IN VITRO INSULINOTROPIC ACTION OF A NEW NON-SULFONYLUREA HYPOGLYCEMIC AGENT, CALCIUM (2S)-2-BENZYL-3-(CIS-HEXAHYDRO-2-ISINDOLINYLCARBONYL) PROPIONATE DIHYDRATE (KAD-1229), IN RAT PANCREATIC B-CELLS

HIDEKI OHNOTA, * MIHO KOBAYASHI, TAKASHI KOIZUMI, KENJI KATSUNO, FUMIYASU SAT0 and TORU AIZAWA †

Central Research Laboratories, Kissei Pharmaceutical Co. Ltd., Nagano-ken; and
†Department of Geriatrics, Endocrinology and Metabolism, School of Medicine, Shinshu University, Nagano-ken, Japan

(Received 7 June 1994; accepted 21 September 1994)

Abstract—We examined the in vitro insulinotropic action of a novel non-sulfonylurea compound, calcium (2S)-2-benzyl-3-(cis-hexahydro-2-isoindolinyl-carbonyl) propionate dihydrate (KAD-1229), which is a succinate derivative, using rat pancreatic islets and perfused pancreas. The sodium salt of KAD-1229 free acid (KAD-1229-Na) stimulated insulin secretion from isolated rat islets and perfused rat pancreas in a concentration-dependent manner at 0.1 to 10 µM. It produced a predominant first phase and a less prominent second phase response in the presence of 5.55 mM glucose. An ATP-sensitive K+ (K+ATP) channel activator, diazoxide, eliminated the insulinotropic effect of KAD-1229-Na. Glucose primed the B-cell in the perfused pancreas, but KAD-1229-Na did not. When the insulinotropic effects of 16.7 mM glucose on isolated rat islets were inhibited submaximally by 1 nM norepinephrine, the addition of 1 nM KAD-1229-Na reversed this inhibition. All of these insulinotropic effects of KAD-1229-Na were qualitatively indistinguishable from those of sulfonylurea compounds. We conclude that KAD-1229-Na acts on K+ATP channels of pancreatic B-cells despite its non-sulfonylurea structure.

Key words: KAD-1229; sulfonylurea; insulin secretion; perfusion of pancreas

We have developed a novel oral hypoglycemic agent, KAD-1229 $ (Fig. 1), which possesses a rapid-onset and short-lasting hypoglycemic effect compared with sulfonylureas in rats and beagles [1]. This compound is expected to suppress post-prandial hyperglycemia in patients with type 2 diabetes mellitus with less hypoglycemic side-effects. An in vitro insulinotropic effect of KAD-1229 was demonstrated in isolated mouse pancreatic islets and HIT T15 insulinoma cell lines in the presence of 5.55 mM glucose [1]. We also found that the drug displaces [3H]glibenclamide from the membrane of HIT T15 cells and inhibits 86Rb+ efflux from these cells. These results indicate that KAD-1229 interacts with the putative sulfonylurea binding site(s), the K+ATP channel. Mogami et al. [2] demonstrated that KAD-1229-Na inhibits K+ATP channels on excised membrane patches from a transgenic mouse B-cell line, MIN 6. All of these results demonstrate that KAD-1229 acts directly on K+ATP channels of the B-cell. In the present study, we examined the insulinotropic action of KAD-1229, using rat pancreatic tissue, in order to further clarify the mechanism of action of this novel succinate derivative.

It is well known that a high concentration of glucose elicits biphasic insulin secretion [3, 4], owing to complex signal transduction [5–10]. An involvement of protein kinase C in the second phase response is also suggested [6]. On the other hand, sulfonylureas, inhibitors of the K+ATP channel [11, 12], have been reported to induce a biphasic or monophasic insulin secretion, depending on the experimental conditions [4, 13, 14]. Accordingly, we compared the temporal profile of insulin secretion induced by KAD-1229-Na, which is likely to be a non-sulfonylurea K+ATP channel inhibitor, with that induced by a high concentration of glucose.

* Corresponding author: Hideki Ohnota, Kissei Pharmaceutical Co. Ltd., 4-63-1 Kashiiwara Hotaka, Minamiazumi Nagano-ken 399-83, Japan. Tel. 81-263-82-8820; FAX 81-263-82-8826.
† Abbreviations: KAD-1229, calcium (2S)-2-benzyl-3-(cis-hexahydro-2-isindolinyl-carbonyl) propionate dihydrate; KAD-1229-Na, sodium salt of KAD-1229 free acid; K+ATP channel, ATP-sensitive K+ channel; KRB, K+Ringer bicarbonate; and IRI, immunoreactive insulin.
**Fig. 2.** Insulinotropic effects of 16.7 mM glucose, KAD-1229-Na and gliclazide (GZ) on isolated rat islets. Rat islets were isolated and preincubated for 45 min in KRB buffer containing 5.55 mM glucose and 0.2% BSA. Batches of 3 islets were then incubated in 1 mL of the same buffer containing stimulant for 30 min. Immunoreactive insulin (IRI) in the culture supernatant was determined by radioimmunoassay kits. Data are expressed as means ± SEM (N = 5-15). Key: (***) significantly different from the control (P < 0.01) by Duncan’s multiple comparison test; and (#) significantly different from the control (P < 0.05) by Student’s unpaired t-test.

ATP-sensitive K⁺ channel activators inhibit the insulinotropic action of various substances [15, 16]. We examined the effect of a K⁺ATP channel activator, diazoxide, on insulin release induced by KAD-1229-Na. The K⁺ATP channel activator is expected to eliminate KAD-1229-Na-induced insulin release if the insulinotropic action of the drug is essentially dependent on K⁺ATP channel closure. In addition, we also investigated the effect of norepinephrine on insulin release induced by KAD-1229-Na. The inhibitory effects of norepinephrine on insulin secretion are well established [17–21]. Norepinephrine activates the inhibitory GTP-binding protein, G₁₁, and causes depression of cyclic AMP [22]. We examined the interaction of KAD-1229-Na and the cAMP branch by the use of norepinephrine.

**MATERIALS AND METHODS**

**Chemicals.** KAD-1229-Na was synthesized in our laboratory. Diazoxide, glibenclamide, yohimbine and collagenase (Type XI) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.), and gliclazide and pentobarbital from Dainihonseiyaku (Osaka, Japan). Norepinephrine was obtained from Sankyo (Tokyo, Japan).

**Isolation of pancreatic islets and static incubation experiments.** Ten- to fifteen-week-old male Sprague-Dawley rats were purchased from SLC (Shizuoka, Japan). The islets were obtained by collagenase dispersion [23]. In brief, the distal end of the common bile duct was clamped, and 15 mL of ice-cold KRB buffer containing 120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 5.55 mM glucose, 25 mM NaHCO₃, 5 mM HEPES, 0.2% BSA (equilibrated with 95% O₂/5% CO₂, pH 7.4), with 4 mg/mL collagenase was injected into the common bile duct to disrupt the exocrine tissue; then the pancreas was removed. Pancreatic tissue was incubated for 160 sec in a shaking water bath at 37°C. The digest was immediately cooled and washed with ice-cold KRB buffer. The islets were handpicked from the digest under a stereomicroscope.

Three islets/well were used in the incubation experiments. After preincubation in KRB buffer at 37°C for 45 min, the islets were incubated for 30 min in 1 mL KRB buffer containing test substances followed by the removal of the buffer, which was stored at −20°C for later determination of insulin.

**Perfusion of isolated rat pancreas.** The perfusion of the pancreas was performed as previously described [24] with minor modifications. Briefly, fed 12- to 15-week-old Sprague-Dawley rats were anesthetized with pentobarbital. All blood vessels located between the pancreas and other organs such as the spleen, the stomach, and the liver were ligated. The superior mesenteric as well as the celiac arteries were cannulated and used as the inlet of perfusion medium. The portal vein was used as the outlet. The common bile duct was also cannulated. The pancreas with an adjacent 5–6 cm of duodenum was excised and transferred to a container maintained at 37°C. Perfusion was begun at a flow rate of 2.0 mL/min.

The perfusion medium was modified KRB buffer containing 118 mM NaCl, 4.0 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5.55 mM glucose, 25 mM NaHCO₃, 4% dextran T-70 and 0.2% BSA. The glucose concentration was 5.55 mM. The perfusion medium was 2.0 mL/min.

The perfusion medium was modified KRB buffer containing 118 mM NaCl, 4.0 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5.55 mM glucose, 25 mM NaHCO₃, 4% dextran T-70 and 0.2% BSA. The glucose concentration was 5.55 mM, unless otherwise stated. After a 20-min stabilizing period, experimental perfusion was initiated, and the effluent was collected into ice-cold plastic tubes. A preparation with a
leakage of more than 3% of the total perfusion volume was discarded. In our system, the dead volume was approximately 3 mL, and the data are expressed with the dead volume uncorrected. Therefore, fractions collected between 7 and 36 min (see Results) contained perfusate from the pancreas exposed to the test substance.

Other procedures. Insulin was measured by commercially available radioimmunoassay kits (Eiken Chemical, Tokyo, Japan) with rat insulin as a standard. Glucagon was determined by radioimmunoassay kits (Daiichi, Tokyo, Japan) with pork glucagon as a standard. Data are presented as means ± SEM. Statistical analysis was performed using the Duncan’s multiple comparison test or Student’s unpaired t-test. P < 0.05 was considered significant.

RESULTS

Insulinotropic action of KAD-1229-Na in the islet incubation experiments (Fig. 2). In the presence of 5.55 mM glucose, KAD-1229-Na stimulated insulin release in a concentration-dependent manner from 0.1 to 100 μM. Gliclazide, a sulfonylurea, caused comparable effects at concentrations ranging from 1 to 100 μM. The maximum concentration of the two agents induced an approximately 10-fold increase in insulin release during a 30 min incubation. Under the same conditions, 16.7 mM glucose caused about a 45-fold increase in insulin release. Insulin release elicited by the combination of a supramaximum concentration (10 μM) of KAD-1229-Na and gliclazide was no more than that produced by either one of the drugs alone.

Insulinotropic action of KAD-1229-Na in pancreatic perfusion experiments. KAD-1229-Na produced a biphasic insulin release from perfused rat pancreas (Fig. 3). Compared with 16.7 mM glucose, the maximum concentration (10 μM) of KAD-1229-Na produced approximately the same magnitude of the first phase response with an attenuated second phase response (Fig. 3 and Table 1). Both phases of insulin release elicited by KAD-1229-Na were concentration dependent. In these experiments, a transient and slight increase in glucagon secretion by KAD-1229-Na was observed (data not shown).

Effects of the K⁺ATP channel activator, diazoxide, on insulin release induced by KAD-1229-Na. In the incubation experiments, 100 μM diazoxide eliminated insulin release induced by KAD-1229-Na, gliclazide and 16.7 mM glucose (Table 2). In perfused rat pancreas, the stimulation of insulin release by KAD-1229-Na was also inhibited completely by 100 μM diazoxide (Fig. 4). Quite interestingly, removal of both the high concentration

---

Table 1. Insulin released from the perfused pancreas in the first phase (7–10 min) and the second phase (11–36 min)

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>N</th>
<th>1st Phase</th>
<th>2nd Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1 mM glucose</td>
<td>9</td>
<td>159 ± 25</td>
<td>1092 ± 176</td>
</tr>
<tr>
<td>KAD-1229, 0.01 μM</td>
<td>3</td>
<td>14 ± 3*</td>
<td>30 ± 10*</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>9</td>
<td>85 ± 13</td>
<td>233 ± 25*</td>
</tr>
<tr>
<td>1 μM</td>
<td>9</td>
<td>104 ± 15</td>
<td>338 ± 54*</td>
</tr>
<tr>
<td>10 μM</td>
<td>8</td>
<td>124 ± 32</td>
<td>449 ± 101*</td>
</tr>
</tbody>
</table>

Each value was calculated from the data shown in Fig. 2. Data are expressed as means ± SEM. * Significantly different from 11.1 mM glucose (P < 0.01).

---

Table 2. Effect of diazoxide on insulin secretion induced by glucose, KAD-1229-Na and gliclazide

<table>
<thead>
<tr>
<th>Incubation buffer</th>
<th>IRI (ng/islet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (100 μM)</td>
<td></td>
</tr>
<tr>
<td>+ Diazoxide</td>
<td></td>
</tr>
<tr>
<td>Basal (5.55 mM glucose)</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>+ 11.1 mM glucose</td>
<td>5.50 ± 0.74*</td>
</tr>
<tr>
<td>+ KAD, 0.1 μM</td>
<td>1.54 ± 0.47*</td>
</tr>
<tr>
<td>+ Gliclazide, 0.3 μM</td>
<td>2.31 ± 0.30*</td>
</tr>
<tr>
<td>+ Gliclazide, 3 μM</td>
<td>2.80 ± 0.68*</td>
</tr>
</tbody>
</table>

Isolated rat islets were incubated for 30 min with various drugs as described in the legend of Fig. 2. Data are expressed as means ± SEM (N = 5). Statistical analysis was performed by Student’s unpaired t-test.

* Significantly different from the basal secretion (P < 0.01 and < 0.05, respectively).

‡, § Significantly different from the respective group without diazoxide (P < 0.01 and < 0.05, respectively).

---

Fig. 3. Insulin release from perfused rat pancreas by 11.1 mM glucose and KAD-1229-Na. Rat pancreata were isolated as described in Materials and Methods. After a stabilizing period of 20 min, portal vein effluent was fractionated and insulin contents were determined. Glucose (11.1 mM) (○, N = 9), 0.01 (○, N = 3), 0.1 (Δ, N = 9), 1 (□, N = 9) or 10 (□, N = 8) μM KAD-1229-Na was added from 6 min to the basal buffer containing 5.55 mM glucose. In this system, stimulated fractions were collected from 7 to 36 min. Each point represents the average of 3–9 observations. Most SEM bars were omitted for clarity.
of glucose and 100 µM diazoxide resulted in a rebound-like transient increase in insulin secretion in perfusion experiments (Fig. 4), which was observed consistently in three independent experiments. Such a "rebound" of insulin secretion upon the removal of diazoxide was not found when KAD-1229-Na or gliclazide was used as a secretagogue (Fig. 4).

**Effects of norepinephrine on insulin secretion induced by KAD-1229-Na and 16.7 mM glucose.**

Norepinephrine inhibited insulin release induced by KAD-1229-Na and glucose (Fig. 5). The minimum effective concentration of norepinephrine was 0.1 µM, and complete suppression was observed at 10 µM for both KAD-1229-Na- and glucose-stimulated insulin release. When insulin release in response to 16.7 mM glucose was inhibited

**KAD-1229-Na-induced recovery of insulin secretion inhibited by 1 µM norepinephrine (NE).** Isolated islets were incubated for 30 min with various combinations of drugs, and insulin contents were determined (N = 5). Data are expressed as means ± SEM. Key: (***) significantly different from the basal insulin secretion (C) (P < 0.01); and (#) and (##) significantly different from 16.7 mM glucose + 1 µM norepinephrine (E) (P < 0.05 and 0.01, respectively).
A novel non-sulfonylurea insulinotropic agent

Table 3. Effects of yohimbine, KAD-1229-Na, gliclazide and glibenclamide on inhibition of insulin release by norepinephrine

<table>
<thead>
<tr>
<th>Incubation buffer</th>
<th>IRI (ng/islet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal (5.55 mM glucose)</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>+ 11.1 mM glucose</td>
<td>5.98 ± 0.59*†</td>
</tr>
<tr>
<td>+ 11.1 mM glucose + 1 μM norepinephrine</td>
<td>1.45 ± 0.22†</td>
</tr>
<tr>
<td>+ 11.1 mM glucose + 1 μM norepinephrine + 0.1 μM yohimbine</td>
<td>2.28 ± 0.27*</td>
</tr>
<tr>
<td>+ 11.1 mM glucose + 1 μM norepinephrine + 1 μM yohimbine</td>
<td>2.67 ± 0.40*†</td>
</tr>
<tr>
<td>+ 11.1 mM glucose + 1 μM norepinephrine + 10 μM yohimbine</td>
<td>5.12 ± 0.52*†</td>
</tr>
<tr>
<td>+ 100 μM yohimbine</td>
<td>5.28 ± 0.50*†</td>
</tr>
<tr>
<td>+ 1 μM KAD-1229-Na</td>
<td>3.38 ± 0.45†</td>
</tr>
<tr>
<td>+ 1 μM KAD-1229-Na</td>
<td>2.91 ± 0.19†</td>
</tr>
<tr>
<td>+ 0.3 μM glibenclamide</td>
<td>2.70 ± 0.34*†</td>
</tr>
</tbody>
</table>

Rat islets were incubated as described in the legend of Fig. 2 with various drugs, and the insulin secreted was determined. Data are expressed as means ± SEM (N = 5).

* Significantly different from basal secretion (P < 0.01).
† Significantly different from basal + 11.1 mM glucose + 1 μM norepinephrine (P < 0.01).
‡ Significantly different from basal secretion (P < 0.05).
§ Significantly different from basal + 11.1 mM glucose + 1 μM norepinephrine (P < 0.05).

submaximally by 1 μM norepinephrine, the addition of 1 or 10 μM KAD-1229-Na significantly reversed this inhibition (Fig. 6). Reversal of norepinephrine-induced suppression of stimulated insulin release was also tested with the sulfonylureas gliclazide and glibenclamide and an α-blocker, yohimbine (Table 3). Ten micromolar gliclazide and 0.3 μM glibenclamide partially reversed norepinephrine inhibition of glucose-stimulated insulin release. On the other hand, yohimbine completely eliminated the inhibitory effect of norepinephrine as previously reported [18] (Table 3).

DISCUSSION

In the present study, we investigated the insulinotropic effect of a new non-sulfonylurea agent, KAD-1229, in rat pancreatic tissue. All of our experiments were performed with a basal glucose concentration of 5.55 mM, which is equivalent to a normal fasting plasma glucose level. By doing this, we attempted to analyze the action of KAD-1229-Na under a normal physiological condition.

In the incubation experiments, the minimum effective concentration of KAD-1229-Na was approximately 3–10 times lower than that of gliclazide. This is in accord with data obtained in mouse pancreatic islets and HIT T15 cells [1]. In that study, KAD-1229 displaced glibenclamide from its binding site(s) with a Kᵢ value 100–1000 times lower than that of gliclazide. The maximum insulin release produced by KAD-1229-Na in the presence of 5.55 mM glucose was smaller than that elicited by 16.7 mM glucose. This was expected because glucose has effects not only on K⁺ATP channels, but also on other sites to cause sustained insulin release [7, 8].

When KAD-1229-Na was added to the maximum concentration of gliclazide, no further increase in insulin secretion was observed. This fact suggests that KAD-1229, a novel succinate derivative, stimulates insulin release through the same mechanism as sulfonylurea, i.e. inhibition of the K⁺ATP channels.

We also examined the temporal profile of KAD-1229-Na-induced insulin release in perfused rat pancreas. The drug produced a first phase response with a small second phase response. It was apparent that the maximum concentration of KAD-1229-Na produced significantly less insulin release than 16.7 mM glucose because a large second phase response was absent in the case of KAD-1229-Na stimulation (Fig. 2 and Table 1). The temporal profile of gliclazide-induced insulin release was similar to that of KAD-1229-Na-induced insulin release (data not shown). When the perfusion was carried out using buffer containing 2.8 mM glucose, KAD-1229-Na and gliclazide produced only a first but not a second phase insulin response (data not shown). Therefore, these drugs cause the second phase insulin release only in the presence of a suprathreshold concentration of glucose. In isolated perfused pancreas, KAD-1229-Na stimulated glucagon release slightly (data not shown), as previously reported with a sulfonylurea [14].

We demonstrated that 100 μM diazoxide, an activator of K⁺ATP channels, eliminated the stimulation of insulin release induced by KAD-1229-Na. This finding indicates that the K⁺ATP channel inhibition is a mandatory step for the stimulation of insulin release by KAD-1229-Na. The absence of glucose, stimulation with 16.7 mM glucose and 100 μM diazoxide, for 30 min, which by itself had no effect on insulin release, was followed by a transient increase in insulin release upon removal of both substances. We interpret this phenomenon as follows. During the 16.7 mM glucose and 100 μM diazoxide treatment, the B-cell is primed with high glucose despite the absence of insulin release, as shown previously [25]. When diazoxide is removed, the membrane potential of B-cells shifts from the hyperpolarized level to the basal level. Such a small degree of membrane depolarization produces a brisk
insulin release in high glucose-primed B-cells. Björklund and Grill [25] reported protective effects of diazoxide on glucose-induced desensitization in vitro. Namely, in the islets perfused with 27 mM glucose plus diazoxide for 190 min, a re-challenge with 27 mM glucose produces a greater insulin release than in those perfused with high glucose alone [25]. A similar sensitizing mechanism may have been responsible for our observation, although pancreata were treated with glucose for a shorter period.

Norepinephrine is an important physiological regulator of insulin secretion. It has been demonstrated that norepinephrine binds to the α-adrenoceptors of the B cell [18, 19], and lowers cytoplasmic cyclic AMP levels and Ca²⁺ concentrations. Cyclic AMP is known to modulate voltage-dependent Ca²⁺ channels in cardiac [26] and B-cells [27]. Furthermore, cyclic AMP-dependent and Ca²⁺-independent potentiation of insulin release was reported [27]. We demonstrated that KAD-1229-Na reverses norepinephrine inhibition of glucose-stimulated insulin release. Gliclazide and glibenclamide showed a similar effect. Therefore, it is likely that K⁺-ATP channel closure is responsible for this effect of KAD-1229-Na. K⁺-ATP channels must have been closed completely with 16.7 mM glucose [28]. Thus, our observations indirectly suggest that norepinephrine inhibited glucose-induced closure of K⁺-ATP channels on B-cells. Alternatively, KAD-1229-Na and sulfonylureas may have some other effect(s), in addition to the inhibition of K⁺-ATP channels, to stimulate insulin release. Further studies are necessary to delineate the nature of the interaction of KAD-1229-Na and norepinephrine.

In summary, we systematically investigated the insulinotropic effect of a new non-sulfonylurea hypoglycemic agent, KAD-1229. The insulinotropic effect of KAD-1229 was pharmacologically distinguishable from that of sulfonylureas, and this agent, as well as sulfonylureas, will be useful in controlling plasma glucose levels.

REFERENCES


25. Björklund A and Grill V, B-cell insensitivity in vitro:

