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Possible Participation of an Islet B-Cell Calcium-Sensing Receptor in Insulin Release

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The calcium-sensing receptor gene was recently shown to be expressed in rat pancreatic islets and purified islet B-cells. In this study, we investigated the possible role of this receptor in the regulation of insulin release from isolated rat pancreatic islets. Poly-L-arginine (0.2–0.3 μ*M*) and poly-L-lysine (0.03–0.1 μM) increased insulin output evoked by D-glucose (8.3 m*M*). This positive effect faded out at higher concentrations of the basic peptides. Likewise, the release of insulin evoked by 8.3 m*M* D-glucose was significantly lower at high (1.0 m*M*) than low (0.05–0.1 m*M*) concentrations of neomycin. The insulinotropic action of Ba^{2+} in Ca^{2+}-deprived islets was potentiated in rats pretreated with pertussis toxin. However, Gd^{3+} inhibited insulin release evoked by D-glucose in islets prepared from normal rats or animals pretreated with pertussis toxin and incubated in the absence or presence of either theophylline or forskolin. Gd^{3+} (0.3 m*M*) failed to affect effluent radioactivity from islets prelabeled with *myo*-[2-^{3}H]inositol and cyclic AMP net production in islets incubated in the absence or presence of forskolin. Gd^{3+} decreased, however, ^{45}Ca efflux from prelabeled islets perifused in the absence or presence of extracellular Ca^{2+}. It is speculated that a negative insulinotropic action mediated by the calcium-sensing receptor, and possibly attributable to a fall in cytosolic Ca^{2+} concentration, may prevent excessive insulin secretion in pathological situations of hypercalcemia.

Key Words: Calcium-sensing receptor; pancreatic islets.

Introduction

It was recently reported that the calcium-sensing receptor gene is expressed in rat pancreas *(1)*, human insulinoma cells *(2)*, and rat pancreatic islets and purified islet B-cells, as well as tumoral islet cells of the RINm5F or BRIN-BD11 lines *(3)*. The primary aim of the present study was to investigate the possible role of this receptor in the regulation of insulin release from isolated rat pancreatic islets. The tools used for such a purpose include mainly several inorganic (Ba^{2+}, Gd^{3+}) and organic (poly-L-arginine, poly-L-leucine, neomycin) polyvalent cations known to be sensed by the calcium-sensing receptor in several cell types, especially parathyroid cells *(4)*. Since pretreatment of these cells with pertussis toxin abolishes their response to the polyvalent cations *(4,5)*, experiments were also conducted in islets isolated from rats injected 3 d before sacrifice with pertussis toxin.

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Results

Effects of Poly-L-arginine and Poly-L-lysine

At 8.3 m*M* D-glucose, the release of insulin averaged 90.4 ± 4.4 μU/islet per 90 min ($n = 81$). Relative to such a control value, poly-L-arginine failed to affect significantly insulin output when tested in the 0.02–0.1 μ*M* range, but increased the secretory response to D-glucose ($p < 0.001$) at concentrations of 0.2–0.3 and 1.0 μ*M* (Table 1). When compared within the same experiments, the release of insulin evoked by 8.3 m*M* D-glucose in the presence of 1.0 μ*M* poly-L-arginine only represented 87.1 ± 3.7% ($n = 24$; $p < 0.05$) of the mean corresponding value found in the presence of 0.3 μ*M* poly-L-arginine (100.0 ± 4.8%; $n = 24$).

At a concentration of 0.01 μ*M* , poly-L-lysine also failed to affect significantly glucose-stimulated insulin release, whereas it augmented insulin output at 0.03 μ*M* ($p < 0.025$) and 0.1 μ*M* ($p < 0.06$). A progressive decline in secretory rate was observed, however, as the concentration of poly-L-lysine was increased from 0.03 to 0.1, 0.3, and 1.0 μ*M* (Table 1), with the latter two concentrations again failing to modify insulin output significantly .

Effects of Ba^{2+}

Over 60 min of incubation, Ba^{2+} (2.0 m*M*) tripled ($p < 0.001$) insulin output by islets prepared from normal rats and incubated in the absence of $CaCl_2$ and the presence of both EGTA (0.1 m*M*) and theophylline (1.4 m*M*). D-Glucose (5.6 m*M*) further enhanced ($p < 0.01$) Ba^{2+}-stimulated

Table 1
Effects of Poly-L-arginine and Poly-L-lysine on Insulin Release Evoked by 8.3 m*M* D-Glucose

Drug dosage	Insulin output (% of control)	
(μ*M*)	Poly-L-arginine	Poly-L-lysine
Nil	100.0 ± 8.1 (36)	100.0 ± 5.7 (45)
0.01–0.02	100.8 ± 7.6 (12)	100.7 ± 8.4 (24)
0.03		124.5 ± 9.7 (24)
0.06–0.10	119.4 ± 9.2 (36)	114.7 ± 5.2 (48)
0.20–0.30	153.5 ± 8.2 (35)	102.1 ± 5.8 (24)
1.00	168.3 ± 8.0 (24)	86.5 ± 6.1 (24)

insulin output (Table 2). In the presence of D-glucose and theophylline, a rise in Mg^{2+} concentration from 1.0 to 21.0 m*M* failed to reproduce the effect of Ba^{2+}, and the release of insulin from the Ca^{2+}-deprived islets exposed to the high concentration of Mg^{2+} was not significantly different from that recorded at normal Mg^{2+} concentration (1.0 m*M*) in the sole presence of theophylline.

Essentially comparable results were obtained when the same experiments were conducted in islets from rats pretreated with pertussis toxin (Table 2). In this case, the secretory response to Ba^{2+} was much higher, however, than in the islets from control rats ($p < 0.001$) and failed to be significantly increased further by D-glucose.

Effects of Gd^{3+} and Neomycin

At a normal concentration of extracellular Ca^{2+} (1.0 m*M*), Gd^{3+} (0.1–0.6 m*M*) failed to enhance insulin release evoked by 8.3 m*M* D-glucose (Table 3, experiment 1). On the contrary, the trivalent cation caused a concentration-related inhibition of glucose-stimulated insulin release, with a significant decrease in insulin output ($p < 0.01$) already observed at a 0.1 m*M* concentration of Gd^{3+} (Table 3, experiment 1).

At a normal Ca^{2+} concentration, neomycin sulfate (0.05–1.0 m*M*) failed to affect significantly the release of insulin evoked by 8.3 m*M* D-glucose (Table 3, experiment 2). Nevertheless, such a release was significantly lower ($p < 0.02$) in the presence of 1.0 m*M* neomycin than at lower concentrations of the drug (0.05 and 0.1 m*M*).

The unexpected finding that Gd^{3+} inhibited glucose-stimulated insulin release led us to compare the effect of the trivalent cation in islets from control rats and animals pretreated with pertussis toxin (Table 4).

In the control rats, Gd^{3+} (0.3 m*M*) inhibited, modestly but significantly ($p < 0.03$), glucose-stimulated insulin output to 80.2 ± 7.8% ($n = 47$) of the mean corresponding reference value (100.0 ± 3.8%; $n = 46$). Both theophylline (1.4 m*M*) and forskolin (10 μ*M*) markedly enhanced the secretory response to D-glucose ($p < 0.001$ in both cases). Whereas Gd^{3+} severely inhibited ($p < 0.001$) insulin release from islets exposed to both D-glucose and forskolin, the trivalent cation failed to decrease significantly insulin output in the presence of D-glucose and theophylline.

The release of insulin caused by 16.7 m*M* D-glucose was much higher ($p < 0.001$) in the rats pretreated with pertussis toxin than in control animals. In relative terms, Gd^{3+} inhibited more severely glucose-stimulated insulin release in the rats pretreated with the toxin than in the control animals. Indeed, in the presence of Gd^{3+}, the output of insulin now averaged 58.8 ± 2.7% ($n = 48$) of the mean corresponding reference value (100.0 ± 4.1%; $n = 48$). This percentage was in fact lower ($p < 0.02$) than that found in control rats. By contrast, the relative magnitude of the enhancing action of either theophylline or forskolin was less pronounced ($p < 0.001$ in both cases) in the toxin-pretreated rats than in the control animals. Thus, relative to the mean value found in the sole presence of D-glucose, the output of insulin recorded in the presence of theophylline and forskolin, respectively, averaged 223.4 ± 15.1 and 246.8 ± 12.2% in islets from control rats, as distinct from only 158.1 ± 7.4 and 171.4 ± 7.8% in islets from toxin-treated animals. The inhibitory action of Gd^{3+} on insulin release evoked by D-glucose and either theophylline or forskolin, however, displayed closely comparable relative values in the two groups of rats (Fig. 1). It averaged 18.4 ± 4.6% (df = 79) in the case of theophylline and 48.2 ± 3.8% (df = 97) in the case of forskolin (pooled data obtained in control and toxin-treated rats).

As illustrated in Fig. 1, the relative magnitude of the inhibitory action of Gd^{3+} (0.3 m*M*) on insulin secretion evoked by D-glucose alone was not significantly different from that found in the concomitant presence of theophylline in control rats ($p > 0.5$) or from that recorded in the concomitant presence of forskolin in toxin-pretreated animals ($p > 0.2$). Inversely, the Gd^{3+}-induced relative decrease in insulin output evoked by D-glucose alone was less pronounced than that found in the concomitant presence of forskolin in control rats ($p < 0.01$) and more marked than that observed in the concomitant presence of theophylline in toxin-pretreated animals ($p < 0.001$).

Since the significance of these results could be obscured by the presence of extracellular Ca^{2+} in the incubation medium, further experiments were conducted in the absence of this divalent cation.

In the absence of $CaCl_2$, D-glucose (16.7 m*M*) augmented five times the insulin output (Table 3, experiment 3). In the Ca^{2+}-deprived islets, Gd^{3+} (0.6 m*M*) failed to affect significantly basal insulin release and suppressed the secretory response to D-glucose ($p < 0.001$).

In a further set of experiments in this series, EGTA (0.1 m*M*) was added to the medium containing no $CaCl_2$, and forskolin (10 μ*M*) was now incorporated to the incubation medium to allow for a sizeable secretory response to D-glucose *(6)*. Under these experimental conditions, D-glucose (16.7 m*M*) indeed augmented ($p < 0.001$) insulin release (Table 3, experiment 4). The insulinotropic action of the hexose was not significantly affected by 0.3 m*M* Gd^{3+}, but

Table 2
Effects of Ba^{2+} and Mg^{2+} on Insulin Release by Islets from Control Rats and Animals Pretreated with Pertussis Toxin

Ca^{2+} (m*M*)	Mg^{2+} (m*M*)	Ba^{2+} (m*M*)	EGTA (m*M*)	Theophylline (m*M*)	D-Glucose (m*M*)	Insulin output (μU/islet per 60 min)	
						Control	Pertussis
Nil	1.0	Nil	0.1	1.4	Nil	19.0 ± 3.0 (15)	6.1 ± 3.2 (15)
Nil	1.0	2.0	0.1	1.4	Nil	59.8 ± 3.8 (15)	209.8 ± 7.8 (14)
Nil	1.0	2.0	0.1	1.4	5.6	84.8 ± 8.2 (15)	226.0 ± 11.8 (14)
Nil	21.0	Nil	0.1	1.4	5.6	25.1 ± 3.7 (15)	9.7 ± 3.0 (14)

Table 3
Effects of Gd^{3+} and Neomycin on Insulin Release in Islets from Control Rats and Animals Pretreated with Pertussis Toxin

Experiment Number	Ca^{2+} (m*M*)	EGTA (m*M*)	Forskolin (m*M*)	D-Glucose (m*M*)	Gd^{3+} (m*M*)	Neomycin (m*M*)	Insulin output (μU/islet per 90 min)
1	1.0	Nil	Nil	8.3	Nil	Nil	138.9 ± 10.3 (10)
	1.0	Nil	Nil	8.3	0.1	Nil	108.2 ± 5.3 (24)
	1.0	Nil	Nil	8.3	0.6	Nil	99.2 ± 7.4 (12)
2	1.0	Nil	Nil	8.3	Nil	Nil	134.2 ± 7.7 (11)
	1.0	Nil	Nil	8.3	Nil	0.05	150.6 ± 8.1 (11)
	1.0	Nil	Nil	8.3	Nil	0.1	149.7 ± 9.1 (11)
	1.0	Nil	Nil	8.3	Nil	1.0	115.7 ± 9.7 (12)
3	Nil	Nil	Nil	Nil	Nil	Nil	16.3 ± 1.2 (15)
	Nil	Nil	Nil	Nil	0.6	Nil	17.1 ± 2.6 (14)
	Nil	Nil	Nil	16.7	Nil	Nil	87.9 ± 8.1 (15)
	Nil	Nil	Nil	16.7	0.6	Nil	29.3 ± 8.7 (14)
4	Nil	0.1	0.01	Nil	Nil	Nil	17.7 ± 1.8 (15)
	Nil	0.1	0.01	16.7	Nil	Nil	42.2 ± 3.1 (35)
	Nil	0.1	0.01	16.7	0.3	Nil	40.1 ± 4.0 (14)
	Nil	0.1	0.01	16.7	0.6	Nil	21.7 ± 2.5 (20)
	Nil	0.1	0.01	16.7	Nil	0.1	32.5 ± 3.1 (35)
5*a*	Nil	0.1	0.01	Nil	Nil	Nil	17.9 ± 2.9 (20)
	Nil	0.1	0.01	16.7	Nil	Nil	53.6 ± 9.8 (30)
	Nil	0.1	0.01	16.7	0.3	Nil	51.7 ± 8.6 (30)
	Nil	0.1	0.01	16.7	Nil	0.1	31.3 ± 5.4 (30)

a Experiment conducted in islets prepared from rats pretreated with pertussis toxin.

Table 4
Effect of Gd^{3+} on Insulin Release Evoked by 16.7 m*M* D-Glucose in Islets Prepared from Control Rats or Animals Pretreated with Pertussis Toxin and Incubated at Normal Ca^{2+} Concentration

Experiment number	Theophylline (m*M*)	Forskolin (m*M*)	Gd^{3+} (m*M*)	Insulin output (μU/islet per 90 min)	
				Control	Pertussis
1	Nil	Nil	Nil	264.3 ± 16.7 (21)	510.3 ± 20.7 (22)
	Nil	Nil	0.3	211.9 ± 42.2 (22)	328.5 ± 20.8 (22)
	1.4	Nil	Nil	584.2 ± 36.9 (21)	801.0 ± 33.3 (22)
	1.4	Nil	0.3	509.5 ± 22.9 (20)	688.5 ± 26.8 (20)
2	Nil	Nil	Nil	225.2 ± 11.8 (25)	435.0 ± 30.1 (26)
	Nil	Nil	0.3	180.6 ± 13.6 (25)	231.0 ± 11.3 (26)
	Nil	0.01	Nil	547.2 ± 24.0 (25)	745.3 ± 33.8 (26)
	Nil	0.01	0.3	272.0 ± 20.7 (25)	401.3 ± 14.7 (25)

was impaired by either 0.6 m*M* Gd^{3+} ($p < 0.001$) or 0.1 m*M* neomycin ($p < 0.03$).

Essentially comparable results were recorded in islets prepared from rats pretreated with pertussis toxin (Table 3, experiment 5). Thus, in the absence of $CaCl_2$ and the presence of both EGTA (0.1 m*M*) and forskolin (10 μ*M*), the release of insulin was increased ($p < 0.05$) by D-glucose (16.7 m*M)* and, in the presence of the hexose, not signifi-

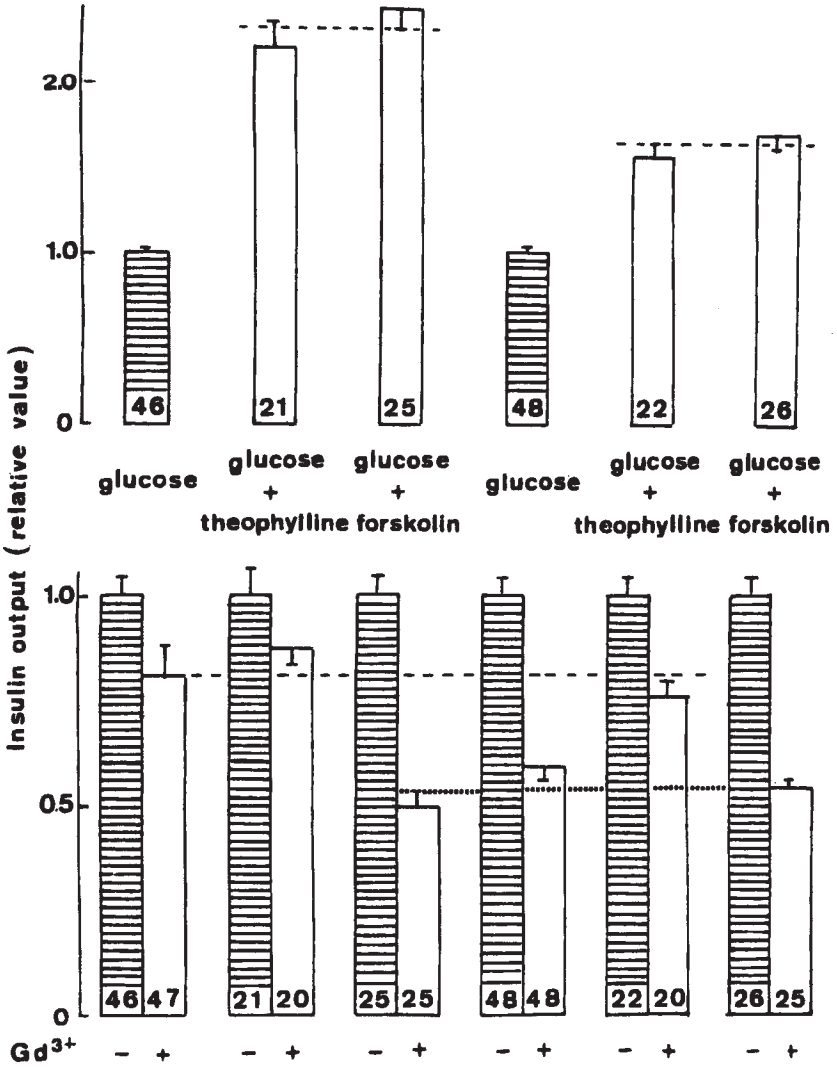

Fig. 1. Insulin release evoked by D-glucose (16.7 m*M*) in islets from control rats **(left)** and animals pretreated with pertussis toxin **(right)**. **(Top)** Effects of theophylline (1.4 m*M*) and forskolin (10 μ*M*) on glucose-stimulated insulin output. All results are expressed relative to the mean reference value (horizontally hatched columns) found within the same experiment(s) in the sole presence of the hexose. (– – –), The lower relative increase in insulin secretion attributable to theophylline or forskolin in the islets from toxin-pretreated rats as compared to control animals. **(Bottom)** Effects of Gd^{3+} (0.3 m*M*) on insulin release under the three experimental conditions. All results being expressed relative to the mean reference value (horizontally hatched columns) found within the same experiment(s) in the absence of Gd^{3+}. (– – –) and (· · ·), The lower relative inhibitory action of Gd^{3+} in islets exposed to theophylline as compared to forskolin in either control or toxin-pretreated rats. Mean values (±SEM) refer to the number of individual measurements indicated at the bottom of each column.

cantly affected by 0.3 m*M* Gd^{3+} but inhibited ($p < 0.06$) by 0.1 m*M* neomycin.

In all the experiments presented so far, the measurement of insulin release was always made after 90 min of incubation. To possibly detect a short-lived effect of Gd^{3+} (0.3 m*M*) on the hydrolysis of polyphosphoinositides, fluxes of Ca^{2+}, or insulin release, a last series of experiments was conducted in perifused islets (Fig. 2). The islets were prelabeled with either *myo*-[2-^{3}H]inositol or ^{45}Ca over 60 min of incubation at 8.3 and 16.7 m*M* D-glucose, respectively. In the presence of 8.3 m*M* D-glucose and whether in the presence of extracellular Ca^{2+} (1.0 m*M*) or in its absence (no $CaCl_2$, EGTA 0.5 m*M*), Gd^{3+} (0.3 m*M*) failed to cause any obvious increase in ^{3}H fractional outflow rate from islets prelabeled with *myo*-[2-^{3}H]inositol. Gd^{3+} also failed to increase ^{45}Ca efflux and, on the contrary, caused a rapid, sustained, and rapidly reversible decrease in ^{45}Ca outflow. Over the 25 min of exposure to Gd^{3+}, the mean integrated ^{45}Ca fractional outflow rate was, in the presence and the absence of extracellular Ca^{2+}, 15.6 ± 2.5 and 19.4 ± 3.1%, respectively ($n = 4$ and $p < 0.01$ in both cases) lower than the paired theoretical value calculated by exponential extrapolation of the initial (min 31–45) and late (min 86–90) measurements. At a normal extracellular Ca^{2+} concentration, Gd^{3+} slightly decreased insulin output; the mean secretory rate during the 25 min of exposure to Gd^{3+} was 60 ± 9 nU/min

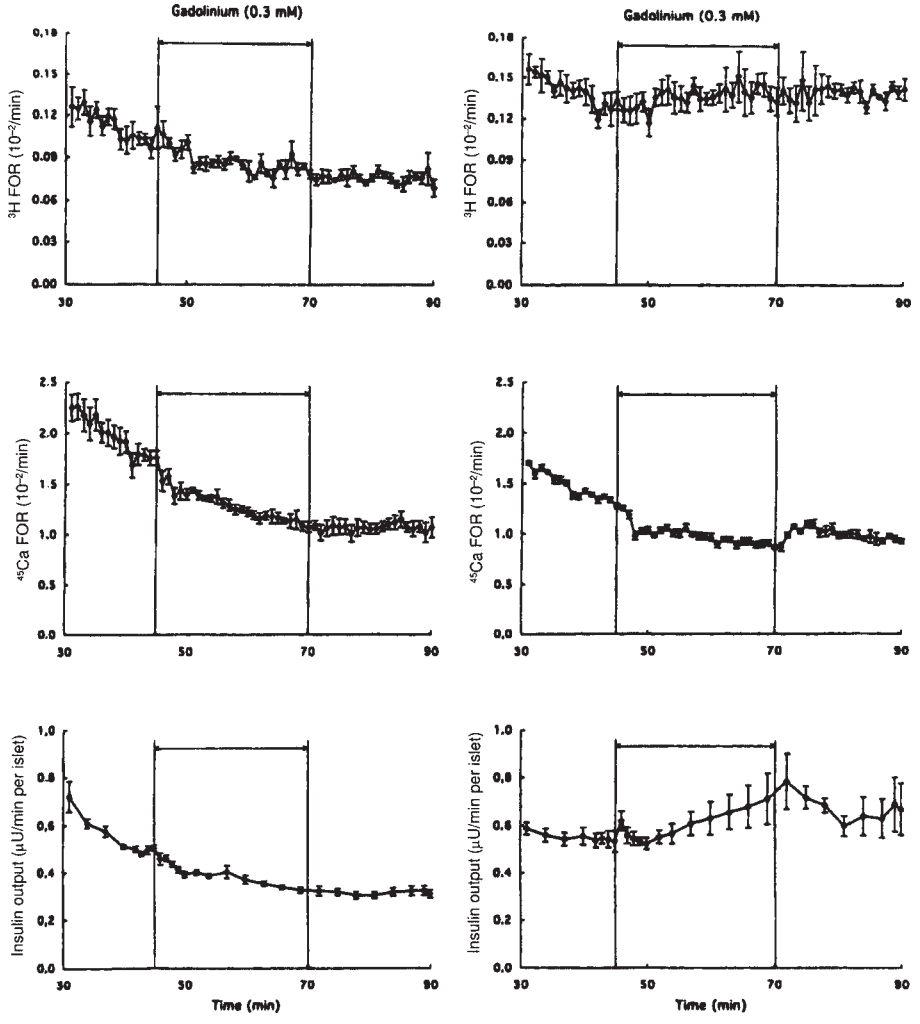

Fig. 2. Time course for the changes in ^{3}H fractional outflow rate for islets prelabeled with *myo*-[2-^{3}H]inositol, effluent radioactivity from islets prelabeled with ^{45}Ca, and insulin release evoked by Gd^{3+} (0.3 m*M*), administered from min 46 to 70, in islets perifused in the presence of 8.3 m*M* D-glucose either at **(left)** normal extracellular Ca^{2+} concentration (1.0 m*M*) or **(right)** in the absence of $CaCl_2$ and presence of EGTA (0.5 m*M*). Mean values (±SEM) refer to four **(top** and **middle)** or eight **(bottom)** individual experiments.

per islet lower ($n = 8$; $p < 0.001$) than the paired theoretical value calculated by exponential extrapolation of the measurements made before (min 31–45) and after (min 84–90) Gd^{3+} administration. In the Ca^{2+}-deprived islets, however, Gd^{3+} apparently caused a modest increase in insulin output (Fig. 2, lower right). Except in two of eight experiments, the output of insulin was indeed higher ($p < 0.02$) at min 69 than at min 45. Such a stimulation failed to occur, however, in the other two experiments, so the overall mean increase in insulin output during exposure to Gd^{3+} did not achieve statistical significance.

As shown in Table 5, Gd^{3+} (0.3 m*M*) also failed to affect significantly the net production of cyclic AMP (cAMP) by islets incubated for 30 min in the presence of extracellular Ca^{2+} (1.0 m*M*) and D-glucose (16.7 m*M*), with or without forskolin (10.0 μ*M*). As expected, the diterpene markedly increased cAMP net production ($p < 0.001$), whether in the absence or presence of Gd^{3+}.

Discussion

The release of insulin evoked by D-glucose in isolated rat pancreatic islets progressively increases as the concentration of extracellular Ca^{2+} is raised in the 0–4.0 m*M* range *(7)*. Under suitable experimental conditions, a rise in extracellular Ca^{2+} concentration can even provoke insulin release from islets deprived of any exogenous nutrient *(8,9)*. It is not easy to decide whether, under these experimental conditions, the secretory response to Ca^{2+} involves the calcium-sensing receptor recently proposed to be present in insulin-producing cells *(3)*. Indeed, such a response coincides with and is currently ascribed to an increased influx of Ca^{2+} into the islet cells *(7)*.

As an alternative approach, the first tool used in the present study to assess the possible functional role of the calcium-sensitive receptors in islet B-cells consisted of the highly basic peptides poly-L-arginine and poly-L-lysine *(4)*. At physiological

Table 5
Effect of Gd^{3+} on cAMP Net Production by Islets from Normal Rats Incubated for 30 min at Normal Ca^{2+} Concentration in the Presence of 16.7 m*M* D-Glucose

Forskolin (m*M*)	Gd^{3+} (m*M*)	cAMP (fmol/islet)
Nil	Nil	6.46 ± 0.83 (15)
0.01	Nil	37.76 ± 3.99 (14)
Nil	0.3	7.55 ± 1.23 (15)
0.01	0.3	40.61 ± 5.32 (14)

concentrations of both D-glucose (8.3 m*M*) and extracellular Ca^{2+} (1.0 m*M*), both poly-L-arginine and poly-L-lysine augmented significantly insulin release when tested at respective concentrations of 0.2–1.0 and 0.03–0.1 μ*M*. The higher range of concentrations required to observe a significant effect of poly-L-arginine, as compared to poly-L-lysine, coincided with the lower molecular weight of the former than the latter basic peptide. In both cases, however, a further rise in peptide concentrations decreased insulin output significantly. At a 1.0 μ*M* concentration, these basic peptides suppress dopamine-stimulated cAMP accumulation in bovine parathyroid cells incubated in the presence of 0.5 m*M* Ca^{2+} *(4)*. The present results suggest that, at high concentrations, these peptides may also display a negative component of their insulinotropic action.

The divalent cation Ba^{2+} was used as the second tool to explore a possible role of the calcium-sensitive receptors in insulin release. The results obtained here in islets from control rats confirm prior observations *(10)*. The insulinotropic action of Ba^{2+}, which was not reproduced by a high concentration of Mg^{2+}, was not suppressed; on the contrary, it was enhanced in islets from rats pretreated with pertussis toxin. Since pretreatment with pertussis toxin abolishes the effect of divalent and trivalent cations, as mediated by the calcium-sensing receptor, in parathyroid cells *(4,5)*, the present results do not suggest that the positive insulinotropic action of Ba^{2+} is mediated by such a receptor. It is indeed currently believed that the stimulation of insulin release by Ba^{2+} depends on its uptake by islet cells with a resulting intracellular redistribution of Ca^{2+} ions *(11)*.

The polycationic antibiotic neomycin mimics the action of di- and trivalent cations in several aspects of parathyroid function *(5)*. It was, therefore, also used to investigate whether the activation of calcium-sensing receptors may affect insulin release. At normal D-glucose and Ca^{2+} concentrations, the release of insulin was significantly lower in the presence of 1.0 m*M* neomycin than at lower concentrations of the antibiotic (0.05 and 0.1 m*M*), a situation reminiscent of that found with the basic peptides previously mentioned. Moreover, in islets from both control rats and animals pretreated with pertussis toxin, as little as 0.1 m*M* neomycin was sufficient to inhibit insulin release evoked by D-glucose in Ca^{2+}-deprived islets exposed to 10 μ*M* forskolin. Under these experimental conditions, the insulinotropic action of the hexose is tightly dependent on the activation of adenylate cyclase by the diterpene *(6)*.

Comparable findings were observed with Gd^{3+}, a trivalent cation currently used in the study of the calcium-sensing receptor *(5,12)*. Gd^{3+} caused a concentration-related inhibition of insulin release from islets of normal rats incubated at physiological concentrations of D-glucose and extracellular Ca^{2+}. When tested at a 0.6 m*M* concentration, Gd^{3+} also inhibited insulin release evoked by the hexose in islets prepared from normal rats and incubated either in the nominal absence of extracellular Ca^{2+} (no $CaCl_2$) or in the absence of $CaCl_2$ but the presence of forskolin.

By comparing the effects of Gd^{3+} on glucose-stimulated insulin release in islets from control rats and animals pretreated with pertussis toxin, further information was gained on the possible mode of action of the trivalent cation in islet B-cells. As expected *(13)*, the release of insulin evoked by D-glucose (16.7 m*M*) at normal extracellular Ca^{2+} concentration (1.0 m*M*) was higher in the toxin-treated than control rats. In the latter animals, it was only slightly decreased by Gd^{3+} (0.3 m*M*). In the toxin-treated rats, however, the inhibitory action of Gd^{3+} on glucose-stimulated insulin release was much more pronounced. Such a high sensitivity to Gd^{3+} was also observed in control rats provided that the islets were incubated in the presence of forskolin (10 μ*M*). Thus, in the presence of the diterpene, the relative extent of the inhibitory action of Gd^{3+} on glucose-stimulated insulin release was comparable in islets from control and toxin-pretreated rats. Theophylline, however, which increases the cAMP content of islet cells by inhibiting phosphodiesterase, rather than by activating adenylate cyclase, did not confer to islets from control rats an increased sensitivity to Gd^{3+}. In fact, the results obtained in toxin-pretreated rats suggest that theophylline, which is thought to cause an intracellular translocation of Ca^{2+} into the cytosol *(14)*, protected the islet B-cells against the inhibitory action of Gd^{3+} on insulin release.

Several of the results discussed so far are apparently compatible with the view that activation of the calcium-sensing receptor in islet B-cells may cause inactivation of adenylate cyclase. This would account for the negative component of the insulinotropic action of basic peptides and neomycin on glucose-stimulated insulin release, since the hexose causes activation of adenylate cyclase at the intervention of Ca-calmodulin *(15)*. It would also account for the increased sensitivity to neomycin found in Ca^{2+}-deprived islets exposed to forskolin. In this respect, no difference was unexpectedly observed, however, between islets from control rats and toxin-pretreated animals. Hence, it should be underlined that certain effects of polyvalent cations may affect cell function by a receptor-independent mechanism *(4)*. Moreover, pertussis toxin does not suppress the functional response mediated by the calcium-sensing receptor in all cell types *(5,16,17)*.

The comparison between the effects of Gd^{3+} on insulin release evoked by D-glucose in islets prepared from control and toxin-pretreated rats and incubated in the absence or presence of either theophylline or forskolin could also suggest that the trivalent cation inhibits adenylate cyclase activity in islet B-cells. The results obtained in perifused islets are also consistent with such a view. They indicated that Gd^{3+} fails to provoke the hydrolysis of polyphosphoinositides as well as to mobilize intracellular Ca^{2+} in islet cells. The results revealed, however, that, at least in the absence of extracellular Ca^{2+} and at a relatively low concentration (0.3 m*M*), Gd^{3+}, like basic peptides, may also display a positive component of its insulinotropic action.

The measurement of net production of cAMP indicated, however, that Gd^{3+} (0.3 m*M*) fails to affect significantly the net production of the cyclic nucleotide in islets incubated in the presence of Ca^{2+} (1.0 m*M*) and D-glucose (16.7 m*M*), with or without forskolin (0.01 m*M*).

Hence, an alternative explanation for the inhibition of insulin release by agents acting on the calcium-sensing receptors could be a lowering of cytosolic Ca^{2+} concentration, as suggested by the Gd^{3+}-induced decrease of ^{45}Ca efflux from prelabeled islets. This alternative hypothesis could account for the greater sensitivity of Ca^{2+}-deprived islets, as distinct from islets incubated at normal Ca^{2+} concentration, to the inhibitory action of neomycin and Gd^{3+} on glucose-stimulated insulin release (Table 3) and the already mentioned protective effects of theophylline against such an inhibitory action.

In conclusion, under close to physiological conditions, i.e., at 8.3 m*M* D-glucose and in the presence of extracellular Ca^{2+}, the major effect of agents thought to activate calcium-sensing receptors could well consist of the inhibition of insulin release by an as yet unknown mechanism. This proposal is consistent with the view that the calcium-sensing receptor may be coupled to different G-proteins in distinct cell types. Further work is required, however, to confirm such a proposal and to explore its possible relevance to the regulation of insulin release in vivo. For instance, the present findings raise the idea that a negative insulinotropic action mediated at the intervention of the calcium-sensing receptor could prevent excessive secretion of insulin in pathological situations of hypercalcemia *(18–20)*.

Materials and Methods

Poly-L-arginine hydrochloride (average mol wt 12,100), poly-L-lysine hydrochloride (average mol wt 22,700), neomycin sulfate, and gadolinium chloride were obtained from Sigma (St. Louis, MO).

Most experiments were performed in pancreatic islets isolated by the collagenase procedure *(21)* from fed female Wistar rats (B & K Limited, Hull, UK). A series of experiments was conducted in islets from rats that had been injected intraperitoneally 3 d before sacrifice with pertussis toxin (Sigma) in saline (2.8 μg in 1.0 mL of saline per rat). These rats (205 ± 3 g; $n = 21$) had gained 4.7 ± 1.4 g ($n = 21$, $p < 0.005$) over the 3 d following the injection of the toxin. At the time of killing, their plasma D-glucose concentration averaged 6.82 ± 0.12 m*M* ($n = 20$), as distinct ($p < 0.01$) from 7.42 ± 0.19 m*M* ($n = 14$) in control animals.

The methods used to measure the plasma concentration of D-glucose *(22)* in blood samples collected at the time of killing of the rats by decapitation, the release of insulin from incubated *(21)* or perifused *(23)* islets, and the fractional outflow rate of radioactivity from perifused islets prelabeled with either *myo*-[2-^{3}H]inositol *(24)* or ^{45}Ca *(25)* were as previously described.

For measuring cAMP net production *(26)*, groups of 10 islets each were preincubated for 60 min at 37°C in 0.3 mL of a bicarbonate-buffered medium *(21)* containing bovine serum albumin (5 mg/mL) and D-glucose (2.8 m*M*). The islets were then washed twice and eventually incubated for 30 min at 37°C in 0.1 mL of the same medium now containing 16.7 m*M* D-glucose and, as required, forskolin (0.01 m*M*) and/or Gd^{3+} (0.3 m*M*). The incubation was halted by the addition of 210 μL of ethanol (96°). Each sample was then sonicated for 15 s and centrifuged for 5 min at 4°C. Aliquot (200 μL) of the supernatant solution were vacuum-dried, and then redissolved in 200 μL of a cAMP assay buffer for acetylation and measurement of the cyclic nucleotide by radioimmunoassay (RIAVEN Camp [^{125}I]RIA Kits; DuPont, Brussels, Belgium).

Statistical Analysis

All results, including those already mentioned, were presented as mean values (±SEM) together with the number of individual observations (n) or degrees of freedom (df). The statistical significance of differences between mean values was assessed by the use of the Student's t-test. The data collected in perifused islets were analyzed as described elsewhere *(27)*.

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References

1. Bruce, J. I. E., Yang, X., Ferguson, C. J., Elliott, A. C., Steward, M. C., Case, R. M., et al. (1999). *J. Biol. Chem.* **274,** 20,561–20,568.
2. Kato, M., Doi, T., Imamura, M., Furutani, M., Hosotani, H., and Shimada, Y. (1997). *Surgery* **122,** 1203–1211.
3. Rasschaert, J., Deng, Y., and Malaisse, W. J. (1998). *Diabetologia* **41(Suppl. 1),** A140.
4. Brown, E. M., Katz, C., Butters, R., and Kifor, O. (1991). *J. Bone Miner. Res.* **6,** 1217–1225.

5. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., et al. (1993). *Nature* **366,** 575–580.
6. Malaisse, W. J., García–Morales, P., Dufrane, S. P., Sener, A., and Valverde, I. (1984). *Endocrinology* **115,** 2015–2020.
7. Malaisse, W. J., Hutton, J. C., Sener, A., Levy, J., Herchuelz, A., Devis, G., et al. (1978). *J. Membr. Biol.* **38,** 193–208.
8. Devis, G., Somers, G., and Malaisse, W. J. (1975). *Biochem. Biophys. Res. Commun.* **67,** 525–529.
9. Devis, G., Somers, G., and Malaisse, W. J. (1977). *Diabetologia* **13,** 531–536.
10. Sener, A. and Malaisse, W. J. (1979). *Eur. J. Biochem.* **98,** 141–147.
11. Berggren, P. O., Andersson, B., and Hellman, B. (1982). *Biochim. Biophys. Acta* **720,** 320–328.
12. Riccardi, D., Park, J., Lee, W. –S., Gamba, G., Brown, E. M., and Hebert, S. C. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 131–135.
13. Malaisse, W. J., Svoboda, M., Dufrane, S. P., Malaisse–Lagae, F., and Christophe, J. (1984). *Biochem. Biophys. Res. Commun.* **124,** 190–196.
14. Brisson, G. R., Malaisse–Lagae, F., and Malaisse, W. J. (1972). *J. Clin. Invest.* **51,** 232–241.
15. Valverde, I., Vandermeers, A., Anjaneyulu, R., and Malaisse, W. J. (1979). *Science* **206,** 225–227.
16. Pearce, S. H. S., Bai, M., Quinn, S. J., Kifor, O., Brown, E. M., and Thakker, R. V. (1996). *J. Clin. Invest.* **98,** 1860–1866.
17. Emanuel, R. C., Adler, G. K., Kifor, O., Quinn, S. J., Fuller, F., Krapcho, K., et al. (1996). *Mol. Endocrinol.* **10**, 555–565.
18. Gibbs, R. and Lebovitz, H. (1970). *Clin. Res.* **18,** 31.
19. Kim, H., Kalkhoff, R. K., Costrini, N. V., Cerletty, J. M., and Jacobson, M. (1971). *J. Clin. Invest.* **50,** 2596–2605.
20. Yasuda, K., Hurukawa, Y., Okuyama, M., Kikuchi, M., and Yoshinaga, K. (1975). *N. Engl. J. Med.* **292,** 501–504.
21. Malaisse–Lagae, F. and Malaisse, W. J. (1984). In: *Methods in diabetes research*. Larner, J. and Pohl, S. L. (eds.). Wiley: New York.
22. Bergmeyer, H. U. and Berndt, E. (1974). In: *Methods of enzymatic analysis*. Bergmeyer, U. (ed.). Academic: New York.
23. Herchuelz, A. and Malaisse, W. J. (1978). *J. Physiol. (Lond.)* **283**, 409–424.
24. Mathias, P. C. F., Best, L., and Malaisse, W. J. (1985). *Cell Biochem. Funct.* **3,** 173–177.
25. Herchuelz, A., Sener, A., and Malaisse, W. J. (1980). *J. Membr. Biol.* **57,** 1–12.
26. Valverde, I., Garcia–Morales, P., Ghiglione, M., and Malaisse, W. J. (1983). *Horm. Metab. Res.* **15,** 62–68.
27. Jijakli, H., Ulusoy, S., and Malaisse, W. J. (1996). *Pharmacol. Res.* **34,** 105–108.