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Stereospecific Regulation of Tyrosine Hydroxylase and Proenkephalin Genes by Short-Chain Fatty Acids in Rat PC12 Cells

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ABSTRACT

Circulating short-chain fatty acids (SCFAs) are primarily derived from bacterial fermentation of carbohydrates in the colon where they function as physiologic modulators of epithelial cell maturation. Butyrate has been shown to induce tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis, and enkephalin neuropeptide gene transcription, suggesting a role in perinatal sympathoadrenal stress-adaptation. We sought to determine whether there were SCFA structural requirements for this effect. Nine biologically relevant SCFAs and butyrate derivatives were tested in an *in vitro* model (PC12, rat pheochromocytoma cells) for their ability to regulate neurotransmitter-related gene expression. Our results revealed that among all the studied SCFAs, only propionate and butyrate increased tyrosine hydroxylase and proenkephalin mRNA levels. The functional activity was selective to the carbon atom chain length and associated with the presence of an ethyl moiety in the carbon atom backbone chain. Modifications or absence of this domain affected the gene induction response, suggesting a receptor-mediated mechanism(s). Moreover, propionate, butyrate, and the drug 4-phenylbutyrate were each shown to regulate transmitter genes via at least three independent mechanisms: histone hyperacetylation, cAMP signaling, or peroxisome proliferator-activated receptor gamma–mediated pathways. Thus, the biologic impact of SCFAs on catecholaminergic and opioid systems depend on the activation of SCFA-specific, dose-specific, and gene-specific molecular mechanisms. We speculate that *1*) circulating levels of SCFAs may influence sympathoadrenal transmitter biosynthesis and hence whole animal stress-adaptive responsiveness after birth, and *2*) the adverse effects of antibiotics on delayed acquisition of postnatal gut flora may affect this apparent evolutionary advantage of gut colonization. **(*Pediatr Res* 55: 847–854, 2004)**

Abbreviations

ppEnk, preproenkephalin
TH, tyrosine hydroxylase (EC 1.14.16.2)
PC12, rat pheochromocytoma
SCFA, short-chain fatty acids
SB, sodium butyrate
PKA, protein kinase A
PPAR, peroxisome proliferator-activated receptor
RT-PCR, reverse transcriptase-PCR
HDAC, histone deacetylase
TSA, trichostatin A
4-PB, phenylbutyrate
2-HB, 2-hydroxybutyrate
3-HB, 3-hydroxybutyrate
RXR, retinoid X receptor
PPRE, peroxisone proliferator response element
PMSF, phenylmethyl sulfenylfluoride

Sympathoadrenal transmitter systems mature to adult capacity a week or more after birth in response to progressive cholinergic innervation, exposure to growth factors, and hormonal influences from both the hypothalamic-pituitary-adrenal cortical and thyroid hormonal systems (1–6). Although the importance of these conventional control mechanisms is well established, the evolutionary significance of other exogenous stimuli can be considered in a broader view of environmentally derived signal-transduction. For example, the delayed period of adrenal transmitter accumulation after birth coincides with the time taken to establish full enteral feedings, colonization of the intestine, and attendant production of SCFA (7). Moreover, in the rat (as well as in humans), this time frame is far longer than the capacity of the chromaffin cell to rapidly induce biosynthesis of either catecholamines or enkephalins, which can occur in a matter of hours (8). Maturational delays occurring up to 2 wk after birth portend the existence of other more gradually acquired regulatory signals.

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In a recent report (9), we hypothesized that gut-derived SCFAs serve a function as "acquired" environmental modulators of the developing sympathoadrenal system. We showed that SB interacted with the classic cholinergic-nicotinic, signal-transduction system and, together, could differentially regulate TH mRNA (the rate-limiting enzyme in catecholamine biosynthesis) and the mRNA for the co-localized neuropeptide transmitter ppEnk (9). Whether other gut-derived or metabolic pathway SCFAs have similar effects as SB and how these influences are mediated at the cellular and molecular level remain largely unknown. Thus, in the present report, we sought to determine whether SCFAs other than SB (or biologically relevant SB derivatives) could modulate classic catecholaminergic or neuropeptide pathways *in vitro*. If so, this would represent new opportunities to therapeutically enhance neonatal adaptive responses to stress. We also examined which intracellular signal-transduction systems are linked to SB effects and whether long-chain fatty acid binding receptors (PPAR) were involved in the signal-transduction cascade.

METHODS

Cell culture. The PC12 cells used in the experiments were originally developed by Greene and Tischler (10). They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% fetal bovine serum, 50 μg/mL streptomycin, and 50 IU/mL penicillin as described earlier (9). Cells were treated with the drugs indicated (see figure legends), harvested after 48 h, and processed for further analysis as described below. Each experiment was repeated more than two times, with six individual replicates per experimental group, to permit proper statistical evaluation of the results.

Northern blot analysis. Total RNA was isolated from each individual Petri dish using RNazol (Tel-Test, Inc., Friendswood, TX, U.S.A.), and Northern blot analysis was performed as previously described by us (11). After transfer to Gene Screen Plus membranes, the filters were hybridized consecutively to labeled rat cDNA probe for TH and a probe for 18S ribosomal RNA. For detection of ppEnk mRNA, an anti-sense RNA probe was prepared by *in vitro* transcription of the ppEnk plasmid kindly provided by SL Sabol (12) using MAXIscript SP6 kit (Ambion, Austin, TX, U.S.A.). Filters were washed under proper stringency and exposed for autoradiography, using Kodak BioMax film (Rochester, NY, U.S.A.). The autoradiographs were scanned and the abundance of each mRNA was expressed relative to concomitantly measured 18S ribosomal RNA levels. The results are presented as fold induction compared with the corresponding control group on the same Northern blot.

RT-PCR analyses. Total RNA isolated from PC12 cells (1 μg) was subjected to RT-PCR analyses using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Denver, CO, U.S.A.) in a two-step, single-tube reaction, and rat PPARγ-gene specific primers [reverse: position 1378 to 1359, 5' GTACGCGGATCAGCATCCCGTC; forward: 5' TGCCAGTACTGCCGTTTCCAC sequence 834–854, Gi:206317 (13)], in a Perkin-Elmer Gene Amplification PCR System 9700. The first (RT) reactions were incubated for 1 h at 42°C. After inactivation of the reverse transcriptase (5 min at 95°C), 25 pmol of both forward and the same reverse primer was added, and the generated cDNA was used directly as a template for polymerization in PCR. After an initial denaturation of 5 min at 95°C, 30 cycles of 1 min at 94°C, 1 min at 48°C, and 2 min at 95°C were performed, followed by elongation for 5 min at 72°C and soak file at 4°C. The resulting PCR products (5 μL from each reaction) were analyzed on 2% agarose gel using PCR molecular weight markers from Promega (Madison, WI, U.S.A.).

Western blot analyses. PC12 cells were homogenized in 20 mM HEPES, pH 7.5; 350 mM NaCl; 25% glycerol; 0.25% NP-40; 1 mM Na_2VO_3; 0.25 mM PMSF; 5 mM $MgCl_2$; 1 μg/mL each of aprotinin, pepstatin, and leupeptin; 1 mM EGTA; and 1 mM DTT. Protein concentration was determined using the Bradford assay. Equal amounts of proteins were separated on 10% SDS-PAGE, electroblotted onto a nitrocellulose membrane (BioRad, Hercules, CA, U.S.A.), and incubated with an anti-PPARγ antibody (1:500, Santa Cruz Biotech, Santa Cruz, CA, U.S.A.). The immune reaction was visualized by enhanced chemiluminescent substrate from Pierce (Rockford, IL, U.S.A.), using a horseradish peroxidase label and Kodak XAR-5 film, as described by the manufacturer. Mouse heart extract (Santa Cruz Biotech.) was used as a positive control.

Statistical analysis. Data are expressed as mean ± SEM and normalized to the values in the control, taken as 1. Differences between the experimental groups from three independent experiments were evaluated by performing ANOVA followed by the Fisher's least significant difference test for experiments with more than two groups. A level of $p \leq 0.05$ was accepted as statistically significant

RESULTS

Structural requirements for the capacity of different SCFAs and their derivatives to alter neurotransmitter-related gene expression. Using physiologic pH and blood level concentrations (14) for treatment, we compared the ability of SCFAs with different carbon chain lengths or biologically relevant structural analogs of SB to induce neurotransmitter-related gene expression in our model system (9). PC12 cells were treated with vehicle or with 1 mM of each SCFA or an SB derivative. Total RNA was isolated from all experimental groups and subjected to Northern blot analysis for the genes of interest as described in "Methods." The results are summarized in Figure 1.

Hybridization of the blots with a radiolabeled TH cDNA probe revealed a 3.5-fold increase in TH mRNA expression for cells treated with SB when compared with the control ($p < 0.05$). The five-carbon atom (C5) SCFA, sodium valerate, showed a 1.5- fold increase, whereas sodium caproate (C6) had no statistically significant effect on TH mRNA levels (Fig. 1*A*). As previously reported by us and by others (9, 15–17), SB (C4) treatment of PC12 cells caused a 4-fold increase in ppEnk mRNA expression when compared with the untreated control group ($p < 0.05$). In contrast, SCFAs having shorter carbon

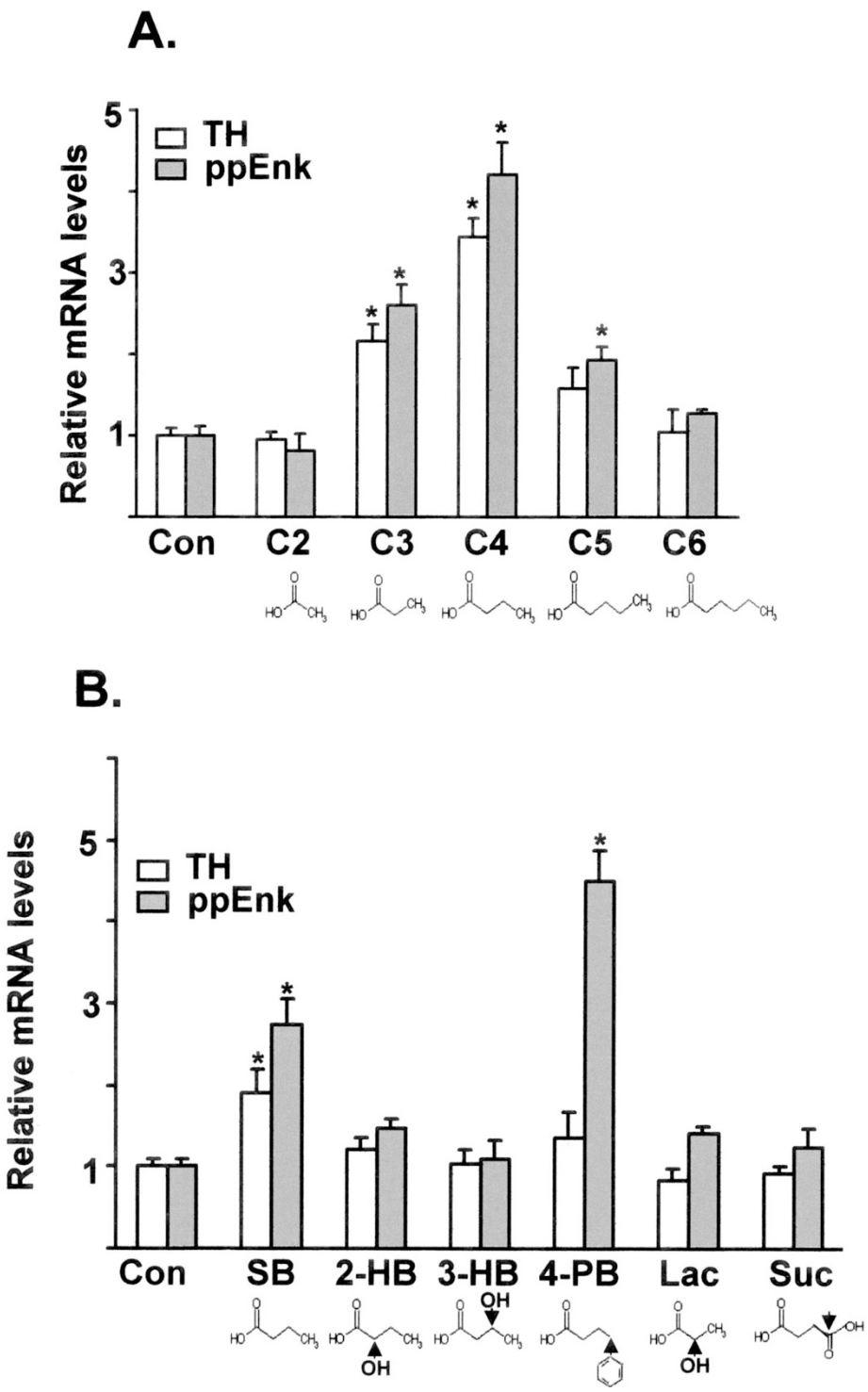

Figure 1. Ability of different SCFAs to regulate neurotransmitter gene expression. *A,* effect of carbon chain length. PC12 cells were treated with 1 mM of each SCFA: acetate (*C2*), propionate (*C3*), SB (*C4*), valerate (*C5*), and caproate (*C6*); *B,* structural modifications of SB and propionate: 1 mM of each SB, 2-HB, 3-HB, 4-PB, lactate (*Lac*), and succinate (*Suc*) were tested. After 48 h, total RNA was isolated and subjected to Northern blot analysis. The changes in the relative mRNA levels are presented as mean ± SEM values relative to those in untreated cells. * $p < 0.05$ *vs* respective control (*Con*) group. $n = 6$ per group.

chains, such as acetate (C2; the most common SCFA present in either the gut or the bloodstream), did not affect ppEnk or TH mRNA levels (Fig. 1*A*). Similarly, further increases in carbon chain length (C5, valerate, and C6, caproate) gradually reduced the capacity of SCFAs to induce these transmitter system genes.

We then sought to determine whether different stereochemical modifications of SB would modify its effects on neurotransmitter-related gene expression. PC12 cells were treated with the indicated SCFA at 1 mM final concentration for 48 h, and Northern blot analyses for the genes of interest were performed as described in the "Methods." Although SB and

4-PB (a lipophilic, pharmacologically stable analog of SB) caused marked increase in the relative steady-state levels of ppEnk mRNA, treatment with either 2-HB or 3-HB failed to result in a statistically significant change in the expression of ppEnk gene (Fig. 1*B*). The effects of SB and 2-HB or 3-HB were similar for both ppEnk and TH genes. Interestingly, the addition of a hydroxyl group on the second carbon atom of propionate (creating lactate) resulted in loss of the transcription regulatory effect for both ppEnk and TH genes. Similarly, treatment with succinate (C4, with two carboxylic groups at either end) had no significant effect on neurotransmitter gene expression, suggesting an ethyl moiety (C_2H_5) in the carbon atom backbone chain is a key to the effect.

In contrast to the common pattern of regulation with naturally occurring SCFAs, a differential pattern of regulation for ppEnk and TH mRNA levels was observed when PC12 cells were treated with a pharmacologic analog of SB, 4-PB, which has been used in humans to treat cancer, hemoglobinopathies, and urea cycle disorders (18). There was a 4.5-fold increase in ppEnk mRNA expression in PC12 cells treated with 4-PB yet no statistically significant effect on TH mRNA levels compared with control. This suggested that the transcriptional control or mRNA stabilization pathways for TH and ppEnk differ as we have previously noted (9, 19).

Rehybridizing the same blots with an 18S ribosomal cDNA probe did not show any differences among the experimental groups in any of the above experiments, indicating a gene-specific effect of all treatments.

Concentration dependency and chromatin structure. To further define the requirements for the effect of different SCFAs on neurotransmitter-related gene expression, we performed concentration-dependence studies. The capacity of SB and propionate to alter neurotransmitter gene expression was compared at low (1 mM), physiologic concentrations (7, 14) and at relatively high (6 mM) doses (Fig. 2). Inasmuch as the effects of these SCFAs in different genetic contexts were closely correlated with the degree of inducible histone hyperacetylation (20–22), increasing concentrations of TSA, a highly specific HDAC inhibitor (23), were also examined. 4-PB was used at doses (millimolar range) similar to the therapeutic plasma concentrations in humans shown to inhibit HDAC *in vitro* [reviewed in Gore and Carducci (18)]. The results of these treatments are summarized in Figure 2.

As we reported previously (9), both the low (1 mM) and the high (6 mM) doses of SB resulted in a similar magnitude of elevation of ppEnk mRNA levels (Fig. 2*A*). In contrast, TH mRNA levels were significantly increased at 1 mM but reduced after exposure to 6 mM SB. The higher concentration of propionate had a similar effect on elevating the steady-state levels of ppEnk mRNA (data not shown); however in contrast to 6 mM SB, it did not significantly alter the basal levels of TH mRNA. Additional evidence for independent regulation of these two neurotransmitter-related genes was provided when increasing concentrations of 4-PB was tested. We found a more than doubling of the rise in the relative ppEnk mRNA levels, whereas the abundance of TH mRNA was essentially unaffected at any doses of the drug studied (Fig. 2*B*). These data further support the notion that a passive diffusion mechanism is not likely to account for the differential effects of the examined SCFA on neurotransmitter-related gene expression and that it may be related to receptorlike or alternative mechanism.

Interestingly, the specific HDAC inhibitor TSA induced the expression of both genes in a dose-dependent manner (Fig. 2*C*). The concentrations of TSA used in these experiments were shown previously by others to result in accumulation of highly acetylated histones (24). This confirmed that HDAC inhibitors can regulate the expression of neurotransmitter-related genes.

However, our results revealed that although all three SCFAs tested share the ability to inhibit HDAC and, hence, alter chromatin structure, each one had a differential dose- and gene-specific effect on neurotransmitter-related gene expression. This suggests that acetylation of histones or of other preexisting molecules is only one component in a cascade of events initiated by SCFAs resulting in alteration of neurotransmitter gene expression. Other, so far unrecognized, drug-specific and gene-specific pathways may also have an impact on neurotransmitter-related gene expression.

PPARγ as a target for SCFAs. PPAR are involved in many of the transcriptional effects of dietary long-chain fatty acids, fatty acid metabolites, and hypolipidemic and anti-diabetic drugs (25, 26). These nuclear receptors are also molecular targets for cell differentiation inducers like phenylacetate and 4-PB (27). Therefore, we hypothesized that members of the PPAR family of receptors may mediate some of the effects of SCFAs on neurotransmitter-related gene expression.

Because PPAR receptors in PC12 cells had never been described and only one recent study reported PPAR expression in the adult rat adrenal (28), Western blot analysis was performed. Total protein extracts from PC12 cells and from the positive-expressing tissue (mouse heart extracts, provided by the vendor) were separated on a 10% SDS-PAGE, electroblotted, and probed with a PPARγ-specific polyclonal antibody. According to the vendor, this antibody is reactive with the two PPARγ isoforms [γ1 and γ2, reviewed in Rosen and Spiegelman (29)] from mouse, rat, and human; and not cross-reactive with PPARα or PPARβ proteins. Typical immunoblot is shown on Figure 3*A*, confirming that detectable levels of PPARγ are expressed in PC12 cells.

In addition, when total RNA from PC12 cells was subjected to RT-PCR analysis, a DNA fragment of predicted size (544 bp) was obtained (Fig. 3*B, lanes 2* and *3*). As a control for genomic DNA contamination, the reverse transcriptase was heat-inactivated in the first step (*lane 4*), and no amplification fragment was observed in this reaction. Thus, both Western blot and RT-PCR analyses indicated detectable levels of expression of PPARγ in PC12 cells.

PPARs function as ligand-activated transcription factors, which heterodimerize with the RXR. Transcriptional activation of target genes occurs after the activated PPAR–RXR complex binds to a response element in the 5' region of the target gene. To test whether ppEnk and TH genes are potential targets for transcriptional regulation by PPARγ, we performed a computer-based sequence homology search of the published rat TH and ppEnk promoter sequences. The Genomatix MatInspector search (30) for PPARγ binding sites (direct repeat of an

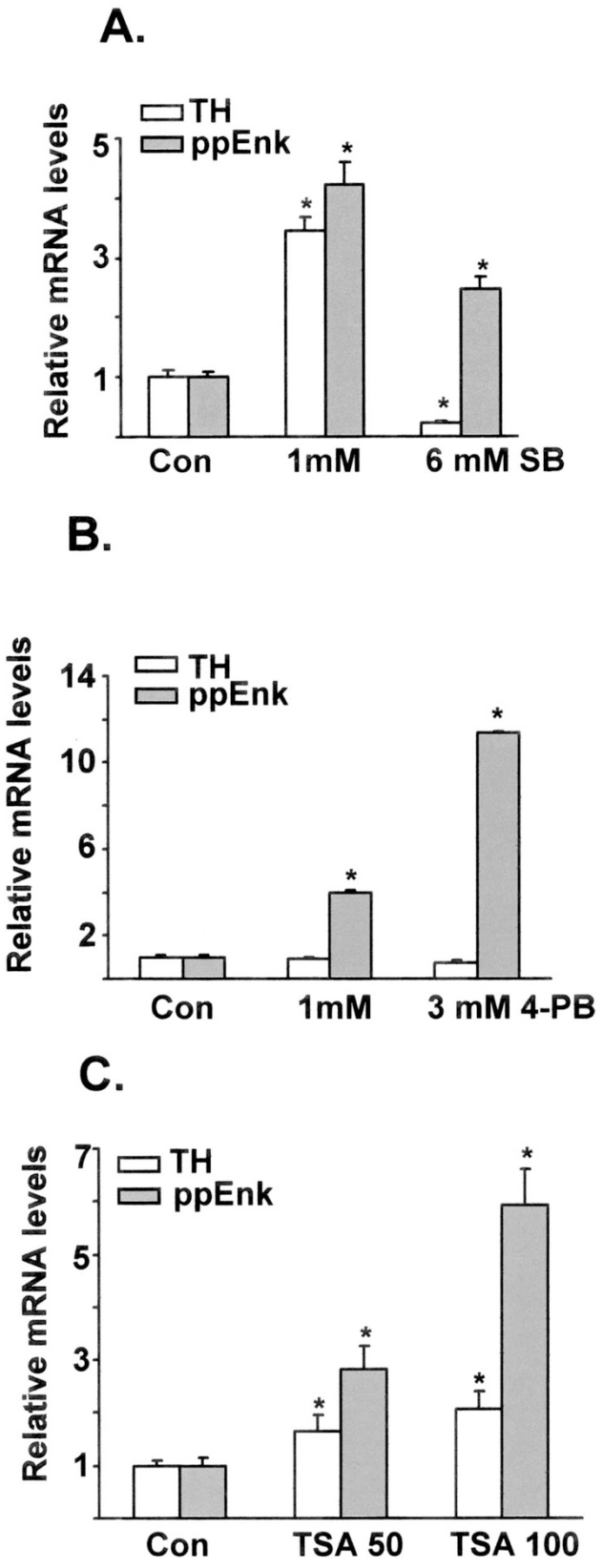

Figure 2. Dose-dependent changes in the response. Increasing concentrations of the tested drugs (*A,* SB; *B,* 4-PB; *C,* TSA) or vehicle were added to PC12 cells. After 48 h, total RNA was isolated and subjected to Northern blot analyses. The data obtained for relative mRNA levels are presented as mean ± SEM values relative to those in control (*Con*) cells. * $p < 0.05$ *vs* respective Con group. $n = 6$ per group.

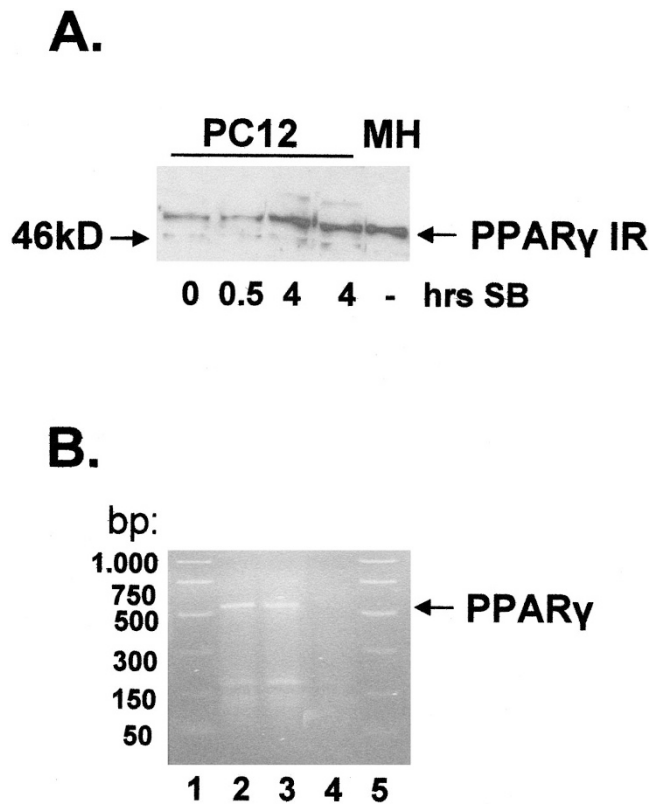

Figure 3. Identification of PPARγ-immunoreactive (*IR*) protein and PPARγ mRNA in PC12 cells. *A,* total homogenates from PC12 cells and from mouse heart extract (*MH*) were subjected to Western blot analyses using an antibody specific for PPARγ. The mobility of the marker proteins and mouse PPARγ-IR protein is indicated. *B,* agarose gel electrophoresis: markers (*lanes 1, 5*); RT-PCR products from total RNA isolated from PC12 cells (*lanes 2, 3*); and control for DNA contamination (*lane 4*). The arrow indicates the PPARγ fragment of expected size.

AGGNCA half-site separated by a 1-bp spacer (29) revealed at least one potential PPRE motif in the upstream region of rat ppEnk gene (position −393 to −374 upstream of the transcription start site (31). Whether this element is able to bind specifically PPARγ–RXR heterodimers remains to be elucidated. No such homologies were found in the rat TH promoter, consistent with the lack of induction of TH mRNA levels by PB (Figs. 1*A* and 2*B*).

To test the hypothesis that PPARγ may mediate the effects of SCFAs, we examined whether diclofenac will antagonize the actions of SB in PC12 cells. Diclofenac is a widely used analgesic, binds to PPARγ at clinically relevant concentrations, and also antagonizes PPARγ transactivation by rosiglitazone in a receptor-mediated manner in 3T3-L1 preadipocyte cells (32). Treatment of PC12 cells with diclofenac alone did not cause a statistically significant change in the relative ppEnk mRNA levels (Fig. 4*A*). However when cells were grown in the presence of both 4-PB and diclofenac, we observed a 50% reduction of the rise of ppEnk mRNA levels triggered by 4-PB alone. In contrast, diclofenac was found to have only marginal (not statistically significant) effects on SB-induced ppEnk gene expression (Fig. 4*B*). Similar results were obtained for TH (data not shown). Thus, in contrast to the drug 4-PB, signaling through PPARγ nuclear receptors does not appear to be the

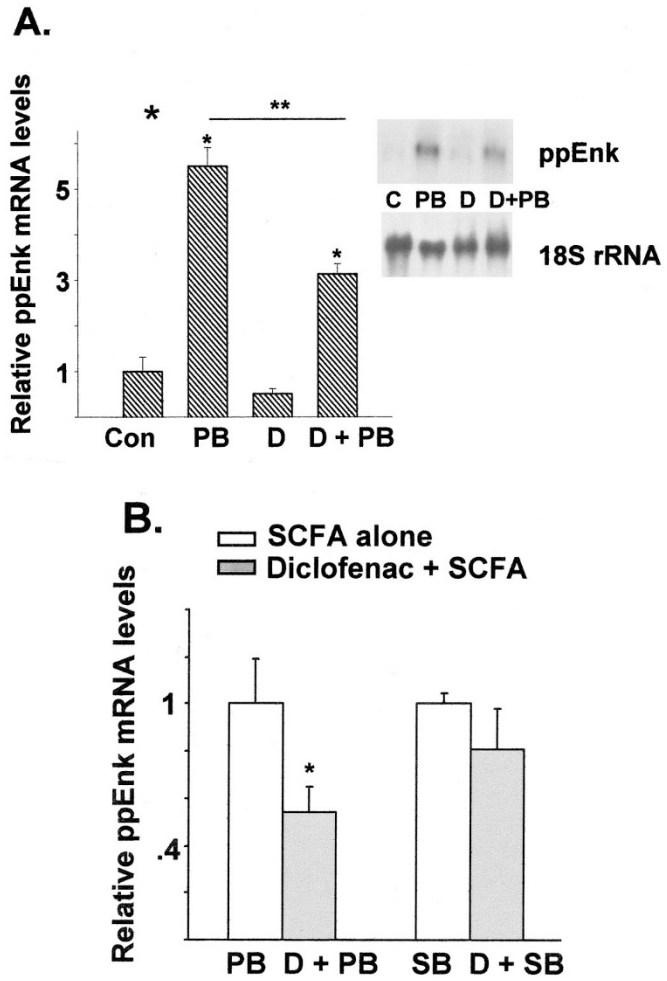

Figure 4. Effect of PPARγ receptor antagonist diclofenac (*D*). *A,* diclofenac antagonizes PB-induced ppEnk gene expression. The effect of 25 μM D on PB-induced ppEnk mRNA levels was compared with that of 1 mM PB alone. The inset shows a typical Northern blot. Error bars indicate SEM. $*p < 0.05$ *vs* corresponding controls (*C; Con*), $n = 6$ per group. *B,* comparison of the effect of D on PB- and SB-induced ppEnk gene expression.

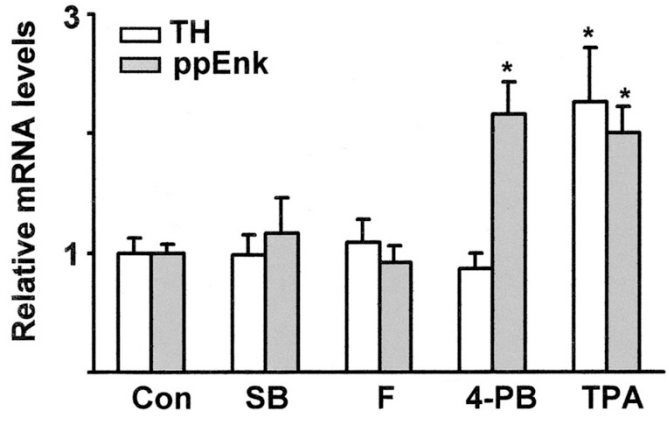

Figure 5. Different requirements for cAMP signaling pathway: The effects of vehicle (*Con*) or 1 mM SB, 20 μM forskolin (*F*), 1 mM 4-PB, and 2 μM 12-O-tetradecanoylphorbol 13-acetate (*TPA*) were examined in PKA-deficient PC12 cells [A123.7 (34)]. The changes in the relative ppEnk and TH mRNA levels were determined by Northern blot analysis and are presented as mean ± SEM values relative to those in vehicle-treated cells. $*p < 0.05$ *vs* respective Con group.

major pathway triggered by gut-derived SB to alter transcription of these neurotransmitter-related genes.

Requirements for an intact PKA signaling pathway. Recently several SCFA were identified as specific agonists for orphan G-protein–coupled receptors GPR41 and GPR43 (33). A large number of hormones, neurotransmitters, and other signaling substances that bind to G-protein–coupled receptors have their signals converge at one sole second messenger, cAMP. On the basis of these data, we examined whether activation of the PKA–cAMP second messenger system is essential for the induction of neurotransmitter-related gene expression by SCFA in our model system.

Genetically engineered PKA-deficient PC12 cells (cell line A123.7) were used in these experiments (34). Treatment of A123.7 cells with SB failed to alter the steady-state levels of mRNA for TH and ppEnk genes (Fig. 5). Forskolin, well known for its ability to activate adenylate cyclase, resulting in increased cAMP levels, activation of PKA pathway, and stimulation of cAMP-responsive genes [including TH and ppEnk (2, 3, 5)] in wild-type PC12 cells, was used as a positive control in these experiments. Addition of forskolin to A123.7 cells did not induce both TH and ppEnk mRNA levels. However, ppEnk mRNA levels were increased in A123.7 cells in response to 4-PB treatments. In contrast, 12-O-tetradecanoylphorbol 13-acetate, a phorbol ester that activates the protein kinase C pathway, still resulted in an increase in both TH and ppEnk mRNA levels, indicating that the cells are viable and responsive to treatments, stimulating a different pathway.

Thus, these data indicate that SCFAs and structural analogs of SB display different requirements for the cAMP–PKA second messenger system for their effects on neurotransmitter-related gene expression: the endogenous physiologic agent (SB) is linked to activation of PKA pathway, whereas the pharmacologic congener (4-PB) is not.

DISCUSSION

In the present report, we determined that a variety of biologically relevant, gut-derived SCFAs affect neurotransmitter-related gene expression in PC12 cells at physiologic blood concentrations. Our results were also consistent with the existence of steric-structural requirements for the stimulatory effect of these SCFAs, suggesting a receptor-like mechanism exists as well as regulatory links to HDAC, cAMP-dependent intracellular mechanisms but not to PPARγ fatty acid receptors.

Propionate (C3) as well as SB (C4) resulted in a statistically significant increase in ppEnk and TH mRNA levels when compared with vehicle-treated controls (Fig. 1, $p < 0.05$ for both). In contrast, SCFAs having shorter carbon chains, like acetate (C2; the most common SCFA present in either the gut or the bloodstream), did not affect ppEnk or TH mRNA levels (Fig. 1). Increases in carbon chain length (C5, valerate, and C6, caproate) gradually reduced the capacity of SCFAs to induce these transmitter system genes. This functional hierarchy is similar to effects on colon carcinoma cells, in which SB is the most potent SCFA in inducing cellular growth arrest, differentiation, and apoptosis, followed by propionate and valerate

(20). G-protein–coupled receptor activation of polymorphonuclear leukocytes follows faithfully to this chain-length cascade as well (35).

On further analysis, we defined additional structural requirements of SCFAs. Specifically, creating ketone bodies (markers of hypoglycemic ketosis) by hydroxylation of the ethyl moiety of the parent SB molecule (2-HB or 3-HB) efficiently eliminated the effects on the neurotransmitter-related mRNA levels (Fig. 1*B*). Moreover, succinate (γ-carboxylbutyrate) or lactate (α-OH-propionate), both naturally occurring metabolites of the oxidative degradation of glucose via the Krebs cycle or carboxylic acid cycle, also had no effect on altering catecholamine or enkephalin mRNA levels. Therefore, it appears that only gut-derived SCFAs (*i.e.* propionate and SB) with a conserved C_2H_5-R (ethyl) moiety can induce TH and ppEnk transmitter-related genes.

These actions of SB are relevant and likely to occur *in vivo* because rats fed a high-fiber diet can have high luminal SB levels [up to 40 mM, see Fitch and Fleming (36)], already shown to be associated with histone hyperacetylation and growth inhibition of colonic epithelial cells (37). Taken together, our results support the interpretation that stereospecific requirements exist for the capacity of SCFAs and their structural analogs to modulate neurotransmitter-related gene expression. This evokes an intriguing argument for the existence of a receptor-mediated SCFA signal-transduction pathway(s) that may also be important in facilitating movement of fatty acids across cell membranes or into the nucleus.

Given the common requirement for an intact PKA intracellular signaling pathway for the stimulatory effects of SCFAs on neurotransmitter gene expression reported here, it is attractive to speculate that G-protein–linked receptors, coupled to the cAMP–PKA cascade, are potential candidates mediating SCFA responses in PC12 cells. In support of this interpretation is the activation of orphan G-protein–coupled receptors GPR41 and GPR43 by SCFAs propionate and SB, respectively (33, 35). Because the pharmacology of GPR43 matched the effects of SCFA on neutrophils, the authors speculated that such receptors may be involved in the development of inflammatory bowel disease, a finding that may also have relevance to the origins of neonatal bowel diseases like necrotizing enterocolitis (38).

In addition to the G-protein–coupled receptor family, we identified four independent lines of evidence to support a functional relationship between PPARγ and transmitter-related gene regulation by 4-PB: *1*) PPARγ mRNA and protein is expressed in PC12 cells at detectable levels; *2*) diclofenac, a nonsteroidal antiinflammatory drug and a known PPARγ blocking agent, antagonizes the effect of 4-PB on neurotransmitter gene expression; *3*) a Genomatix MatInspector search for PPARγ binding sites revealed at least one potential PPRE motif in the upstream region of rat ppEnk gene; and *4*) no putative PPARγ promoter elements were found in the TH gene and no effect of 4-PB on TH mRNA was observed. The identification of PPARγ in nonadipose tissues suggests novel functions for this receptor family that are distinct from its well-characterized long-chain fatty acid metabolic regulatory activity (39, 40). One of them could be a potential role of PPAR signaling in the development of the peripheral nervous system, its regulation and functions mediated via catecholamine or opiate peptide transmitter systems. Indeed, dietary C20 fatty acids have been reported to affect catecholamine synthesis in the CNS (41).

CONCLUSIONS

In conclusion, although the SCFAs found to affect neurotransmitter-related gene expression all share the ability to inhibit HDAC (18, 20–22), their final impact at the level of gene expression appears to be determined by activation of gene-specific signal-transduction pathways and the relative importance of pharmacologic properties of the treatments, including drug type and dose. Furthermore, our data implicate both G-protein orphan and PPARγ receptors as being involved in the independent regulation of catecholaminergic and opioid pathways by SCFAs and by related drugs like 4-PB. Taken together, our findings link disparate physiologic functions into a new view of interactive systems in which dietary intake and lipid metabolism may also be influenced by environmental signals governing sympathoadrenal stress-adaptation and cardiovascular pathophysiology via catecholaminergic pathways (42).

We speculate that *1*) circulating levels of SCFAs may influence sympathoadrenal transmitter biosynthesis and hence whole animal stress-adaptive responsiveness after birth, and *2*) the adverse effects of antibiotics on delayed acquisition of postnatal gut flora may affect this apparent evolutionary advantage of gut colonization. Further work will be necessary to test these hypotheses in living animals.

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