# Alterations in $Ca^{2+}$ Signaling, and *c-fos* and *nur77* Expression are Associated with Sodium Butyrate-Induced Differentiation of C6 Glioma Cell

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## Abstract

**Sodium butyrate is well known in stimulating growth and differentiation of cancer cells. In the present study, butyrate treatment caused decreases in thymidine incorporation in the early passages (45-60) of C6 glioma cells. In addition, butyrate also caused decreases in inositol incorporation and transient ATP-stimulated $Ca^{2+}$ mobilization suggesting that butyrate altered general mechanisms of $Ca^{2+}$ signaling in these cells. To gain direct insight into the crosstalk between sodium butyrate and $Ca^{2+}$ signaling in transcriptional regulation, we investigated the induction of the $Ca^{2+}$-sensitive immediate early genes (IEGs), *c-fos*, *nur77* and *c-myc*. Sodium butyrate per se enhanced the expression of *c-fos* mRNA, and the enhanced levels were maintained for 24 h, but over the same time period, the initially increased levels of *nur77* expression tailed off, while *c-myc* expression was slightly reduced. Increasing intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) by thapsgargin and A23187 induced the expression of both *c-fos* and *nur77* mRNA expression, and synergistic effects were observed when cells were incubated with sodium butyrate plus thapsgargin and A23187. However, removal of both extracellular $Ca^{2+}$ by EGTA, or intracellular free $Ca^{2+}$ with BAPTA did not affect the sodium butyrate-induced *c-fos* and *nur77* mRNA. These results suggest that although sodium butyrate altered $Ca^{2+}$ signaling which is an important regulatory mechanism for *c-fos* and *nur77* expression, nevertheless the sodium butyrate-induced *c-fos* and *nur77* expression may be not in fact mediated through $Ca^{2+}$ signaling.**

**Key Words: ATP, $Ca^{2+}$ signaling, $Ca^{2+}$ release, C6 glioma cells, *c-fos*, *c-myc*, *nur77*, sodium butyrate, thapsgargin**

## Introduction

Sodium butyrate (NaB), a 4-carbon fatty acid, has been shown to inhibit growth and induce differentiation in a variety of carcinoma cells, for example colon cancer cells (1, 55), hepatoma cells (44), HeLa cells (7), leukemia cells (5, 19, 21, 27, 28), glioma cells (50, 51) and neuroblastoma cells (42, 43). In C6 glioma cells, earlier reports indicated that the actions of butyrate were associated with morphological and protein level changes (14, 57), post-transcriptional regulation of the glial marker, glutamine synthetase (23), and inhibition of S100 protein (18). These reports demonstrated that sodium butyrate has a wide range of actions and complex biological effects on cells, but the exact biochemical mechanisms involved remained unclear.

Recent findings indicated that it seems butyrate and calcium has complex influences on cells (4, 17, 46). We have demonstrated that the sodium butyrate-induced differentiation in the older passages of C6 glioma cells (>300 passages) was associated with decreases in the synthesis of phosphoinositides, histamine-stimulated $IP_3$ production (50), and $Ca^{2+}$ mobilization (40). These results suggest that the action of butyrate may involve alterations in the

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general mechanisms of phosphoinositide (PI) turnover and $Ca^{2+}$ signaling. Furthermore, it is well documented that $Ca^{2+}$ is an important regulator for *c-fos* transcription (12, 35, 45, 58). In order to further elucidate the biochemical mechanisms of butyrate's action, to examine the early cellular biochemical responses, and to gain direct insight into the interaction of butyrate and $Ca^{2+}$ signaling in transcriptional regulation, we investigated the ATP-stimulated $Ca^{2+}$ mobilization, and the expression of the $Ca^{2+}$-sensitive immediate early genes (IEGs), *c-fos*, *nur77* and *c-myc* in younger C6 glioma cells (passage 45-60).

IEGs were first identified as a set of growth-related genes superinduced by growth factors in the presence of cycloheximide (26). Since then, the expression of IEGs has been implicated in the signal transduction by which extracellular stimuli modulate gene expression, leading to prolonged changes in cells. The expression of *c-fos* has been studied extensively, and became an important experimental model for studies of stimulus-transcription coupling because it is activated within minutes by many different stimuli in the nervous system (36). Recently, induction of *nur77* has been shown to involve in the differentiation of neuroblstoma cells (42). Sodium butyrate has been shown to induce the expression of *c-fos* in PC 12 cells (38), in F98 glioma cells (51, 52) and in human CACO-2 colon carcinoma cell (47). Conversely, it was found to inhibit *c-fos* expression in HCC-M hepatoma cells (44). Butyrate also reduced the expression of *c-myc* in PLC/PRF/S human hepatoma cells (44), SW837 rectal carcinoma cells (16), and ovarian carcinoma cells (24). However, evidence of any direct effect of sodium butyrate on $Ca^{2+}$ signaling and the expression of *nur77* in glial cells is lacking. In this study, we found that butyrate altered ATP-stimulated $Ca^{2+}$ signaling, and induced *c-fos* and *nur77* expression. The effect of butyrate on the expression of *c-fos* and *nur77* mRNA were probably mediated through a $Ca^{2+}$-independent mechanism. This is the first report on the involvement of butyrate in *nur77* expression.

## Materials and Methods

### *Materials*

Adenosine triphosphate (ATP), bovine serum albumin (BSA, calcium mobilization agents, thapsgargin and A23187 were purchased from Sigma (St. Louis, MO, USA). Ham's F10 media was purchased from GIBCO (Grand Island, NY, USA) and fetal bovine serum from Biological Industries (Kibbuta Beit Haemek, Israel). Fura-2-AM was purchased from Molecular Probe (Eugene, OR, USA), and T-25 and T-75 plastic tissue culture flasks, 10 cm and 6-well culture dishes, 15 and 50 ml polypropylene centrifuge tubes were purchased from Corning Glass Inc. (Corning, NY, USA).

### *Cell Culture*

C6 glioma cells originally purchased from ATCC, were cultured in culture medium (F10 medium supplemented with 10% FBS) and gentamicin (50 μg/ml) as described previously (40, 50). Cells (passage 45-60) normally were maintained in T-75 flasks or 10 cm dishes (Corning, NY, USA). In a typical experiment, 1 X $10^6$ cells were subcultured into 10 cm dishes and cultured for 2 days.

### *Thymidine and Inositol Incorporation Assay*

The cells were cultured in the presence of 0, 1, 2.5 or 5 mM sodium butyrate in culture media for 24 h. The cells were then removed by trypsin, counted and then subcultured into 6-well plates at a density of $1 \times 10^6$/well for thymidine incorporation and $6 \times 10^5$/well for inositol incorporation. The cells were then incubated with 1 μCi $^3$H-thymidine or 2 μCi $^3$H-inositol in a 5% $CO_2$ incubator at 37°C for 2 h. The thymidine incorporation reaction was terminated by aspiration, and the cells were scraped into a test tube containing 500 μl 1% Triton X-100 plus 5 ml 6% TCA and incubated for 10 min at room temperature. Following incubation, the samples were transferred to a filtration unit with GF/C filter papers and rinsed 5 times with 6% TCA and once with 70% ethanol. The radioactivities of the filter papers were determined by scintillation spectrophotometry.

For the inositol incorporation assay, the labeled medium was aspirated, 1.3 ml ice-cold methanol was added and most of the cells were scraped into a test tube. One ml water was added into each well, the remaining cells scraped, and these two were transferred into the same test tube and then 2.7 ml chloroform was added into each test tube to extract the phospholipids. After phase separation, the lower chloroform layer containing all the phospholipids was transferred to another test tube. The chloroform was then dried by a speed vac (Savant Instrument Inc, Farmingdale, NY) and the radioactivities of the phospholipids determined by scintillation spectrophotometry (LS6500, Beckman, Palo Alto, CA, USA).

### *Measurement of $[Ca^{2+}]_i$*

The $[Ca^{2+}]_i$ were measured as described earlier (40) according to the original method of Grynkiewicz et al. (13). C6 cells cultured either in the presence or the absence of 2.5 mM of sodium butyrate in culture

medium for 24 h were washed, harvested and resuspended in culture medium at a density of $1 \times 10^9$ cell/ml and incubated with Fura-2/AM (5 μM/ml) for 30 min at 37°C. The cell suspension was then rinsed twice with serum free F10 culture medium to remove the excess Fura-2/AM, and resuspended in culture medium at a density of $4 \times 10^6$/ml. It was then incubated for 20 min at room temperature to allow the entrapped ester to completely hydrolyze. The cell-suspension (0.5 ml) was then washed, resuspended in 2.5 ml loading buffer and then transferred to a 3-$cm^3$ cuvette positioned in the thermostat-regulated (37°C) sample chamber of a dual-excitation beam spectrofluorometer (SPEX, Model CM1T111). The loading buffer consisted of 150 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 5 mM glucose; 10 mM HEPES, pH 7.4; with or without 2.2 mM $CaCl_2$. The cell suspension was continually stirred with a circular stir bar driven by a motor placed beneath the sample chamber.

The membrane-permeant Fura-2/AM was hydrolyzed by nonspecific cytoplasmic esterases to Fura-2. Fura-2 binds cytosolic free $Ca^{2+}$ to become Fura-2-$Ca^{2+}$, which can then be used as a fluorescent indicator of calcium concentration inside the cells. The Fura-2-loaded cells were sequentially illuminated with light at 340 nm and 380 nm, the excitation wavelengths for Fura-2-$Ca^{2+}$ and Fura-2, respectively, and Fura-2 fluorescence emission was measured at 505 nm every second. The excitation and emission band passes were both 1 nm, and the agonist, ATP (100 μM), was added after 60 sec. The net increase in $[Ca^{2+}]_i$ was calculated by subtracting the basal level of $[Ca^{2+}]_i$ from the peak levels ($C_{max}$-$C_{basal}$) and the graph drawn by Sigma plot (49).

*RNA Isolation and Northern Blot Analysis*

Total RNA was extracted from C6 cells cultured in the presence or the absence of 2.5 mM sodium butyrate for various lengths of times (0, 15, 30, 60 min; 2, 4, 12 and 24 h) by the single step acid guanidium thiocyanate-phenol-chloroform extraction method as described earlier (20). For Northern blot analysis, RNA samples (5-20 μg/lane) were applied to 1.2% agarose gel in the presence of 2.2 M formaldehyde. After electrophoresis, gels were transblotted onto Nytran membranes (Amersham Life Science, Buckinghamshire, England). The membranes were then prehybridized at 60°C in a solution containing 1% SDS, 1M NaCl, 10% dextran sulfate and 100 μg/ml of sheared salmon sperm DNA. Complimentary DNA probes of *c-fos* (6, 32), *nur77* (15), *c-myc* (25) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (11) were labeled with [$^{32}$P]-dCTP using the random priming method (Amersham Life Science, Buckinghamshire, England). Radioactive probes ($1 \times 10^6$ cpm/ml) were added directly to the prehybridization solution. Following hybridization incubation 24-48 h at 60°C, membranes were washed twice in 2X SSC at room temperature for 5 min each, follow by two 30 min washes at 60°C in 2X SSC/1%SDS and two 30 min washes at 60°C in 0.1XSSC. Each membrane was then exposed to Hyperfilm-MP (Amersham Life Science, Buckinghamshire, England). The radioactive bands in the film were quantified by a densitometer (Molecular Dynamic, CA, USA). The membrane was then rehybridized to a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (11) as internal control for RNA loading.

*Effect of $[Ca^{2+}]_i$ on IEG Expression*

To examine whether the sodium butyrate-induced *c-fos*, *nur77*, and *c-myc* expression were associated with increases in $[Ca^{2+}]_i$, the cells were incubated with thapsgargin (1 μM) to inhibit $Ca^{2+}$-ATPase on the endoplasmic reticulum or A23187 (1 μM) to induce $Ca^{2+}$ influx. To confirm the effect of increases in $[Ca^{2+}]_i$ on sodium butyrate-induced *c-fos* and *nur77* expression, the cells were then incubated with 20 μM BAPTA (40, 54) to chelate intracellular free $Ca^{2+}$; alternatively, they were incubated in nominal $Ca^{2+}$-free buffer system in the presence of 2 mM EGTA to chelate extracellular $Ca^{2+}$. The RNA was then isolated and Northern blots of *c-fos* and *nur77* analyzed.

*Quantitative Analysis of IEG Expression*

Each experiment was performed three times and the gene expression was quantitatively analyzed by phosphoimage-densitometer scan (Molecular Dynamic, CA, USA). After Northern blot analysis of the particular gene, each blot was stripped and hybridized again with probe for GAPDH. The expression of IEGs was standardized with the respective GAPDH. The experiment was performed three times with three separate cultures. The difference between two means was calculated by Student's t-test and considered statistically significant when $P \leq 0.05$.

## Results

*Treatment of C6 Cells with Sodium Butyrate Decreased $^3$H-thymidine and $^3$H-inositol Incorporation*

To elucidate the relationship of sodium butyrate-inhibited growth of early passages (45-60) of C6 glioma cells and signal transduction pathways, we measured the $^3$H-thymidine and $^3$H-inositol incorporations in cells pretreated with 0, 1, 2.5, and 5

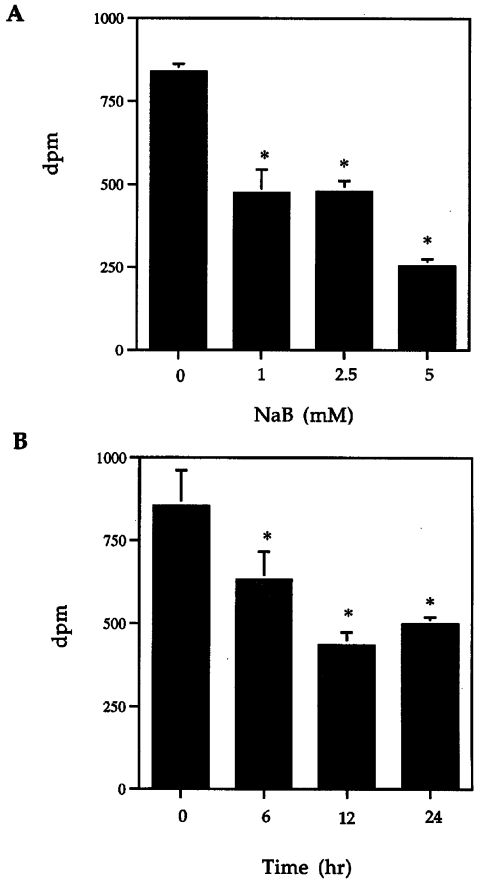


Fig. 1. Effect of sodium butyrate on thymidine incorporation. C6 glioma cells were pretreated **A**. with 0-5 mM sodium butyrate for 24 h, and **B**. with 2.5 mM sodium butyrate for 0-24 h, harvested, counted and seeded $1\times10^5$ cells per well in the presence of $^3$H-thymidine and further cultured for 2 h. Values are means ± SD from six determinations and the symbol * indicate significant different means ($P\leq0.05$) as compared with the controls.

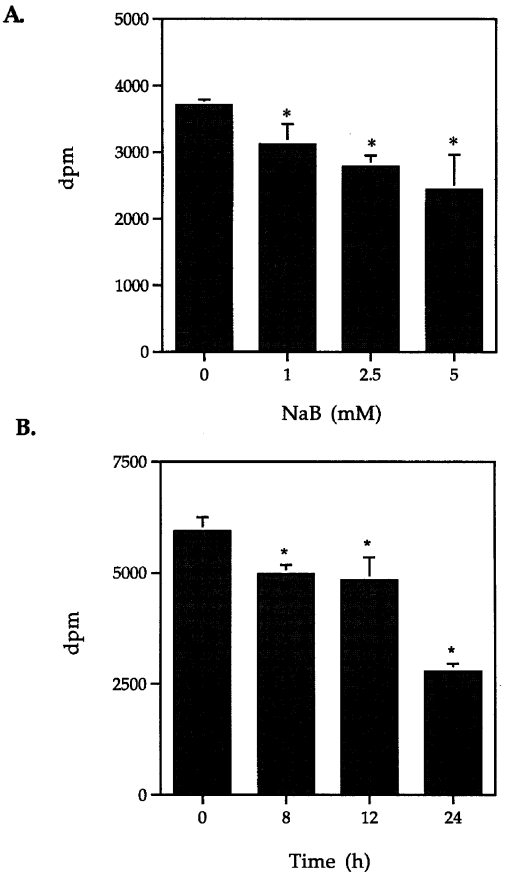


Fig. 2. Effect of sodium butyrate on inositol incorporation. C6 glioma cells were pretreated **A**. with 0-5 mM sodium butyrate for 24 h, and **B**. with 2.5 mM sodium butyrate for 0-24 h. harvested, counted and seeded $1\times10^5$ cells per well in the presence of $^3$H-inositol and further cultured for 2 h. Values are means ± SD from six determinations and the symbol * indicate significant different means ($P\leq0.05$) as compared with the controls.

mM sodium butyrate for 24 h. As shown in figure 1A, sodium butyrate decreased $^3$H-thymidine incorporation into DNA in a concentration-dependent manner. In addition, the butyrate-decreased $^3$H-thymidine incorporation was observed in cells pretreated with butyrate for 6 to 24 h (Fig. 1B). As shown in Figure 2A, 1-5 mM butyrate caused significant decreases in inositol incorporation into chloroform extracts of C6 glioma cells. Furthermore, the decrease was found in a time-dependent manner (Fig. 2B). Thus, sodium butyrate decreased inositol incorporation in a similar dose- and time-dependent manner in these cells (Fig. 2A & 2B).

*Sodium Butyrate Decreased ATP-Stimulated $Ca^{2+}$ Mobilization and Net Increases in $[Ca^{2+}]_i$*

In the present study, younger C6 glioma cells purchased from ATCC at passage 45-60 were used. Initial screening showed that ATP was the most potent agonist to stimulate the rapid rise in intracellular calcium concentration ($[Ca^{2+}]_i$) in these cells; these cells did not respond to histamine (data not shown), as was the case in an older C6 cell line (40, 50). To elucidate whether the decreased inositol uptake is associated with agonist-stimulated $Ca^{2+}$ mobilization, the cells were preloaded with Fura-2-AM and the ATP-stimulated $Ca^{2+}$ mobilization was measured. As shown in Figure 3, the ATP-stimulated increases in $[Ca^{2+}]_i$ was decreased in sodium butyrate-treated cells (2.5 mM, 24 h) as compared with the controls. In addition, the basal levels $[Ca^{2+}]_i$ for control and sodium butyrate-treated cells were 55.6±8.2 (n=5) and 77.3±15 (n=4) nM, respectively. Thus, sodium butyrate caused a significant 40% increase in basal level of $[Ca^{2+}]_i$. The net ATP-stimulated increase in $[Ca^{2+}]_i$ was calculated by $C_{max}$-$C_{basal}$. As shown in figure 3B, the ATP-stimulated increases in $[Ca^{2+}]_i$ for control and sodium butyrate-treated cells were 294±35 (n=3) and 148±15 nM (n=3), respectively. Thus

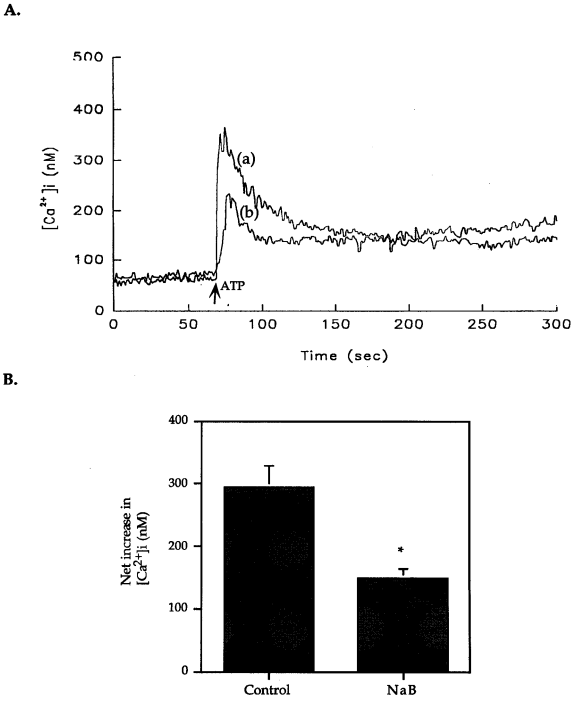


Fig. 3. Effect of sodium butyrate on ATP-stimulated $Ca^{2+}$ mobilization. C6 glioma cells were cultured in the presence (Control) of 2.5 mM sodium butyrate for 24 h, and harvested, preloaded with Fura-2/AM for 30 min, washed and reincubated in buffer containing 2.2 mM $Ca^{2+}$. **A**. The Sigma plot drawn graphs of the ATP-stimulated (100 μM) increases in intracellular $Ca^{2+}$ concentrations were recorded and **B**. net increases in intracellular $Ca^{2+}$ concentrations measured and means calculated. The data represents means ± SD from three determinations and the symbol * indicate significant different means (P≤0.05) as compared with the controls.

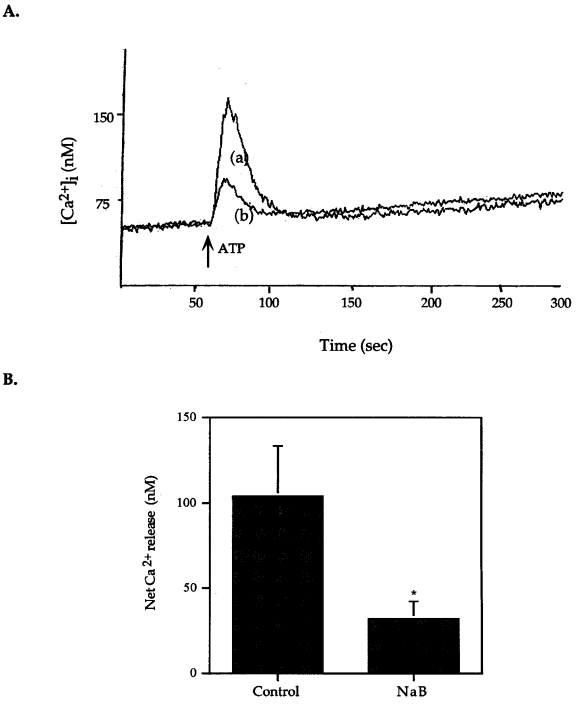


Fig. 4. Effect of sodium butyrate on ATP-stimulated $Ca^{2+}$ release. C6 glioma cells were cultured in the presence (Control) of 2.5 mM sodium butyrate for 24 h, and harvested, preloaded with Fura-2/AM for 30 min, washed and reincubated in a $Ca^{2+}$-free buffer system. **A**. The Sigma plot drawn graphs of the ATP-stimulated (100 μM) increases in intracellular $Ca^{2+}$ concentrations were recorded and **B**. net increases in intracellular $Ca^{2+}$ concentrations measured and means calculated. The data represents means ± SD from three determinations and the symbol * indicate significant different means (P≤0.05) as compared with the controls.

butyrate caused a 50% decrease in ATP-induced net increases in $[Ca^{2+}]_i$ in these cells. These results confirmed our earlier findings that sodium butyrate inhibited agonist-stimulated $Ca^{2+}$ mobilization and decreased the net agonist-induced increase in $[Ca^{2+}]_i$.

To elucidate whether butyrate treatment decrease receptor-mediated $Ca^{2+}$ release from intracellular stores, cells were incubated in $Ca^{2+}$-free loading buffer and monitored for ATP-stimulated $Ca^{2+}$ release. As shown in Figure 4A, ATP stimulated $Ca^{2+}$ release, characterized by a rapid rise and followed by a decline. The net $Ca^{2+}$ releases were 104±29 nM for control cells (Fig. 4B), while the net $Ca^{2+}$ releases for sodium butyrate-treated cells were 32±10 nM. Butyrate caused a 70% decrease in $Ca^{2+}$ release in these cells.

*Sodium Butyrate Induced c-fos and nur77 mRNA Expression*

To analyze whether sodium butyrate affect stimulus-transcriptional coupling in C6 glioma cells, we examine the effect of butyrate on the expression of three immediate early genes (IEGs; *c-fos*, *c-myc* and *nur77*). Without treating cells with butyrate the expression of *c-fos* mRNA (Fig. 5A) was almost not detectable, whereas a lower level of *nur77* (Fig. 6A) and a much higher level of *c-myc* mRNA (Fig 7A) were detected in control cells. Detectable levels of *c-fos* mRNA were induced within 30 min, and reached the maximal level (about 4-fold) in 2 h, with high levels being maintained for 24 h (Fig. 5). Sodium butyrate also stimulated increases in *nur77* expression which then tailed off (Fig. 6A). The *nur77* was induced by butyrate in 30 min, a maximal level of about 3-fold was observed at 2 h, and although the level then declined gradually, a 2-fold expression was still observed 24 h after treatment (Fig. 6B). In contrast, butyrate stimulated a slight decline (about 30%) in the *c-myc* expression after treatment for 1 h, but the decline was gradually reversed after 4 h treatment (Fig. 7). Taken together, sodium butyrate stimulated both *c-fos* and *nur77* expression in C6

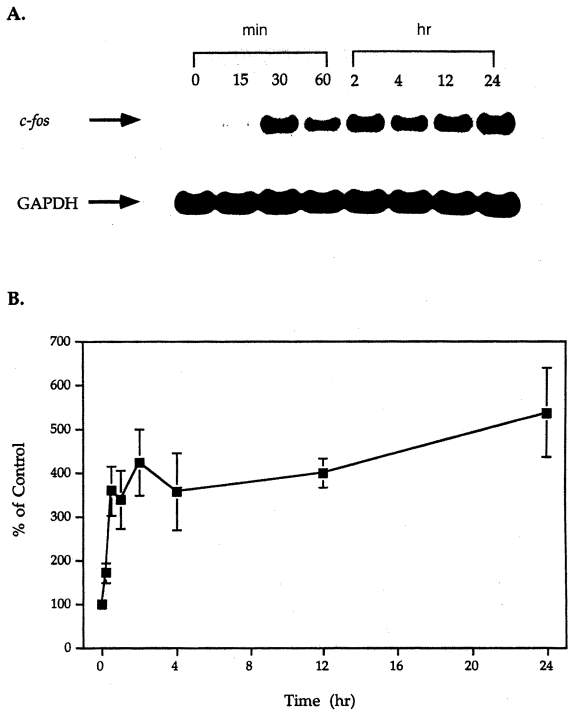


Fig. 5. Effect of sodium butyrate on *c-fos* mRNA expression. C6 glioma cells were cultured on 100-mm dishes and treated with 2.5 mM sodium butyrate for 0-24 h. Total RNA extracted and Northern blot analyzed for *c-fos* and GAPDH. **A**. Autoradiographs for sodium butyrate-stimulated *c-fos* and GAPDH expression, and **B**. quantitative analysis determined by phosphoimage densitometry analysis and the ratio of *c-fos*/GAPDH calculated. Data represent % of control ratio of mean ± SD from three separate cultures.

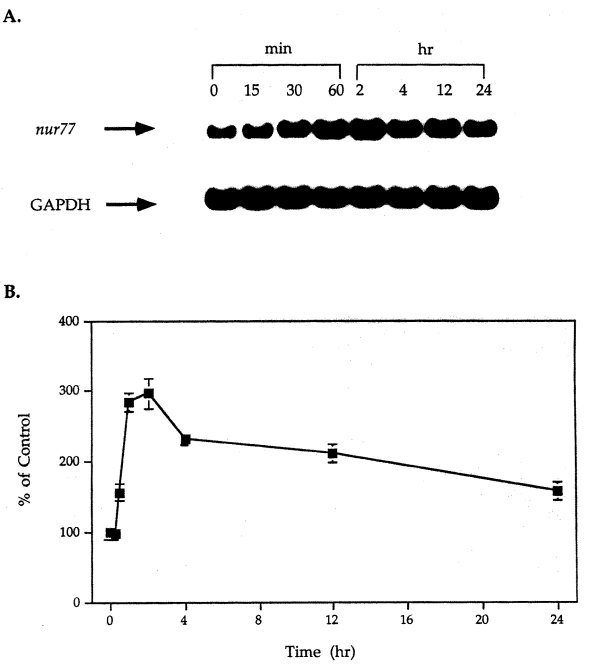


Fig. 6. Effect of sodium butyrate on *nur77* mRNA expressions. C6 glioma cells were cultured on 100-mm dishes and treated with 2.5 mM sodium butyrate for 0-24 h. Total RNA extracted and Northern blot analyzed for *nur77* and GAPDH. **A**. Autoradiographs for sodium butyrate-stimulated *nur77* and GAPDH expression, and **B**. quantitative analysis determined by phosphoimage densitometry analysis and the ratio of *c-fos*/GAPDH calculated. Data represent % of control of mean ± SD from three separate cultures.

glioma cells but the time courses of the expression patterns were different.

### *$Ca^{2+}$ Mobilization Agents Enhanced Sodium Butyrate-induced IEG Expression*

To elucidate whether the butyrate-stimulated *c-fos* and *nur77* expression are associated with $Ca^{2+}$ signaling, the effect of $Ca^{2+}$ on *c-fos* and *nur77* expression was examined. Addition of 1 μM A23187 or thapsgargin (TG) enhanced *c-fos* and *nur77* expression, suggesting that increases in cytosolic $[Ca^{2+}]_i$ constitute an important mechanism for regulating the expression of these IEGs. Incubation of these cells with TG or A23187 in the presence of sodium butyrate further enhanced the *c-fos* and *nur77* expression indicating that the effect of butyrate may be independent of changes in $[Ca^{2+}]_i$ (Fig. 8A).

### *Chelate Extracellular and Intracellular $Ca^{2+}$ Caused No Effect on Sodium Butyrate-Induced IEG Expression*

To verify whether the sodium butyrate-induced *c-fos* and *nur77* mRNA levels were mediated through a $Ca^{2+}$-independent pathway, cells were treated with EGTA or BAPTA to chelate extracellular or intracellular $Ca^{2+}$, respectively. As shown in figure 8B, EGTA and BAPTA per se caused no effect on the mRNA levels of either gene. In addition, the sodium butyrate-induced *c-fos* and *nur77* mRNA expressions were not affected by either $Ca^{2+}$ chelator suggesting that the butyrate-induced *c-fos* and *nur77* expression are probably not due to alterations in $[Ca^{2+}]_i$.

## Discussion

The cellular growth and differentiation of C6 glioma cells is interesting, in so far as these cells express both oligodendrocyte and astrocyte marker proteins (2, 61). Other workers (29, 34, 41) have reported a time-dependent differentiation of C6 cells from oligodendrocyte to astrocytes. Our previous results by using older passages of C6 cells (40, 50) (>300 passages) demonstrated that the mechanisms of butyrate induced C6 differentiation is associated with increases in arachidonic acid incorporation into phosphatidylcholine (PC) and decreases in that of phosphoinositides (PIs), as well as decreases in inositol

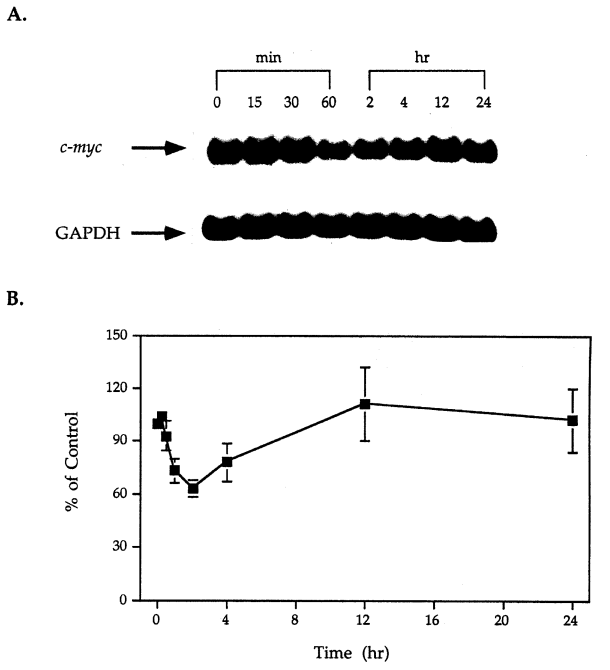


Fig. 7. Effect of sodium butyrate on *c-myc* mRNA expression. C6 glioma cells were cultured on 100-mm dishes and treated with 2.5 mM sodium butyrate for 0-24 h. Total RNA extracted and Northern blot analyzed for *c-myc* and GAPDH. **A**. Autoradiographs for sodium butyrate-stimulated *c-myc* and GAPDH expression, and **B**. quantitative analysis determined by phosphoimage densitometry analysis and the ratio of *c-myc*/GAPDH calculated. Data represent % of control of mean ± SD from three separate cultures.

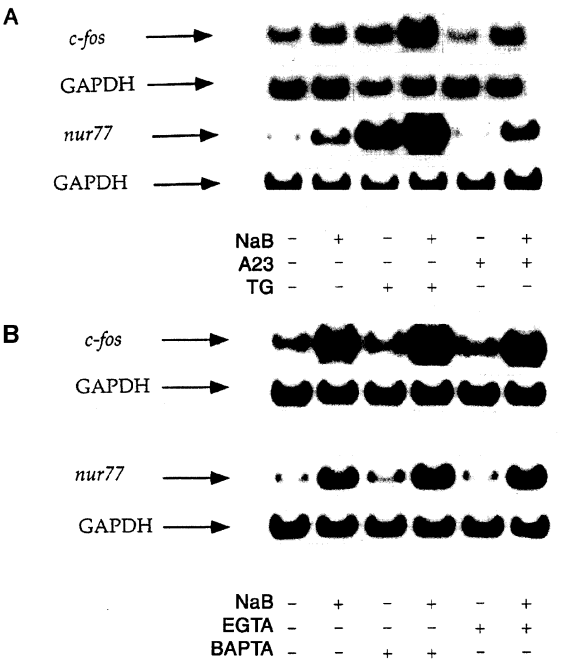


Fig. 8. Effect of $Ca^{2+}$ on sodium butyrate-stimulated *c-fos* and n*ur77* mRNA expression. C6 glioma cells were grown on 100-mm dishes in culture media in the presence or the absence of 2.5 mM, and with or without **A**. the $Ca^{2+}$ mobilization agents; A23187 (A23) or thapsgargin (TG), and **B**. the $Ca^{2+}$ chelators; BAPTA or EGTA and further cultured for 2 h. Total RNA extracted and Northern blot analyzed. Experiments were performed three times by using three different batches of cells and a typical autoradiograph of *c-fos* and *nur77* expressions from one experiment is shown.

incorporation into PIs. Further, the subsequent parameters related to PI turnover signal transduction pathway i.e. histamine-stimulated inositol monophosphate (IP) accumulation, inositol triphosphate ($IP_3$) production, and $Ca^{2+}$ mobilization were all decreased in these cells. The younger C6 cells do not possess histamine receptors, therefore the effect of sodium butyrate on growth, signal transduction pathways and transcription coupling mechanism were examined. The present study reports similar findings that sodium butyrate decreased thymidine incorporation, inositol incorporation, and ATP-stimulated $Ca^{2+}$ mobilization. In these cells, decreases in ATP-stimulated net increases in $[Ca^{2+}]_i$ were 146 nM and 72 nM by using $Ca^{2+}$-added and $Ca^{2+}$-free buffer system, respectively. Thus, in association with decreased inositol incorporation, $Ca^{2+}$ release, butyrate may cause decreases in other $Ca^{2+}$ mobilization pathways, such as $Ca^{2+}$ influx.

A small increase (about 20 nM) in basal levels of $[Ca^{2+}]_i$ was observed in the sodium butyrate-treated C6 cells as compared with the controls. This is probably related to butyrate's ability to change in intracellular pH, which causes changes in $Ca^{2+}$ current (46). In the present study, we used a much lower concentration of butyrate than Shimigol et al. (46) to treat C6 cells, 2.5 mM as compared to 20 mM, and this slightly increased $[Ca^{2+}]_i$ probably contributed only a small fraction (around 10%) to the large decrease in ATP-stimulated net increases in $[Ca^{2+}]_i$. Most of the decrease would thus have been due to $Ca^{2+}$ mobility being disabled.

To further examine the earlier cellular responses, we then analyzed the levels of mRNA of *c-fos*, *c-myc* and *nur77*, and the cross talk between sodium butyrate and $Ca^{2+}$ signaling in the expression of these IEGs. Multiple mechanisms are known in the $Ca^{2+}$ stimulation of these IEGs (10, 12), and quantitative effects of $Ca^{2+}$ on *c-fos* expression have been demonstrated (58). In the present study, we demonstrated that although sodium butyrate altered $Ca^{2+}$ signaling, the sodium butyrate-stimulated *c-fos* and *nur77* expression is not related to increased basal levels of $[Ca^{2+}]_i$. A similar result has been demonstrated before in PC12 cells, i.e. that induction of *c-fos* by sodium butyrate is independent of the $Ca^{2+}$-calmodulin signal transduction pathway (38). There were no report on *nur77* expression and sodium

butyrate-induced *nur77* expression in C6 glioma cells. The different time courses of sodium butyrate-stimulated *c-fos* and *nur77* expression (Fig. 5 & 6) suggest that a different regulatory mechanism intrinsic to each gene might be involved in the down regulation of these two genes in C6 glioma cells. The persistent induction of *c-fos* by butyrate in PC12 cells has been shown to relate to a mechanism whereby the butyrate-induced c-Fos protein was not able to negatively trans-regulate the *c-fos* promoter (38). Thus, different mechanisms may be involved in the negative regulatory process of butyrate-induced *nur77* expression. A report by Enslen and Soderling (8) showed that inhibition of $Ca^{2+}$-sensitive calmodulin-dependent protein kinase and phosphatase (calcineurin) resulted in decreases in transcription of *nur77* in PC 12 cells. Similarly, a decrease in $Ca^{2+}$ signaling could also produce an agonistic or antagonistic effect on *nur77* expression through inhibition of certain kinases and phosphatases. Recently, sodium butyrate has been found to inhibit the phosphorylation of retinoblastoma gene product in mouse fibroblasts (3), whereas it stimulated the phosphorylation of tau protein in neuroblastoma cells (39). A report by Thompson et al. (53) indicated that *c-fos* transcription was regulated by both $Ca^{2+}$-mediated phosphorylation and dephosphorylation. Thus, crosstalk among $Ca^{2+}$ signaling, kinases and phosphatases for the regulation of a gene may be one of the targets of sodium butyrate's action.

Thirty minutes after added, sodium butyrate rapidly induced *c-fos* and *nur77* mRNA accumulation, and the peak levels of *c-fos* (about 4-fold) were maintained as long as butyrate was present (24 h), whereas the level of *nur77* began to decline after 4 h treatment (Fig. 5 & 6). The expression pattern of butyrate-induced *c-fos* was similar to those found by Souleimani and Asselin (47), who have reported that sodium butyrate stimulated *c-fos* expression in a colon carcinoma cell line, CACO-2, cultured in serum-free condition, as well as in PC 12 cells (38). In F-98 glioma cells, on the other hand, the butyrate-induced *c-fos* expression declined after 2 h (51). Souleimani and Asselin (47) demonstrated that butyrate affected both the post-transcriptional mechanism, and the level of transcriptional initiation of *c-fos*. Naranjo et al (38) showed that butyrate induced loss of autoregulatory function in *c-fos*. Thus, similar mechanisms may also exist in C6 cells, with butyrate affecting a specific target of *c-fos* and *nur77* genes.

Although F-98 (22) and C6 glioma cells (2) were originally both cloned from nitrosourea-induced rat glioma, discrepancies in butyrate-induced *c-fos* expression have emerged. Earlier reports indicated that butyrate increased glial fibrillary acidic protein (GFAP) expression in F-98 cells (22, 56). Conversely, butyrate decreased GFAP levels in a human glioma-derived cell line (RF), and in C6 glioma cells (16, 24). We have also found that mRNA and protein levels of GFAP were both decreased when C6 cells were treated with butyrate for more than 12 h (data not shown). Although these results could suggest that the discrepancy is probably due to cell-specific regulation of the genes. It could also being argued that it is due to the culture conditions and the state of the cells in each study. Moreover, C6 cells are known to exhibit differential enzyme expression with cell passages and are considered to be a more immature glia (34 ).

Elevated expression of *c-myc* mRNA is frequently found in carcinoma cells, and sodium butyrate reduces the expression of *c-myc* mRNA (16, 24. However, in our cells sodium butyrate caused only a slight (40%) albeit rapid (1-2 h) reduction in *c-myc* mRNA expression (Fig. 7). After 4 h butyrate treatment, the levels of *c-myc* mRNA began to rise again, and reached the control level at 12 h. In addition, TG stimulated a maximal *c-myc* expression, whereas sodium butyrate inhibited the TG-induced *c-myc* expression (data not shown). This result is similar to that of Krupitza et al. (24), who concluded that butyrate's effect on *c-myc* is probably due to interference with $Ca^{2+}$ mobilization in the signal transduction pathway.

Whether sodium butyrate-induced *c-fos* and *nur77* expression is mediated through the activation of a specific kinase or through induction of a protein kinase inhibitor/phosphatase remains unknown at this moment. Promoter analysis demonstrated that butyrate acted on nucleotide region -757 to -402 on *c-fos* promoter in F-98 cells (52). Others have reported that butyrate targeted the AFT-CRE binding site of the *c-fos* transcription start site in CACO-2 colon carcinoma cells (47), and induced loss of autoregulatory function of *c-fos* promoter in PC12 cells (38). In particular, Nur77 protein was found to play a pivotal role in T cell receptor-mediated apoptosis (30, 59, 60). Furthermore, recent studies revealed that the mechanisms underlie of Nur77-regulated apoptosis is targeting on mitochondria but not on DNA binding (31). In the present study, prolonged treatment of C6 cells with butyrate caused DNA fragmentation (data not shown). Taken together with our findings, this suggests that induction of *c-fos* and *nur77* may be the upstream regulatory mechanism of butyrate-induced cell differentiation or apoptosis, and the induction is not related to butyrate-induced changes in $Ca^{2+}$ signaling in C6 cells.

To summerize: thymidine and inositol incorporation, and ATP-stimulated $Ca^{2+}$ mobilization were inhibited in sodium butyrate-treated C6 glioma cells. Sodium butyrate also stimulated the expression of *c-fos* and *nur77*, and this was probably mediated

through a $Ca^{2+}$-independent regulatory pathway.

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