tube containing aprotinin (1,000 U per tube) and EDTA-2Na (2 mg per tube). The samples were immediately frozen and stored at −20°C until assay of pancreatic hormones.

Measurement of Hormones

Immunoreactive insulin, glucagon, and somatostatin were determined by a double-antibody radioimmunoassay using commercial kits (respectively: Pharmacia, Uppsala, Sweden; Daiichi Radio Isotope, Tokyo, Japan; and Incstar, Stillwater, MN). The calculated values are expressed as the mean ± SEM. Statistical analysis was performed using an unpaired Student's *t* test.

Measurement of $[Ca^{2+}]_i$ in Rat Pancreatic β Cells

$[Ca^{2+}]_i$ measurements in single-islet β cells were performed according to a previously described method[18] with some modification. In brief, islets were isolated from Wistar rats by collagenase digestion and further dispersed into single cells under a Ca^{2+}-depleted condition with 1 mmol/L EGTA. The single cells were plated on cover slips and maintained in short-term culture for up to 4 days in Eagle's minimum essential medium containing 5.6 mmol/L glucose. The single cells were incubated with 1 μmol/L fura-2/acetoxymethylester (AM)[19] for 30 minutes at 37°C. Fura-2–loaded cells were mounted in a superfusion chamber on the stage of an inverted TMD microscope (Nikon, Tokyo, Japan) and were superfused at 37°C at 1 mL/min with Krebs Ringer bicarbonate buffer (KRB) in the absence of BSA with a basal glucose concentration (3 mmol/L). Fura-2 fluorescence at 510 nm following excitation at 340 and 380 nm every 3 seconds was detected by an intensified change-coupled device camera, and the ratio was produced using an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan).[20] The ratio was converted to $[Ca^{2+}]_i$ according to calibration curves. We took the data from cells that exhibited increases in $[Ca^{2+}]_i$ in response to glucose and tolbutamide, responses typical of β cells.[20]

Chemicals

A-4166 and nitrendipine (NTD) were synthesized in our laboratory. Tolbutamide and diazoxide (DAZ) were obtained from Sigma (St Louis, MO). Fura-2–free acid, fura-2/AM, EDTA-2Na, and EGTA were purchased from Dojin Chemical (Kumamoto, Japan). Fetal bovine serum was obtained from Gibco (Grand Island, NY), and dextran was from Wako Pure Chemical (Osaka, Japan). BSA was purchased from Boehringer (Mannheim, Germany).

RESULTS

Effects of A-4166 and Tolbutamide on Hormone Release From Perfused Rat Pancreas

The effects of A-4166 on hormone release from perfused pancreas were investigated and compared with those of tolbutamide. In the presence of basal glucose (3 mmol/L), both A-4166 and tolbutamide markedly stimulated insulin release in a dose-dependent manner. Upon exposure to the agent, insulin release took place rapidly, peaked at 1 to 3 minutes, and then gradually declined toward baseline (Fig 1A and B). The threshold concentration of A-4166 required to stimulate insulin release appeared to exist at approximately 0.03 μmol/L, and the maximally effective concentration at approximately 0.3 μmol/L, and those of tolbutamide were at 0.3 and 3 μmol/L, respectively (Fig 2A). Thus, A-4166 was one log-order more potent than tolbutamide. In contrast, A-4166 and tolbutamide failed to elicit a clear, dose-related effect on glucagon release in the presence of 3 mmol/L glucose, although a slowly progressive elevation took place (Fig 1C and D). In addition, a rapid elevation of glucagon release, although small and transient, was observed with A-4166 and high-dose tolbutamide. Both A-4166 and tolbutamide dose-dependently stimulated somatostatin release, which occurred in a longer-lasting manner than insulin release (Fig 1E and F). At equipotent concentrations of A-4166 and tolbutamide, A-4166 elicited approximately a threefold greater stimulation of somatostatin release than tolbutamide (Fig 2B). As the glucose concentration increased, A-4166 at lower doses stimulated insulin release (data not shown).

Inhibition of A-4166–Induced Insulin Release From Perfused Pancreas and $[Ca^{2+}]_i$ Increase in β Cells by a Ca^{2+} Channel Blocker

The stimulated insulin release by 3 μmol/L A-4166 was inhibited by NTD, a blocker of voltage-dependent L-type Ca^{2+} channels[21] (Fig 3A). Inhibition took place with NTD at 1 to 10 μmol/L, the concentration range at which the drug was previously shown to abolish the peptide-stimulated insulin release and $[Ca^{2+}]_i$ increase in β cells in a reversible manner.[18,20] Inhibition by NTD was statistically significant at 2 minutes after A-4166 stimulation when the response peaked ($P < .05$ *v* A-4166 alone by unpaired Student's *t* test). Thus, a stimulated Ca^{2+} influx through dihydropyridine-sensitive L-type Ca^{2+} channels was suggested.

A crucial role for cytosolic free Ca^{2+} in the control of insulin release from β cells has been well demonstrated.[22,23] We examined the effect of A-4166 on $[Ca^{2+}]_i$ in β cells under the same experimental conditions as used for the study of hormone release. Under superfusion in KRB with 3 mmol/L glucose and without BSA, A-4166 induced an increase in $[Ca^{2+}]_i$ in single rat pancreatic β cells (Fig 3B), and a maximal effect was obtained at 3 μmol/L, the concentration at which a maximal stimulation of insulin release occurred. In the presence of 1 μmol/L NTD, the $[Ca^{2+}]_i$ response to 3 μmol/L A-4166 in individual β cells was either markedly attenuated in amplitude (four of seven cells) or completely inhibited (three of seven cells) in a reversible manner (Fig 3B). The results indicated that A-4166–stimulated insulin release was mediated by the increase in $[Ca^{2+}]_i$ in β cells due to a stimulated Ca^{2+} influx through L-type Ca^{2+} channels.

Inhibition of A-4166–Induced Insulin Release From Perfused Pancreas and $[Ca^{2+}]_i$ Increase in β Cells by an ATP-Sensitive K^+ Channel Opener

The ATP-sensitive K^+ channel is known to be a major determinant of the resting membrane potential of pancreatic β cells, thereby controlling the activity of voltage-dependent L-type Ca^{2+} channels, $[Ca^{2+}]_i$, and insulin secre-

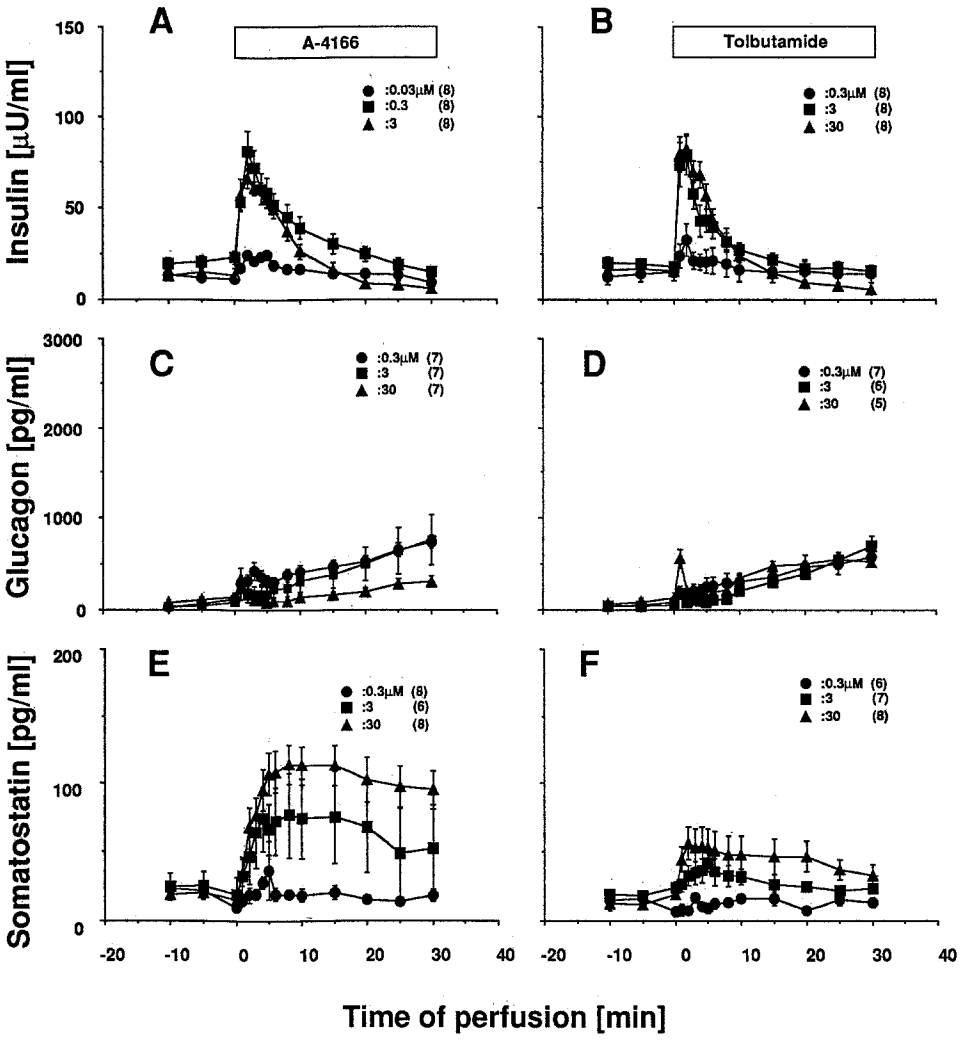

Fig 1. Effects of A-4166 and tolbutamide on hormone release from isolated rat pancreas. (A) and (B) A-4166 and tolbutamide stimulated insulin release in a concentration-dependent manner. (C) and (D) A-4166 and tolbutamide elevated the level of glucagon release; however, the change lacked a clear concentration-dependent property. (E) and (F) A-4166 and tolbutamide concentration-dependently stimulated somatostatin release. Horizontal bars above the graphs indicate the duration of exposure to A-4166 or tolbutamide. All data are expressed as the mean ± SEM.

tion.[24,25] To examine a possible involvement of this channel in the action of A-4166, the effect of DAZ, an opener of the ATP-sensitive K^+ channel, was tested. In the presence of DAZ (400 μmol/L), stimulated insulin release by 3 μmol/L A-4166 was completely inhibited ($P < .05$ *v* A-4166 alone for the response peak at 2 minutes; Fig 4A). The $[Ca^{2+}]_i$ response to 3 μmol/L A-4166 in β cells was also completely inhibited by 400 μmol/L DAZ (Fig 4B). The results suggested that a closure of ATP-sensitive K^+ channels may be involved in triggering the Ca^{2+}-dependent secretory process.

Inhibition of A-4166–Induced Somatostatin Release From Perfused Pancreas by a Ca^{2+} Channel Blocker and an ATP-Sensitive K^+ Channel Opener

The stimulated somatostatin release by 3 μmol/L A-4166 was inhibited by NTD at 1 μmol/L ($P < .05$ *v* A-4166 alone for the response peak at 10 minutes) and also by DAZ at 400 μmol/L ($P < .05$ *v* A-4166 alone for the response peak at 10 minutes), concentrations at which the drugs abolished insulin release (Fig 5). The results suggested that a closure of ATP-sensitive K^+ channels and an activation of L-type Ca^{2+} channels may be involved in A-4166–stimulated somatostatin release from pancreatic δ cells.

DISCUSSION

The present study demonstrated that a nonsulfonylurea oral hypoglycemic agent, A-4166, in the presence of basal 3 mmol/L glucose stimulated insulin and somatostatin release from perfused rat pancreases in a concentration-dependent manner. Tolbutamide also stimulated the release of insulin and somatostatin. A-4166 and tolbutamide elevated the rate of glucagon release; however, the change lacked a clear, concentration-related property. This is in accord with previous reports that sulfonylureas stimulate pancreatic secretion of insulin and somatostatin, whereas their effect on glucagon release varies in the literature, presumably due to differences in experimental conditions.[9] Insulin release was maximally stimulated by A-4166 at 3 μmol/L and by tolbutamide at 30 μmol/L, indicating that A-4166 was one log-order more potent. The maximal increment in insulin release was the same for the two agents.

Somatostatin release was extensively stimulated by A-4166 at 3 μmol/L but only mildly by tolbutamide at 30 μmol/L,

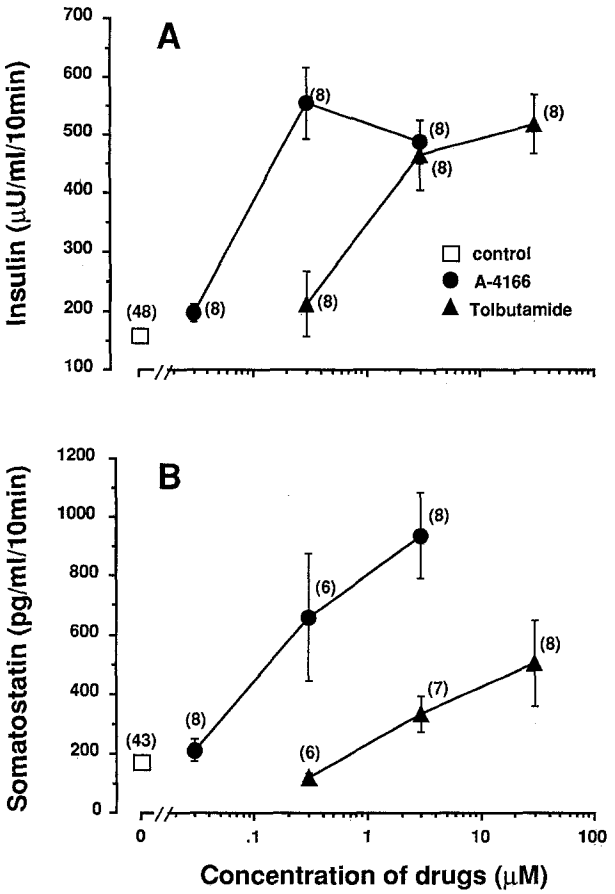

Fig 2. Concentration-response characteristics for A-4166 and tolbutamide to stimulate insulin release (A) and somatostatin release (B). Each point represents the mean ± SEM. Numbers in parentheses indicate the number of experiments.

concentrations at which the two agents exhibited equivalent and maximal effects on insulin release. It has thus been revealed that A-4166 is a notable somatostatin secretagogue, as well as an insulin secretagogue. It is known that somatostatin, despite its inhibitory effect on insulin release, counteracts the progression of hyperglycemia in some diabetics.[10,15] It is suggested that the action of somatostatin to attenuate hyperglycemia is due to (1) inhibition of release of glucagon, a hormone sometimes linked to hyperglycemia in some diabetics,[10,16] (2) delay of absorption of carbohydrate by the intestine,[13] and (3) inhibition of glucagon-stimulated[12] and nerve-stimulated[26] glucose output from the liver. It was also shown that somatostatin 14, the predominant form present in pancreatic islet δ cells,[27] inhibits glucagon release more potently than insulin release.[28] Taken together, the action of A-4166 to stimulate somatostatin release from the pancreas could represent its potential advantage as a hypoglycemic agent.

A-4166–stimulated insulin release was completely inhibited by 400 μmol/L DAZ, an opener of the ATP-sensitive K^+ channel.[24,25] ATP-sensitive K^+ channels play a crucial role in the regulation of insulin secretion, and the major action of sulfonylurea is believed to be closure of this channel.[25,29] On the other hand, inhibition of ATP-sensitive K^+ channels and subsequent insulin release have also been found to occur with a nonsulfonylurea compound, linoglride,[30,31] as well as with the nonsulfonylurea moiety of parent compounds, including HB699 derived from glibenclamide,[32] UL-DF9 derived from gliquidone,[33] and AZ-DF-265.[34] Therefore, it is not surprising that A-4166 could also cause insulin secretion by closing ATP-sensitive K^+ channels in pancreatic β cells.

We have previously shown that A-4166 increases $[Ca^{2+}]_i$ in rat pancreatic β cells, and suggested a possible link between the $[Ca^{2+}]_i$ increase and insulin release. However, concentrations of A-4166 and BSA used in the $[Ca^{2+}]_i$ study were different from those in the insulin-release study.[8] In

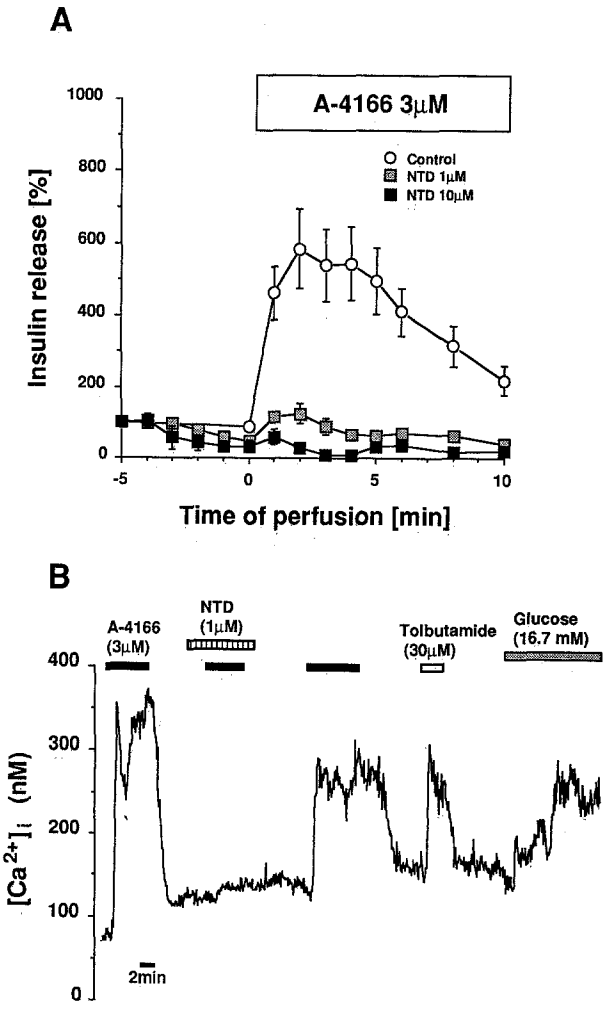

Fig 3. Inhibition of A-4166–induced insulin release and $[Ca^{2+}]_i$ increase by a Ca^{2+}-channel blocker, NTD. (A) Stimulation of insulin release from perfused rat pancreases by 3 μmol/L A-4166 was inhibited by 1 to 10 μmol/L NTD. Each point represents the mean ± SEM for 8 experiments in control, 4 in 1-μmol/L NTD, and 3 in 10-μmol/L NTD. Baseline release rate at −5 minutes, when treatment with NTD started, was normalized as 100%. (B) A-4166 at 3 μmol/L increased $[Ca^{2+}]_i$ in single rat pancreatic β cells under superfusion condition with 3 mmol/L glucose. A-4166–induced increase in $[Ca^{2+}]_i$ was inhibited by 1 μmol/L NTD, followed by a recovery upon washing out the drug. Cells subsequently responded to tolbutamide and high glucose. This is representative of 7 similar experiments.

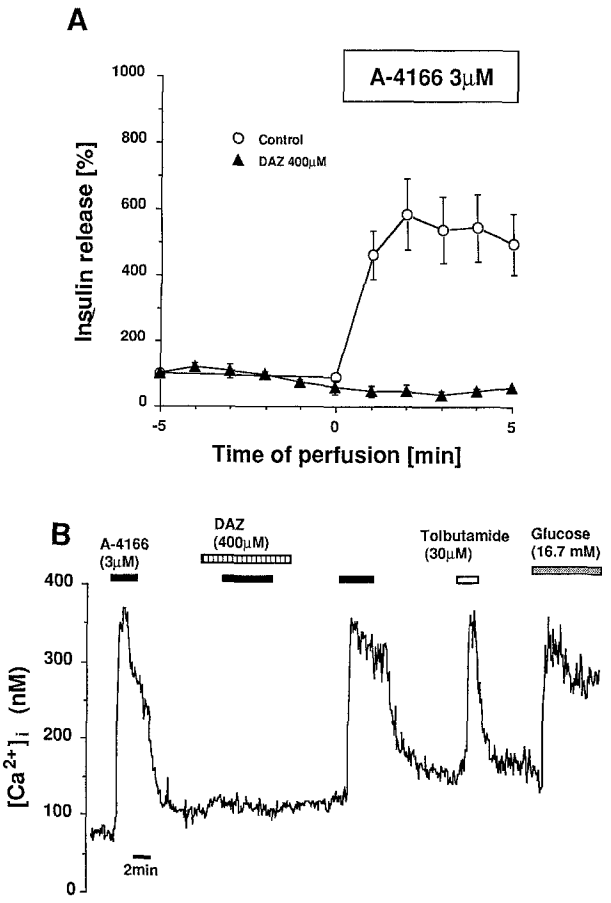

Fig 4. Inhibition of A-4166–induced insulin release and $[Ca^{2+}]_i$ increase by an ATP-sensitive K^+ channel opener, DAZ. (A) Stimulation of insulin release from perfused rat pancreas by 3 μmol/L A-4166 was inhibited by 400 μmol/L DAZ. Each point represents the mean ± SEM for 8 experiments in control and 3 in DAZ. Baseline release rate at −5 minutes, when treatment with DAZ started, was normalized as 100%. (B) 3-μmol/L A-4166–induced increase in $[Ca^{2+}]_i$ in single rat pancreatic β cells under superfusion condition was inhibited by 400 μmol/L DAZ. This is representative of 6 similar experiments.

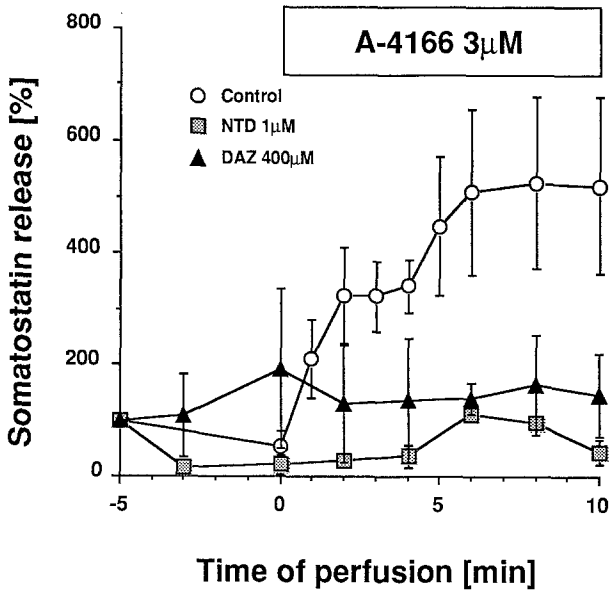

Fig 5. Inhibition of A-4166–induced somatostatin release by NTD and DAZ. Stimulation of somatostatin release from perfused rat pancreases by 3 μmol/L A-4166 was inhibited by 1 μmol/L NTD and by 400 μmol/L DAZ. Each point represents the mean ± SEM for 3 experiments. Baseline release rate at −5 minutes, when treatment with NTD and DAZ started, was normalized as 100%.

the present study, we found that A-4166 at the same concentration (3 μmol/L) increased both insulin release and $[Ca^{2+}]_i$ under the same perfusion condition with 3 mmol/L glucose and without BSA. Moreover, when the $[Ca^{2+}]_i$ response was inhibited by a Ca^{2+} channel blocker, NTD, and by an ATP-sensitive K^+ channel opener, DAZ,[24] the insulin secretory response was also inhibited. It has thus been proved that the increase in $[Ca^{2+}]_i$, due to a stimulated Ca^{2+} influx through L-type Ca^{2+} channels in the β-cell plasma membrane, mediates insulin release in response to A-4166.

A remarkable finding of this study is that somatostatin release induced by A-4166 was completely inhibited by NTD, an inhibitor of L-type Ca^{2+} channels, and by DAZ, an opener of ATP-sensitive K^+ channels. The inhibition by NTD suggests that a stimulated extracellular Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels may be involved in somatostatin release by A-4166. In accordance with this observation, it has been shown that somatostatin release by glibenclamide[35,36] and veratridine[37] strictly required extracellular Ca^{2+}, and that a voltage-dependent Ca^{2+} influx might be involved in somatostatin release induced by veratridine.[37] Inhibition of somatostatin release by DAZ and NTD occurred at concentrations of these agents at which insulin release was inhibited (Fig 5 *v* Figs 3 and 4). The present result suggests a key role of ATP-sensitive K^+ channels and L-type Ca^{2+} channels not only in insulin secretion but also in somatostatin secretion from pancreatic δ cells. It could provide a mechanistic basis for the long-postulated similarity between insulin secretion and somatostatin secretion reflected by notably common secretagogues, including glucose, sulfonylureas, leucine, glucagon, and glucagon-like peptide-1(7-36) amide.[11,35,38]

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