The extracellular, G protein-linked Ca\(^{2+}\)-sensing receptor (CaSR), first identified in the parathyroid gland, is expressed in several tissues and cells and can be activated by Ca\(^{2+}\) and some other inorganic cations and organic polycations. Calcimimetics such as NPS (R)-(3-phenylpropyl)-α-methyl-3-methoxybenzylamine hydrochloride (R-467), a phenylalkylamine, are thought to activate CaSR by allosterically increasing the affinity of the receptor for Ca\(^{2+}\). When tested for its effect on insulin release in C57BL/6 mice, R-467 had no effect under basal conditions but enhanced both phases of glucose-stimulated release. The βIC9 cell also responded to R-467 and to the enantiomer S-467 with a stimulation of insulin release. In subsequent studies with the βIC9 cell, it was found that the stimulatory effect was due to activation of a nonspecific cation channel, depolarization of the β-cell, and increased Ca\(^{2+}\) entry. No other stimulatory mechanism was uncovered. The depolarization of the cell induced by the calcimimetic could be due to a direct action on the channel or via the CaSR. However, it appeared not to be mediated by G\(_{\alpha}\), G\(_{\alpha}\), or G\(_{\beta\gamma}\). The novel mode of action of the calcimimetic, combined with the glucose-dependence of the stimulation on islets, raises the possibility of a totally new class of drugs that will stimulate insulin secretion during hyperglycemia but which will not cause hypoglycemia.

The G protein-linked, extracellular Ca\(^{2+}\)-sensing receptor (CaSR)\(^1\) from the bovine parathyroid gland was first cloned and characterized in 1993 (1). Subsequently, the receptor was found to be expressed in nerve terminals (2) and several other cells and tissues. These include thyroid C cells (3), kidney (4), hippocampus (5), stomach (6), intestine (7), lens epithelial cells (8), human insulinoma (9), and rat islets (10, 11). Although the primary role of the CaSR is assumed to be the regulation of serum Ca\(^{2+}\) levels by parathyroid hormone and calcitonin, its wide distribution suggests the possibility that it subserves several and perhaps many additional functions.

The CaSR was identified in a human insulinoma by RT-PCR (9) and in rat pancreatic islets by immunofluorescence and RT-PCR (10, 11). Interestingly, it has been known for some time that high extracellular Ca\(^{2+}\) stimulates insulin release from human insulinoma. This effect is used clinically in the selective intraarterial calcium injection test for the detection of small insulinomas (12, 13), and in the investigation of persistent hyperinsulinemic hypoglycemia of infancy (14). In view of these findings, we examined the potential significance of the CaSR in pancreatic β-cell function. To do this we employed the phenylalkylamine calcimimetic drug (R)-(3-phenylpropyl)-α-methyl-3-methoxybenzylamine hydrochloride (R-467). This compound is an agonist at the CaSR and is thought to act by an allosteric increase in the affinity of the receptor for Ca\(^{2+}\) and other interacting cations (15–17). We report here that R-467 stimulates insulin secretion in isolated pancreatic islets from C57BL/6 mice and in βHC9 cells. Because R-467 has a novel mechanism of action, and because in islets it only stimulates insulin release in the presence of elevated glucose concentrations, there is the potential for development of new drugs for the treatment of diabetes which are unlikely to cause hypoglycemia.

**EXPERIMENTAL PROCEDURES**

**Materials—**R-467 and its enantiomer S-467 were obtained from NPS Pharmaceuticals (Salt Lake City, UT) as was a polyclonal antibody against the C-terminal region (214–235 of the hCaSR protein (courtesy of Drs. Allen Spiegel and Paul Goldsmith). Staurosporine and Ro-31-8220 were from Calbiochem (La Jolla, CA). Nitrendipine was from RBI. Diazoxide, N-methylglucamine, and tolbutamide were from Sigma. Maitotoxin was obtained from Alexis Corp., San Diego, CA.

**Isolation of Pancreatic Islets—**C57BL/6 mice (22–25 g) and Harlan Sprague-Dawley rats (200–250 g) were used in this study and had access to food and water throughout. After CO\(_2\) asphyxiation, the pancreas was surgically removed and the islets isolated by collagenase digestion (18).

**Insulin Secretion under Perfusion Conditions—**A Krebs-Ringer bicarbonate buffer (KRB) containing (in mM) 129 NaCl, 5 NaHCO\(_3\), 4.8 KCl, 1.2 KH\(_2\)PO\(_4\), 1 CaCl\(_2\), 1.2 MgSO\(_4\), 10 HEPES at pH 7.4 and 0.1% bovine serum albumin was used for the insulin secretion studies with islets. A protocol slightly modified from that originally described (19) was used. Briefly, 20 rodent islets were placed into 70-μl perfusion chambers. They were equilibrated by perfusion at 1 ml/min for 40–45 min with KRB and 2.8 mM glucose at 37 °C. This was followed by the test period and collection of samples for radioimmunassay at 1-min intervals (still at a flow rate of 1 ml/min). The glucose concentration in the various experiments is specified in the legends, and the term basal glucose refers to a concentration of 2.8 mM glucose.

**Cell Culture—**βHC9 cells were cultured in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 1 mM pyruvate, 2.5% fetal bovine serum, and 15% horse serum. The medium was supplemented with 100 μg/ml streptomycin and 100 units/ml penicillin. The cells were kept at 37 °C in a 95% air and 5% CO\(_2\) atmosphere. The buffer used for the measurement of insulin secretion, intracellular Ca\(^{2+}\) (fura-2) and membrane potential (bisoxonol) experiments was identical to the KRB used for the islet studies with the exception that gelatin was used

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\(^1\) The abbreviations used are: CaSR, Ca\(^{2+}\)-sensing receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; PTX, pertussis toxin; KRB, Krebs-Ringer bicarbonate buffer; R-467, (R)-(3-phenylpropyl)-α-methyl-3-methoxybenzylamine hydrochloride; S-467, (S)-(3-phenylpropyl)-α-methyl-3-methoxybenzylamine hydrochloride; PL, phospholipase; K\(_{\text{ATP}}\), ATP-sensitive K\(^+\).

\(^2\) The paper is available on line at http://www.jbc.org
instead of albumin for the bioboxonol experiments. Cells ranging from passage 25 to 35 were used for the experiments.

**Insulin Secretion Measurements**—Under static incubation conditions, βHC9 cells (20) were plated in 16-mm-diameter wells at a density of 0.4 × 10^6 and cultured for 5–8 days. In all static experiments, the cells were preincubated with KRB for 30 min at a basal glucose concentration of 0.1 mmol/liter. This was followed by a 30-min exposure to the test compounds. At the end of this test period, aliquots of the medium were removed and frozen at −20 °C until radioimmunoassay. N-Methylglucamine was substituted for Na+ in the KRB for the Na+-free studies.

**Measurement of Intracellular Free Ca2+ Concentrations**—βHC9 cells, grown in 75-cm² flasks, were trypsinized and resuspended in KRB (pH 7.4) containing 1 μM fura-2-acetoxymethylester and 0.25 mM sulfonpyrazone. The loading took place in a shaking water bath at 37 °C for 30 min. After three washes in KRB, the cells were resuspended in a final volume of 12 ml and then transferred into heated quartz cuvettes in a spectrofluorometer (Perkin-Elmer Cetus Instruments LS-5) under continuous stirring. Excitation and emission wavelengths were set at 340 and 510 nm, respectively (21).

**Membrane Potential Measurements**—Membrane potential was monitored fluorimetrically using the fluorescent dye bisoxonol (22). βHC9 cells were suspended in KRB with 100 mM bisoxonol at a concentration of 10⁶ cells/ml. Gelatin (0.05%) was substituted for the bovine serum albumin to obviate interference with the assay. Three ml of the cell suspension was used for each cuvette and continuously stirred at 37 °C in the spectrofluorometer (Perkin-Elmer Cetus Instruments LS-5) until equilibrated. The excitation and emission wavelengths used were 530 and 580 nm, respectively. Test agents were introduced into the cuvettes when the emission signals were stable.

**Sample Preparation for Immunoblot Analysis**—For the preparation of whole cell lysates, cells were rinsed twice in ice-cold PBS, scraped off and transferred into Eppendorf tubes containing lysis buffer (in mM) 50 NaCl, 15.7 NaHPO₄, 1.47 KH₂PO₄, 2.68 KCl, 1 dithiothreitol, 1% Nonidet P-40, and protease inhibitors (50 μM leupeptin, 25 μg/ml aprotime, 10 μg pepstatin A and 100 μg/ml 4-(2-aminophenyl)-benzenesulfonfyllfluoride hydrochloride (Pefabloc, Roche Molecular Biochemicals). After a 20-min incubation in lysis buffer at 4 °C with shaking, cell debris was removed by centrifugation and the supernatant was stored at −80 °C for subsequent Western blot analysis. Parathyroid and kidney cells were prepared in the same buffer after the tissue was cut into small pieces and disrupted using 20–30 strokes with a Dounce homogenizer.

Crude plasma membranes were isolated from βHC9 and kidney membranes by homogenization in a hypotonic Tris buffer (20 mM, pH 7.4), containing 2 mM EDTA and 2 mM EGTA and the same protease inhibitors as above. After homogenization and cell debris sedimentation in a low speed spin, the supernatant was transferred to an ultracentrifuge and centrifuged for 1 h at 51,000 rpm at 4 °C. The resulting pellet was dissolved in Tris buffer containing 1% Triton X-100. Protein content was determined by Bradford assay.

**Western Blot Analysis**—Samples were separated by electrophoresis on a 7.5% polyacrylamide gel and transferred onto an Immobilon-N membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% bovine serum albumin and then incubated with the monoclonal ADD antibody at a dilution of 1:32,000 for 1 h at room temperature. Following several washes in Tris-buffered saline plus Tween (0.1%), the membrane was incubated for another 1 h in peroxidase-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech) at a dilution of 1:2000 and, after repeated washing steps, the protein of interest was detected with an ECL system (Amersham Pharmacia Biotech).

**Cyclic AMP Measurements**—Cyclic AMP was determined by means of the Biotrak cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

**Electrophysiological Recording**—The whole cell current recording configuration of the patch clamp technique was used (23). Pipettes were pulled from borosilicate glass capillary tubes to a tip diameter of 2–3 μm using a List L/M-3P-A pipette puller. Pipette resistance was typically 2–3 megohms. The pipette solution contained (in mM) 140 KCl, 35 KOH, 10 HEPES, 11 EGTA, 1 CaCl₂, 2 MgCl₂, and 5 tetraethylammonium chloride buffered to pH 7.3 (unless otherwise indicated). For experiments measuring currents in the absence of K⁺ channel effects, the pipette solution contained (in mM) 95 CsCl, 7 MgCl₂, and 5 HEPES (pH adjusted to 7.4 with NaOH). The extracellular solution contained (in mM) 128.8 NaCl, 4.8 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1 CaCl₂, 5 NaHCO₃, 10 HEPES, 2 glucose, 5 tetraethylammonium chloride, and 5 μM nitrendipine buffered to pH 7.4. For experiments requiring sodium-free buffer, N-methylglucamine was substituted for NaCl in the extracellular solution. For experiments employing high extracellular Ca²⁺, the extracellular solution contained (in mM) 90 CaCl₂, 6 KCl, 1.2 MgCl₂, 5 KHCOr, and 10 HEPES, pH 7.4. All solutions were filtered through a 0.2-μm sterile syringe filter prior to use. Recordings were obtained using an Axopatch 200B amplifier and passed through an internal low pass filter with a cutoff frequency of 1 KHz before being digitized at a sampling frequency of 10 KHz on a Gateway 2000 PC. PClamp6 (Fetchex) was used to analyze the data. All recordings were obtained at a holding potential of −60 mV at a temperature of 37 °C between 2 and 5 days after cell passage. Culture medium was removed from the 35-mm culture dish and extracellular solution added immediately prior to study. Studies were performed within 30 min of addition of extracellular solution. The current/voltage relationship of leak currents was determined by manually stepping the membrane potential to different levels. R-467 was then added over a 20-s period to a final concentration of 10 μM. A current/voltage relationship was then determined for the cell in the presence of R-467. Leak currents were then subtracted from the currents obtained in the presence of R-467, and the reversal potential calculated by linear regression of the current/voltage relationship of the currents corrected for leak.

**Statistical Analysis**—Data are expressed as mean ± S.E. Statistical significance was evaluated by using Student’s t test analysis for paired or unpaired samples as appropriate.

**RESULTS**

**Effects of R-467 on Isolated Pancreatic Islets of the Mouse**—The effects of R-467 on insulin release from isolated mouse pancreatic islets are shown in Fig. 1. From the data shown in the lower part of the figure, it can be seen that 10 μM R-467 failed to elicit any response from the islets under basal conditions in the presence of 2.8 mM glucose. However, when insulin release was stimulated by a higher glucose concentration, a marked potentiation of the release rate was seen (upper traces). The submaximally effective concentration of 11.1 mM glucose induced a prompt first phase of secretion followed by a second phase, which was characterized by a low plateau of stimulated release. In the presence of R-467, the islets showed increased rates of insulin release over both the first and second phases of the response. The release rate potentiated by R-467 was 2.6-

**FIG. 1.** The effect of 10 μM R-467 on insulin release by islets from C57BL/6 mice. All islets were pre-perifused with 2.8 mM glucose for 45 min prior to the zero time point shown on the figure. In the lower trace (filled squares), the islets were perfused with 2.8 mM glucose throughout and exposed to R-467 from 5 to 30 min. In the upper two traces, the islets were perfused with 8.3 mM glucose in the absence (open circles) or presence (closed circles) of 10 μM R-467. n = 4.
fold that of the 11.1 mM glucose-stimulated rate at the peak of the first phase, and 5–6-fold that of the second phase.

R-467 had no effect on insulin secretion from Harlan Sprague-Dawley rat islets when tested under both basal and glucose-stimulated conditions (data not shown).

Experiments on the Mechanism of Action of R-467—To facilitate these studies on the mechanism of action of R-467, experiments were performed on the mouse-derived βHC9 cell line (19). The effects of different concentrations of the enantiomers R-467 and S-467 were examined on insulin secretion from βHC9 cells under static incubation conditions. The results are presented in Fig. 2. In the presence of 16.7 mM glucose, R-467 stimulated insulin secretion at concentrations from 1 to 30 µM. At 10 and 30 µM, the rate of glucose-stimulated insulin release was doubled. At 100 µM, R-467 failed to stimulate release or, in some experiments, decreased the rate of release (data not shown). Similar data were obtained when S-467 was examined over the same concentration range showing that there is no stereoselectivity in the responses (Fig. 2). R-467 stimulated insulin secretion in the βHC9 cell at 0.1 mM glucose. Insulin release was 12.1 ± 0.7 pg/1000 cells/30 min at 0.1 mM glucose and 26.4 ± 1.5 pg/1000 cells/30 min in the presence of 10 µM R-467 ($p < 0.02$, $n = 4$).

Because the calcium receptors in the parathyroid gland and the parafollicular C cells mediate their effects via heterotrimERIC G proteins, the possibility of a link to $G_i$ or $G_o$ (pertussis toxin-sensitive G proteins) was tested. βHC9 cells were incubated for 24 h in the presence of 50 ng/ml pertussis toxin (PTX) and subsequently tested for their responses to 16.7 mM glucose and R-467. As can be seen from the results shown in Fig. 3, 16.7 mM glucose-stimulated insulin secretion was enhanced after PTX treatment, as anticipated from previous data (24). However, R-467 stimulated insulin secretion to a similar extent, regardless of whether they had been treated with PTX. In positive control experiments, treatment with PTX blocked the effect of norepinephrine to inhibit release. Thus, $G_i$ and $G_o$ proteins are not involved in the action of R-467.

We next investigated the effect of R-467 on intracellular Ca$^{2+}$ ([Ca$^{2+}$]) using βHC9 cells and the fura-2 technique. At 1 µM R-467, the threshold concentration for the stimulation of insulin secretion, no increase in [Ca$^{2+}$]$_i$ was detected. At concentrations that caused a marked stimulation of insulin secretion, between 3 and 30 µM, R-467 increased [Ca$^{2+}$]$_i$ at all concentrations tested. Examples of the increase in [Ca$^{2+}$]$_i$ caused by 3 and 10 µM R-467 are shown in Fig. 4 (A and B). R-467 did not increase [Ca$^{2+}$], in the absence of extracellular Ca$^{2+}$ (Fig. 4C), nor did it increase [Ca$^{2+}$], in the presence of the voltage-dependent L-type Ca$^{2+}$-channel blocker nitrendipine (Fig. 4D). Thus, R-467 is increasing [Ca$^{2+}$], by activating, directly or indirectly, the L-type voltage-dependent Ca$^{2+}$ channels in the β-cell. In order to activate voltage-dependent Ca$^{2+}$ channels, R-467 must either depolarize the cell or act as an L-type channel agonist as does the pharmacologic agent BayK 8644. Thus, the effect of R-467 on the membrane potential of βHC9 cells was monitored with the membrane potential-sensitive indicator bisoxonol. It was found that R-467 caused a prompt depolarization of the βHC9 cell, thus accounting for the activation of voltage-dependent Ca$^{2+}$ channels and increased [Ca$^{2+}$]$_i$. In Fig. 5A is shown the depolarizing effect of 10 µM R-467. The next question was how does R-467 depolarize the cell? To answer this, the effect of R-467 on the membrane potential of βHC9 cells was examined using bisoxonol under several different conditions. The results of some of these experiments are shown in Fig. 5. It can be seen that R-467 depolarizes the cell (Fig. 5A), even after hyperpolarization by a maximally effective concentration of diazoxide (Fig. 5B), and also after depolarization by 500 µM tolbutamide, which is a maximally effective concentration for inhibition of the ATP-sensitive K$^+$ (K$_{ATP}$) channel (Fig. 5C). Thus, R-467 is not depolarizing the cell by closing the K$_{ATP}$ channels. Depolarization was also observed in the presence of 100 µM triethylammonium (data not shown), so that the unlikely action to block voltage and Ca$^{2+}$-activated K$^+$ channels is ruled out. The possibility of a direct action on Ca$^{2+}$ channels and increased Ca$^{2+}$ entry was also ruled out because R-467 depolarized in the absence of extracellular Ca$^{2+}$ and presence of EGTA (Fig. 6A). The next possibility to be tested was that R-467 might be activating a cation channel other than a Ca$^{2+}$ channel, with a channel carrying Na$^+$, the most prevalent extracellular cation, being the most likely candidate. Thus, membrane potential was monitored under conditions in which Na$^+$ was absent from the extracellular medium and isotopically replaced by N-methylglucamine. The results are shown in Fig. 6B. In the absence of extracellular Na$^+$, R-467 failed to depolarize the βHC9 cell. Thus, Na$^+$ appears to be the charge-carrying source of the depolarization under normal conditions. As depolarization in the presence of Na$^+$ was not affected by tetrodotoxin (Fig. 6C), the most likely explanation for these data is that R-467 activates a nonspecific cation channel that carries mostly Na$^+$. This was confirmed by experiments with maitotoxin, a com-
pound that is known to activate nonspecific cation channels in the \( \beta \)-cell (25–27). In Fig. 7 are shown the results of these studies. Maitotoxin (1 nM) depolarized the cells and prevented any further depolarization by R-467 (Fig. 7A). In paired cells under control conditions, R-467 (10 \( \mu \)M) depolarized the \( \beta \)HC9 cells as expected.

It has been reported that glucagon-like peptide 1 and pituitary adenylate cyclase activating polypeptide activate a nonspecific channel in \( \beta \)-cells and cloned cell lines (25–27). Additionally, it is known that these peptides act via their specific heptahelical receptors, the activation of Gs, and subsequent increase in cyclic AMP levels. Consequently, cyclic AMP was measured in the \( \beta \)HC9 cells in the absence and presence of R-467, and in the presence of forskolin as a positive control. The results are shown in Table I. In the presence of 0.1 mM glucose, 3, 10, and 30 \( \mu \)M R-467, concentrations that depolarize the \( \beta \)HC9 cell and stimulate insulin release, had no effect on the cellular cyclic AMP content. In contrast, 1 \( \mu \)M forskolin used as a positive control induced a large increase in cyclic AMP. Thus, activation of the nonspecific cation channel by R-467 is not due to elevated cyclic AMP levels.

In additional studies on the mechanism of action, staurosporine and Ro-31-8220, two inhibitors of protein kinase C, had no effect on the stimulation of insulin release by R-467. Therefore, an action to depolarize the \( \beta \)-cell by activation of PKC, as has been noted previously in the RINm5F cell line (28), appears to be unlikely.

Further evidence corroborating the idea that R-467 depolarizes the cell, increases \([Ca^{2+}]_i\), and stimulates insulin secretion by activation of a nonspecific cation channel was obtained when the effect of R-467 on \([Ca^{2+}]_i\) was examined in \( Na^+ \)-free medium. Under these conditions, in which R-467 failed to depolarize the cell, the compound had no effect on \([Ca^{2+}]_i\) (data not shown). The possibility that R-467 could stimulate insulin release by a mechanism (or mechanisms) in addition to its ability to depolarize the cell was tested by seeking an effect of R-467 to enhance insulin secretion under conditions in which the cell was depolarized by 40 mM KCl. The results of these experiments are shown in Fig. 8. \( \beta \)HC9 cells were stimulated by depolarization with 40 mM KCl in the presence of 150 \( \mu \)M

![Fig. 3. The effect of R-467 on insulin secretion by \( \beta \)HC9 cells stimulated with 16.7 mM glucose under normal conditions and after treatment with 50 ng/ml pertussis toxin for 24 h. \( n = 4 \).](image)

![Fig. 4. The effect of R-467 on \([Ca^{2+}]_i\) in \( \beta \)HC9 cells. A and B, the effect of 3 and 10 \( \mu \)M R-467, respectively, on \( \beta \)HC9 cells under control conditions. C and D, the lack of effect of R-467 on \([Ca^{2+}]_i\), in the absence of extracellular Ca\(^{2+}\) and in the presence of nitrendipine, respectively. The panels are representative of several such experiments.](image)
Diazoxide (to block any effects of glucose on the K<sub>ATP</sub> channels) and in the presence of 0.1 and 16.7 mM glucose. Under these conditions, R-467 had no effect on insulin secretion. Thus, it is possible to conclude that R-467 has no action other than depolarization of the β-cell that could contribute to the stimulation of insulin secretion.

Electrophysiological studies were performed to confirm the finding that R-467 activates a nonspecific cation channel. The resting membrane potential of βHC9 cells varied between −257 and −261 mV. Application of 10 μM R-467 induced an inward current in 81% of cells studied in extracellular buffer containing 128.8 mM NaCl (Fig. 9A, n = 26). Preliminary experiments suggest that this current is eliminated when cells are perfused with Na<sup>+</sup>-free buffer after R-467 application. The mean peak current was 1.15 ± 0.25 nA (S.E., n = 21). The current/voltage relationship of this inward current was roughly linear with a reversal potential of approximately 3.5 mV (Fig. 9B).

To investigate the contribution of Na<sup>+</sup> to the R-467-induced current, βHC9 cells were studied using Cs<sup>+</sup>-containing pipette buffer and Na<sup>+</sup>-free external buffer containing 90 mM Ca<sup>2+</sup>. All of these conditions, R-467 had no effect on insulin secretion. Thus, it is possible to conclude that R-467 has no action other than depolarization of the β-cell that could contribute to the stimulation of insulin secretion.

Electrophysiological studies were performed to confirm the finding that R-467 activates a nonspecific cation channel. The resting membrane potential of βHC9 cells varied between −57 and −61 mV. Application of 10 μM R-467 induced an inward current in 81% of cells studied in extracellular buffer containing 128.8 mM NaCl (Fig. 9A, n = 26). Preliminary experiments suggest that this current is eliminated when cells are perfused with Na<sup>+</sup>-free buffer after R-467 application. The mean peak current was 1.15 ± 0.25 nA (S.E., n = 21). The current/voltage relationship of this inward current was roughly linear with a reversal potential of approximately 3.5 mV (Fig. 9B). To investigate the contribution of Na<sup>+</sup> to this current further, extracellular NaCl was replaced with N-methylglucamine. R-467 application did not induce an inward current under these conditions (n = 4), suggesting that Na<sup>+</sup> is a carrier of this current. In experiments using Cs<sup>+</sup>-containing pipette buffer to inactivate K<sup>+</sup> channels, R-467 still induced inward currents in cells studied in Na<sup>+</sup>-containing extracellular buffer (n = 4, mean peak current = 2.57 ± 0.81 nA) and no inward current in cells studied in Na<sup>+</sup>-free extracellular buffer (n = 2), providing further evidence for Na<sup>+</sup> permeability of the channel. To investigate the contribution of Ca<sup>2+</sup> to the R-467-induced current, βHC9 cells were studied using Cs<sup>+</sup>-containing pipette buffer and Na<sup>+</sup>-free external buffer containing 90 mM Ca<sup>2+</sup>. All of these conditions, R-467 had no effect on insulin secretion. Thus, it is possible to conclude that R-467 has no action other than depolarization of the β-cell that could contribute to the stimulation of insulin secretion.
cells showed inward current (0.93 ± 0.4 nA, n = 3) upon R-467 application, suggesting that the channel is permeable to both Na\(^+\) and Ca\(^{2+}\). These data are consistent with the presence of a Na\(^+\)- and Ca\(^{2+}\)-permeable inward channel that can be activated by R-467 in βHC9 cells.

Western blot analyses were performed on the βHC9 cells under denaturing and non-denaturing conditions in order to identify the CaSR. The results are shown in Fig. 10. Kidney medulla cells and parathyroid cells were used for comparison. In the first two lanes, Western blots of two concentrations of a whole cell lysate of rat parathyroid gland prepared under non-denaturing conditions are shown. A dense band at around 220 kDa was detected as anticipated. In lanes 3 and 4, bands in the region of 300 kDa were seen for membranes from kidney medulla and the βHC9 cells, respectively, indicating the presence of multimeric forms of the glycosylated CaSR. Under reducing conditions, bands in the region of 100–140 kDa were observed as the monomeric forms of the CaSR. These findings are similar to those of others who have detected the CaSR at several different molecular weights depending upon the extent of glycosylation of the receptor and whether the CaSR is seen as a monomer or a multimer. In these experiments it is clear that CaSR in the βHC9 cell behaves on SDS gels like the CaSR in the kidney medulla.

DISCUSSION

These studies demonstrate that R-467, a calcimimetic drug that acts on the CaSR, stimulates insulin secretion in pancreatic islets of C57BL/6 mice and in the mouse-derived βHC9 cell line. Importantly, R-467 and the enantiomer S-467 have a novel mechanism of action to depolarize the β-cell by activation of a nonspecific cation channel and stimulates insulin release from the islet only in the presence of stimulatory glucose con-
concentrations. Because of these two features, the potential exists for the development of a new class of pharmacologic agents for the treatment of type II diabetes which will reduce hyperglycemia but not cause hypoglycemia.

The CaSR is a heptahelical G protein-coupled receptor belonging to group 2 of the C family of G protein-coupled receptors (29, 30). The C family comprises three groups, the group 1 metabotropic glutamate receptors (receptors 1–8), the group 2 putative pheromone receptors, and the group 3 γ-aminobutyric acid type B receptors. The CaSR couples to different G proteins and second messenger systems depending upon the cell type. In parathyroid cells, as in CaSR-transfected HEK293 cells, CaSR activates phospholipase C (PLC), most likely via the activation of Gq/11, and the products of increased PLC activity stimulate cyclic AMP has been seen in the parathyroid and transfected HEK293 cells, suggesting the activation of Gq and/or Gq proteins. When CaSR is expressed in Xenopus oocytes, there is a pertussis-sensitive component to the activation of PLC, which also suggests the additional involvement of Gq (1), in thyroid parafollicular cells, increased influx of extracellular Ca2+ is responsible for the stimulation of calcitonin secretion (41). Similar to the differences in the choice of second messengers that are activated by CaSR in different cells, so also are there differences in the pharmacology of the activating ligands (30).

Western blot analysis of the CaSR is interesting in that the receptor has been detected at several different molecular weights, exists in both monomeric and multimeric forms, and differs in the extent of its glycosylation at several sites on the large extracellular domain. Thus, the variable glycosylation and the multimeric nature of the receptor account for its several different molecular weights (32–35). In the βHC9 cell examined here, the receptor was detected in dimeric and monomeric forms, both of which exhibited a range of molecular weights, presumably due to the different amounts of glycosylation. By polyacrylamide gel electrophoresis the βHC9 cell CaSR behaved in a similar manner to the CaSR from the kidney medulla and differently from the parathyroid CaSR. Under conditions favoring the multimeric forms of the receptor, the receptors from kidney and βHC9 cells had molecular masses of around 300 kDa. Under conditions favoring the monomeric forms, the molecular masses for both kidney and βHC9 cells was in the range of 100–140 kDa. Other similarities between the rat β-cell CaSR and the kidney CaSR exist. The sequence of the RT-PCR products from the β-cell has more than 99% homology with the cDNA of the rat kidney CaSR (11). Of interest is the fact that activation of the kidney CaSR, like the β-cell CaSR, results in a depolarization-induced increase in [Ca2+]i, which may well be due to activation of a nonspecific cation channel (36). Additionally, the kidney CaSR is not activated by neomycin, Gd3+, or Mg2+ as we found also for the β-cell (data not shown). Information on the similarity between the kidney CaSR and the β-cell CaSR with respect to enantiomer specificity is not available. The differences between the CaSR in the kidney and β-cell on the one hand, and in the parathyroid gland and parafollicular cells on the other, merit emphasis. They exhibit different behavior on polyacrylamide gel electrophoresis, and are activated by a different spectrum of agonists. However, in all four of these tissues (this report and Refs. 36, 40, and 41), and in rat hippocampal cells (37), HEK293 cells (38, 39), and rat osteoclasts (42), the activation of a nonspecific cation channel is a common feature. The existence of nonsel ective cation channels in β-cells has been demonstrated previously (26, 43, 44), and maitotoxin, an activator of these channels, has been shown to stimulate insulin release (26).

Finally, the question arises as to whether the effect of the calcimimetic R-467 can activate a nonspecific cation channel and stimulate insulin secretion in the β-cell is due to an interaction with the CaSR or whether the compound directly activates the channel. Evidence in favor of an interaction with the receptor is the similarity of the βHC9 cell receptor on SDS gels with the kidney CaSR, activation of which also results in a depolarization, activation of voltage-dependent calcium channels, and increased [Ca2+]i. In favor of a direct interaction is the lack of stereosepecificity between R- and S-467. However, as mentioned above, the stereospecificity of the CaSR in kidney is not known. These two receptors may well have a different pharmacology from the originally described Ca2+ sensing receptors with their main property of sensing small changes in extracellular Ca2+. The purpose of the CaSR on the β-cell is unknown, but it will be important to seek endogenous ligands that might activate the receptor and modulate insulin secretion. Finally, whether the calcimimetics act on the CaSR or directly on the nonspecific ion channels, the enhancement of glucose-stimulated insulin secretion is a very important property of the drug. This conclusion is emphasized by the fact that hormones that increase insulin release under physiological conditions, for example glucagon-like peptide 1 and pituitary adenylate cyclase activating polypeptide, have been implicated in the activation of nonsel ective cation channels (25–27). Furthermore, the lack of stereoeospecificity on the β-cell is an advantage in the development of drugs for the stimulation of insulin release in that the S-enantiomer is less effective on those tissues that do exhibit stereospecificity. Thus, the nonspecific cation channels have both physiologic and pharmacologic importance to the control of insulin release.

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The Calcimimetic R-467 Potentiates Insulin Secretion in Pancreatic β Cells by Activation of a Nonspecific Cation Channel

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