Endocrine, *vol. 30, no. 3, 353–363, December 2006* 0969–711X/06/30:353–363/$30.00
ENDO (Online) ISSN 1559-0100

Stimulus-Secretion Coupling of Hypotonicity-Induced Insulin Release in BRIN–BD11 Cells

Renaud Beauwens,[1] Len Best,[2] Nicolas Markadieu,[1] Raphael Crutzen,[1] Karim Louchami,[3] Peter Brown,[2] Allen P. Yates,[2] Willy J. Malaisse,[3] and Abdullah Sener[3]

Laboratories of [1]Cell and Molecular Physiology and [3]Experimental Hormonology, Brussels Free University, Brussels, Belgium; and [2]Department of Medicine, Manchester University, Manchester, UK

The stimulus-secretion coupling for hypotonicity-induced insulin release was investigated in BRIN–BD11 cells. A 50 m*M* decrease in extracellular NaCl caused a twofold increase in insulin release. The release of insulin evoked by hypotonicity progressively decreased in an exponential manner. The response to extracellular hypotonicity displayed a threshold value close to 20 mOsmol/L and a maximal response at about 70 mOsmol/L. Hypotonicity also caused a rapid increase in cell volume followed by a regulatory volume decrease (RVD), cell membrane depolarization with induction of spike activity, and a rise in cytosolic Ca^{2+} concentration. 5-Nitro-2-(3-phenylpropylamino)benzoate inhibited the secretory response to hypoosmolarity, failed to affect the early increase in cell volume but prevented the RVD, and suppressed the hypotonicity-induced plasma membrane depolarization. Insulin release provoked by hypotonicity was inhibited by verapamil, absence of Ca^{2+}, thapsigargin, furosemide, tributyltin, and diazoxide. On the contrary, tolbutamide augmented modestly insulin release recorded in the hypoosmolar medium. Last, a rise in extracellular K^+ concentration, while augmenting basal insulin output, failed to affect insulin release in the hypoosmolar medium. Thus, the insulin secretory response to hypotonicity apparently represents a Ca^{2+}-dependent process triggered by the gating of volume-sensitive anion channels with subsequent depolarization and gating of voltage-sensitive Ca^{2+} channels.

Key Words: Hypotonicity; insulin release; BRIN–BD11 cells.

Introduction

Exposure of insulin-producing cells to a medium of low osmolarity provokes insulin release *(1–6)*. The mechanism(s) involved in such a process are not well understood. To cite only one example, it remains a matter of debate whether the secretory response to hypotonicity is causally linked to the activation of chloride channels *(6)*. The major aim of the present study was to gain further insight in the stimulus-secretion coupling for hypotonicity-induced insulin secretion. In order to explore all variables specifically in insulin-producing cells, the experiments were conducted in BRIN–BD11 cells, an insulin-secreting cell line established by electrofusion of normal pancreatic B-cells from New England Deaconess Hospital rats with immortalized RINm5F cells *(7)*.

Results

Insulin Release

Comparison Between Three Insulin-Producing Cell Lines

In a first set of experiments, the secretory response to hypotonicity, as provoked by a decrease in NaCl concentration by 50 m*M*, was examined in three lines of insulin-producing cells. The results of these experiments are summarized in Table 1.

In MIN-6 cells exposed to the hypotonic medium, the release of insulin over 30 min incubation averaged 118.3 ± 1.9% ($n = 12$; $p < 0.001$) of the mean corresponding value found within the same experiment(s) under isotonic conditions (100.0 ± 1.4%; $n = 12$). The former percentage was lower ($p < 0.001$) than that found in either INS-1 cells (174.4 ± 10.8%; $n = 12$; $p < 0.001$ vs isotonic control values) or BRIN–BD11 cells (265.5 ± 26.9%; $n = 18$; $p < 0.001$ vs isotonic control values). The latter two mean percentages were also significantly different from one another ($p < 0.02$). Despite lower absolute values for insulin output (expressed per cell), the BRIN–BD11 cells thus offered the advantage of a relatively greater secretory responsiveness to hypotonicity (Table 1; upper panel).

In some of these pilot experiments, the ratio between insulin output under hypotonic/isotonic conditions was also measured at increasing concentrations of D-glucose (1.1,

Received November 21, 2006; Revised December 15, 2006; Accepted December 21, 2006.

Author to whom all correspondence and reprint requests should be addressed: Prof. W. J. Malaisse, Laboratory of Experimental Hormonology, Brussels Free University, 808 Route de Lennik, B-1070 Brussels, Belgium. E-mail: malaisse@ulb.ac.be

Table 1
Secretory Response to Hypotonicity and D-Glucose In Three Insulin-Producing Cell Lines

Cell line	MIN-6		INS-1		BRIN–BD11	
Osmolarity	Iso	Hypo	Iso	Hypo	Iso	Hypo
Insulin output (μU/10^6 cells)	62.8 ± 5.5 (12)	74.1 ± 6.4 (12)	86.7 ± 6.1 (12)	153.4 ± 17.1 (12)	11.0 ± 0.8 (18)	28.6 ± 2.6 (18)
Insulin output (% of Iso)	100.0 ± 1.4 (12)	118.3 ± 1.9 (11)	100.0 ± 2.9 (12)	174.4 ± 10.8 (12)	100.0 ± 6.5 (18)	265.5 ± 27.0 (18)
Ratio (%)	Hypo/Iso		Hypo/Iso		Hypo/iso	
D-glucose (1.1 m*M*)	115.5 ± 1.3 (2) [100.0 ± 0.0][a]		145.3 ± 15.3 (2) [100.0 ± 0.0]		240.8 ± 17.2 (4) [100.0 ± 0.0]	
D-glucose (5.0 m*M*)	124.8 ± 3.4 (2) [108.1 ± 4.1]		154.3 ± 0.9 (2) [107.5 ± 12.0]		297.8 ± 41.9 (4) [122.8 ± 14.6]	
D-glucose (10.0 m*M*)	113.4 ± 6.2 (2) [98.1 ± 4.2]		145.9 ± 15.3 (2) [102.7 ± 21.4]		334.7 ± 10.3 (4) [140.5 ± 7.4]	

[a]The values shown in square brackets refer to the Hypo/Iso ratio expressed relative to the paired value found within the same experiment(s) in the presence of 1.1 m*M* D-glucose.

5.0, and 10.0 m*M*). As indicated in the lower part of Table 1, no significant effect of the hexose concentration upon the secretory responsiveness to hypotonicity was detected in either MIN-6 or INS-1 cells. Indeed, the hypotonic/isotonic ratio in insulin output recorded in the presence of 5.0 and 10.0 m*M* D-glucose averaged, in MIN-6 and INS-1 cells, respectively, 103.1 ± 3.8% and 105.1 ± 10.1% (n = 4 and $p > 0.5$ vs unity in both cases) of the paired value found within the same experiment(s) in the presence of only 1.1 m*M* D-glucose. A different situation prevailed, however, in BRIN–BD11 cells. Thus, in the latter cells, the hypotonic/isotonic ratio increased from 240.8 ± 17.2% to 334.7 ± 10.3% (n = 4 in both cases; $p < 0.005$) as the concentration of D-glucose was raised from 1.1 to 10.0 m*M*. As a matter of fact, in the BRIN–BD11, there was a significant positive correlation (r = 0.705; n = 12; $p < 0.02$) between the values for the hypotonic/isotonic ratio (expressed relative to the reference value found within the same experiment at 1.1 m*M* D-glucose) and the concentration of the hexose. Such was also the case (r = 0.626; n = 12; $p < 0.05$) when the absolute values for the hypotonic/isotonic ratio were compared to the concentration of D-glucose.

These findings indicate that the BRIN–BD11 cells not only display a relatively greater secretory response to hypotonicity but also positive modulation of such a response by the extracellular concentration of D-glucose. All further experiments were, therefore, conducted only in BRIN–BD11 cells.

Control Values

The basal release of insulin recorded over 30 min incubation at 1.1 m*M* D-glucose in BRIN–BD11 cells exposed to a salt-balanced medium averaged 25.6 ± 2.4 μU/10^6 cells in 30 min (n = 37).

A rise in D-glucose concentration from 1.1 to 11.1 m*M* augmented insulin release from the BRIN–BD11 cells by 23.0 ± 7.7% (n = 15; $p < 0.01$).

The release of insulin recorded in the hypotonic medium, i.e., when the concentration of NaCl was decreased by 50 m*M*, averaged 229.7 ± 16.8% (n = 37; $p < 0.001$) of the paired basal value.

The time course for the secretory response to hypoosmolarity, as assessed in a series of four experiments, is illustrated in Fig. 1. The increment in insulin output attributable to hypoosmolarity above the corresponding basal value decreased in an exponential manner, there being a highly significant correlation (r = –0.942; $p < 0.005$) between the logarithmic values for such an increment and the time after decreasing the osmolarity of the incubation medium. Such was not the case, however, for the difference in secretory rate between the values measured under hypotonic conditions in the presence of 0.1 m*M* NPPB and corresponding basal values. Indeed, such differences failed to display any significant correlation with time and averaged no more than 3.2 ± 1.6 μU/15 min (n = 6).

Effect of NPPB

The inhibitor of volume-sensitive anion channels NPPB (0.1 m*M*) slightly enhanced basal insulin output to 115.9 ± 7.6% (n = 8; $p < 0.05$) of paired control value (Table 2). In the presence of NPPB, the release of insulin recorded in the hypotonic medium was only 31.0 ± 11.9% (n = 8; $p < 0.025$) higher than the paired value found in the salt-balanced medium, as distinct ($p < 0.01$) from an increment of 159.7 ± 52.2% (n = 8; $p < 0.005$) provoked, within the same experiments, by the hypotonic medium in the absence of NPPB. In other words, when the cells were exposed to the hypotonic medium, the release of insulin found in the presence of

Table 2
Insulin Release (μU per 30 min) from BRIN–BD11 Cells Incubated Under Isotonic or Hypotonic Conditions in the Absence (Control) or Presence (Experimental) of Different Tested Agents

Exp Nr	Tested agent	Isotonicity		Hypotonicity[a]	
		Control	Experimental	Control	Experimental
1/2	NPPB (0.1 m*M*)	15.9 ± 0.8 (8)	18.5 ± 1.0 (8)	47.3 ± 7.9 (8)	24.3 ± 1.5 (8)
3	Verapamil (10 μ*M*)	15.6 ± 4.6 (4)	26.3 ± 8.7 (4)	42.5 ± 6.7 (4)	25.9 ± 4.1 (4)
3	No Ca^{2+} + EGTA (0.5 m*M*)	15.6 ± 4.6 (4)	1.2 ± 1.2 (4)	42.5 ± 6.7 (4)	15.2 ± 6.9 (4)
4	Thapsigargin (1.0 μ*M*)	31.3 ± 2.7 (4)	29.7 ± 2.1 (4)	44.3 ± 1.1 (4)	29.0 ± 2.7 (4)
4	Diazoxide (0.1 m*M*)	31.3 ± 2.7 (4)	23.9 ± 0.7 (4)	44.3 ± 1.1 (4)	32.8 ± 1.6 (4)
5	Tolbutamide (10 μ*M*)	23.3 ± 4.2 (4)	19.6 ± 0.4 (4)	64.8 ± 2.3 (4)	79.3 ± 3.4 (4)
6	K^+ (30.0 m*M*)	31.5 ± 5.1 (4)	45.1 ± 7.7 (4)	56.9 ± 5.2 (4)	53.3 ± 4.3 (4)
7/8	Gluconate[b]	17.1 ± 4.4 (6)	3.0 ± 0.9 (6)	40.5 ± 1.7 (4)	21.9 ± 2.2 (4)
1/6/8/9	Furosemide (0.1 m*M*)	23.8 ± 2.7 (12)	21.2 ± 3.0 (12)	56.5 ± 3.5 (12)	44.9 ± 4.6 (12)
5	Acetazolamide (1.0 m*M*)	23.3 ± 4.2 (4)	18.0 ± 0.8 (4)	64.8 ± 2.3 (4)	58.1 ± 2.0 (4)
10	Tributyltin (1.0 μ*M*)	25.3 ± 2.9 (6)	26.0 ± 2.4 (6)	48.9 ± 5.6 (6)	32.5 ± 2.6 (6)
10	Tributyltin (2.5 μ*M*)	25.3 ± 2.9 (6)	47.8 ± 4.6 (6)	48.9 ± 5.6 (6)	48.6 ± 3.3 (6)

[a]Hypotonicity was provoked by the omission of 50 m*M* NaCl.
[b]Partial substitution of Cl^- by gluconate during both preincubation (180 min) and incubation (30 min); such a substitution amounted to 118 m*M* during preincubation and during incubation under isotonic conditions and 68 m*M* during incubation under hypotonic conditions.

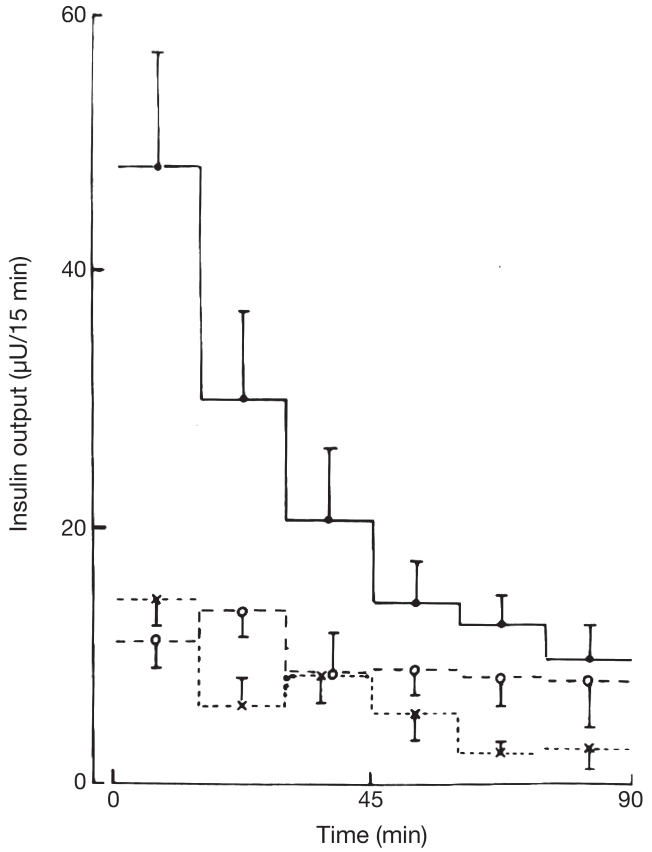

Fig. 1. Time course for the changes in basal insulin output (crosses and dotted line), and insulin secretion found under hypotonic conditions (50 m*M* decrease in NaCl concentration) in the absence (closed circles and solid line) or presence of 0.1 m*M* NPPB (open circles and dashed line). Mean values (± SEM) refer to four experiments.

NPPB (0.1 m*M*) only represented 58.5 ± 11.0% ($n = 8$; $p < 0.025$) of the paired value found in the absence of NPPB.

Ca^{2+} Dependency of the Secretory Response to Hypoosmolarity

Two sets of data were obtained to indicate that the secretory response to hypoosmolarity represents a Ca^{2+}-dependent process.

First, verapamil (10.0 μ*M*) decreased ($p < 0.04$) the output of insulin recorded in the hypotonic medium to 61.0 ± 8.4% ($n = 4$) of the paired value found in the absence of the organic calcium-antagonist. In the presence of verapamil, the release of insulin from cells exposed to the hypotonic medium indeed only averaged 119.1 ± 33.9% ($n = 4$; $p > 0.5$ vs unity) of the paired basal value recorded in cells exposed to a salt-balanced medium, as distinct ($p < 0.05$) from 304.5 ± 70.9% ($n = 4$; $p < 0.02$ vs unity) in the absence of verapamil.

Second, in the absence of extracellular Ca^{2+} and presence of EGTA (0.5 m*M*), the output of insulin recorded in the hypotonic medium was decreased to 17.8 ± 17.8% ($n = 4$; $p < 0.02$) of the paired value found at normal extracellular Ca^{2+} concentration (1.0 m*M*). In absolute terms, the paired increment in insulin secretion caused by hypoosmolarity averaged 6.7 ± 6.7 μU per 30 min ($n = 4$) in the Ca^{2+}-depleted medium, as distinct ($p < 0.05$) from 26.8 ± 3.5 μU per 30 min ($n = 4$) at normal extracellular Ca^{2+} concentration. In considering these results, it should be underlined that the omission of Ca^{2+} also decreased the basal output of insulin recorded at normal osmolarity by 14.5 ± 4.6 μU per 30 min ($n = 4$; $p < 0.04$), such a basal output averaging 15.6

± 4.6 μU per 30 min in the presence of Ca^{2+} (1.0 m*M*) and 1.2 ± 1.2 μU per 30 min ($n = 4$) in its absence.

The basal release of insulin was not affected significantly ($p > 0.6$) by thapsigargin (1.0 μ*M*), averaging 31.3 ± 2.7 and 29.7 ± 2.1 μU per 30 min in its absence and presence, respectively ($n = 4$ in both cases). However, in the hypotonic medium, thapsigargin decreased insulin output to 64.6 ± 7.3% ($n = 4$; $p < 0.04$) of the paired value found in its absence. This finding is consistent with a process of Ca^{2+}-induced Ca^{2+} release from intracellular organelles in the cells stimulated by hypoosmolarity.

Role of ATP-Sensitive K^+ Channels

Three sets of experiments were conducted to explore the possible participation of ATP-sensitive K^+ channels in the secretory response to hypoosmolarity.

First, the effects of diazoxide (0.1 m*M*) were investigated under both basal and hypotonic conditions. At normal osmolarity, diazoxide decreased basal insulin release to 76.9 ± 5.3% ($n = 4$; $p < 0.04$) of its paired control value. Likewise, the output of insulin found in the hypotonic medium only represented, in the presence of diazoxide, 73.9 ± 5.0% ($n = 4$; $p < 0.04$) of the paired value found within the same experiment(s) in its absence. This suggests that, in relative terms, the gating of ATP-sensitive K^+ channels by diazoxide plays a comparable modulatory role ($p > 0.65$) upon insulin output under basal and hypotonic conditions.

Second, the effect of tolbutamide (10 μ*M*) upon both basal and hypotonicity-stimulated insulin release was examined. Tolbutamide failed to affect significantly basal insulin output (d.f. = 6; $p > 0.4$) at the low concentration of D-glucose (1.1 m*M*) used in the present experiments with mean values of 23.3 ± 4.2 and 19.6 ± 0.4 μU per 30 min in the absence and presence of the hypoglycaemic sulfonylurea. However, in the hypoosmolar medium, the release of insulin was increased ($p < 0.02$) by tolbutamide from 64.8 ± 2.3 to 79.3 ± 3.4 μU per 30 min ($n = 4$ in both cases). The latter finding indicates that in cells engaged in an active process of insulin secretion, the hypoglycemic sulfonylurea augmented, modestly but significantly, the output of insulin recorded in a hypotonic medium, possibly by minimizing the fall in insulin secretion otherwise characterizing the secretory response to hypoosmolarity.

Last, a rise in extracellular K^+ concentration to 30 m*M*, while increasing insulin release at normal osmolarity to 146.7 ± 16.6% ($n = 4$) of paired basal value, decreased the increment in insulin output attributable to hypoosmolarity to 25.4 ± 20.1% ($n = 4$; $p < 0.05$) of the paired control value found at normal extracellular K^+ concentration. The latter two percentages being vastly different from one another ($p < 0.005$), they indicate that the membrane depolarization caused by the high extracellular K^+ concentration augmented, as expected, insulin output at normal osmolarity, but apparently prevented hypoosmolarity to provide a further depolarization of the plasma membrane. This interpretation is borne out by the finding that, in the hypoosmolar medium, the absolute value for insulin output averaged, in the presence of 30 m*M* K^+, 95.2 ± 13.0% ($n = 4$; $p > 0.7$ vs unity) of the paired value recorded at normal extracellular K^+ concentration.

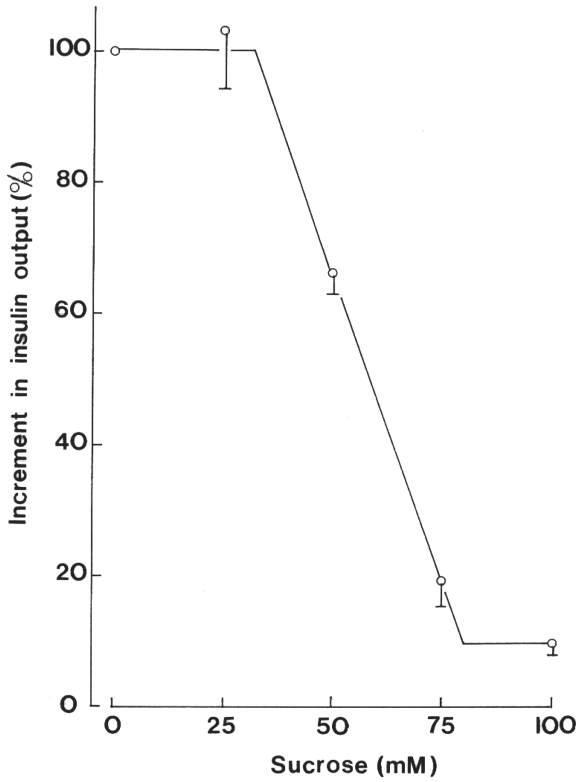

Fig. 2. Effect of increasing concentrations of sucrose upon the increment in insulin output evoked by a decrease in NaCl concentration of 50 m*M*, the estimated osmolarity ranging from about 200 to 300 mOsmol/L. Mean values (± SEM) refer to four individual determinations and are expressed in percentage of the paired increment found in the absence of sucrose. In this set of experiments, such a control increment averaged 45.6 ± 8.9 μU per 30 min.

Concentration Dependency of the Response to Hypoosmolarity

A decrease in NaCl concentration of 25 m*M* yielded an increment in insulin output above paired basal value representing 55.0 ± 14.1% ($n = 4$; $p < 0.005$) of that recorded within the same experiment(s) when the NaCl concentration was decreased by 50 m*M*.

Likewise and as illustrated in Fig. 2, the addition of sucrose to the hypotonic medium (deprived of 50 m*M* NaCl) provoked a concentration-related decrease in the secretory response to hypoosmolarity. As judged from these data, a decrease in osmolarity of 75 mOsmol/L was sufficient to provoke a maximal secretory response. Indeed, the increment in insulin output above basal value recorded in the presence of 25 m*M* sucrose averaged 103.3 ± 9.1% ($n = 4$) of that found in the absence of sucrose.

In the presence of 50 m*M* sucrose, however, the increment in insulin output was decreased ($p < 0.005$) to 66.1 ±

3.0% ($n = 4$) of that recorded in the absence of the disaccharide. Further increases in the concentration of sucrose to 75 and 100 m*M* provoked further decreases ($p < 0.05$ or less) of the increments in insulin secretion above basal value otherwise caused by NaCl depletion (Fig. 2).

Nevertheless, even in the presence of 100 m*M* sucrose, i.e., in an isoosmotic medium, the output of insulin remained significantly higher ($p < 0.02$) than basal value, whether judged from the paired differences in absolute values for insulin secretion (+ 6.7 ± 1.5 μU per 30 min; $n = 4$) or the increments in insulin release relative to those recorded in the hypotonic medium in the absence of sucrose (9.6 ± 1.8%; $n = 4$).

Substitution of Cl^- by Gluconate

When the cells were both preincubated for 180 min and incubated for 30 min in Cl^--depleted media (substitution of 118 m*M* Cl^- by an equimolar amount of gluconate), the basal release of insulin only represented 20.6 ± 7.1% ($n = 6$; $p < 0.001$) of paired control value. Under hypotonic conditions (decrease of osmolarity by 100 mOsmol/L), however, the output of insulin from cells both preincubated and incubated in the presence of gluconate represented 55.0 ± 7.4% ($n = 4$; $p < 0.01$) of the paired control value found in cells preincubated and incubated in the presence of Cl^- rather than gluconate. The latter percentage was significantly higher ($p < 0.02$) than that found for the basal release of insulin. These findings indicate that the decrease in extracellular Cl^- concentration affects less severely insulin secretion under hypoosmolar conditions than under isotonic conditions.

Nevertheless, even after preincubation at normal Cl^- concentration (120 m*M*), the increment in insulin output attributable to hypotonicity (reduction of osmolarity by 100 mOsmol/L) and recorded when 68 m*M* of Cl^- was substituted by an equimolar amount of gluconate during the first incubation remained 14.7 ± 3.4 μU per 30 min ($n = 4$; $p < 0.025$) lower than that recorded in the absence of gluconate (24.9 ± 5.9 μU per 30 min; $n = 4$).

Effect of Furosemide

After 60–180 min preincubation and during 30 min incubation in the presence of furosemide (0.1 m*M*), the inhibitor of the Na-K-2Cl cotransporter failed to affect significantly ($p > 0.6$) basal insulin output, which averaged 103.3 ± 6.2% ($n = 10$) of paired control basal value. Furosemide, however, decreased the output of insulin recorded under hypoosmolar conditions to 78.7 ± 7.7% ($n = 12$; $p < 0.02$) of the paired value recorded in its absence. In these experiments, the secretion of insulin found in the hypotonic medium in the absence of furosemide averaged 276.7 ± 35.5% ($n = 12$; $p < 0.001$) of paired basal value.

Effect of Acetazolamide

The basal release of insulin was not affected significantly ($p > 0.2$) by 1.0 m*M* acetazolamide, averaging 23.3 ± 4.2 and 18.0 ± 0.8 μU per 30 min ($n = 4$ in both cases) in the absence and presence of the carbonic anhydrase inhibitor. Likewise, the increment in insulin output attributable to hypoosmolarity averaged 41.5 ± 6.0 and 40.2 ± 1.5 μU per 30 min ($n = 4$ in both cases; $p > 0.8$) in the absence and presence of acetazolamide, respectively. These findings suggest that the HCO_3^- anion does not participate, to any significant extent, in the insulin secretory response to hypoosmolarity.

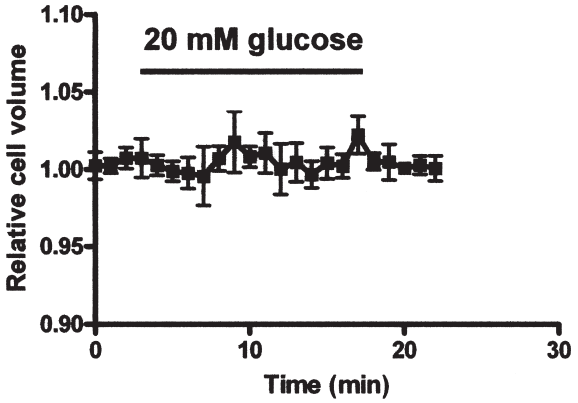

Fig. 3. Effect of 20 m*M* D-glucose on the relative cell volume of BRIN–BD11 cells. Mean values (± SEM) refer to three experiments. The reference cell volume before rising the D-glucose concentration averaged 4.7 ± 1.7 pL ($n = 3$).

Effect of Tributyltin

At a 1.0 μ*M* concentration, tributyltin failed to affect significantly ($p > 0.8$) the basal output of insulin, which averaged 25.3 ± 2.9 and 26.0 ± 2.4 μU per 30 min ($n = 6$ in both cases) in the absence and presence of the Cl^- ionophore, respectively. At the same concentration, however, tributyltin decreased the increment in insulin output attributable to hypoosmolarity to 24.3 ± 8.0% ($n = 6$; $p < 0.001$) of the paired value recorded in its absence. When the concentration of tributyltin was increased to 2.5 μ*M*, the output of insulin was virtually identical ($p > 0.8$) at normal osmolarity (47.8 ± 4.6 μU per 30 min, $n = 6$) and in the hypotonic medium (48.6 ± 3.3 μU/min; $n = 6$). As indicated by the former value, this situation coincided with the fact that tributyltin (2.5 μ*M*) almost doubled ($p < 0.005$) basal insulin release, an effect conceivably attributable to the lowering of Cl^- concentration in the insulin-producing cells. In any case, the present results support the view that the Cl^- ionophore, by decreasing or abolishing the chemical gradient of Cl^- across the plasma membrane, decreased or abolished the secretory response to hypoosmolarity.

Cell Volume

The basal value for cell volume averaged 3.04 ± 0.46 pL ($n = 13$). A rise in D-glucose concentration from 1.1 to 20.0 m*M* failed to cause any obvious change in cell volume (Fig. 3).

A decrease in extracellular osmolarity, as caused by a reduction of NaCl concentration by 50 m*M*, however, provoked

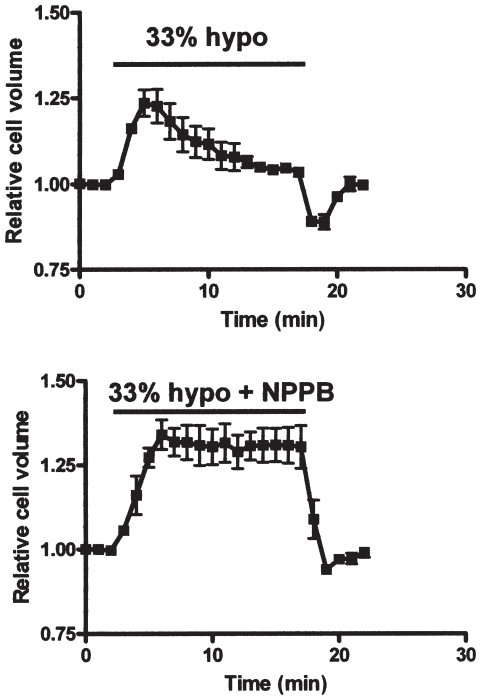

Fig. 4. Time course for the changes in volume of BRIN–BD11 cells exposed (horizontal bar) to a hypotonic medium in the absence (upper panel) or presence (lower panel) of 0.1 m*M* NPPB. Mean values (± SEM) are derived from four (upper) or three (lower) experiments. The reference cell volume before decreasing extracellular osmolarity averaged 2.3 ± 0.3 pL (n = 7).

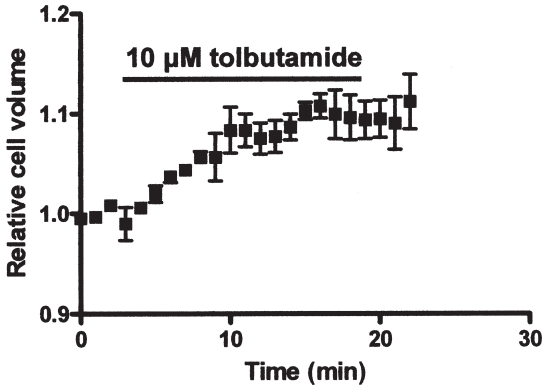

Fig. 5. Effect of 10 µ*M* tolbutamide on relative cell volume of BRIN–BD11 cells. Mean values (± SEM) refer to three experiments. The reference cell volume before addition of tolbutamide averaged 3.0 ± 0.2 pL (n = 3).

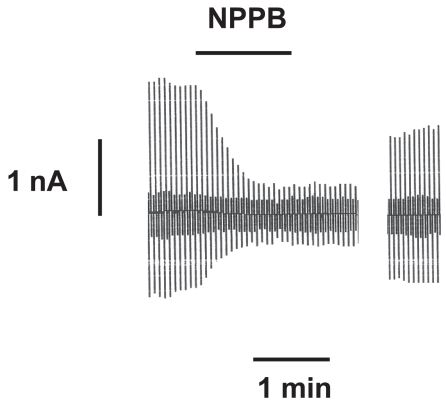

Fig. 6. Effect of 0.1 m*M* NPPB (horizontal bar) upon volume-regulated anion channel activity. Conventional whole-cell recording with hypertonic intracellular medium to induce BRIN–BD11 cell swelling were obtained using a 0 mV, 50 ms holding potential. Voltages pulses of ± 100 mV were applied at 2 s intervals. The gap in the trace corresponds to approximately 2 min.

a rapid increase in cell volume followed by a regulatory volume decrease (Fig. 4, upper panel). The peak value reached shortly after lowering extracellular osmolarity was close to a 25% increase in cell volume. Upon restoration of normal extracellular osmolarity, the cell volume recovered its basal value, after a transient decrease below such a value.

When the same experiments were repeated in the presence of NPPB (0.1 m*M*), the initial increase in cell volume caused by a decrease in extracellular osmolarity was as pronounced and even slightly higher than that found in the absence of NPPB. The latter drug prevented, however, the later regulatory volume decrease otherwise observed during exposure to the hypotonic medium (Fig. 4, lower panel).

As already observed in normal islet B-cells *(5)*, tolbutamide (10 µ*M*) caused a progressive and not rapidly reversible increase of cell volume in BRIN–BD11 cells incubated in the absence of D-glucose (Fig. 5).

Inhibition by NPPB of Volume-Regulated Anion Channel Activity

The findings so far presented led us to assess whether NPPB (0.1 m*M*) indeed inactivate volume-regulated anion channels in the BRIN–BD11 cells. As illustrated in Fig. 6, NPPB decreased in a rapid and reversible manner, both the inward and outward currents provoked by ± 100 mV voltage pulses. The inward currents were decreased ($p < 0.01$) by NPPB from a control value of –6.3 ± 0.3 nA to –3.2 ± 0.5 nA, while the outward currents were decreased ($p < 0.01$) from 15.6 ± 1.2 nA to 4.0 ± 0.7 nA (all n = 4).

Membrane Potential

The resting membrane potential averaged –58.7 ± 1.8 mV (n = 4) in the BRIN–BD11 cells.

A rise in D-glucose concentration from 1.1 to 11.0 m*M* D-glucose only caused on occasion a depolarization of the plasma membrane with induction of spike activity (Fig. 7).

Exposure of the cells to a hypotonic medium (decrease of NaCl by 50 m*M*) provoked immediate or progressive depolarization and, on occasion, induction of spiking activity (Figs. 8 and 9). The latter response was rapidly reversible in some (Fig. 8) but not all (Fig. 9, upper panels) cases upon restoration of a normal extracellular osmolarity. Nevertheless, NPPB (0.1 m*M*) provoked a rapid and rapidly reversible repolarization with suppression of spiking activity (Fig. 8 and Fig. 9, lower panel).

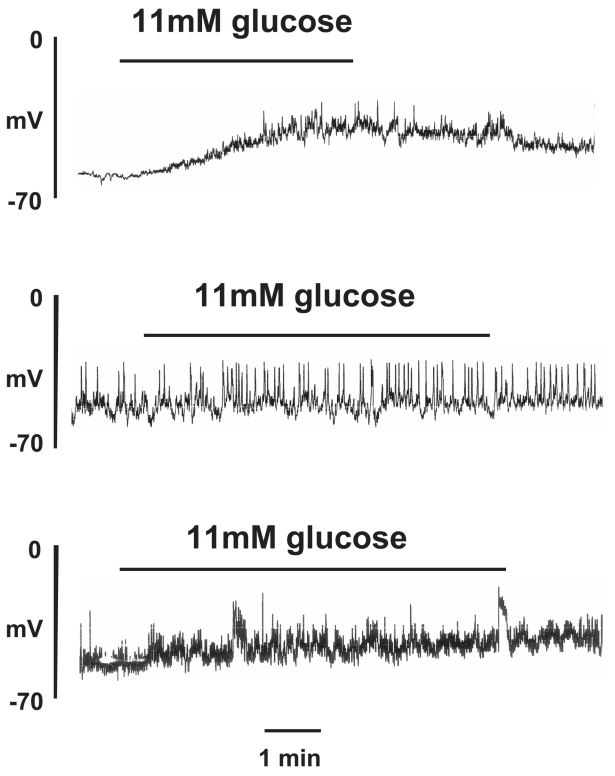

Fig. 7. Effect of 11 m*M* D-glucose on membrane potential in three different BRIN–BD11 cells.

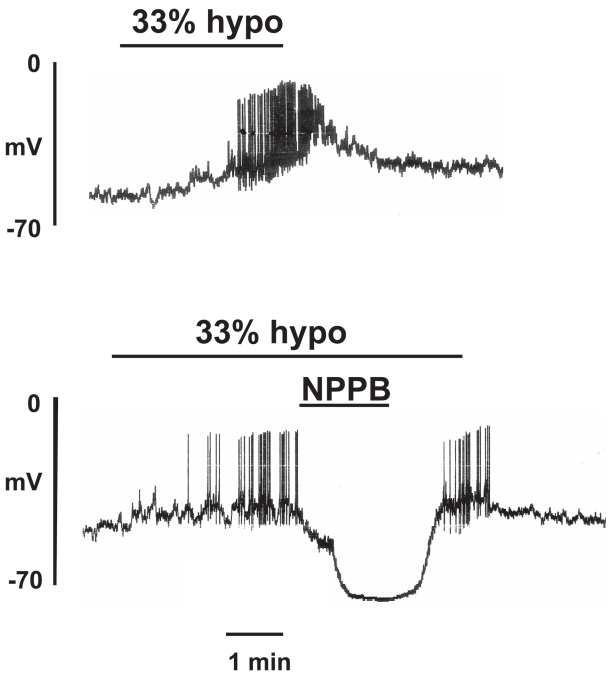

Fig. 8. Effects of hypotonicity (33% decrease in osmolarity) and NPPB (0.1 m*M*) on membrane potential in BRIN–BD11 cells.

Cytosolic Ca^{2+} Concentration

Exposure of the BRIN–BD11 cells to the hypotonic medium provoked a rapid, sustained, and reversible increase in cytosolic Ca^{2+} concentration (Fig. 10). The effect of NPPB upon cytosolic Ca^{2+} concentration was not examined, because the latter drug displays strong fluorescence.

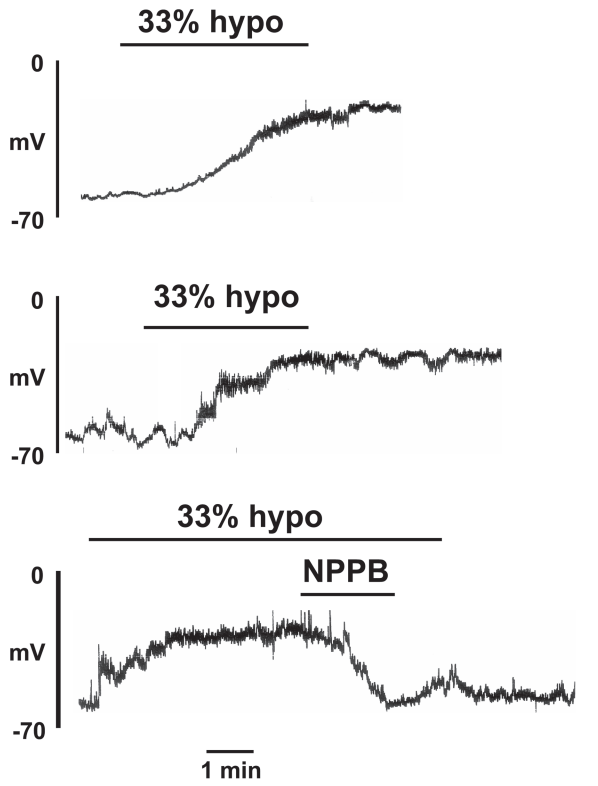

Fig. 9. Effects of hypotonicity (33% decrease in osmolarity) and NPPB (0.1 m*M*) on membrane potential in BRIN–BD11 cells.

Discussion

The BRIN–BD11 cells used in the present and prior studies displayed a modest secretory response to D-glucose, this coinciding with a pattern for the metabolism of the hexose distinct from that found in normal insulin-producing cells *(8)*. A much larger stimulation of insulin was recorded in response to hypotonicity.

The selection of BRIN–BD11 cells for the present work was indeed motivated by the three following considerations. First, among three lines of insulin-producing cells, the BRIN–BD11 cells displayed the greater secretory responsiveness to hypotonicity. Second, such a secretory response was modulated by the extracellular concentration of D-glucose. Third, the use of a homogeneous population consisting solely of insulin-producing cells allowed one to avoid the limitations otherwise prevailing when using non-purified normal islet cells. Moreover, the present approach is also favorable in the perspective of obtaining large numbers of cells for isolation and characterization of the volume-sensitive anion channels.

The time course for hypotonicity-induced insulin release was similar to that reported in experiments conducted in cells such as normal rat pancreatic islets *(2)*, HIT–T15 cells *(5)*, and βHC9 cells *(6)*. It was indeed characterized by a rapid onset, early peak value, and, thereafter, an exponential decay.

It coincided with an early increase in cell volume followed by a regulatory volume decrease, plasma membrane

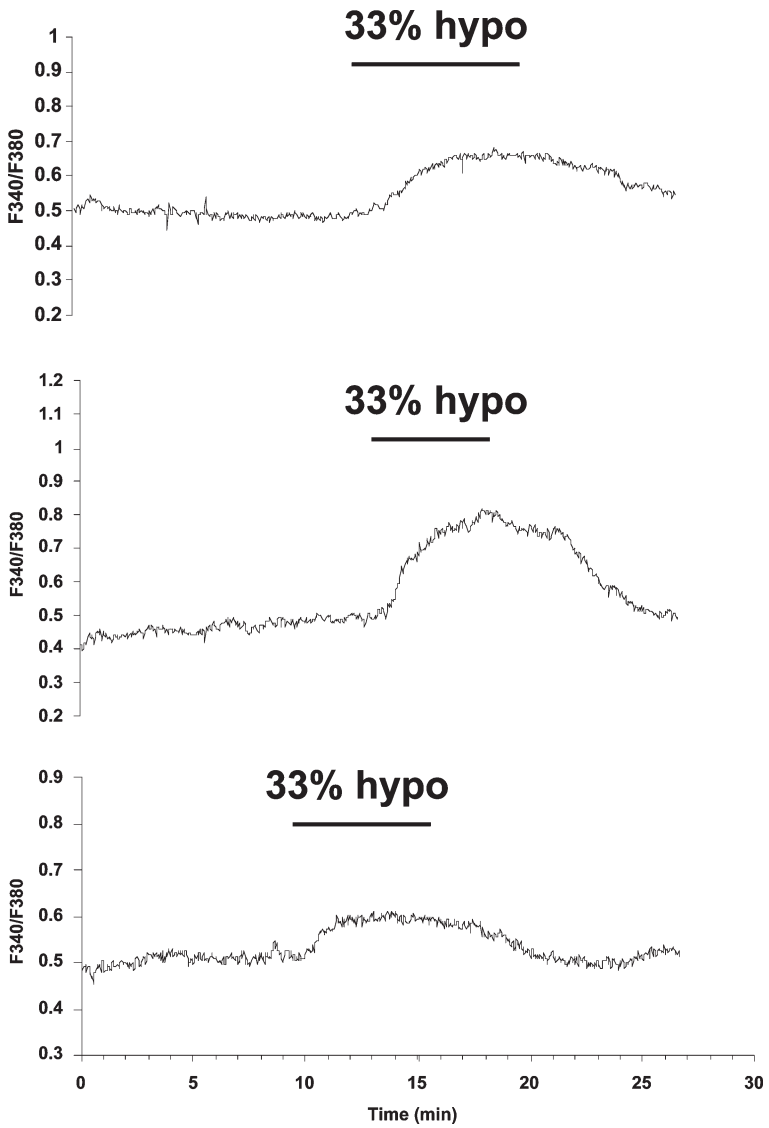

Fig. 10. Effect of hypotonicity (33% decrease in osmolarity) upon cytosolic Ca^{2+} concentration in three different BRIN–BD11 cells, as judged from the F340/F380 ratio for emission fluorescence at excitation wavelengths of 340 and 380 nm.

depolarization with induction of spiking activity, and a rapid, sustained and reversible increase in cytosolic Ca^{2+} concentration. These findings are also in fair agreement with those reported in prior publications *(1,3,5,9,10)*.

In the present experiments, the inhibitor of volume-sensitive anion channel NPPB abolished the regulatory volume decrease otherwise recorded in response to extracellular hypoosmolarity. This finding is again in fair agreement with a prior report documenting inhibition of regulatory volume decrease by NPPB in RINm5F cells exposed to a hypotonic medium *(10)*. NPPB also reversibly inhibited the activity of the volume-regulated anion channels in BRIN–BD11 cells in which activation of these channels was induced by intracellular hypertonicity. Comparable findings were already collected in tumoral RINm5F cells and HIT-T15 cells *(10)*. Moreover, NPPB opposed the effect of extracellular hypoosmolarity on plasma membrane potential, bioelectrical spiking activity, and insulin release. These findings indicate that activation of volume-sensitive anion channels is critical for hypotonicity-induced insulin release. The effect of NPPB on $[Ca^{2+}]_i$ could not be investigated because of its own fluorescence.

If any, the participation in the regulatory volume decrease of the activation of a K^+ conductance other than that regulated by ATP appears quite restricted, in view of the observed hypotonicity-induced depolarization and its suppression, as well as that of the regulatory volume decrease, by NPPB.

In the presence of extracellular Ca^{2+}, osmotic cell swelling increases $[Ca^{2+}]_i$. This was first documented in mouse islet β-cells *(3)*, and here confirmed in BRIN–BD11 cells. Conflicting results were reported, however, concerning the

Ca^{2+}-dependency of the sequence of events leading to hypotonicity-induced insulin release. Best and colleagues first indicated that the amplitude of swelling-activated currents in insulinoma cells was not influenced by the presence of an intracellular Ca^{2+} chelator or replacement of extracellular Co^{2+} by Ca^{2+}, suggesting that this Cl^- conductance is neither Ca^{2+}-dependent nor Ca^{2+}-activated *(10)*. Drews et al. *(3)* then reported that, in mouse islet β-cells, omission of extracellular Ca^{2+} or addition of D600 abolished the inward current carried by Cl^- and triggered by osmotic cell swelling. Likewise, while the present investigations afford three independent sets of data in support of the Ca^{2+}-dependency of hypotonicity-induced insulin release, based on the use of verapamil, EGTA, and thapsigargin, it was claimed, in a recent study conducted by Straub et al. *(6)* in βHC9 cells, that the release of insulin due to a hypotonic shock was higher in the absence of extracellular Ca^{2+} than in its presence. Yet, the same authors observed that the L-type voltage-dependent Ca^{2+} channel blocker nitrendipine (10 μ*M*) largely suppressed the secretory response to hypotonicity in the presence of extracellular Ca^{2+} (1.0 m*M*), but not so in its absence. They concluded that extracellular hypotonicity may provoke insulin release by two distinct mechanisms operative in either the presence or absence of extracellular Ca^{2+}. Moreover, the same authors reported that, in the presence of Ca^{2+}, the secretory response of the βHC9 cells to hypotonicity was not affected by the chloride channel blocker DIDS (4,4-diisothiocyanate-stilbene-2,2'-disulfonic acid) tested at a 0.1 m*M* concentration, at variance with prior results on the inhibition by DIDS of (i) swelling-induced activation of anion-permeable Cl^- channels in tumoral *(5, 10)* and normal *(3)* insulin-producing cells, (ii) the regulatory volume decrease recorded in RINm5F cells exposed to a hypotonic medium *(10)*, (iii) the hypotonicity-induced stimulation of taurine and L-aspartate efflux from prelabeled INS-1 cells *(4)*, and (iv) hypotonically induced insulin secretion in HIT–T15 cells *(5)*. Straub et al. *(6)* concluded from their work that, in the presence of extracellular Ca^{2+}, the increased influx of Ca^{2+} and subsequent stimulation of insulin release provoked by extracellular hypoosmolarity could not be attributed to activation of chloride channels. Alternatively, they proposed that, in the presence of extracellular Ca^{2+}, the influx of water or stretching of the plasma membrane activates L-type Ca^{2+} channels, the activation of chloride channels being redundant to hypotonicity-induced insulin release. To say the least, these considerations duly document that the mechanism for the latter secretory response remains a matter of debate.

Another issue considered in this report refers to the possible participation of ATP-sensitive K^+ channels in the secretory response to extracellular hypoosmolarity. Our data suggest that a closing of these channels is not involved in hypotonicity-induced insulin release. Indeed, the relative extent of the inhibitory action of diazoxide on insulin release was virtually identical under isotonic and hypotonic conditions. Moreover, the closing of ATP-sensitive K^+ channels by tolbutamide was still able to enhance insulin release from BRIN–BD11 cells exposed to a hypotonic medium.

The release of insulin caused by an increase in extracellular K^+ concentration and that provoked by a decrease in extracellular osmolarity apparently represent two comparable modalities for stimulation of insulin secretion, involving both plasma membrane depolarization. The secretory response to each of these two procedures was indeed comparable to that recorded when the BRIN–BD11 cells were simultaneously exposed to a high extracellular K^+ concentration and low extracellular osmolarity.

As already reported by several investigators *(5,6)*, the stimulation of insulin release provoked by a decrease in extracellular NaCl concentration is prevented by equiosmolar replacement of NaCl by another osmolyte, e.g., sucrose in the present experiments. These experiments also document the concentration-dependency of the secretory response to hypoosmolarity.

As a rule, the former observation is interpreted to indicate that the hypotonicity-induced stimulation of insulin release is not attributable to a decrease in extracellular Na^+ or Cl^- concentration. However, our data reveal that, upon isoosmolar substitution of NaCl by sucrose, the output of insulin remained slightly but significantly higher than basal value. The latter finding suggests either that sucrose exerts a modest but significant positive insulinotropic action, that a lowering of Na^+ concentration by 50 m*M* favors insulin secretion independently of any change in extracellular osmolarity, or that a decrease in Cl^- concentration also by 50 m*M* likewise increases insulin output under isoosmolar conditions. The first of these three hypotheses is compatible with the proposal that insulin-producing cells are equipped with the same G proteins as those identified in taste buds *(11)*. The second hypothesis is compatible with the observation that equimolar substitution of Na^+ by choline augments glucose-stimulated insulin release *(12)*. It should be emphasized, however, that equimolar substitution of Na^+ by either Li^+ or Tris inhibits glucose-stimulated insulin release *(12)*. The third hypothesis is consistent with the view that a lowering of extracellular Cl^- concentration favors the outflow of Cl^- by volume-sensitive anion channels. Further experiments in this study precisely aimed at investigating the role of Cl^- in the secretory response to hypoosmolarity.

In this respect, our findings suggest that the decrease in extracellular Cl^- concentration only plays a modulatory role in the secretory response to hypoosmolarity. Nevertheless, a prolonged preincubation at low Cl^- concentration may impair the response to hypoosmolarity, and even more so that to D-glucose, pointing to the adverse effect of intracellular Cl^- depletion in the secretory process. Moreover, any decrease in insulin output found at low extracellular Cl^- concentration is reminiscent of the role attributed to this

and other extracellular anions (e.g., OH^-) in the anion-osmotic process for exocytosis of insulin-containing secretory granules *(13)*.

The present findings on the effect of acetazolemide upon hypotonicity-induced insulin release suggest that, at the low concentration of D-glucose used in these experiments, the efflux of HCO_3^- anions plays little, if any, role in the insulin secretory response to extracellular hypoosmolarity.

Last, the data collected in the presence of tributyltin, are consistent with the view that a high intracellular concentration of Cl^- anions, as presumably achieved in insulin-producing cells at the intervention of the Na^+-K^+-$2Cl^-$ co-transporter specifically expressed in rat β, as distinct from α, islet cells *(14)*, is required to allow the process of hypotonicity-induced insulin release. This view is further supported by the present finding that furosemide decreases insulin release from BRIN–BD11 cells exposed to a hypotonic extracellular medium. In HIT–T15 cells, furosemide also suppresses the increase in cell volume otherwise provoked by extracellular hypoosmolarity, and even causes shrinkage of cells maintained under isotonic conditions *(5)*. Likewise, Majid et al. *(14)* indicated that bumetanide, another inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter, while failing to prevent rat islet cells shrinkage in response to extracellular hypertonicity reduces the subsequent volume recovery otherwise recorded in the absence of bumetanide. Thus, whenever expressed, the Na^+-K^+-$2Cl^-$ cotransporter appears critical to raise intracellular Cl^- concentration above electrochemical equilibrium and, hence, to provide the driving force for Cl^- exit upon opening of chloride channels such as the volume-sensitive anion channel.

The hypotonicity-induced activation of volume-regulated anion channels is further supported by the finding that a decrease in extracellular osmolarity increases tritiated taurine efflux from prelabeled rat pancreatic islets *(15)*, tritiated taurine and D-aspartate outflow from prelabeled INS-1 cells *(4)* and the release of unlabeled taurine and GABA from perifused rat islets *(2)*.

The identity of these Cl^- channels remains to be unambiguously established. As underlined by Kinard et al. *(5,16)*, they may differ somewhat from CLC-3, e.g., by their halide sequence ($Br^- > Cl^- > I^-$), which is shared with the cystic fibrosis transmembrane conductance-regulator, and by their response to cyclic AMP.

The possible participation of these Cl^- channels in the process of glucose-stimulated insulin release requires further investigations *(17)*. It is supported, however, by the fact that a rise in D-glucose concentration above the threshold value for its insulinotropic action causes β-cell swelling *(18)* and increases $^{36}Cl^-$ efflux from prelabeled islets *(19,20)*, and by the observation that the secretory response to the hexose is abolished by all volume-regulated anion channel inhibitors so far tested *(21–23)*. The present finding that a rise in D-glucose concentration amplifies the secretory response to hypotonicity also supports such a view.

Likewise, hypoglycemic sulfonylureas, known to close K_{ATP} channels, may, in addition, potentiate the volume-regulated anion channel current in insulin-producing cells. Best et al. *(24)* proposed that this second action of hypoglycemic sulfonylureas may be causally linked to their effect to increase β-cell volume, as here also observed in BRIN–BD11 cells.

In conclusion, therefore, a sound understanding of the sequence of events involved in activation of volume-sensitive anion channel may be relevant to both the physiology and pharmacology of insulin secretion, as provoked by either D-glucose and other nutrient secretagogues or hypoglycemic sulfonylureas.

Materials and Methods

Tissue culture materials and media were obtained from Sarstedt (Numbrecht, Germany), and In Vitrogen Life Technologies (Carlsbad, CA, USA), respectively. All pharmacological agents were purchased from Sigma (St. Louis, MO, USA).

BRIN–BD11 cells kindly provided by Prof. A. Herchuelz (Laboratory of Pharmacology, Brussels Free University, Brussels, Belgium) were grown at 37°C in a humidified incubator gassed with 5% CO_2 in air, and cultured in RPMI 1640 medium (cat. no. 21875-034, Gibco) supplemented with 10% (v/v) heat-inactivated FBS, 50 IU/mL penicillin and 50 µg/mL streptomycin. Groups of 10^6 cells each were subcultured onto 6-well plates (10 cm^2) 24 h before the experiments. Some experiments were also conducted in either MIN-6 or INS-1 cells, kindly provided by Prof. J.-C. Henquin (Unité d'Endocrinologie et Métabolisme, Université Catholique de Louvain, Brussels, Belgium) and Prof. C. B. Wollheim (Division de Biochimie Clinique, University of Geneva, Geneva, Switzerland), respectively.

For measuring insulin release, the BRIN–BD11 cells were preincubated for 30–90 min and eventually incubated for 30 min at 37°C. Except if otherwise mentioned, the incubation medium used to measure insulin release (1.0 mL) contained 10 m*M* Hepes (pH 7.4), 111 m*M* NaCl, 24 m*M* $NaHCO_3$, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1.1 m*M* D-glucose, and 0.5 mg/mL bovine serum albumin, with an estimated osmolarity close to 300 mOsmol/L. It was equilibrated against a mixture of O_2/CO_2 (19/1; v/v). As a rule, hypotonicity was achieved by decreasing the NaCl concentration to 61 m*M*. In order to rise to K^+ concentration from 5 to 30 m*M*, NaCl (25 m*M*) was replaced by an equimolar amount of KCl. In low chloride medium, NaCl (111 m*M*), KCl (5 m*M*), and $MgCl_2$ (1 m*M*) were replaced by equimolar amounts of the corresponding gluconate salts, and the concentration of $CaCl_2$ was raised to 1.5 m*M*. The insulin content of the incubation medium was measured by a method previously described *(25)*.

Changes in BRIN–BD11 cell volume were measured using a video-imaging technique as described previously

(10). Activity of the volume-regulated anion channel was assessed using the conventional whole-cell recording technique, with cell swelling induced by the use of a hypertonic pipette solution *(22)*. Plasma membrane potential was recorded using the perforated patch recording technique *(21)* and the cytosolic concentration of Ca^{2+} assessed by fluorescence of cells loaded with fura-2 essentially as described earlier *(9)*.

All results are presented as mean values (± SEM) together with the number of individual determinations (*n*) or degree of freedom (d.f.). The statistical significance of differences between mean values was assessed by use of Student's *t*-test.

Acknowledgments

This study was supported in part by the Wellcome Trust, the Fonds Alphonse et Jean Forton and the Belgian Foundation for Scientific Medical Research (grant 3.4.581.02). We are grateful to C. Demesmaeker for secretarial help.

References

1. Best, L., Miley, H. E., and Yates, A. P. (1996). *Exp. Physiol*. **81,** 927–933.
2. Bustamente, J., Lobo, M. V. T., Alonso, F. J., et al. (2001). *Am. J. Physiol*. **281,** E1275–E1285.
3. Drews, G., Zempel, G., Krippeit-Drews, P., et al. (1998). *Biochim. Biophys. Acta* **1370,** 8–16.
4. Grant, A. C. G., Thomson, J., Zammit, V. A., and Shennan, D. B. (2000). *Mol. Cell. Endocrinol*. **162,** 203–210.
5. Kinard, T. A., Goforth, P. B., Tao, Q., et al. (2001). *Diabetes* **50,** 992–1003.
6. Straub, S. G., Daniel, S., and Sharp, G. W. G. (2002). *Am. J. Physiol*. **282,** E1070–E1076.
7. McClenaghan, N. H., Barnett, C. R., Ah-Sing, E., et al. (1996). *Diabetes* **45,** 1132–1140.
8. Rasschaert, J., Flatt, P. R., Barnett, C. R., McClenaghan, M. H., and Malaisse, W. J. (1996). *Biochem. Mol. Med*. **57,** 97–105.
9. Sheader, E. A., Brown, P. D., and Best, L. (2001). *Mol. Cell. Endocr.* **181,** 179–187.
10. Best, L., Sheader, E. A., and Brown, P. D. (1996). *Pflügers Arch*. **431,** 363–370.
11. Malaisse, W. J. and Malaisse-Lagae, F. (1997). *Biochem. Mol. Int.* **43,** 1367–1371.
12. Malaisse, W. J., Malaisse-Lagae, F., and Brisson, G. (1971). *Horm. Metab. Res*. **3,** 65–70.
13. Somers, G., Sener, A., Devis, G., and Malaisse, W. J. (1980). *Pflügers Arch.* **388,** 249–253.
14. Majid, A., Speake, T., Best, L., and Brown, P. D. (2001). *Pflügers Arch.* **442,** 570–576.
15. Jijakli, H., Zhang, Y., Sener, A., and Malaisse, W. J. (2006). *Endocrine* **29,** 331–339.
16. Kinard, T. A. and Satin, L. S. (1995). *Diabetes* **44,** 1461–1466.
17. Best, L., Brown, P. D., and Tomlinson, S. (1997). *Exp. Physiol*. **82,** 957–966
18. Miley, H. E., Sheader, E. A., Brown, P. D., and Best, L. (1997). *J. Physiol*. **504,** 191–198.
19. Malaisse, W. J., Zhang, Y., Louchami, K., and Jijakli, H. (2004). *Endocrine* **25,** 23–25.
20 Sehlin, J. (1978). *Am. J. Physiol*. **235,** E501–E508.
21. Best, L. (1997). *Diabetologia* **40,**1–6.
22. Best, L. (2002). *Cell. Signal.* **14,** 69–73
23. Best, L., Brown, P. D., Sheader, E. A., and Yates, A. P. (2000). *J. Membr. Biol*. **177,** 169–175.
24. Best, L., Davies, S., and Brown, P. D. (2004). *Diabetologia* **47,** 1990–1997.
25. Leclercq-Meyer, V., Marchand, J., Wousen-Colle, M. C., et al. (1985). *Endocrinology* **116,** 1168–1174.