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# Opposite Effects of Sodium Butyrate on CCK mRNA and CCK Peptide Levels in RIN Cells

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**The effects of the differentiation-inducing agent sodium butyrate on cholecystokinin (CCK) expression was investigated in the pancreatic islet tumor cell line RIN 1056E, which contains high levels of CCK-like immunoreactivity (CCK-LI). Exposure to butyrate for 24 h dose-dependently inhibited cell proliferation and increased the cell content in CCK-LI over the concentration range 0.1–8 m*M*. With 2 m*M* butyrate, cell proliferation was decreased by 50% and CCK-LI content was increased by 300%, whereas the level of steady-state CCK mRNA was reduced by 75%. Cycloheximide (10 μg/mL) abolished the sodium butyrate-induced increase in CCK-LI content. This article reports the novel finding that butyrate exerts opposite effects on CCK mRNA and immunoreactivity. The butyrate-induced increase in cellular CCK-LI content is entirely dependent on continuing protein synthesis.**

**Key Words:** Butyrate; CCK immunoreactivity; CCK mRNA; (RIN 1056E cells).

## Introduction

Peptide hormone production is an example of differentiated function that may require the coordinate regulation of a number of correlated genes, such as the one encoding the peptide of interest and those involved in the proteolytic processing, packaging, and secretion. Sodium butyrate is a differentiation-inducing agent that stimulates insulin gene transcription and increases insulin cell content and secretion in cultured insulin-producing cell lines, such as the transplantable rat insulinoma (RIN) (Philippe et al., 1987a; Karlsen et al., 1991). Similarly, butyrate stimulates somatostatin gene expression and secretion in RIN cells (Green and Shields, 1984). In contrast, butyrate was shown to inhibit gene transcription in other systems (Philippe et al., 1987a; Lazar, 1990; Ormandy et al., 1992). This was accompanied by a reduction in the level of the corresponding posttranslational products (Lazar, 1990; Ormandy et al., 1992). Taken together, these data indicate that butyrate-induced modifications of gene expression are accompanied by a parallel variation in the level of posttranslational products. The present article reports that butyrate may exert anticoordinate effects on gene expression and accumulation of posttranslational products. The mechanism was evidenced in a cell line that produces cholecystokinin (CCK), a regulatory peptide of the brain–gut axis displaying a variety of effects on several gut and brain functions. Butyrate decreased the steady-state CCK mRNA levels and increased the cellular content in CCK-like immunoreactivity (CCK-LI). This last effect was shown to be entirely dependent on continuing protein synthesis.

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## Results

Cells incubated for 24 h with increasing concentrations of sodium butyrate over the range 0.1–8 m*M* showed a decreased proliferation rate, as assessed by cell DNA content (Fig. 1A). This decrease in cell DNA content became significant with 0.5 m*M* sodium butyrate. A more than 50% decrease was obtained with 8 m*M* sodium butyrate. At the same time, a clear dose-dependent enhancement of cellular CCK-LI was observed (Fig. 1B). This increase became also significant with 0.5 m*M* sodium butyrate.

CCK secretion in the culture medium was not significantly affected by sodium butyrate treatment, even at the concentration of 8 m*M* (Fig. 1C). For subsequent experiments, we used 2 m*M* of sodium butyrate, which produced the half-maximal effects on cell proliferation and CCK-LI content. The effects of 2 m*M* sodium butyrate were recorded during 24 h following addition of the drug. The increase of cellular CCK-LI became significant only 24 h after sodium butyrate treatment (Fig. 2A), whereas CCK secretion in the culture medium was never modified on sodium butyrate treatment (Fig. 2B).

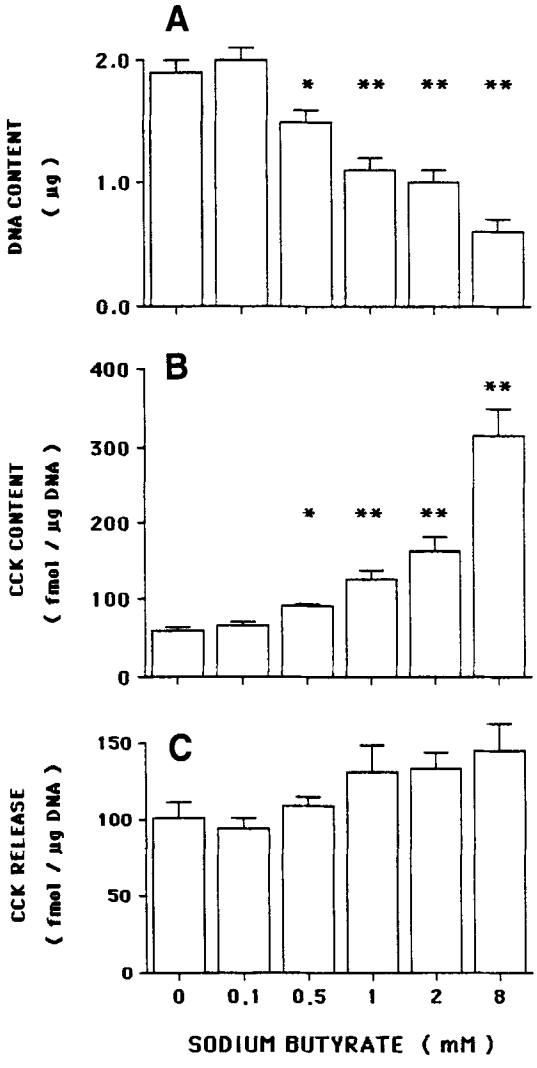


**Fig. 1.** Effects of increasing concentrations of sodium butyrate on DNA synthesis **(A)**, cellular content of CCK-LI **(B)**, and CCK release **(C)** in rat islet tumor RIN 1056E cells. The RIN cells were exposed to sodium butyrate for 24 h and then treated for cell proliferation and CCK-LI measurements as described in Materials and Methods. All results are corrected for amount of DNA/culture well and expressed as mean values ± SEM ($n = 6$). *$p < 0.02$ vs control, **$p < 0.01$ vs control.

To investigate whether the effects of sodium butyrate on cellular CCK-LI were related to an increase in CCK gene expression, cells were incubated with 2 m*M* sodium butyrate for various time lengths. Total RNA was subsequently extracted and analyzed by Northern blot. As shown in Fig. 3, the Northern blot analysis revealed a single band of CCK mRNA, which was approx 750 nucleotides in size. With 2 m*M* butyrate, no significant changes in CCK mRNA levels were observed until 6 h of incubation. In cells exposed for 12 h and 24 h to sodium butyrate, the CCK mRNA levels were reduced by 60 and 75%, respectively, with little additional effect between 24 and 48 h (Fig. 3).

To investigate the mechanism involved in the sodium butyrate-induced increase in CCK cell content, cells were incubated with either 2 m*M* butyrate alone or a mixture of

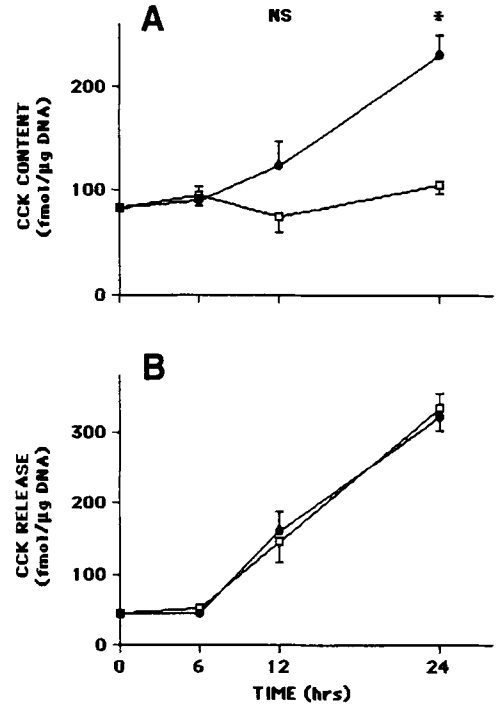


**Fig. 2.** Time-course study of sodium butyrate effects on cellular CCK-LI content **(A)** and CCK release **(B)**. Cells were exposed to sodium butyrate (2 m*M*) for 6, 12, and 24 h. Control cells: open squares; sodium butyrate-treated cells: full circles. All results are corrected for amount of DNA/culture well and expressed as mean values ± SEM ($n = 6$). *$p < 0.01$ vs control, NS (not significant).

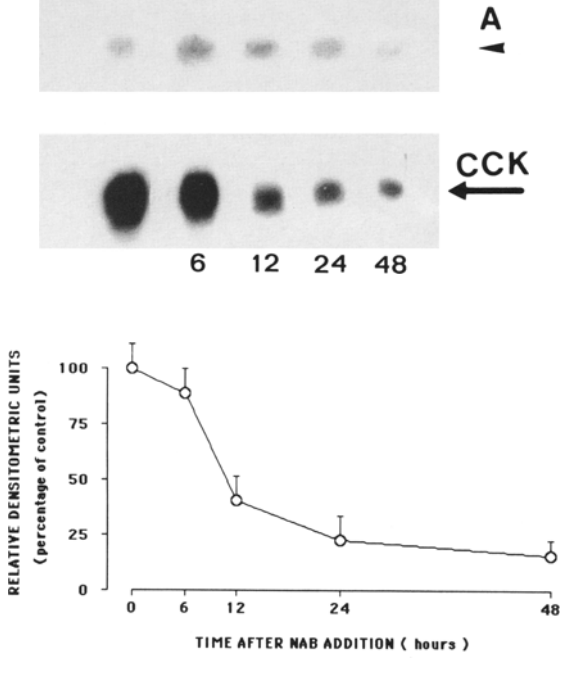


**Fig. 3.** Time-course study of sodium butyrate effects on CCK mRNA levels. Cells were exposed to sodium butyrate treatment for 6, 12, 24, and 48 h. Top: Representative Northern blot (A = β-actin). Bottom: Densitometric quantification of CCK mRNA levels after normalization to β-actin. Results are mean ± SEM of five independent experiments.

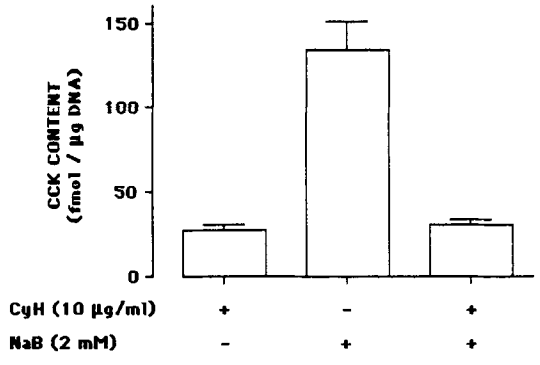


**Fig. 4.** Effect of cycloheximide on sodium butyrate-induced increase in cellular content of CCK-LI in RIN 1056E cells. The RIN cells were plated in 24-well plates at a density of $5.10^5$ cells/well. After a 24-h culture period, the medium was replaced by RPMI supplemented with 0.5% fetal calf serum. Sodium butyrate (NaB, 2 m*M*) and cycloheximide (CyH, 10 µg/mL) were added 1 h later. After a further 24-h incubation period, cells were extracted as described in Materials and Methods for subsequent CCK radioimmunoassay. All results are corrected for amount of DNA/culture well and expressed as mean values ± SEM of six experiments. CyH vs NaB, $p < 0.05$; CyH vs CyH + NaB, NS (not significant).

butyrate and cycloheximide (10 µg/mL). Cycloheximide abolished the increase in cell content of CCK induced by butyrate (Fig. 4). This result indicates that sodium butyrate increased cellular CCK content through a step involving *de novo* protein synthesis.

## Discussion

The short-chain fatty acid, sodium butyrate, is a differentiation-inducing agent because it has been shown (1) to alter proliferation rate and morphology in a variety of cell lines and (2) to increase expression of differentiation-associated gangliosides in pancreatic islet cell lines (Bartholomeusz et al., 1989). Additionally, sodium butyrate increased insulin, glucagon, and somatostatin gene expression in insulin-producing cell lines (Green and Shields, 1984; Philippe et al., 1987a; Powers et al., 1988; Karlsen et al., 1991). In contrast, butyrate inhibited angiotensinogen gene expression, which was ectopic in the insulinoma cells (Philippe et al., 1987a). The present article reports that butyrate reduced the proliferation rate and CCK steady-state mRNA content of the RIN 1056E cells, which produce fully processed CCK peptides (Aucouturier et al., 1994). At this time, the ectopic nature of CCK expression in pancreatic cells cannot be stated, since gut regulatory peptides of the gastrin/CCK family have been identified in the fetal pancreas (Brand and Fuller, 1988; Bardram et al., 1990), and immunoreactive CCK material was found in islets of Langerhans of adult rats (Shimizu et al., 1994).

The mechanisms of the butyrate-induced decrease of CCK mRNA levels are unknown. However, the fact that CCK mRNA levels do not change for 6 h after addition of sodium butyrate, but rapidly decrease between 6 and 12 h may suggest modifications in mRNA half-life. Sodium butyrate is known to affect gene expression through changes in transcription rates possibly owing to alterations in histone acetylation. One potential mechanism by which sodium butyrate might affect CCK gene expression could thus be indirect, by altering the synthesis of a specific RNase involved in the degradation of CCK mRNA. Direct measurements of CCK-specific transcription rates and mRNA stability, in the presence and absence of butyrate, should prove informative.

Unexpectedly, the variation of cellular content of CCK-LI in butyrate-exposed RIN 1056E was opposite to that of CCK gene expression, as shown by Northern blot analysis. After a 24-h incubation with 2 m*M* butyrate, the cellular content in CCK-LI was increased by approx 300% compared to controls. Cycloheximide abolished the butyrate-induced increase in cellular content of CCK, thus clearly indicating that continuing protein synthesis is an absolute requirement to increase cellular content of CCK. Although the identity of these proteins is presently unknown, it may be speculated that they include enzymes involved in the maturation of the posttranslational products of the CCK gene. Several enzymes, e.g., proconvertases, involved in the processing of regulatory peptides have been recently cloned. The possibility that butyrate induced the expression of one of them is currently being explored in our laboratory.

## Materials and Methods

### *Materials*

RPMI medium and additive were obtained from Gibco BRL (Eragny, France). Sodium butyrate and cycloheximide were purchased from Sigma Chemicals (St. Quentin Fallavier, France). The cDNA probe for the rat CCK, a 575-bp fragment of the coding region of prepro-cholecystokinin (Deschenes et al., 1984), was kindly provided by J. E. Dixon.

### *Cell Culture*

The pancreatic RIN 1056E cell line is a subclone of the original rat islet cell tumor (RIN-r) obtained following total body X-irradiation to an inbred albino NEDH rat (Gazdar et al., 1980; Philippe et al., 1987b). This cell line expresses high levels of CCK mRNA (Brand and Wang, 1988), fully processes CCK peptides, but does not contain gastrin-like immunoreactivity (Aucouturier et al., 1994). The cells were grown in RPMI 1640 medium supplemented with 10% calf serum as previously described (Aucouturier et al., 1994). Twenty-four hours after plating in 24-well plates at a density of $5–10^5$ cells/well, the medium was replaced by RPMI supplemented with 0.5% fetal calf serum. Sodium butyrate or cycloheximide was added 1 h later from stock solutions prepared in sterile water.

### *RNA Extraction and Northern Blot Analysis*

Total RNA was extracted from cells by homogenization in guanidine thiocyanate and sedimentation through a cesium chloride cushion (Chirgwin et al., 1979).

Twenty milligrams of total RNA from each sample were size-fractionated on a 1.4% agarose gel after denaturation in glyoxal, followed by electrotransfer to a nylon membrane (Nylon, Schleicher and Schull, Kane, NH). Blots were baked for 2 h at 80°C, prehybridized in 1*M* NaCl/1% SDS/10% Dextran sulfate at 50°C and hybridized at the same temperature for 24 h after addition of the radiolabeled cDNA probes as previously described (Philippe et al., 1987b). They were then washed at 55°C twice in 1X SSC (0.15*M* NaCl/0.015*M* Na citrate)/1% SDS and exposed for varying times at –70°C with an intensifying screen. The autoradiograms were quantitated by scanning densitometry.

### *CCK Extraction and Radioimmunoassay*

Cultured cells were homogenized in 2 mL extraction medium (2*N* acetic acid and 20 m*M* HCl) at 4°C. The homogenate was sonicated and boiled at 100°C for 10 min. After centrifugation (4000*g*, 10 min, 4°C), the pellet was re-extracted with 2 mL extraction medium. The supernatants were pooled, neutralized with $NH_4OH$, and stored at –20°C until analysis.

The CCK assay was performed as previously described (Chery-Croze et al., 1985) using antibody 39A, which cross-reacts at 100% with CCK 33 and CCK 8, at 12% with sulfated gastrin 17, at 5% with unsulfated gastrin 17, and at <0.1% with unsulfated CCK 8, unsulfated CCK 7-gly and gastrin 34.

Tissue CCK concentration was expressed as fmol/μg DNA. DNA content was measured by fluorometry (Labarca and Paigen, 1980).

### *Statistical Analysis*

Statistical significance was assessed using the *t*-test for unpaired data. Variations were significant if the *p* value was <0.05.

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