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BUTYRATE AND TGF-β DOWNREGULATE Na,K-ATPase EXPRESSION IN CULTURED PROXIMAL TUBULE CELLS

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SUMMARY: Attempts were made to examine the effect of growth inhibitors on regulation of Na,K-ATPase in primary cultures of renal proximal tubule cells. We observed that both TGF-β and butyrate induced dose dependent decrease of Na,K-ATPase activity. The time course studies showed that the effect of TGF-β preceded the effect of butyrate on inhibition of Na,K-ATPase activity. Both butyrate- and TGF-β-induced inhibition of this enzyme were in general mediated by transcriptional decreases of α and β mRNA and therefore also protein abundance. Like its effect on Na,K-ATPase activity, TGF-β compared to butyrate induced much earlier decline in Na,K-ATPase α and β mRNA and protein abundance. Butyrate did not affect gene expression of TGF-β and anti-TGF-β antibody had no effect on butyrate-induced inhibition of DNA synthesis. Obviously, both butyrate and TGF-β can inhibit the expression of Na,K-ATPase α and β mRNA, but TGF-β does not mediate butyrate's effect.

Na,K-ATPase, known as the sodium pump, is an integral membrane protein found in all animal cells. This enzyme pumps Na^+ out of the cell and K^+ into the cell coupled to the hydrolysis of ATP, thus, establishing transmembrane ionic gradients of these two cations. In kidney, the sodium pump is composed of an α catalytic subunit and β glycosylated subunit (1, 2, 3). During the development from neonatal to adult stages, Na,K-ATPase activity in renal tubules is enhanced by 2- to 4-fold and thus has been viewed as an differentiated marker for kidney tubules (4, 5). Several pieces of evidence showed that peptide growth factors stimulated Na,K-ATPase activity in MDCK cells (6) and α and β subunit mRNA in clone 9 cells (7). However, whether growth inhibitors exert effects on Na,K-ATPase regulation has not yet been probed. To elucidate whether peptide growth factors or inhibitors affected the regulation of Na,K-ATPase in kidney epithelial cells, we employed primary culture of proximal tubule cells. TGF-β, a dimeric peptide, is capable of regulating cell proliferation, differentiation and other functions in most epithelial cell types in culture (8, 9) and butyrate resembles TGF-β in its cellular effects (9, 10). Because both butyrate and TGF-β are potent growth inhibitors for proximal tubule cells, we

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examined whether they downregulated Na,K-ATPase activity through decreasing gene expression and protein subunit abundance. Our findings were in the affirmative. Yet, while TGF-β mRNA and bioactivity were expressed in cultured proximal tubule cells (11, 12), butyrate-induced effects on inhibition of Na,K-ATPase and cell growth was found not to be mediated by autocrine release of TGF-β.

MATERIALS AND METHODS

Culture techniques

Our methodology of primary rabbit proximal tubular cultures has been previously published (13). In brief, kidneys were removed from pentobarbitol-anesthetized adult New Zealand white rabbits (4-6 lbs), and the cortices were dissected, minced and digested by collagenase (1 mg/ml). Highly purified proximal tubules were isolated by centrifugation through a 48% Percoll density gradient. Freshly isolated tubules were cultured in serum-free hormonally-defined media as described (13). The standard culture medium used was low glucose (5.5 mM) Dulbecco's modified Eagle's medium (DME), supplemented with penicillin (192 IU/ml), streptomycin (200 ug/ml), insulin (5 ug/ml), hydrocortisone ($5x10^{-8}$ M) and transferrin (5 ug/ml). The culture dishes were maintained in an incubator under 20% O_2/5%CO_2 atmosphere at 37°C. The medium was changed every other day until the culture was terminated.

Measurement of cell growth

Proliferation rate of cultured proximal tubules were assessed by determining the cell number, total protein content and DNA synthesis by thymidine incorporation in cultures in 60-mm dishes.

Preparation of cell homogenates

Cultured proximal tubules, at specified ages, were rinsed 3 times with ice cold phosphate buffered saline, scraped with rubber policeman and centrifuged at 1600 X g for 3 min. The supernatant was removed and cell pellets were stored at -70°C prior to use. Homogenization was performed by sonication with Branson sonifier at settings of 2.5 50% duty cycle, 20 pulses in homogenization buffer (5% sorbitol, 5 mM histidine-imidazole buffer, pH 7.5, 0.5 mM Na_2EDTA, and 0.1 mM phenylmethyl-sufonylfluoride).

Na, K-ATPase enzymatic activity

The Na,K-ATPase activity in cell homogenates was determined by the method of Lo et al (14), in which ouabain sensitive inorganic phosphate liberation from ATP was quantitated and expressed as μmoles phosphate per mg protein per hour.

Immunoblot analysis of Na,K-ATPase subunit abundance

The immunoblotting method used to determine Na,K-ATPase subunit abundance has been described (15). In brief, 50 μg protein was resolved by SDS-PAGE, and electrophoretically blotted onto nitrocellulose paper. The nitrocellulose paper was incubated with mouse anti-rabbit Na,K-ATPase α and β antisera (1:10,000 dilution), obtained from Dr. M. Kashgarian (Yale University, New Haven, CT), and immune

complexes detected with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1: 1,000 dilution), and finally the immune complexes were visualized by fluorograghy with enhanced chemiluminescence (ECL) detection kit (Amersham International PLC, UK).

Cytoplasmic RNA extraction and Northern analysis

The cytoplasmic RNA was extracted from two confluent 100-mm dishes by treatment with detergent, followed by phenol extraction and ethanol precipitation as described previously (15) with the inclusion of vanadyl ribosyl complex (5%, v/v). Yields of RNA were stored in 10 mM tris(hydroxymethyl)aminomethane HCl, pH 7.0, and1 mM EDTA in liquid nitrogen until use. The RNA concentration was determined spectrophotometrically at the absorbance of 260 nm. RNA (7 μg total) from control or TGF-β-treated sample was resolved by electrophoresis in formaldehyde/formamide 1% agarose gels with inclusion of 2 μg/ml ethidium bromide in each sample. The RNA was transferred to nitrocellulose filters and ethidium bromide-stained 18 and 28S ribosomal species were made visible by ultraviolet light illumination to make sure the same amount of RNA was loaded on each lane. The specific mRNA immobilized on nitrocellulose filters was detected by hybridizing with ^{32}P-labelled cDNAs. The Na,K-ATPase cDNAs correspond to rat kidney full length α1 and β1 subunit (2, 15). TGF-β cDNA, a gift from Dr. M.-C. Hung (Baylor Medical College), corresponds to partial DNA sequence of human TGF-β (16). In addition, mRNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also assessed as internal control for RNA quantitation.

Statistics

All data were expressed as mean ± the standard error of the mean (SE). Differences between groups were determined according to Student's T-test, multi-group comparison was determined by one way ANOVA and considered significant when p values were less than 0.05.

RESULTS

Effect of EGF and IGF-I on Na,K-ATPase activity

Cultured proximal tubule cells at the confluent phase were incubated with medium respectively containing 1 or 10 ng/ml EGF or 10 or 100 ng/ml IGF-I for 24 hr and then Na,K-ATPase activity was assessed. Both EGF and IGF-I are known mitogens for cultured proximal tubule cells. Na,K-ATPase acitivity was 3.76±0.39 μmole Pi/mg protein/hr in control (n=6) and 3.69 ±0.52, 3.41±0.45, 3.70±0.42, 3.60±0.34 μmole Pi/mg protein/hr (n=6, 5, 6, 6) in 1 ng/ml EGF, 10 ng/ml EGF, 10 ng/ml IGF-I, 100 ng/ml IGF-I treated samples, respectively. It is thus evident that neither EGF nor IGF-I alter Na,K-ATPase activity in cultured proximal tubule cells.

Effect of butyrate and TGF-β on cell proliferation

To assess the effect of butyrate or TGF-β on growth of proximal tubule cells, 5 mM butyrate or 5 ng/ml TGF-β was added to primary cultures at day 1 after tubule cells had firmly attached to the dishes. The cell number and total protein content

were evaluated at the subsequent days in culture as the indicator for the quantity of cells. Compared to the control, there were significantly lower total protein levels (Fig 1) and cell number in cells cultured in medium supplemented with butyrate or TGF-β. The result of cell number is similar to that of total protein contents and thus is not shown. Obviously, both butyrate and TGF-β completely inhibited the growth of proximal tubule cells in primary culture.

Effect of butyrate and TGF-β on Na,K-ATPase activity, α and β protein and mRNA abundance

Cultured proximal tubule cells at the subconfluent phase were incubated with 0.25, 2.5 and 5 ng/ml TGF-β or 2 and 5 mM butyrate for 24 h and Na,K-ATPase activity were assessed. As shown in Fig 2a and 2b, butyrate and TGF-β dose dependently decreased Na,K-ATPase activity. To compare the effect of butyrate and TGF-β on Na,K-ATPase activity, cultured proximal tubule cells at the confluent phase were treated with butyrate (5 mM) or TGF-β (5 ng/ml) for 8, 12, or 24 h and then Na,K-ATPase activity was evaluated. As shown in Fig 3, TGF-β reduced Na,K-ATPase activity by 25±6%, 24±2% and 49±7% by 8, 12, and 24 h, respectively, whereas butyrate caused a significant decrease in Na,K-ATPase activity at 24 h only. Besides, the inhibitory effect of TGF-β on Na,K-ATPase activity appeared earlier and was at all time points more pronounced than butyrate.

The time course effect of butyrate (5 mM) and TGF-β (5 ng/ml) on Na,K-ATPase α and β subunit protein abundance of cultured proximal tubular cells was furthermore assessed. As shown in Fig 4, both butyrate and TGF-β induced coordinated decreases in α and β protein abundance. TGF-β significantly decreased Na,K-ATPase α protein abundance within 8 h, and β abundance within 12 h treatment. In contrast, butyrate coordinately decreased Na,K-ATPase α and β subunit abundance within 12 h

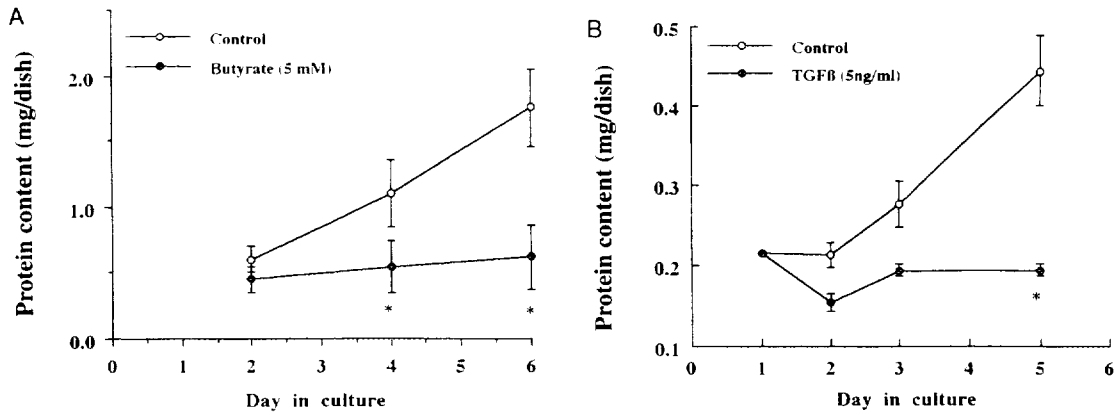


Fig 1. Growth curve of primary culture of rabbit proximal tubules under standard (control) media and supplemented with (A) butyrate (5 mM) or (B) TGF-β (5 ng/ml). (A) and (B) depict the results from cells grown on 100-mm and 60-mm dishes, respectively. Each point represents the mean ± SE of 3 experiments in duplicate or triplicate (*: $p<0.05$, vs. time control).

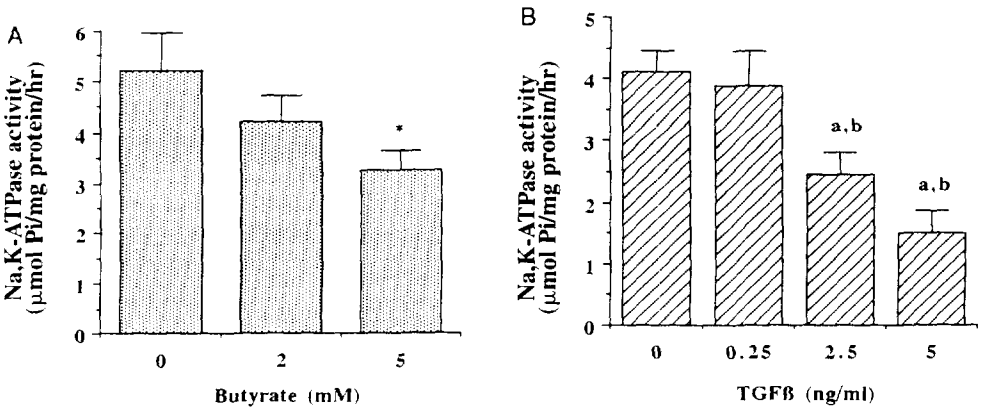


Fig 2. Effects of butyrate (A) and TGF-β (B) on Na,K-ATPase activity in cultured proximal tubule cells. Proximal tubule cells at the subconfluent phase were treated with different doses of butyrate or TGF-β for 24 hr and Na,K-ATPase activity was assessed. Each bar represents mean ± SE of 3 experiments in duplicate. (*: $p<0.05$, vs. control; a: $p<0.05$, vs control; b: $p<0.05$, vs 0.25 ng/ml)

treatment. These results indicate that butyrate- and TGF-β- induced decrease in Na,K-ATPase activity may be reflected by coordinated decrease in α and β protein levels.

The time course changes of Na,K-ATPase α and β mRNA abundance induced by butyrate (5 mM) and TGF-β (5 ng/ml) were analyzed by Northern blotting. As shown in Fig 5, TGF-β inducesd coordinate decrease in both α and β mRNA abundance within 4 h and butyrate decreased both α and β mRNA abundance within 8 h treatment. The inhibitory effect of TGF-β on Na,K-ATPase gene expression appeared earlier than that of butyrate.

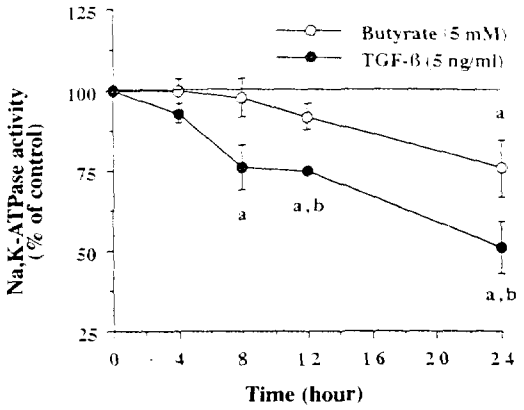


Fig 3. Time course of butyrate- and TGF-β-induced changes on Na,K-ATPase activity. Cultured proximal tubule cells at the confluent phase were treated with butyrate (5 mM) or TGF-β (5 ng/ml), and Na,K-ATPase activity was assessed at 4, 8, 12, and 24 hr. Each point represents mean ± SE of 4 experiments in duplicate. TGF-β significantly decreases Na,K-ATPase activity within 8 hr, while butyrate decrease Na,K-ATPase activity within 24 hr. (a: $p<0.05$, vs control; b: $p<0.05$, TGF-β vs butyrate)

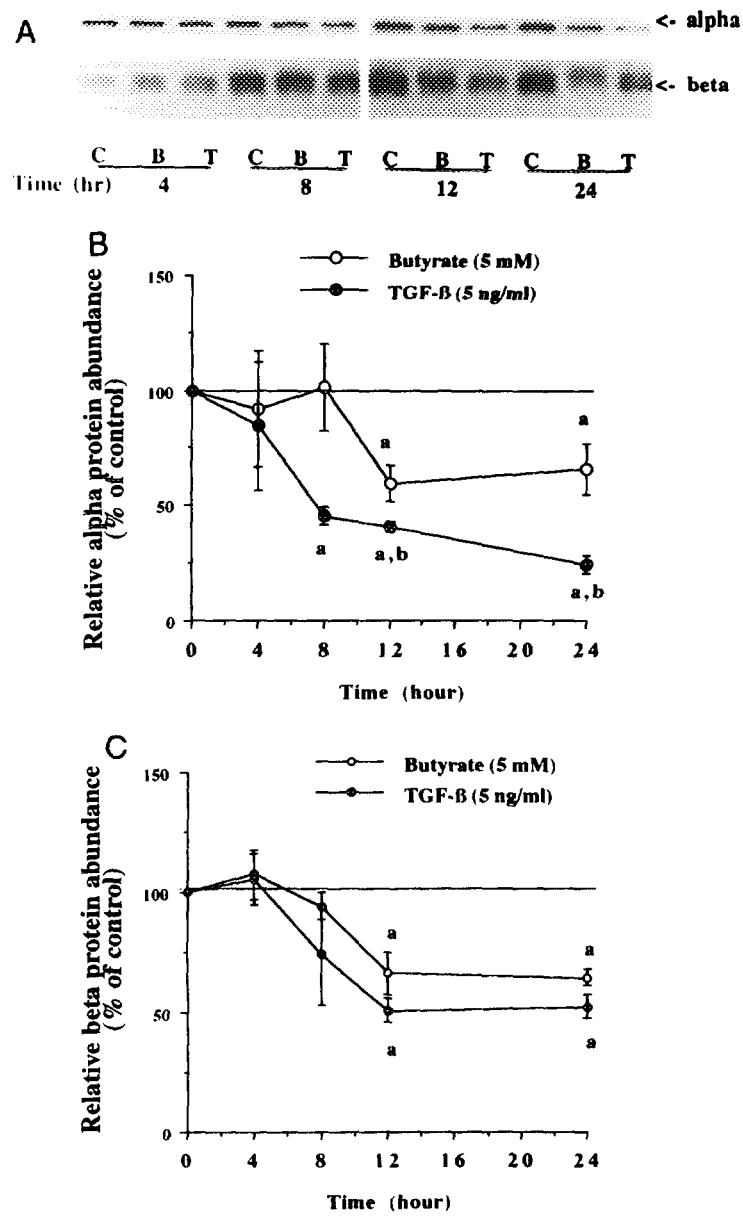


Fig 4. Time course of butyrate- and TGF-β induced changes on Na,K-ATPase α and β subunit protein abundance. Cultured proximal tubule cells at the confluent phase were treated with media containing butyrate (5 mM) or TGF-β (5 ng/ml), and Na,K-ATPase α and β subunit protein abundance was assessed by Western blot analysis at 4, 8, 12, and 24 hr. (A) is a representative autoradiogram from Westen blot analysis. C= control, B= butyrate, and T= TGF-β. (B) and (C) depicts the time course changes of Na,K-ATPase α and β protein abundance, respectively, as determined by scanning densitometry of Western blot autoradiograms. Each point represents for mean ± SE from 4 experiments. (a: $p<0.05$, vs control; b: $p<0.05$, TGF-β vs butyrate)

Whether TGF-β mediated butyrate's effects

To elucidate whether TGF-β mediated butyrate-induced inhibition of Na,K-ATPase gene expression and cell proliferation, we examined whether butyrate affects

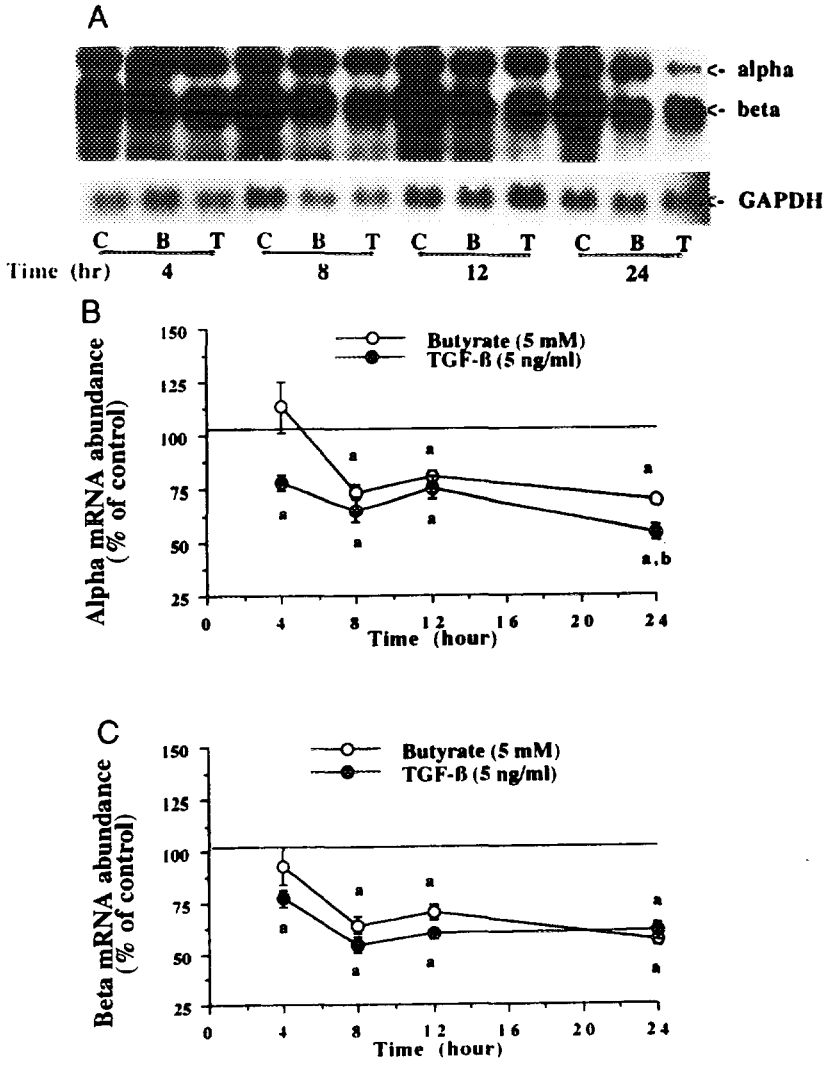


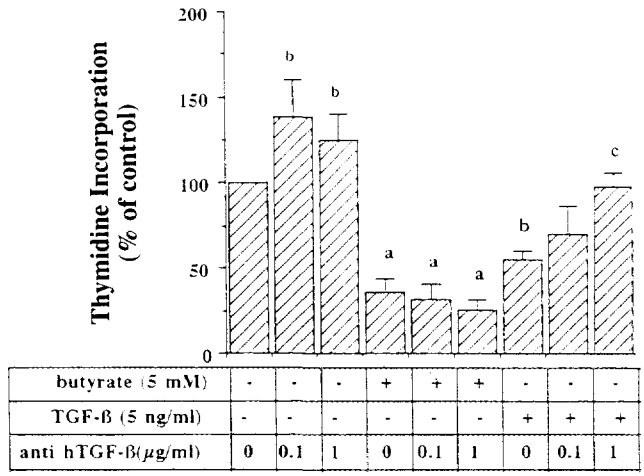
Fig. 5. Time course of butyrate- and TGF-β induced changes on Na,K-ATPase α and β mRNA abundance. Cultured proximal tubule cells at the confluent phase were treated with media containing butyrate (5 mM) or TGF-β (5 ng/ml), and Na,K-ATPase α and β subunit mRNA was assessed by Northern blot at 4, 8, 12, and 24 hr. (A) is a representative result of Northern blot. C, B, and T indicates control, butyrate and TGF-β, respectively. (B) and (C) depicted the time course changes of Na,K-ATPase α and β mRNA abundance, respectively, as determined by scanning densitometry of Northern blot autoradiograms. (mean ± SE; a: vs control, $p<0.05$; b: TGF-β vs butyrate, $p<0.05$)

TGF-β mRNA. Cultured proximal tubule cells were treated with 5 mM butyrate for 4, 8, 12, and 24 h and TGF-β mRNA was assessed. However, butyrate did not affect TGF-β mRNA levels during the 24-h time course of treatment (data not shown). Furthermore, we determined whether anti-TGF-β1 antibody, which has been shown to neutralize TGF-β1 sufficiently (R&D Systems, Minneapolis, MN), could prevent butyrate-induced decrease of DNA synthesis. Cultured proximal tubule cells at the

subconfluent phase were treated with butyrate ± anti-TGF-β1 antibody, or TGF-β ± anti-TGF-β1 antibody for 24 hr and DNA synthesis rate was assessed by 4-h [^{3}H]-thymidine incorporation assay. As shown in Fig 6, anti-TGF-β1 antibody induced a small but significant increase of thymidine incorporation, suggesting TGF-β1 was secreted into culture media. TGF-β (5 ng/ml) induced a 45±6% decrease in thymidine incorporation, which could be completely neutralized by 1 μg/ml anti-TGF-β1 antibody. In contrast, butyrate induced a 62±8% decrease in thymidine incorporation, but anti-TGF-β1 antibody could not block butyrate's effect in this respect.

DISCUSSION

The present study was the first report to demonstrate that two potent growth inhibitors of epithelial cells, butyrate and TGF-β, induced downregulation of Na,K-ATPase activity. Western blot analysis revealed that both α and β protein levels were coordinately decreased by butyrate and TGF-β, it is thus confirmed that the decreased activity of Na,K-ATPase exerted by butyrate and TGF-β has resulted from a coordinate decrease in the quantity of both protein subunit. Northern blot analysis revealed that both Na,K-ATPase α and β mRNA levels were coordinately decreased in cultured proximal tubules by either butyrate or TGF-β, indicating pretranslational regulation is involved. These data indicate butyrate and TGF-β can inhibit α and β gene



butyrate (5 mM)	-	-	-	+	+	+	-	-	-
TGF-ß (5 ng/ml)	-	-	-	-	-	-	+	+	+
anti hTGF-ß(μg/ml)	0	0.1	1	0	0.1	1	0	0.1	1

Fig 6. Effect of anti-hTGF-β antibody on butyrate or TGF-β induced decreased thymidine incorporation in cultured proximal tubule cells. Cultured proximal tubule cells at the subconfluent phase were treated with butyrate ± anti-hTGF-β antibody, or TGF-β ± anti-hTGF-β antibody for 24 hr and 4 hr thymidine incorporation was evaluated. Each bar represents for mean ± SE of 3 experiments in duplicates. (a: $p<0.01$ vs. control; b: $p< 0.05$ vs. control; c: $p<0.05$ vs 5 ng/ml TGF-β.) The anti-hTGF-β neutralizing antibody (1 μg/ml) sufficiently blocks TGF-β1-, but not butyrate-induced decreased thymidine incorporation.

expression and thereby coordinately decrease α and β protein subunit abundance which further leads to decreased Na,K-ATPase activity.

Evidence is available indicating that peptide growth factors stimulate Na,K-ATPase activity in cultured epithelial cells. For example, in MDCK cells, hepatocyte growth factor (HGF) stimulates a 63% increase in the Na,K-ATPase activity (6), but whether HGF affects α or β mRNA has not been studied. In a nontransformed line of hepatocyte (clone 9), peptide growth factors, such as PDGF, EGF and bFGF, induced 0.5- to 1-fold increase in the abundance of Na,K-ATPase α mRNA and 1- to 2-fold increase in β mRNA (7). Although effects of these factors on the cell growth were not looked into, these studies demonstrated that cell growth is associated with upregulation of Na,K-ATPase. In our study, we also tested effects of two potent mitogens for proximal tubule, EGF and IGF-I. However, EGF and IGF-I in our hands failed to affect Na,K-ATPase activity, suggesting that different cells may respond to growth factors differently.

In renal proximal tubule cells, upregulation of Na,K-ATPase can be acchieved by factors such as thyroid hormone (17), glucocorticoid (15, 18), or ionic stimulation (i.e. low K^+) (2, 19). However, substances which can chronically depress Na pump activity of cells have not been decribed. Kennedy and lever demonstrated that differentiation inducer hexamethylene bisacetamide (HMBA) and dimethylsulfoxide (DMSO) reduced ouabain binding to the MDCK cells, but did not alter the Na,K-ATPase activity of the cell extracts (20, 21). Their reports reflected that differentiation agents induced a change in turnover of Na,K-ATPase without a change in number of total pump sites. On the other hand, our study demonstrated that butyrate and TGF-β inhibited the total number of the Na,K-ATPase and its gene expression in cultured cells. Our reports indicate for the first time that there is close relationship between growth inhibition and downregulation of Na,K-ATPase expression in epithelial cells.

In vivo experiments have demonstrated that the expression of Na,K-ATPase α and β genes is markedly elevated during the early process of renal regeneration after acute tubular necrosis (ATN) (22). Exactly what causes the elevation of Na,K-ATPase α and β mRNA abundance has not been identified, but it could be resulted from signals inducing renal cell hyperplasia. It is also not known whether growth inhibitors are required to turn off renal regeneration and/or the elevated Na,K-ATPase gene expression in the recovery of renal failure. In view of the potent effect of TGF-β exerted on inhibition of proliferation and Na,K-ATPase gene expression in proximal tubule cells, we propose TGF-β may play important roles in the recovery of renal proximal tubule during renal regeneration after ATN. This hypothesis awaits further investigation.

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