Expression of the Electrogenic $\text{Na}^+\text{-HCO}_3^-$ -Cotransporter NBCe1 in Tumoral Insulin-Producing BRIN-BD11 Cells

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Key Words

Insulin-producing BRIN-BD11 cells • $\text{Na}^+\text{-HCO}_3^-$ cotransporters NBCe1-A and NBCe1-B • Insulin secretion • Na^+ handling

Abstract

Background/aims: The expression of the electrogenic $\text{Na}^+\text{-HCO}_3^-$ -cotransporter NBCe1 was recently documented in rat pancreatic islet B-cells, it being speculated that such a protein participates in the extrusion of bicarbonate generated by the oxidative catabolism of nutrients from insulin-producing cells. Considering the prevalence of a Crabtree effect in tumoral insulin-producing cells, the possible presence of NBCe1 was now investigated in BRIN-BD11 cells, an insulin-producing cell line established by electrofusion of normal pancreatic B-cells with immortalized RINm5F cells. **Methods:** The possible presence of NBCe1 in BRIN-BD11 cells was investigated by RT-PCR, western blot analysis and immunocytochemistry. The release of insulin and net uptake of $^{22}\text{Na}^+$ were also measured in the BRIN-BD11 cells. **Results:** RT-PCR, western blot analysis and immunocytochemistry documented the presence of NBCe1 in BRIN-BD11 cells. A reported inhibitor of NBCe1, i.e. tenidap, (50–100 μM), inhibited basal and hypotonicity-induced insulin release from the BRIN-BD11

cells, whilst increasing the net uptake of $^{22}\text{Na}^+$ by the same cells. The latter effect was, in relative terms, more pronounced in the presence than absence of ouabain. **Conclusion:** BRIN-BD11 cells, like normal pancreatic islet B-cells, express NBCe1, with predominance of the B variant of this electrogenic $\text{Na}^+\text{-HCO}_3^-$ -cotransporter.

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Introduction

It was recently reported that rat pancreatic islet cells express both the two major variants NBCe1-A and NBCe1-B of the electrogenic $\text{Na}^+\text{-HCO}_3^-$ -cotransporter NBCe1, with a preferential localization of NBCe1-B in insulin-producing cells [1]. The major aim of the present study was to investigate whether a comparable situation prevails in tumoral 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 [2].

The BRIN-BD11 cells selected for such a purpose differ from normal islet cells in such respects as the pattern of glucose metabolism and, hence, the effect of hexose on cell ATP content [3, 4]. For instance, the fractional contribution of anaerobic glycolysis relative to total

glucose utilization is much higher in BRIN-BD11 cells than in normal islet cells [3]. It was recently proposed that, in rat pancreatic islets, the production of bicarbonate accounts for the major fraction of the carbon dioxide generated by the oxidative catabolism of nutrient insulin secretagogues. Taking into account the possible role of the electrogenic $\text{Na}^+\text{-HCO}_3^-$ -cotransporters NBCe1 in bicarbonate efflux from insulin-producing cells and the operation of a typical Crabtree effect in the tumoral BRIN-BD11 cells, it was considered of potential interest to explore the expression of these cotransporters and to investigate their eventual participation in sodium handling and insulin secretion in the BRIN-BD11 cells, taken as an example of B-cell dysfunction. Limited information was also collected in two other tumoral insulin-producing cell lines, namely INS-1 and MIN6.

Materials and Methods

BRIN-BD11 cells were grown as described elsewhere [5]. Some experiments were also conducted in INS-1 [6] and MIN6 cells [7].

Reverse transcription-polymerase chain reaction

RNA was isolated from BRIN-BD11 and INS-1E cultured cells and fresh rat tissue, using AURUM™ total RNA fatty acid and tissue kit (BioRad Laboratories, Hercules, CA). Quantification of the purified total RNA was performed on Nanodrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Quality of the total RNA was checked using an Experion automated Electrophoresis system (BioRad). Reverse transcription to cDNA was performed on 1 µg of total RNA in a final 20 µl reaction volume using High Capacity cDNA Reverse Transcription kit Applied BioSystem. The RT-PCR reactions were carried out in a 20 µl final volume containing: 1 µl of cDNA added to 4 µl Go Taq Green 5X buffer, 0.2 mM dNTP, 0.5 µM of each primer, 0.5 U GoTaq™ DNA polymerase (Promega, Madison, WI), using i-Cycler System (Bio-Rad). The primers used were: isoform NBCe1-A forward 5'-AGT TTG GCT CCC AGG CAC A-3' and reverse 5'-CAC CCT GTT CCA CTT TCT CTT-3' and for NBCe1-B forward 5'-TCA CAT TCG GTC GCC AAA-3' and reverse 5'-CCC TGT TCC ACT TTC TCT TCA-3'. Thermocycling conditions were: denaturation at 95°C for 90 s, followed by 33 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 1 min), terminated by a final elongation at 72°C of five minutes. Amplification of NBCe1, regardless of splice variation, coined "all" NBCe1, were obtained using the forward primer 5'-CTC ACT TCT CCT GTG CTT GCC T-3' and reverse primer 5'-GTG GTT GGAAA TAG CGG CTG G-3', and thermocycling conditions were: denaturation 95°C for 90 s, 35 cycles (95°C for 20 s, 61°C for 20 s, 72°C for 30 s), with a final elongation at 72°C of five min. PCR products were analyzed by agarose gel electrophoresis in the presence of 0.5 µg/mL of ethidium bromide and visualised by UV

transillumination using a GelDoc apparatus (Bio-Rad).

Western blot analysis

Crude plasma membranes from rat kidney and Brin-BD11 cells were prepared as described previously [8] except that a membrane pellet of 17,000 g centrifugation was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). BRIN-BD11 cells (20 µg) and kidney extract (5 µg) were loaded on 6 % polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride membranes and immunolabeled using the anti-NBC1 antibody (AB3204, Chemicon, Temecula, CA) at a dilution of 1:2500. The bound antibodies were then detected using the enhanced chemiluminescence method (ECL kit, Amersham, Piscataway).

Immunofluorescence

Immunofluorescence detection method was used for the immunohistological analysis. After fixation of BRIN-BD11, INS-1 and MIN6 cells with PAF (4 %, w/v), the cells were blocked for 1h at room temperature with 1:20 normal goat serum (Vector Labs, Belgium) in PBS for non-specific reactions. Incubation with primary antibodies was performed overnight at 4°C. Antibodies against NBC1 which were different in their aminoacid sequence (AB-3204, AB-3208 and AB3212, Chemicon, Temecula, CA), were used at a concentration of 1/100 in PBS/normal goat serum 1/20. Purified rabbit IgGs were used as negative controls. The secondary antibody, a fluorescein goat anti-rabbit (Vector Labs, Belgium) was applied at a dilution of 1/100 for 30 min at room temperature. The slides were mounted and DNA was counterstained with DAPI (Prolong®Gold antifade, Molecular Probes). The staining patterns were observed with an Axioplan and recorded with an Axiocam (Carl Zeiss, Germany). The slides were unmounted in PBS at 37°C. Anti-insulin (I2018, Sigma-Aldrich, Belgium) was applied at a dilution of 1/3000 overnight, at 4°C. The secondary antibody was a Rhodamine Red X-conjugated goat anti-mouse IgG (H+L) (115-295-146, Jackson ImmunoResearch Laboratories, PA) diluted at 1/100 in PBS/normal goat serum 1/20. The slides were mounted and DNA was counterstained with DAPI (Prolong®Gold antifade, Molecular Probes).

Insulin Release

The method for measuring insulin release from BRIN-BD11 cells was identical to that described elsewhere [5]. Briefly, the cells were preincubated for 30 min at 37°C in a salt-balanced isotonic medium containing D-glucose (1.1 mM) and bovine serum albumin (0.5 mg/ml) in the absence or presence of a reported inhibitor of NBCe1, i.e. tenidap (5-chloro-2-hydroxy-3-(thiophene-2-carbonyl)indole-1-carboxamide; 50 µM to 1.0 mM) which was kindly provided by Pfizer, Groton, CT, USA and then further incubated for 30 min at the same temperature and same tenidap concentration. The results are expressed as µU of insulin released by BRIN-BD11 cells grown at confluence in 1.0 ml of incubation medium.

Sodium uptake

For measuring the net uptake of ^{22}Na by dispersed BRIN-BD11 cells, groups of 10^5 cells were incubated for 5, 10 or 20

min at 37°C in 0.1 ml of the same salt-balanced medium containing D-glucose (16.7 mM), bovine serum albumin (5 mg/ml), ^{22}Na (1.5 nCi/ μl) and, as required, ouabain (1.0 mM) and/or tenidap (0.1 mM). After incubation the cells were separated from the incubation medium over 1 min centrifugation at 5,000 g, 0.15 ml of a mixture of dibutylphthalate and diisononylphthalate being then added to each tube, which was again centrifuged for 3 min at 4°C and 5,000 g. The bottom of the tube containing the cell pellet was then cut and placed in a counting vial for measuring its radioactive content by liquid scintillation [9].

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

Results

RT-PCR

As shown in Fig. 1, the BRIN-BD11 cells expressed mainly the NBCe1-B variant, whilst in INS-1 cells both NBCe1-B and, to a lesser extent NBCe1-A, provided sizeable amplification signals. Such was also the case in pancreas and kidney.

Western blotting

Western blot analysis documented the presence, in both BRIN-BD11 cells and kidney, of a predominant NBCe1 band with a molecular weight close to 130 kDa (Fig. 2).

Immunocytochemical localization of NBCe1

Immunohistochemical labeling with a fluorescein labelled secondary antibody revealed the presence of NBCe1 in BRIN-BD11, INS-1 and MIN6 cells (Fig. 3). The immunodetection of NBCe1 in BRIN-BD11 cells was confirmed by use of three distinct antibodies differing in their amino acid sequence (Fig. 4). In the BRIN-BD11 cells, NBCe1 was found to co-localize with insulin (Fig. 5).

Insulin release

In the 50-100 μM range, tenidap decreased ($p < 0.005$) basal insulin output, as measured in the presence of 1.1 mM D-glucose, to 72.2 ± 5.0 % ($n = 5$) of paired control value (26.7 ± 7.7 μU per 30 min; $n = 5$). When the concentration of NaCl was decreased by 50 mM, the release of insulin averaged 215.3 ± 11.6 % ($n = 5$; $p < 0.001$) of paired basal value (Table 1). In such a hypotonic medium, tenidap (50-100 μM) lowered insulin secretion ($p < 0.001$) down to 39.4 ± 5.6 % ($n = 5$; $p < 0.001$) of paired control value (60.5 ± 21.2 μU per 30

Fig. 1. RT-PCR of BRIN-BD11 cells, INS-1 cells, pancreas and kidney.

Fig. 2. Western blot analysis of kidney and BRIN-BD11 cell extracts.

min; $n = 5$) or 85.0 ± 12.2 % ($n = 5$) of paired basal value (recorded in the isoosmotic medium and in the absence of tenidap). The latter percentage was no more significantly different ($df = 8$; $p > 0.3$) from that recorded, also in the presence of tenidap, in the isotonic medium. In other words, the relative magnitude of the inhibitory action of tenidap was higher ($p < 0.01$) in the hypotonic medium than in the isotonic one, with a paired hypertonic/isotonic ratio averaging 220.0 ± 36.3 %, resulting in the suppression of the secretory response to extracellular hypoosmolality.

Incidentally, at higher concentrations (0.5 and 1.0 mM), tenidap augmented basal insulin release (μU per 30 min) from a control value of 42.7 ± 2.0 ($n = 6$) to, respectively, 101.7 ± 6.0 ($n = 3$; $p < 0.001$) and 357.2 ± 29.7 ($n = 3$; $p < 0.001$). Such a concentration-related increase ($p < 0.005$) was also observed ($p < 0.02$) in cells exposed to the hypotonic medium (50 mM decrease in NaCl concentration), in which case, the release of insulin increased from a control value of 135.0 ± 7.7 ($n = 6$) to 144.6 ± 21.1 and 496.9 ± 90.2 ($n = 3$ in both cases) at 0.5 and 1.0 mM tenidap, respectively. The latter two values were no more significantly different from those recorded,

Fig. 3. Immunodetection of NBCe1 (AB-3204) in BRIN-BD11 cells (A), in INS-1 cells (B) and in MIN6 cells (C). Immunohistochemical labelling was performed using fluorescein-labelled secondary antibody. Both manipulations were conducted at a 1:100 dilution.

Fig. 4. Immunodetection of NBCe1 in BRIN-BD11 cells. Immunohistochemical labelling was performed using fluorescein-labelled secondary antibody. The NBCe1 antibodies used for labelling differed in their amino acid sequences; (A) AB-3204, (B) AB-3208 and (C) AB-3212. All manipulations were conducted at a 1:100 dilution.

Fig. 5. Co-localization studies of NBCe1 and insulin in BRIN-BD11 cells. Double labelling with anti-NBCe1 (A) and anti-insulin (B) demonstrating NBC (green) and insulin (red) positive cells. Panel (C) represents the merged image of NBCe1 and insulin.

at the same concentration of tenidap, in the cells exposed to the basal isotonic medium ($p > 0.1$ or more). In the presence of tenidap (0.5 and 1.0 mM), the relative magnitude of the increment in insulin output attributable to hypotonicity indeed represented no more than $14.1 \pm 5.7\%$ ($n = 6$) of that recorded within the same experiment in the absence of tenidap.

²²Na net uptake

After 10 min incubation in the absence of tenidap and ouabain, the net uptake of ²²Na averaged 100.6 ± 14.4 pmol/ 10^3 cells ($n = 14$). As illustrated in Fig. 6, the net uptake of ²²Na progressively increased during incubation ($r = 0.217$, $df = 166$; $p < 0.007$) in an exponential manner. The time course for such an uptake, as depicted in Fig. 6, was compatible with an extracellular contamination close to 45.2 pmol/ 10^3 cells, corresponding to an extracellular space close to 0.32 nl/ 10^3 cells and a steady-state intracellular ²²Na content close to 71.5 ± 9.0 pmol/ 10^3 cells (Fig. 6, inset). Taking into account the intracellular ³HOH space of BRIN-BD11 cells (1.44 nl/ 10^3 cells), the readings recorded after 20 min incubation in the absence of both ouabain and tenidap (94.7 ± 14.6 pmol/ 10^3 cells; $n = 14$) would then correspond to an apparent Na⁺ intracellular concentration of 34.3 ± 10.1 mM.

As expected, ouabain (1.0 mM) increased ($p < 0.03$) the net uptake of ²²Na to $122.7 \pm 8.7\%$ ($n = 80$) of the

Osmolarity	Iso	Hypo
Expmt. 1		
Control	100.0 (5) ^a	215.3 $\pm$ 11.6 (5)
Tenidap (50-100 μ M)	72.2 $\pm$ 5.0 (5)	85.0 $\pm$ 12.2 (5)
Expmt. 2		
Control	100.0 (6) ^a	315.1 $\pm$ 26.1 (6)
Tenidap (0.5 mM)	223.1 $\pm$ 8.6 (3)	311.6 $\pm$ 40.6 (3)
Tenidap (1.0 mM)	892.6 $\pm$ 132.2 (3)	1205.2 $\pm$ 236.9 (3)

Table 1. Effects of tenidap upon insulin release in an isoosmolar or hypoosmolar medium. ^aResults expressed relative to paired basal value recorded in cells exposed to an isoosmolar medium in the absence of tenidap. Such a reference value averaged 35.5 ± 4.3 μ U per 30 min ($n = 11$).

mean corresponding values ($100.0 \pm 5.1\%$; $n = 80$) recorded within the same experiment(s), under the same experimental conditions (presence or absence of tenidap) and at the same time of incubation in the absence of the cardiac glycoside (Fig. 7). In the absence of ouabain, tenidap (0.1 mM) failed to increase significantly ($p > 0.2$) ²²Na net uptake (Fig. 7). In the presence of ouabain, how-

Fig. 6. Time course for ^{22}Na net uptake by BRIN-BD11 cells. Mean values ($\pm$ SEM) refer to 56 separate determinations at each time point. The inset illustrates, in semi-logarithmic coordinates, the time course for the difference (Δ) between the close-to-equilibrium value (min 20) and actual value (min zero to min 10) for the intracellular ^{22}Na content of the BRIN-BD11 cells (as calculated after correction for extracellular contamination); the oblique line corresponds to the regression line.

ever, such an uptake averaged in the presence of tenidap 160.7 ± 19.2 ($n = 42$; $p < 0.005$) of the mean corresponding value recorded within the same experiment(s) and at the same time of incubation in BRIN-BD11 cells exposed to only ouabain ($100.0 \pm 7.7\%$; $n = 42$). The normalized values for ^{22}Na net uptake found in the presence of tenidap failed to differ significantly ($p > 0.08$) in the cells exposed to ouabain ($160.7 \pm 19.2\%$; $n = 42$) and those incubated for 10–20 min in the absence of the cardiac glycoside ($116.8 \pm 11.3\%$; $n = 28$). Pooling together these two sets of results yielded an overall mean percentage of $143.1 \pm 12.6\%$ ($n = 70$), as compared ($p < 0.003$) to a reference mean value of $100.0 \pm 5.9\%$ ($n = 70$).

Discussion

The present findings are, in most respects, similar to those recently collected in rat isolated pancreatic islets or dispersed islet cells [1].

First, BRIN-BD11 cells, as well as INS-1 cells, were found by RT-PCR to express NBCe1-B and to a lower extent NBCe1-A.

Second, western blot analysis confirmed the presence of a NBCe1 band in BRIN-BD11 cells.

Third, immunocytochemical investigations documented the presence of NBCe1 in all lines of tumoral insulin-producing cells examined for such a purpose, i.e. in BRIN-BD11, INS-1 and MIN6 cells. The NBCe1 colocalized with insulin in the BRIN-BD11 cells.

Fig. 7. Effects of ouabain (O) and tenidap (T) upon ^{22}Na net uptake. Mean values ($\pm$ SEM) are expressed relative to the control values (C) recorded within the same experiment(s) and at the same time of incubation in the absence of either the cardiac glycoside or tenidap, and refer to the number of separate determinations indicated at the bottom of each column.

Furthermore, in our previous study, tenidap (3 to 100 μM) caused a concentration-related inhibition of insulin secretion evoked over 90 min incubation by D-glucose (16.7 mM) in rat isolated islets, with a half-maximal inhibition at a tenidap concentration close to 50 μM [1]. In the present study, tenidap (50–100 μM) decreased basal insulin output from BRIN-BD11 cells exposed to 1.1 mM D-glucose and abolished the increment in insulin secretion provoked by extracellular hypoosmolarity. In the BRIN-BD11 cells, higher concentrations of tenidap (0.5 to 1.0 mM) augmented insulin release whether under conditions of extracellular isoosmolarity or hypoosmolarity, whilst still suppressing the secretory response to a decrease in extracellular osmolarity. Likewise, in rat isolated pancreatic islets exposed to 5.6 mM D-glucose, increasing the concentration of tenidap to 0.3 and 1.0 mM significantly increased insulin release ($p < 0.005$) from a control value of $33.5 \pm 2.7 \mu\text{U}/90 \text{ min}$ per islet to respectively 49.6 ± 3.8 and $491.8 \pm 24.3 \mu\text{U}/90 \text{ min}$ per islet ($n = 19$ –20). Such dramatic increases of insulin release from either BRIN-BD11 cells or rat isolated pancreatic islets exposed to high concentrations of tenidap might well correspond to an unspecific damaging effect on insulin-producing cells.

The handling of ^{22}Na by BRIN-BD11 cells and the effects of tenidap thereupon also yielded results comparable to those previously collected in rat dispersed islet cells examined under comparable experimental conditions [1]. First, the estimated intracellular Na^+ concentration averaged $34.3 \pm 10.1 \text{ mM}$ in the BRIN-BD11 cells, as

compared to 35.9 ± 5.7 mM in rat dispersed islet cells [1]. Second, the fractional turnover rate of the intracellular Na^+ pool, as calculated from the data illustrated in the inset of Fig. 6, amounted to 22.2 percent/min in BRIN-BD11 cells, as compared to 18.3 ± 1.3 percent/min in prelabelled perfused rat islets [10]. Last, relative to the mean control values (no tenidap) for ^{22}Na net uptake, those recorded in the presence of tenidap (0.1 mM) were also comparable in BRIN-BD11 cells and rat dispersed islet cells, averaging in these two cell types respectively 116.8 ± 11.3 and 126.5 ± 13.0 % in the absence of ouabain, and 160.7 ± 19.2 and 145.6 ± 19.6 in the presence of the cardiac glycoside [1]. Under each of these two distinct experimental conditions, no significant difference ($p > 0.5$ in both cases) was thus observed when comparing BRIN-BD11 cells and normal rat dispersed islet cells, even though the just-mentioned results ignore the dispersion of separate determinations for the corresponding reference values (100 %) found in the absence of tenidap. Moreover, in either BRIN-BD11 cells or rat dispersed islet cells, no significant difference was observed between the normalized results recorded in the presence of tenidap and either presence or absence of ouabain. When pooling together the results recorded in the absence and presence of ouabain, the overall mean value found in the presence of tenidap averaged in BRIN-BD11 cells 143.1 ± 12.6 % ($n = 70$) of the mean corresponding reference value (100.0 ± 5.9 %; $n = 70$) as compared to

135.2 ± 11.3 % ($n = 66$) of the mean corresponding reference value (100.0 ± 6.3 %; $n = 64$) in rat dispersed islet cells. In this respect, there was again no significant difference ($p > 0.6$) between BRIN-BD11 cells and rat dispersed islet cells. If advantage is taken from these close analogies between BRIN-BD11 cells and rat dispersed islet cells by pooling together results referring to the same experimental conditions, it appears that, even in the absence of ouabain, tenidap significantly increases ($p < 0.05$) ^{22}Na net uptake from a mean reference value of 100.0 ± 6.3 % ($n = 62$) to 122.3 ± 8.8 % ($n = 64$), and that the relative effect of tenidap is less pronounced ($p < 0.06$) in the absence of ouabain than its presence, in which case the ^{22}Na net uptake recorded in the presence of tenidap represents 154.4 ± 13.8 % ($n = 72$) of its mean reference value (100.0 ± 5.9 %; $n = 72$).

In conclusion, BRIN-BD11 cells and, apparently, other tumoral insulin-producing cells such as INS-1 and MIN6 cells, are equipped, like normal islet B-cells, with the electrogenic $\text{Na}^+\text{-HCO}_3^-$ -cotransporter NBCe1, predominantly its B variant.

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